An Investigation of the effect of Immune Complexes on Non-Infected and HIV-Infected Mononuclear Phagocytes

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Summary

The aims of this project were to investigate the effect of immune complexes on mononuclear phagocytes in the presence or absence of infection with the human immunodeficiency virus (HIV). Mono Mac 6(MM6) cells and human monocyte-derived macrophages (MDM) were used to compare the effect of chemical stimulants, cytokines and immune complexes on surface antigen expression. The same experiments were performed using HIV-infected cells to determine the effect of HIV infection on these parameters. Some of the stimulants especially IL-6, IL-10, TNF-α and HIV increased CD80 expressions, the effect being greater in MDM than MM6 cells. This has implications for antigen presentation. Cytokines caused the differentiation of MM6 cells and significantly increased or decreased surface antigen expressions. The MM6 cells express surface antigens at lower levels then MDM this indicates that MM6 cells need to differentiate before expressing surface antigens. The high standard deviations obtained meant that no significant change in surface antigen expression was seen in all HIV non-infected and infected MDM incubated with various immune complexes and HIV-sera.

Comparing whole blood incubated with KLH and rabbit anti-human IgG showed that both seemed to produce IL-10 and IL-6 early. Only rabbit complexes stimulated TNF-α release. MDM incubated with IL-6, IL-10, and TNF-α showed increased expression of CD16 and CD80 only at day 3. However, HIV proteins (CHO) incubated in whole blood caused a significantly release of IL-6 at 8 hours with no detection of IL-10 and TNF-α.

MM6 infected with HIV-1_Ba-L for 5 days showed increased expression of CD16 and CD11c, but reduced expression of the other antigens examined. Significant levels of IL-10 were released at 8 and 12 hours with a slight increase in IL-6, and TNF-α. HIV protein-containing (CHO) immune complexes slightly increased IL-6 secretion at 8 hours at which point, IL-10 production was high. HIV-infected cells incubated with HIV-sera showed a lack of TNF-α release but IL-6 and IL-10 was detected at 12 and 8 hours respectively. The detection of high levels of IL-10 and the inhibition of TNF-α production may stimulate progression to full blown AIDS.
ABBREVIATIONS

cAMP - Cyclic adenosine monophosphate
ADCC - Antibody-dependent cellular cytotoxicity
AIDS - Acquired Immunodeficiency Syndrome
BCG - Bacilli Calmette-Guerin
CA - Capsid
CC - Chemokines
CMV - Cytomegalovirus
CR - Complement receptor
EBV - Epstein-Barr virus (EBV)
EDTA - Ethylenediamine tetraacetic acid
FCS - Foetal calf serum
GM-CSF - Granulocyte macrophage colony-stimulating factor
HLA-DR - Human leukocyte antigens-DR
ICAM-1 - Intracellular adhesion molecule-1
IFN-\(\gamma\) - Interferon gamma
IL- - Interleukin
IN - Integrase
ITAMs - Immunoreceptor tyrosine-base activation motifs
KLH - Keyhole limpet hemocyanin
KS - Kaposi's Sarcoma
LAS - Lymphadenopathy
LFA-1 - Lymphocyte function associated molecule-1
LTR - Long terminal repeat
LTNP - Long-term, non-progresso’s
MA - Protein matrix
MACS - Magnetic cell sorter
M-CSF - Macrophage colony-stimulating factor
MIP - Macrophage-inhibiting protein
MHC - Major histocompatibility complex
MM6 - Mono Mac 6 cells
MPS - Mononuclear phagocyte system
<table>
<thead>
<tr>
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<th>Definition</th>
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<tr>
<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Pneumocystis carinii pneumonia</td>
</tr>
<tr>
<td>PPD</td>
<td>Adjuvant peptide</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin A</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMNs</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute 1640 Medium</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SU</td>
<td>Surface glycoprotein</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>7TM</td>
<td>Seven-transmembrane-domain receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>V</td>
<td>Variable region</td>
</tr>
<tr>
<td>Vit D₃</td>
<td>1,25-dihydroxyvitamin D₃ (1,25-[OH] 2D₃)</td>
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ACKNOWLEDGEMENTS

I would like to thank Dr L.-J. Reynolds for her invaluable advice and support throughout the course of this work.

I also like to thank my friends and family for their constant support and especially a big “thank you” to my mother for supporting me throughout this course.
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1.0. INTRODUCTION

1.1. GENERAL INTRODUCTION

At the end of 1979, a large number of cases of mononucleosis-like syndrome accompanied with high fever, weight loss, and swollen lymph nodes were reported in the Gay community of California. This community also exhibited a high incidence of other unusual diseases including infection caused by Cytomegalovirus (CMV), Pneumocystis carinii pneumonia (PCP), Toxoplasma gondii and the cancer, Kaposi's sarcoma (Grmek, 1990). These opportunistic disorders indicated suppression of the immune system and the condition became known as the Acquired Immunodeficiency Syndrome (AIDS; Siegal et al., 1981).

In 1983, Barre-Sinoussi et al., isolated a reverse transcriptase containing virus from the lymph node of a patient with persistent Lymphadenopathy syndrome (LAS). Since viral infections are often accompanied by enlarged lymph nodes, it was presumed that LAS resulted from a human virus such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV). The virus isolated from the LAS patient (known as the lymphadenopathy-associated virus or LAV) demonstrated characteristics similar to those of the human T-cell leukaemia virus (HTLV). However, Montagnier and co-workers (1983) pointed out that the LAS agent, although similar to HTLV in infecting CD4+ lymphocytes, destroyed the cells rather than immortalising them. Around the same time, a team in the United States of America (USA) led by Robert Gallo isolated a human retrovirus from the peripheral blood mononuclear cells (PBMC) of adult and paediatric AIDS patients which was distinct from HTLV (Gallo et al., 1984). It was called HTLV-III. This virus exhibited lymphotropic and cytopathic properties showed cross reactivity with some of the proteins found in HTLV-I and HTLV-II, especially the p24 core proteins. Later it was confirmed that LAV and HTLV-III were effectively the same virus.

In 1986, the International Committee on the Taxonomy of Viruses recommended giving the AIDS virus the name "Human Immunodeficiency Virus" or HIV. Since it had been shown that the AIDS virus showed greater genetic homology with equine infectious anaemia virus and other lentivirus, rather than the human T cell leukaemia virus. Subsequently another subtype of HIV was isolated.
in Western Africa and the viruses became known as HIV-1 and HIV-2 respectively. Although HIV-2 can cause AIDS, the development of overt disease may be very protracted compared to that caused by HIV-1 (Levy, 1993).

The World Health Organisation (WHO) estimated that 13 million people would be infected with HIV by the end of 1993, representing an increase of 1 million new infections since 1992 (WHO, 1993; Hughes and Rutherford, 1995). The rural regions in sub-Saharan Africa and South and Southeast Asia, remain vulnerable to new HIV infections because by the year 2000, an estimated 40 million people will be infected with HIV and 10 million will have died of AIDS (WHO, 1991; Murray, 1996).

HIV infection is initially established in lymphoid organs that serve as major reservoirs for HIV. Indeed, lymphoid organs may exhibit active and progressive HIV disease during clinically latent periods. Destruction of the lymphoid tissue and the loss of the patient's ability to respond to HIV and/or other pathogens are caused by chronic stimulation of the immune system due to persistence of the virus in lymphoid organs. Some patients with primary HIV infection show expansions of restricted Vβ subsets of CD8+ T-cells that represent HIV-specific cytolytic T cells (Pantaleo and Fauci, review, 1995). Patients who are long-term, non-Progresso's (LTNP) and who have been HIV-infected for about 10 years have normal lymph nodes, HIV-specific humoral and cell mediated responses and high, stable CD4+ T cell counts over a number of years. These patients express only a low-level viremia, although virus derived from the mononuclear cells of such patients are found to be replication competent and infectious (Pantaleo and Fauci, review, 1995).

The depletion of CD4+ T-lymphocytes in an AIDS patient with lymphadenopathy (Barre-Sinoussi et al., 1983) and the ability of monoclonal anti-CD4 antibodies to block HIV infection of CD4+ T-lymphocytes in vitro (Dalgleish et al., 1984; Klatzman et al., 1984b), indicated that CD4 is a natural receptor for HIV (Klatzman et al., 1984a; Popovic et al., 1984a). However, conclusive evidence came from Maddon et al (1986) and Evans et al (1988) that the receptor for HIV-1 and HIV-2 is CD4. HIV infects cells with higher expression of CD4 such as a subpopulation of T-lymphocytes, macrophages and, to a lesser extent, blood monocytes (Asjo et al., 1987; Sattentau and Weiss, 1988).
In vitro isolation of HIV from infected T-lymphocytes was demonstrated using mitogen-generated syncytia (multinucleated giant cells) which died releasing viral progeny (Lifson et al., 1986; Yoffe et al., 1987). In vitro, cytopathic changes are shown to be minimal in HIV infected mononuclear phagocytes, with a low level of virus released into the supernatant (Lifson et al., 1986; Yoffe et al., 1987; Gendelman et al., 1988). It is assumed that cells of the mononuclear phagocyte lineage are involved in the continuous production and transmission of virus to a new host (Pearce-Pratt and Phillips, 1993).

HIV only infects activated and proliferating T-lymphocytes that express the nuclear factor-κB (NF-κB) responsible for the up-regulation of transcription of certain genes by binding to distinct sequences in gene promoters (Stevenson et al., 1990). The replication of HIV-1 in T-lymphocytes is dependent upon the viral long terminal repeat (LTR) which binds NF-κB (Nabel and Baltimore, 1987, Duh et al., 1989. By contrast, human monocytes (either activated, quiescent or γ-irradiated) and in vitro differentiated human macrophages do not need to express NF-κB for HIV-1 to replicate (Bernstein et al., 1991; Weinbery et al., 1991; Schuitemaker et al., 1990).

Host proteins may play a role in the viral life cycle e.g. by incorporating into the virion during virus assembly or by playing a role in early infection (Ott, 1997). Briant et al., 1996 showed that the binding of gp120-anti-gp120 immune complexes to infected, resting, PBMCs stimulated viral replication.

1.2. CLINICAL FEATURES OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION

1.2.1. Infections and tumours associated with AIDS

Opportunistic infections associated with late stages of AIDS which include *Candida albicans*, *Cryptococcus*, *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Pneumocystis carinii*, *Mycobacterium avium intracellular*, *Herpes Simplex virus (HSV)* and *Cytomegalovirus (CMV)* begin to emerge as a patient condition worsens (Horsburgh, 1991). Typically, HIV-infected adults who develop *Pneumocystis carinii pneumonia* (PCP) have CD4 counts<200/mm³ indicating a
severe immune deficiency. The type of infection involved may vary geographically. For example, *Penicillium marneffei* is found in patients in Northern Thailand and visceral leishmaniasis is seen in various parts of the Mediterranean and Latin America (Beiser, 1997). *Mycobacterium avium* infection occurs in half of all people who develop AIDS in North America. In addition, cytomegalovirus disease affects 45% of patients with HIV infection (Hoover et al., 1993). Cytomegalovirus retinitis is a typical manifestation of infection which often results in blindness (Beiser, 1997).

Tuberculosis is the commonest opportunistic infection globally and the leading cause of death in HIV infection. Although treatment with the drugs such as isoniazid and rifampin has been found to be effective in patients with HIV, (Beiser, 1997; Murray, 1996) multidrug resistant tuberculosis affects about 19% of HIV patients (Beiser, 1997).

Kaposi's Sarcoma (KS) was formerly found predominantly in elderly males but is now frequently associated with HIV infection (Hymes et al., 1981). Recent studies have shown that KS is caused by a new herpesvirus which was detected in the peripheral blood mononuclear cells of patients with HIV, its presence correlating with low CD4 counts (Beiser, 1997). HIV patients who lacked clinical evidence of KS but had detectable KS-associated herpesvirus in peripheral blood cells showed a higher chance of developing the disease. The virus may be sexually transmitted and is commonly found in the genital tissues of healthy adults especially in semen (Beiser, 1997). Although KS is associated with HIV, its incidence in homosexual males has decreased sharply due to safe sex practice but has increased among intravenous drug abusers and heterosexuals of both genders (Murray, 1996).

HIV has been shown to cause neurological dysfunction and the incidence of AIDS-related dementia has increased (Sharer, 1992).

### 1.2.1.1. Epidemiology of HIV Infection

Epidemiological studies have demonstrated that the route of transmission of HIV is by blood or blood products (Ammann et al., 1983). High-risk groups were identified which included homosexuals, intravenous drug abusers and blood product recipients, especially haemophiliacs, (Stoneburner et al., 1990). In
homosexuals, it has been demonstrated that HIV-1 is present in the semen during and immediately after primary infection (Tindall et al., 1992). Infected, heterosexual women produce HIV-1 in cervico-vaginal secretions in both cell-associated and cell-free forms. This shedding of infectious virus is more likely in HIV-infected women with genital inflammation. In addition, seronegative women with genital tract inflammation are susceptible to HIV-1 infection via intercourse because lymphocytes and macrophages are found at the site of inflammation (Mayer and Anderson, 1995).

In Africa and Asia, the high transmission of HIV in adults is largely heterosexual due to frequent, unprotected sexual contact (W.H.O. report, 1993; Fischl et al., 1987; Mastro et al., 1994). Epidemiological studies suggest susceptibility to HIV infection per sexual contact is higher in women than men. However, the long incubation period complicates the estimation of the rate of sexual transmission, as many persons do not know they are infected until years after exposure (Mayer and Anderson, 1995).

Infants can be infected via breast-feeding from infected mothers (Kimball et al., 1995). Between 15 and 35% of infants born to seropositive mothers are infected but factors influencing transmission are uncertain (Ryder et al., 1989).

The introduction of preventative programmes has reduced the risk of infection from infected blood products, needle sharing (among intravenous drug users), needle-stick injury and exposure to open wounds or mucous membranes (Ammann et al., 1983).

1.2.1.2. Clinical features of HIV infection

Persons infected with HIV may be asymptomatic or may exhibit a range of clinical signs and symptoms including lymphadenopathy, opportunistic infections and cancers (Metroka et al., 1983). At the time of infection, about 30% to 60% of people exhibit a syndrome resembling infectious mononucleosis (Cohn, 1997). Acute infection appears 2-6 weeks after exposure to the virus. Typical symptoms include fever, myalgia, arthralgia, lethargy, lymphadenopathy, pharyngitis, nausea, headaches, photophobia and rashes. HIV infected persons enter a latent asymptomatic phase that may last as long as 14 years before the emergence of
full-blown AIDS. A person with full-blown AIDS has a poor prognosis of maybe 1-2 years (Schuitemaker et al., 1992b).

A large decline in CD4 cells in the first six months after seroconversion is associated with rapid progression to AIDS or death (Cohn, 1997). Similarly, plasma levels of 10,000 copies or more of HIV-RNA within one year of seroconversion indicates a similar prognosis (Mellors et al., 1995; Saag et al., 1996). Infected patients with a low viral load and plasma HIV RNA levels ≤ 4530 copies/ml have an 8% probability of developing AIDS in five years whilst patients with >38,270 RNA copies/ml have a 62% probability of doing so (Mellors et al., 1996).

1.3. The human immunodeficiency virus

HIV is a retrovirus that belongs to the lentivirus family. It is typically associated with a long period of latency between infection and onset of clinical symptoms (Gelderblom et al., 1987). Studies on the structure of the isolated virus showed a cone-shaped core and a viral capsid. The latter being composed of identical subunits arranged with an icosahedral symmetry, that provide protection for the viral nucleic acid and enzymes required for replication (Figure 1.1; Gelderblom et al., 1987). The virus comprises two identical strands of RNA that are surrounded by a nucleocapsid (p7) and associated with reverse transcriptase. This is enclosed by the polypeptides that comprise the capsid (p24) of the mature virion. The outer structure of the virus is glycoprotein (gp160) arranged as 72, knob-like structures (Ozel et al., 1988; Pinter et al., 1989; Earl et al., 1990; McCune et al., 1988). The gp160 comprises gp120 (responsible for HIV-1 binding to CD4 on the host cells) that noncovalently interacts with gp41 (Ott, 1997).

The RNA-dependent, DNA polymerase (reverse transcriptase, RT), converts the virus RNA into DNA. Reverse transcriptase is cleaved by the Pol gene products (enzymes which are vital for infectivity) which also cleave the protease and integrase enzymes (Haseltine, 1991; Cullen, 1991).

The HIV-1 genome encodes four accessory proteins (Figure 1.2) Nef, Vif, Vpr and Vpu (see Section 1.3.1, Ott, 1997). It has been suggested that Vif encodes regulatory proteins that play a role in the final stages of packing the viral
Figure 1.1. Structure of human immunodeficiency virus-1 (HIV-1)
The structure of the human immunodeficiency virus (HIV), using the nomenclature adopted by Leis et al., (1988). The spikes of the transmembrane glycoprotein (TM, gp41) and surface glycoprotein (SU, gp120) project through the lipid envelope. These surface spikes initiate the viral infection cycle by binding to specific receptors on the surface of host cells. A protein matrix (MA, p17) surrounds the capsid, which in turn is covered with a lipid bilayer (derived from the host cells during budding). The viral RNA (in association with nucleocapsid, NC, p7), viral reverse transcriptase (RT) and integrase (IN) enzymes are present inside the viral capsid (CA). The CA consists of repeating subunits of p24 and is responsible for the cone-shaped core of virions.
Figure 1.2. The genome of HIV-1 is the causitive agent of AIDS

The standard retroviral genes (green)- gag, pol and env- are present along with several other genes coding for regulatory product (red). The long terminal repeat (LTR) elements, which are characteristic of retroviral proviruses, and are crucial to the regulated expression of the virus are shown in blue. The diagram is adapted from that of Eales (1997).
nucleoprotein core and can influence infectivity of progeny virions (Hoglund et al., 1994).

1.3.1. Binding of HIV to CD4

The gp120 knobs projecting from HIV virions bind to the CD4 molecule (Figure 1.3) on host T-lymphocytes and mononuclear phagocytes (Klatzman et al., 1984b; Sattentau and Weiss, 1988). Peripheral blood monocytes that express a higher level of CD4 decline at the same rate as T-cells during disease progression (Lucey et al., 1991). Whilst gp120 is responsible for CD4 recognition, gp41 (which is noncovalently linked to gp120) anchors HIV to the host cell membrane (Geleziunas et al., 1994).

CD4 is a member of the immunoglobulin (Ig) supergene family and consists of four external Ig-like domains, (D1-D4 or V1-V4), a transmembrane region and a cytoplasmic tail linked to p56Lck protein tyrosine kinase. The binding of HIV-1 gp120 occurs at the V1 region (Geleziunas et al., 1994). The V1 and a non-contiguous region on CD4 bind to the fourth conserved portion of gp120 near the C terminal (Arthos et al., 1989; Cordonnier et al., 1989; Maddon et al., 1986). Alterations in the conformation of gp120 allows the virus to demonstrate different cell tropism's (Koito et al., 1994). Interaction of gp120 with CD4 exposes a fusion domain in gp41 that may be the first step of HIV entry (Sattentau and Moore, 1991). Studies indicate that gp41 alone can mediate HIV entry and syncytium formation between CD4+ and CD4- cells (Marcon and Sodroski, 1994; Perez et al., 1992).

Once infected with HIV, CD4 surface expression is down regulated (Hoxie et al., 1986; Poulin et al., 1991; Geleziunas et al., 1994). This is associated with expression of HIV-1 auxiliary proteins Nef and Vpu which respectively are expressed during the early regulatory and structural phases of HIV-1 gene expression (Cullen, 1991). The Vpu gene encodes a 16-KDa protein whilst Nef encodes a 27-KDa myristilated, membrane-associated phosphoprotein vital for HIV-1 replication in vitro (Geleziunas et al., 1994). Down-regulation of CD4 surface expression on thymocytes of transgenic mice or CD4+ cell lines has been shown to be due to the expression of Nef which does not however affect the levels of CD4 mRNA or protein. Nef also affects CD8 expression on murine but not human cells (Garcia et al., 1993, Skowronski et al., 1993).
The virus replication cycle is initiated when viral glycoprotein spikes bind to a specific receptor on the host cell surface. CD4 is the main receptor, and binding of gp120 to CD4 is thought to cause a conformational change that exposes gp41. The mechanisms by which the virus enters the cells and uncoats are still unclear, but probably involve fusion of the viral and host cell membranes. Once inside the cell, the virus forms pre-integration complexes and reverse transcribes its RNA genome. The DNA copies of the viral genome move to the nucleus where they integrate randomly into the host chromosomes. When the cell is activated, viral proteins are transcribed and progeny virions are produced. Virions mature by budding either through the host cell membrane, or in myeloid cells, into intracellular vesicles (adapted from Cook, 1994).
Lymphocytes and monocytes both transfected with gp160 were shown to contain CD4-gp160 complexes. In the absence of syncytium formation, the expression of gp160 causes cytopathic effects in CD4\(^+\) but not CD4\(^-\) cells. This is associated with CD4, P56\(^{\text{lck}}\) PTK found in gp160-CD4 complexes which inhibit maturation and transport of gp160 and CD4 molecules. The complexes are formed and retained in the endoplasmic reticulum (ER) (Geleziunas et al., 1994).

1.3.1.1. Co-receptors for HIV

For over a decade researchers have looked for other receptors that facilitate HIV infection. In 1996 several groups showed that the chemokine receptors CCR5 and CXCR4 fulfilled these roles. The co-receptors were identified as members of seven-transmembrane-domain (7TM) receptors that form a group of structurally related proteins. The chemokine receptors have two cysteines, one in the NH\(_2\)-terminal domain and the second in the third extracellular loop, that form a disulphide bond important for ligand binding (Baggiolini et al., 1997). These molecules act as co-receptors for both non-syncytium-inducing and syncytium-inducing HIV-1 strains. Other chemokine receptors (e.g. CCR3 and CCR2b) can be used by some HIV-1 strains but only in association with CCR5 and CXCR4 (Clapham, 1997). The CCR5 is for 'macrophage-tropic' (M-tropic) HIV-1 strains and CXCR4 for 'T-cell tropic' (T-tropic) HIV-1 strains. The M-tropic strains replicate in macrophages and in CD4\(^+\) T cells but do not form syncytia in T cell lines (Moore et al., 1997).

The V3 loop region of gp120 is vital for interaction with CCR5 or CXCR4 although the nature of the CCR5 and CXCR4 binding sites on gp120 are not yet understood (Paxton et al., 1996; Choe et al., 1996; Cocchi et al., 1996). Mutant gp120 in which the V3 loop has been deleted is unable to bind to CCR5 but it has been suggested that V3 might not be the only binding site (Wu et al., 1996, Trkola et al., 1996). However, deletion of the V1-V2 loop structure from gp120 does not prevent gp120-CCR5 interaction (Wu et al., 1996). It is feasible that CCR5 and/or CXCR4 can bind at multiple points on conserved structures in the V3 loop. Also, CD4 residues may be involved in the gp120-CCR5 interaction since MIP-1\(\beta\) binding to CCR5 on CCR5-transfected cells can be inhibited by the amino-terminal domains (D1, D2) of CD4 (Wu et al., 1996). Adapted strains of HIV-2 and SIV, when used to infect CD4\(^+\) human cells in vitro were able to replicate, indicating that these strains may use CXCR4 for entry, in the absence of CD4. Indeed, it has been shown that a mAb against
CXCR4 inhibited CD4-dependent and CD4-independent CXCR4-mediated infection (Endres et al., 1996).

1.3.1.2. Entry and uncoating of HIV

The initial stage of infection depends upon virus glycoprotein binding to specific cellular receptors as described in previous sections. Following this, the virus envelop fuses with the cell membrane and uncoating is encouraged by the acidic environment of intracellular endocytic vesicles which causes the release of viral nucleic acid (Maddon et al., 1986). Other studies have suggested that cellular entry of HIV is unaffected by pH both in T cells (Stein et al., 1987; McClure et al., 1988) and CD4− cells (Tateno et al., 1989). Other factors have been suggested as instrumental in HIV infection of myeloid cells. These include antiviral antibodies and specific Fc receptors (Robinson et al., 1991; Haubrich et al., 1992) and the binding of complement-coated virions to CR3 and CD11b/CD18 (Reisinger et al., 1990).

1.3.1.3. Formation of syncytia

In 1955, Black and Melnick recognised that a virus could spread from one cell to another. Early blocking experiments using cocultures of peripheral blood lymphocytes (PBL) from HIV seropositive patients and mitogen-stimulated PBL from uninfected people showed that azidothymidine and virus-neutralizing antibodies had no effect on cell-to-cell transmission of HIV. However, antibodies to the CD4 receptor HIV prevented fusion between infected and uninfected cells. This suggested the importance of CD4 receptor in HIV entry into uninfected cells (Gupta et al., 1989). Later experiments, by Sato et al., (1992) demonstrated that within hours of incubating HIV-infected H9 cells with T-cells, synthesis of new viral protein and unintegrated DNA were detected. Observations using light microscopy indicate that one hour after infection numerous small syncytia were observed followed by large syncytia at 2-4 hours and giant syncytia by 8 hours (Sato et al., 1992). In HIV infection, the induction of syncytium formation is caused by surface expressed gp120 binding CD4 on cells. This has been confirmed since syncytium formation and viral spreading is blocked by antibody to the viral gp120 (Phillips, 1994).
1.3.1.4. Cell to cell transmission of HIV

The transmission of virus from one mononuclear cell to another involves cell adhesion molecules that mediate adherence between infected and virgin cells. Adherence is followed by shedding of the virus into the space between the two cells and its subsequent attachment to the virgin cell. The cytokines produced by these cells may influence the secretion of HIV (Kupfer et al., 1986; Young and Cohn, 1986). Additionally, cell-to-cell transmission may occur through uninfected dendritic cells passively transferring HIV to CD4+ lymphocytes. The infected lymphocytes form syncytia and undergo cytolysis releasing HIV (Cameron et al., 1992). Epithelial cells that line organ systems allow the passage of some pathogens by transcytosis. Infected epithelial cells do not serve as reservoirs of HIV but are infected and die even though the cells do not express CD4. Anti-CD4 antibodies did not block infection of these cells (Tateno et al., 1989). Culture supernatants from HIV-infected, transformed T-cells was less successful in infecting epithelial cell lines than those from chronically infected T cell or monocyte lines infect (Phillips and Tan, 1992; Tan et al., 1993). It was found that 15 minutes after adding infected cells, HIV budding could be observed at the point of contact with the epithelium. T-cells were also observed to adhere to epithelium for a few minutes then detach and bind to another site on the epithelium (Pearce-Pratt and Phillips, 1993). It has been shown that the integrin molecule protein complex CD11a-c/CD18 is involved in the infection and cytopathic effect of HIV-1 in vitro in addition to CD4.

1.3.1.5. Reverse transcription and integration of viral nucleic acid

Following entry, the viral RNA is released in the cytoplasm and is reverse transcribed to give double-stranded, linear, DNA (cDNA) proviral intermediates. The association of virion gag proteins facilitates the migration of this viral nucleoprotein into the cell nucleus. Long terminal repeat (LTR) elements generated by reverse transcription are important for regulating viral expression (Bukrinsky et al., 1992). Integrase, a single, viral, 32 KDa protein helps the DNA integrate into the host chromosomes (Farnet and Haseltine, 1991). This integration is a random process and is vital for productive infection (Hong et al., 1993).

Productive infection has been shown to be associated with cellular activation. In quiescent cells, only a small portion of the viral genome is transcribed and there is no production of viral protein. Infection is aborted after 3 to 5 days if the cells are not activated.
and expressing HLA-DR. Peripheral blood monocytes express >90% HLA-DR but are not as susceptible to HIV infection as are macrophages. Differentiated macrophages have less CD4 expression than non-differentiated monocytes. Peripheral blood, CD4+ monocytes maintained in suspension and inoculated with HIV, produces a continuous, low level of virus as they differentiate into macrophages. HIV remains quiescent in non-differentiated cells, replication occurring after differentiation in the tissues (Levy, 1993).

1.3.1.6. Late stages of the viral replication cycle

Translated as polypeptides, which are cleaved by a viral protease, retroviral proteins undergo myristolation or phosphorylation and glycosylation by host enzymes. Precursor proteins and the mature gp160 are heavily glycosylated (Mizouchi et al., 1988), deglycosylation resulting in reduced infectivity and capacity to form syncytia (Kozarsky et al., 1989). During release of the virus by budding, the host cell membrane is incorporated in the envelope. Phosphorylation regulates HIV-1 specific Vpu which enhances viral detachment from the cell membrane and degradation of CD4 in the endoplasmic reticulum (Schubert and Strebel, 1994).

Infection of human cells by HIV varies depending on the strain of the virus. Successful entry may be accompanied by a latent phase due to failure of integration of viral nucleic acids into the host chromosome, or to lack of transcription of the viral proteins. Once integrated, viral DNA can remain in a latent state for long periods. However, once activated, replicating CD4+ T-lymphocytes are a major source of HIV (Zack et al., 1990). Production of HIV by cells of the mononuclear phagocyte system is quite distinct from that seen in T cells. Mononuclear phagocytes form a reservoir of virus, low levels of which are constantly released. In contrast to this, follicular dendritic cells of the lymph nodes are associated with high levels of virus production (Pantaleo et al., 1993). The mechanism of release of mature virus from myeloid cells differs to that seen in T-lymphocytes since HIV is found in large intracellular vacuoles that restricts their release (Popovic and Gartner, 1987; Schuitemaker et al., 1991).

In the early stages of infection, asymptomatic individuals produce non-syncytium-inducing virus that is tropic for primary cells but not for transformed T cell lines (Popovic and Gartner, 1987; Schuitemaker et al., 1991). In the later stages of the disease, viruses are produced that show a high rate of replication and the capacity to induce syncytia (Fenyo et al., 1988). This may be because in the early stages of infection the immune response
suppresses the highly virulent strains of HIV whilst the less virulent escape immune surveillance. Also, the persistent infection in macrophages goes unnoticed during the asymptomatic period of the disease (Fenyo et al., 1988).

1.3.1.7. Host proteins in HIV

The complicated life cycle of HIV is carried out by a limited set of proteins. Thus the virus depends on host proteins for survival. These may play a role in the viral life cycle either by incorporation into the virion during virion assembly or in early infection (Ott, 1997).

Studies have shown that budding HIV-1 contains components from the host cell plasma membrane in its envelope (Zavada, 1982). Neutralisation studies showed the presence of HLA-DR antigen on all virions produced from an HLA-DR⁺ cell line (Arthur et al., 1992). Other studies have demonstrated the presence of this antigen on viruses isolated in vivo (Bastiani et al., 1997; Saarloos et al., 1997). Recently it has been shown that HLA-DR increases viral infectivity in vitro and in vivo. However, it is not vital to infection since host cells can be infected in vitro by HIV-1 produced from HLA-DR⁻ cells (Arthur et al., 1992; Cantin et al., 1997). In T cell activation, HLA-DR-dependent intracellular signals are required to ensure cell division. Without such signals, activation results in programmed cell death (apoptosis). Earlier studies indicated that the interaction between free virus and CD4⁺ provided this signal. It has been suggested that the observed decline in virus specific CD4⁺ T cells could be due to HLA-DR on the surface of virions inducing apoptosis. Recent work demonstrated that HLA-DR on virions interacts with T cells and presents superantigen to resting T cells in vitro (Ott, 1997). HLA class I is present at low levels on HIV and does not affect its infectivity (Benkirane et al., 1994).

Cell adhesion proteins also have been detected on HIV-1. CD44, a receptor for hyaluronic acid increases in vivo infectivity. LFA-1 (CD11a) and it counter receptor ICAM-1 has been found on the surface of HIV. These adhesion molecules can bind to CD4⁺ cells allowing infection. Other surface proteins found on HIV are two-complement cytolysis inhibiting proteins, CD55 and CD59. Other host cell components associated with HIV include Cyclophilin A, an abundant cellular protein produced from epithelial cells and involved in T cell activation. It has been demonstrated bound to Gag at a site in the centre of p24 (Luban, 1996; Franke et al., 1994). Also, actin and cytoskeleton components which play an active role during budding are found incorporated into the virion (Ott, 1997). Another
component found in HIV is ubiquitin, a protein which is implicated in protein turnover, cell cycle control, antigen presentation, DNA repair, and the heat shock response (Finley and Chau, 1991). A single ubiquitin molecule is conjugated to a small amount of the p6 protein in mature HIV-1 suggesting a role in HIV budding. The host and virus interaction offers potential targets for therapeutic intervention (Ott, 1997).

1.3.1.8. HIV infection of mononuclear phagocytes

As mentioned earlier, replication of HIV in cells of the mononuclear phagocyte series is quite distinct to that seen in T cells. The stage of maturation and differentiation is vital for HIV-1 replication in these cells (Gazzolo and Mace, 1990). Studies have shown that, owing to their heterogeneity, the use of human mononuclear phagocytes in studying viral replication poses some difficulties. Therefore monocytic cell lines such as THP-1 and U937 have been instead (Kitano et al., 1990). Other studies have shown that human monocytes infected with HIV-1 in vitro for 2 days show enough virus production to measure reverse transcriptase (RT) activity in culture supernatants whilst infected, differentiated, macrophages yield only low levels of extracellular virus as detected by a p24 assay (Valentin et al., 1991).

Biologically active chemicals such as vitamin D₃, Dimethyl sulfoxide (DMSO), dibutyryl, cyclic adenosine monophosphate (cAMP), and Tetradecanoylphorbol-13-acetate (TPA) induce differentiation of both monocytic cell lines and human peripheral blood monocytes (Kitano et al., 1990). These chemicals are thought to enhance replication of both monocytotropic and lymphotropic variants of HIV-1 (Skolnik et al., 1991).

Mononuclear phagocytes (which include tissue macrophages and blood monocytes) are primary targets for HIV infection. They act as a reservoir for viral replication and are resistant to virus-induced apoptosis. Blood monocytes aid in the dissemination of HIV into various tissues such as the brain (Meltzer et al., 1990). Macrophages are the predominant cell type infected with HIV and play a vital role in the pathogenesis of HIV-related encephalopathy (AIDS dementia complex). The resident macrophages of the brain, microglia, are able to support productive HIV infection and may possess between 500 and 1500 copies of HIV RNA per cell; about 10 times more than in blood leukocytes (Bornemann et al., 1997).

The interaction between HIV and the CD4 receptor on mononuclear phagocytes requires an appropriate HIV strain tropic for CD4+ cells (O’Brien, 1995). Conformational
changes take place in the gp120 when monocyte CD4 binds, thus exposing V3 determinants and allowing the entry of macrophage tropic strains (Koka et al., 1995). The entry of viral DNA into the nucleus is similar in both T cells and macrophages although the rate of reverse transcription varies. The lack of division and a limitation in intracellular nucleotide pools may cause a slow rate of reverse transcription in macrophages (O, Brien, 1994).

The high HIV copy number in macrophages may be attributed to the non-cytopathic character of HIV in these cells and the budding of large amounts of virus into intracellular vacuoles. The latter are derived from the Golgi complex and provide an environment for virus assembly. HIV produced in macrophages may disseminate throughout the body. This process is aided by cytokines that regulate and modulate macrophage activity and virus replication (Bomemann et al., 1997).

1.4. The mononuclear phagocyte system

In order to understand the unique role of the cells of the mononuclear phagocytes system in the spread of HIV, it is necessary to have a detailed understanding of the cells themselves. Mononuclear phagocytes are capable of synthesising over 100 products, processing and presenting antigens and communicating with other cells via the secretion of cytokines. They originate from granulocyte-macrophage colony forming units (GM-CSF) that are stem cells present in the bone marrow. These go through a number of divisions, differentiating into fully mature monocytes in the bone marrow (Figure 1.4). After 24hrs, these mature cells are released to the circulation (Metcalf, 1971). In response to inflammation, injury, allograft or tumour rejection cytokines are released that encourage the production of monocytes from the bone marrow (van Furth et al., 1973).

Surface antigens expressed on monocytes include the lymphocyte function associated molecule-1 (LFA-1, CD11a/CD18) that binds to the intracellular adhesion molecule-1 (ICAM-1, CD54) on endothelial cells allowing migration of monocytes. The monocytes migrate from the blood vessel between the endothelial cells into the subendothelial space by a process termed diapedesis (Rothlein et al., 1986). This process allows monocyte migration to a particular site of inflammation in response to interleukin-1 (IL-1) and interferon-γ (IFN-γ) that upregulate expression of ICAM-1 (Dustin et al., 1986). When monocytes migrate into the tissues they mature into macrophages. The latter can
remain in the tissues for days or even years and show distinct phenotypic characteristics depending on the tissue in which they are found e.g. alveolar macrophages in the lungs and Kupffer cells in the liver (van Furth, 1989; Lasser, 1983).

1.4.1. Monocyte/macrophage characteristics

Cells of the MPS are a functionally and phenotypically diverse group. Monocytes are small (10-12um) uniformly rounded cells. In vitro, blood monocytes will adhere, flatten and spread in the presence of serum. They will develop a heterogeneous appearance after 6-8 days. Activation of monocytes/macrophages by the addition of IFN-γ or 1,25-dihydroxyvitamin D₃ in culture causes an increase in cell size which is accompanied by an increase in the cytoplasmic to nuclear ratio and, increased numbers of cytoplasmic granules and lipid inclusions (Andreesen et al., 1990).

1.4.1.1. Enzyme activity

Certain cell types can be identified by their enzymes such as α-naphthyl acetate esterase (α- NAE) and α-naphthyl butyrate esterase found in mononuclear phagocytes (Kaplow, 1975). Blood monocytes have peroxidase activity in their primary lysosomes that is lost as they mature but reappears in association with the nuclear envelope and rough endoplasmic reticulum, as exhibited in tissue macrophages (Bos et al., 1988). Lysosomal myeloperoxidase activity (which makes up 5% of the total cellular protein in monocytes) decreases as they mature. This enzyme can catalyse the oxidation of hydrogen peroxide, in the presence of chloride ions to give hypochlorous acid that is toxic to microorganisms (Edwards and Swan, 1986). Myeloperoxidase also plays a role in the metabolism of leucotriene C₄ (LTC₄) (Neill et al., 1985).

1.4.1.2. Respiratory burst activity

The respiratory burst occurs as a result of increased oxygen uptake during which glucose is metabolised by the hexose monophosphate shunt. This is associated with the production of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), which are toxic to pathogenic bacteria and fungi (Figure1.5; Babior, 1984). Superoxide is converted to H₂O₂ by the cellular
Figure 1.4. The path of monocytes differentiating to tissue macrophages
Based on information from Van Furth et al. (1973; 1979) and Whitelaw (1966).
Figure 1.5. Respiratory Burst
This occurs as the result of stimulated phagocytes that produces oxygen metabolites and other microbicidal compounds. Diagram is adapted from that of Eales, 1997.
enzyme superoxide dismutase (SOD). H$_2$O$_2$ reacts with halide ions to produce hypohalous acid, a reaction catalysed by myeloperoxidase metabolites (Nakagawara et al., 1981).

Free radicals are effective microbicidal agents due to their instability but their production is regulated to prevent adverse toxicity to host cells (Nakagawara et al., 1981).

1.4.1.3. Non-oxidative microbial killing

Macrophages contain granule-associated proteins such as collagenases, deoxyribonucleases, sulphatases, lipases, and elastase, phosphatases and polysaccharidases that have antimicrobial activity. Also, exposure of MPS to IFN-γ causes the degradation of cellular reserves of tryptophan that are vital for microbial growth (Mintz et al., 1988; Ozaki et al., 1987).

1.4.1.4. Secretory activity

Mononuclear phagocytes (MNPs) can generate prostaglandins, leucotrienes and hydroxyeicosatetraenoic acid (HETE) through the oxidative metabolism of arachidonic acid in response to a variety of stimuli. Synthesis of cyclooxygenase and the production of prostaglandin by MNPs have been shown to be stimulated by endotoxin (Fu et al., 1990). However, quiescent human peritoneal macrophages have been shown to synthesise and release PGE$_2$ and thromboxane B$_2$ (Mackenzie et al., 1992). MNPs, without stimulation, can synthesise large amounts of cytokines, in particular IL-1. Secretion of this cytokine may be stimulated by a number of factors including bacterial endotoxin. IL-1 is detected in lysosomal fractions and exists in two forms; IL-1α that is associated with the plasma membrane and IL-1β, the secreted form (Bakouche et al., 1987). IL-1 induces inflammation and activates T- and B-cells (Manetti et al., 1996).

Tumour necrosis factor-α is also produced by macrophages. It is involved in the regulation of the immune system and acts as an effector molecule in macrophage-mediated tumour cell cytotoxicity (Carswell et al., 1975; Urban et al., 1986). Human peripheral blood monocytes isolated by adherence to plastic release macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF, Bennett et al., 1992).
MNPs are one of the chief sources of IL-12. It is produced by neutrophils, dendritic and Langerhans' cells. Phagocytic cells produce IL-12 in response to various bacteria, bacterial products, other intracellular pathogens and viruses. The production of IL-12 during infection is followed by the production of IFN-γ resulting in activation of phagocytic cells, which in response, produce higher levels of IL-12 (Trinchieri, 1997).

IL-10 that is produced by macrophages and other cells, inhibits the production of IL-12. It has been suggested that the delayed expression of IL-10 compared to IL-12 and other proinflammatory cytokines, both in vivo and in vitro, makes IL-10 an effective downregulator of the IL-12 response (Trinchieri, 1997).

1.4.1.5. Chemotaxis, chemokines and their receptors

Monocytes use their receptors to sample the environment and use a process called chemotaxis to allow them to move along concentration gradients of stimulatory molecules. Chemicals called chemokines (Synderman et al., 1986) enhance this process. The chemokines are composed of a large family of small cytokines with four cysteines linked by disulphide bonds. There are two subfamilies of chemokines CXC and CC that differ by one amino acid. Both groups of chemokines are important because of their actions on leukocytes and their involvement in inflammation and immunity (Baggiolini et al., 1997). In humans, the genes coding for CXC chemokines are on chromosome 4 and those for CC chemokines coded for by genes on chromosome 17 (Baggiolini et al., 1997). Table 1 shows chemokines and their selective chemokine receptors.

Chemokines act through a group of structurally related protein receptors which have a characteristic seven-transmembrane-domain (7TM, Baggiolini et al., 1994; Murphy, 1994). Two receptors for IL-8, CXCR1 and CXCR2 are expressed on all neutrophils and monocytes and at low levels on CD8⁺ T cells and natural killer cells (NK). The receptors are not present on CD4⁺ T cells and B cells. CXCR1 is specific for IL-8 whilst CXCR2 shows affinity for IL-8 and other CXC chemokines. CXCR3 is expressed on IL-2-activated T lymphocytes but not on resting T lymphocytes, B-lymphocytes, monocytes, or granulocytes. CXCR4 is distributed on leukocytes and a variety of tissue cells (Baggiolini et al., 1997). It has been identified as a co-receptor, (with CD4), for the infection of cell lines or blood lymphocytes by lymphocyte-tropic, syncytium inducing, HIV-1 strains (Feng et al., 1996).
### Table 1. The Ligand selectivity of chemokine receptors (adapted from Baggiolini et al., 1997)

<table>
<thead>
<tr>
<th>Chemokin</th>
<th>Receptors</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC</td>
<td>CXCR1</td>
<td>IL-8</td>
</tr>
<tr>
<td></td>
<td>CXCR2</td>
<td>IL-8, GROα,β,γ, NAP-2, ENA78, GCP-2</td>
</tr>
<tr>
<td></td>
<td>CXCR3</td>
<td>IP10, Mig</td>
</tr>
<tr>
<td></td>
<td>CXCR4</td>
<td>SDF-1</td>
</tr>
<tr>
<td>CC</td>
<td>CCR1</td>
<td>RANTES, MIP-1α, MCP-2, MCP-3</td>
</tr>
<tr>
<td></td>
<td>CCR2α/β</td>
<td>MCP-1, MCP-2, MCP-3, MCP-4</td>
</tr>
<tr>
<td></td>
<td>CCR3</td>
<td>Eotaxin, RANTES, MCP-3, MCP-4</td>
</tr>
<tr>
<td></td>
<td>CCR4</td>
<td>RANTES, MIP-1α, MCP-1</td>
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<tr>
<td></td>
<td>CCR5</td>
<td>RANTERS, MIP-1α, MIP-1β</td>
</tr>
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</table>
Two receptors for CC chemokines, CCR1 and CCR2 (originally designated MIP-1α/RANTES and MCP-1 receptor) are expressed on monocytes, their genes being located on chromosome 3p21. They recognise the chemokines MCP-2, MCP-3 and MCP-4 (Baggiolini et al., 1997). CCR3 (or eotaxin receptor) recruits cells in allergy and is mostly expressed on eosinophils (Daugherty et al., 1996). The CCR4 and CCR5 chemokine receptors bind RANTES and MIP-1α. CCR4 is expressed on basophils, T and B-lymphocytes, and monocytes (Power et al., 1995). The gene for CCR5 is located on chromosome 5 and has been shown to be a co-receptor for cellular entry of monocyte/macrophage-tropic HIV-1 strains (Baggiolini et al., 1997).

1.4.1.6. Surface antigen expression

Surface antigen expression on monocytes may change during maturation to macrophages and thus may be used as an indicator of cellular activation and differentiation (Wright et al., 1990). Blood monocytes express CD4, CD54 (ICAM-1) CD64 (FcyRI) but not CD16 (FcyRIII) which is found on monocyte-derived macrophages (Ravetch and Kinet, 1991).

CD14

Monocytes characteristically express CD14, a receptor that binds complexes of lipopolysaccharide (LPS) and LPS-binding protein (Wright et al., 1990). It is a phospholipid-linked protein whose expression is modulated by bacterial LPS, IFN-γ, and phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187 and anti-CD14 monoclonal antibody. Monocytes in vitro shed soluble CD14 that may play a role in regulating CD14 surface expression (Bazil and Strominger, 1991). Human monocytes cultured in-vitro for 5 days with 10% human serum showed a decrease in CD14 expression (Ziegler-Heitbrock et al., 1993).

Adhesion molecules

Human monocytes express CD11a/11b/18 and CD32 (Valentin et al., 1991). The integrin molecule CD11c/CD18 (CR4, p150, 95) is found on activated myeloid cells and T cell subsets. Studies showed that incubating these cells with PMA increased the expression of CD11c. Human peripheral blood monocytes incubated for 15 days in a cytokine and
serum-free system showed reduced expression of CD11c compared to fresh monocytes (Bennett et al., 1992).

**Major histocompatibility complex class II (MHC class II)**

Monocytes and macrophages have been shown to express products of the Major histocompatibility complex class II genes (MHC Class II) that are important in antigen presentation to T cells (Andreesen et al., 1990). Human monocytes cultured for 5 days express high levels of HLA-DR, one of the MHC class II gene products (Tokuda and Levy, 1996). IL-4 induces HLA-DR expression on human monocytes via a G protein-dependent system (Vassiliadis and Papatheakis, 1992). Antigen presentation to T-lymphocytes requires the direct interaction of T cell CD4 with MHC Class II molecules on macrophages (Gay et al., 1987) and the interaction of CD28 and CD80, one of the dominant costimulatory pathways (Lenschow et al., 1996).

**CD80**

T-cell activation requires costimulatory signals from CD28 that are dependent upon the binding of CD80 (B7-1/B7, Matulonis et al., 1995). CD80 was first identified as a B cell activation molecule and the ligand for CD28 and later for CTLA-4 (Yokochi et al., 1982). Subsequently, this molecule has been detected on antigen presenting cells including dendritic cells, Langerhans cells, activated monocytes, activated T cells and a variety of tumour lines. The human and murine CD80 genes have been cloned and found to be members of the immunoglobulin supergene family (Tivol et al., 1997). CD80 expression on B cells can be increased by cytokines such as IL-2 and IL-4 whilst IFN-\(\gamma\) treatment increases its expression on monocytes (Hathcock and Hodes, 1995). A second B7 family member, CD86 (B7-2), was identified and found to be structurally similar to CD80. CD86 has an extracellular domain containing two Ig-like domains, a transmembrane domain, and a cytoplasmic tail that contains three sites for protein kinase C phosphorylation. Resting B cells express low levels of CD86 and no CD80 but both are upregulated following B cell activation with agents such as lipopolysaccharide (LPS), the mitogen Concanavalin A (Con A), or cyclic adenosine monophosphate (cAMP). The induction of CD86 on B cells with IL-4 occurs within 6hrs of stimulation, with maximal expression occurring between 18 and 24h. By contrast, CD80 expression on B-cells is only detected 24h post stimulation and reaches a maximum 48 to 72h later (Stack et al., 1994; Lenschow et al., 1996). Also, IFN-\(\gamma\)
increases the expression of CD86 on B cells, peritoneal macrophages and peripheral blood monocytes. By contrast, IL-10 blocks the upregulation of CD80 and CD86 on human peritoneal macrophages (Bulens et al., 1995, Willems et al., 1994).

The binding of its ligand by CD80 expressed on antigen-presenting cells is thought to promote the development of T helper 1 (Th1)- rather than a Th2- type responses. IL-4 and IL-10, which cause the development of Th2 immune responses, down regulate CD80 expression on resting CD14+ human monocytes. IFN-γ, responsible for the development of a Th1-type immune response, enhances the expression of CD80 and CD86 isoforms (Creery et al., 1996). Other cytokines tested including IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor (TGF)-α have not been shown to modulate CD80 expression on resting human monocytes or B cells. The expression of CD80 on resting B cells is higher (21.1± 2.5%) than that on resting human monocytes (4.8± 0.35%; Creery et al., 1996).

The affinity of the CD80/CD86 molecules for CD28 and CTLA-4 may influence which interaction will predominate during an immune response. If these molecules were expressed at low levels, the high-affinity, inhibitory receptor, CTLA-4 may be engaged. CTLA-4 expression is up regulated following T cell activation and the inhibitory nature of the CD80/CD86-CTLA-4 interaction will terminate T cell activation (Tivol et al., 1997).

**CD23**

CD23, a low affinity receptor for IgE (FcεRII) is found on a variety of hematopoietic cell types including the human monocytic cell line U937 (after stimulation with IL-4), human monocytes and Langerhans cells (Conrad, 1990, Alderson et al., 1992). The FcεRII is involved in IgE-dependent cytotoxicity and in promoting phagocytosis of IgE-coated particles by macrophages and eosinophils (Conrad, 1990). IL-3 and GM-CSF have been shown to enhance the expression of CD23 on monocytes and to increase the level of soluble CD23 (sCD23) in monocyte-culture supernatants. GM-CSF and IFN-γ enhance surface and soluble CD23 production by the monocytic cell line U937 (Alderson et al., 1992). Treatment of the human monocytic cell line Mono Mac 6 for up to 3 days with prostaglandin E2 enhanced the expression of CD23 whilst lipopolysaccharid (LPS) and tetradecanoylphorbol-13-acetate (TPA) treatment did not (Ziegler-Heitbrock et al., 1994).
1.4.1.7. CD16, CD32, and CD64

There are three major classes of receptor for immunoglobulin G which have been identified as CD16, CD32, and CD64. Most human and murine FcR are members of the immunoglobulin superfamily (IgSF). The biological properties of membrane FcR are dependent upon the presence or absence of one or several intracytoplasmic activation motifs similar to the BCR and TCR signal transduction subunits (Daeron, 1997; Reth, 1989). The presence of these motifs mean that FcRs are able to trigger cell activation (Reth, 1989). There are three classes of FcγR - FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64) all of, which mediate phagocytosis of IgG-opsonized materials (Jones et al., 1996).

CD16

FcγRIII (CD 16) is a low affinity receptor with a Mr between 50-80 KDa. It exists in two forms - FcγRIIIA and FcγRIIIB. The latter is encoded by a single gene with two alleles located on chromosome 1 (1q23-24, Qiu et al., 1990). The receptor FcγRIIIA is expressed on natural killer (NK) cells (where it regulates their cytotoxic and non-cytotoxic functions), monocytes cultured in vitro, and tissue macrophages. The FcγRIIIB is expressed in neutrophils (Masuda and Roos, 1993). FcγRIIIA activates the src and syk tyrosine kinases (Daeron, 1997).

CD32

FcγRII (CD 32) is a 40-KDa glycoprotein with low affinity for ligands and interacts with complexed IgG. In humans, CD32 is encoded by a minimum of three genes FcγRIIA, B, and C. The FcγRIIA, and FcγRIIC mRNA is expressed in monocytes, macrophages and neutrophils. FcγRIIB mRNA is expressed in monocytes, macrophages, and B-lymphocytes (Masuda and Roos, 1993). Cell activation resulting from FcγRIIA engagement is mediated by ITAMs (immunoreceptor tyrosine-base activation motifs) located in the cytoplasmic region which is capable of interacting with the FcR γ-chain resulting in a change in its signalling behaviour (Masuda and Roos, 1993; van den Herik-Oudijk et al., 1995).

In monocytic cell lines, ITAMs phosphorylation is induced by aggregation of FcγRIIA (Ghazizadeh et al., 1994). Engagement of CD32 also activates the protein tyrosine kinases belonging to the src and syk family of kinases. In infected cells, HIV-1 transcription may be induced via FcγRIIA engagement (Tsitsikov et al., 1995). FcγRIIB have no ITAMs and
belong to a family of single-chain, low-affinity IgG receptors encoded by a single gene located on chromosome one (1q23-24; Hogarth et al., 1991). Alternative splicing generates several isoforms including FcγRIIB1 and FcγRIIB2. Cells of hematopoietic origin express FcγRIIB with FcγRIIB1 on lymphoid and FcγRIIB2 on myeloid cells. FcγRIIB do not activate cells but, when aggregated by multivalent ligands, are involved in capping, endocytosis and phagocytosis (Daeron, 1997).

**CD64**

The high affinity FcγRI (CD64) is a 72-KDa glycoprotein receptor which is expressed on neutrophils, monocytes and macrophages and which binds to monomeric IgG. Interferon-gamma (IFN-γ) increases the expression of CD64 on human neutrophils, monocytes and some monocytic cell lines by up to 20-fold (Perussi et al., 1983, Looney et al., 1986). It has been shown using the myeloid cell line U937, that the receptor is associated with actin (Masuda and Roos, 1993).

In humans, the three genes that encode the α subunits of FcγRi are clustered, on the long arm of chromosome 1 (1q21.1; Daeron, 1997; Morton et al., 1995). In order to bind IgG with high affinity, FcγRI must associate with the γ chain (Miller et al., 1996). It has been shown (using macrophage cell lines) that the aggregation of CD64 occurs after the ITAMs of the receptor molecules are phosphorylated (Duchemin et al., 1994). This phosphorylation coincides with the activation of several sets of cytoplasmic protein tyrosine kinases that belong to the src and slyk family of kinases. To prevent FcγR from activating human monocytes and human neutrophils, CD45 co-aggregates with CD64 (Daeron, 1997).

Macrophages and monocytes mediate ADCC via CD64 which triggers superoxide production and the secretion of inflammatory cytokines (Daeron, 1997). In human monocytes, NF-κB is induced by FcγRI aggregation which, in HIV infection, activates transcription of the virus (Tsitsikov et al., 1995).

**1.4.1.8. FcγR-mediated signalling**

The Fc domain of IgG simulates phosphorylation of tyrosine residues within ITAMs located in the cytoplasmic domain of FcγRII or in the γ-chains associated with FcγRI or FcγRIIIA. This induces receptor clustering causing FcγR activation. The src- and sylk-family kinases cause phosphorylation of conserved tyrosine residues.
The activation of FcγRs involves other phosphotyrosine containing proteins such as paxillin which co-localises with F-actin beneath nascent phagosomes and is phosphorylated by syk (a PI 3-kinase-associated protein that links the FcγR pathway to PI 3-kinase; Greenberg et al., 1994; Nada et al., 1994; Ninomiya et al., 1994). Other tyrosine phosphorylated proteins, phosphatidylinositol specific phospholipase Cγ1 and PI-PLCγ2, upon FcγR activation, caused phosphatidylinositol-4, 5-bisphosphate hydrolysis and, intracellular Ca^{2+} mobilisation (Kiener et al., 1993, Liao et al., 1992).

1.5. Phagocytosis

The term “professional phagocyte” refers to polymorphonuclear neutrophils (PMNs), monocytes and macrophages (Rabinovitch, 1995). These cells ingest and clear large particles (>0.5 μm) including infectious agents, senescent cells and cellular debris by phagocytosis (Allen and Aderem, 1996). Two models of this process have been proposed. These are the zipper and trigger models (Figure 1.6). The zipper model originated from studies involving the phagocytosis of erythrocytes and bacteria mediated by macrophage Fc and complement receptors. For phagocytosis to occur a particle must initiate a response that involves the Fc-dependent cross-linking of FcRs (Griffin and Silverstein, 1974). Studies on phagocytosis have indicated that the process requires receptors other than those initially involved in particle binding. It was shown that macrophages could phagocytose opsonised erythrocytes but the removal of the opsonin or the occupied receptor adjacent to the initial binding site prevented particle ingestion (Griffin et al., 1975). Further studies showed that lymphocytes uniformly coated with IgG were completely ingested by macrophages whilst those opsonized over only one hemisphere of their surface were incompletely ingested as the macrophage pseudopodia extended no further than the IgG cap on the lymphocytes. These studies indicate that the zipper model is under local control due to the close contact between the phagocyte and particle. This encourages pseudopodia to form tight-fitting phagosomes through the continuous ligation of new receptors (Swanson and Baer, 1995).
Figure 1.6. The zippering and triggering models

Initial binding is enough for complete ingestion of the particle by the trigger model while the zipper model, requires additional interactions between opsonins and receptors. (a) Particles attached to the cell surface; (b) Ligands outside the attachment zone removed experimentally; (c) 'Triggering' mechanism; (d) 'Zippering' mechanism. Diagram adapted from Swanson and Baer, 1995.
In the trigger model, bacteria indiscriminately enter epithelial cells and macrophages via macropinosomes. The release of growth factors such as monocyte-macrophage colony-stimulating factor increases surface ruffling and the formation of macropinosomes in these cells (Racoosin and Swanson, 1989). After trapping bacteria, ruffles form with the aid of actin microfilaments and fold back against the cell surface thus forming macropinosomes that are large endocytic vesicles that trap extracellular fluid. Ruffles are unguided pseudopodia and are larger than the bacteria they trap. Thus, the response is disproportionate to the size of the particle. A study showed that the attachment of S. typhimurium to epithelial cells and macrophages stimulated ruffling and macropincytosis thus aiding invasion by S. typhimurium (Swanson and Baer, 1995). The surfaces of macrophages exhibit ruffling in many places. Since it is not restricted to the site of binding, phagosomes are formed that are not closely adjacent to the bacteria. The latter enter by random capture (Swanson and Baer, 1995).

1.5.1.1. The role of the complement receptor 3 (CR3)

Adhesion and phagocytosis of particles opsonized with complement is mediated by CRs on leukocytes. CR1 mediates adhesion to C3b on complement-opsonized particles causing Factor-I to initiate CR1 conversion of C3b to iC3b. This process reduces the affinity of the opsonised particles for CR1 and increases their affinity for CR3. The CR3 is a heterodimer whose engagement initiates a transient increase in cytosolic Ca\(^2+\) (Schmitz et al., 1997; Janconi et al., 1991).

Monocytes, macrophages and polymorphonuclear cells express CR1 (CD35) and CR3 (\(\alpha_\text{IIb}/\beta_3\) integrin, CD11b/18, MAC-1) (Schmitz et al., 1997). Differences exist between complement- and FcR-mediated phagocytosis. CR3-mediated phagocytosis happens in cells activated by inflammatory mediators or extracellular matrix proteins while FcyR-mediated phagocytosis occurs constitutively (Greenberg, 1995).

1.5.1.2. The role of phagocytosis in antigen processing and presentation

Major histocompatibility complex (MHC) molecules associate with small peptides to produce peptide-MHC complexes that are presented to T cells. The recognition of peptide-MHC complexes stimulates T cells to mediate cellular immune reactions and/or to regulate
B cell production of antibody (Harding, 1995). Two distinct classes of molecules present antigens, MHC class I (MHC-I) and MHC class II (MHC-II; Germain, 1994). MHC-II is associated with an invariant chain that blocks the binding of peptides in the endoplasmic reticulum (ER). The cytoplasmic tail of the invariant chain directs the MHC-II-invariant complex from the Trans Golgi network to the endocytic compartments. The invariant chain is proteolytically cleaved as it reaches the endosome and MHC-II molecules bind antigenic peptides (Germain, 1994). It has been shown that a minimum of 20-30 min is required for phagosome-lysosome fusion and phagolysosomal degradation of bacteria and presentation of peptide-MHC-II complexes at the cell surface (Pfeifer et al., 1992).

MHC-I molecules are free to bind peptides in the ER and are not associated with an invariant chain. Also they do not move to the endocytic compartment. Peptides which have been cleaved from antigens in the cytosol by proteasomes are transported into the lumen of the ER where they bind to the MHC-I molecules (Germain, 1994). Some pathogens not detected by the vacuolar system reside in the cytosol where they escape by lysing vacuolar membranes (Pamer, 1993, Brunt et al., 1990). Endogenous antigens or antigens that escape into the cytosol are primarily presented by MHC-I molecules (Collins et al., 1992, Zhou et al., 1992; Harding, 1995).

An alternative to the conventional pathway for MHC-I associated antigen processing and presentation is thought to exist primarily in macrophages (Collins et al., 1992, Zhou et al., 1992). It was shown that in the alternative MHC-I pathway, Brefeldin A, which inhibits transport through the Golgi complex to the cell surface in the conventional pathway, did not inhibit processing of antigen. This indicates that the alternative pathway does not involve a transporter for loading of peptides onto MHC-I molecules in the ER (Harding and Song, 1994). This means that peptides released from phagocytic compartments in the cytoplasm may associate with MHC-I on the cell surface (Pfeifer et al., 1993; Harding and Song, 1994). Alternatively, peptides may bind to MHC-I molecules in post-Golgi compartments or MHC-I molecules may be internalised in phagosomes from the plasma membrane where they may bind peptides produced in the phagolysosomes.

1.5.1.3. Initiation of an antigen-specific response

The recognition of antigen associated with MHC by the TCR along with the co-stimulatory signal derived from the interaction between CD80/86 and their ligands CD28/CTLA4 is vital for T cell activation. An initial signal is received from the T cell receptor...
(TCR) complex and its co-receptors CD4 or CD8 interacting with antigen presented by MHC-class II or I antigens respectively. A second signal is derived from the interaction of CD28 with CD80/86 and is essential for initiating antigen-specific T cell responses, upregulating cytokine expression and promoting T cell proliferation and differentiation (Lenschow et al., 1996).

1.5.1.3.1. CD28/CTLA4

CD28, a glycoprotein, is expressed on the surface of 80% of human T cells. It is present at high levels on developing thymocytes and its expression increases following T cell activation (Lenschow et al., 1996). CD28 is a disulphide-linked homodimeric glycoprotein that can exist as a monomeric protein (Walunas et al., 1994). As a result of ligation, the production of various cytokines is induced including IL-1, IL-2, IL-4, IL-5, TNF, and IFN-γ. CD28 plays an early role in the development and differentiation of Th1 and Th2 subsets and the absence of this signal causes naive T cells to differentiate toward the Th1 phenotype (Lenschow et al., 1996). By contrast, CTLA-4 is not constitutively expressed on T cells, its expression only occurs following T cell activation, peaking 48 h after activation and returning to background levels by 96 h. Expression of CTLA-4 is maximal when CD28 expression is down regulated or impaired (Lenschow et al., 1996). Regulation of expression is a complex process as evidenced by the fact that anti-TCR antibody stimulation of human T cells did not upregulate CTLA-4 but soluble ICAM-1 (a ligand for LFA-1) did so to a slight extent (Linsley et al., 1992; Damle et al., 1994). It has been suggested that CTLA-4 expression is post-transcriptionally regulated or CTLA-4-associated proteins are required for transport and cell surface expression (Lenschow et al., 1996).

CTLA-4 is a disulphide-linked homodimeric glycoprotein that can exist as a monomeric protein (Walunas et al., 1994). Antibodies to human CTLA-4 block the interaction of CD28/CD80 and prevent the production of IL-4 (Seder et al., 1994).

1.6. Human monocytic cell lines

Monocytic cell lines provide a relatively homogeneous group of cells to enable examination of the effect of external agents on monocytes/macrophage characteristics.
One of the problems with these cell lines is that they are relatively immature and may require treatment with various agents to promote maturation.

1.6.1. Mono Mac 6 cell line

The Mono Mac 6 (MM6) cell line was first established by culturing cells isolated from the blood of a patient with monoblastic leukaemia (Ziegler-Heitbrock et al., 1988). MM6 was isolated and assigned to the monocyte lineage according to morphology, cytochemical and immunological criteria. The cells express NaF-sensitive non-specific-esterase, produce reactive oxygen intermediates, express CD14 and are capable of phagocytosing antibody-coated erythrocytes. The expression of certain surface antigens has been enhanced by treatment of MM 6 with IFN-γ. (Ziegler-Heitbrock et al., 1988).

As mentioned above, the MM6 cell line is CD14*, that a marker characteristic of mature peripheral blood monocytes and tissue macrophages (Ziegler-Heitbrock et al., 1994). The cells also produce cytokines such as IL-1α, IL-1β, TNF and IL-6 after stimulation with lipopolysaccharide (Ziegler-Heitbrock et al., 1994, Quentmeier et al., 1995).

Prostaglandin E\(_2\) (PG E\(_2\)), lipopolysaccharide (LPS) and tetradecanoylphorbol-13-acetate (TPA) cause MM6 cells to differentiate as evidenced by reduced cellular proliferation and expression of CD33 and increased phagocytosis of Staphylococcus sp. and M-CSF mRNA expression. MM6 cells constitutively express CD23 but it can be up regulated by PG E\(_2\), rIL-4 and rIL-6 (Ziegler-Heitbrock et al., 1994).

The human Mono Mac 6-cell line has many of the characteristics of mature blood monocytes and expresses the CD14 molecule. Treating Mono Mac 6 cells with LPS and PGE2 increased surface CD14 expression twofold and was accompanied by a rise in soluble CD14 and enhancement of CD14 mRNA. In contrast, Tetradecanoylphorbol-13-acetate (TPA) decreased surface CD14 expression twofold and CD14 mRNA while sCD14 remained the same (Ziegler-Heitbrock et al., 1994). Studies involving the blockade of CD14 or inhibition of protein kinase C indicate that both are involved in signal transduction pathways which results in TNF-α secretion from Mono Mac 6 cells (Steube and Drexler, 1995). MM6 cells have been shown to adhere to human umbilical vein endothelial cells (HUVEC) which are unstimulated or stimulated with TNF-α (Erl et al., 1995). A maximum of 6.2 MM6 cells bind per HUVEC, a similar level to freshly isolated human blood monocytes.
but 34% less than the level observed with U937 cells. To investigate this interaction further, monoclonal antibodies (mAb) directed against E-selectin, VCAM-1 and ICAM-1 on HUVEC and CD11b or CD14 on MM6 cells were used. The decrease in cellular adhesion after 24h correlated with a reduction in E-selectin expression on HUVEC (Erl et al., 1995).

Differentiation of MM6 cells induced can be by the active metabolite of vitamin D3 (1,25 (OH) vitamin D3) as evidenced by the enhanced the activity of phospholipase A2 (PLA2), which is important in the differentiation and growth of monocytic cells (Aepfelbacher et al., 1995).

1.6.1.1. THP-1 cell line

The THP-1 cell line was derived from cells isolated from the blood of a 1-year-old boy suffering from acute monocytic leukaemia (Tsuchiya et al., 1980). The cells retain the appearance of leukaemic cells and grow in suspension as loose clumps. They show lysozyme and NaF-inhabitable α-naphthyl butyrate esterase activity. THP-1 express CD64, CD32 and C3b receptors also MHC antigens HLA-A2, -A9, -B5, -DR1, -DR2. They do not express surface or cytoplasmic immunoglobulins. THP-1 cells have a diploid (46 + X, Y) number of chromosomes (Tsuchiya et al., 1980).

Phorbol esters induce maturation in THP-1 cells resulting in the expression of phenotypic and functional characteristics associated with macrophages (Tsuchiya et al., 1982). PMA (Phorbol 12-myristate 13-acetate) causes cells to adhere, stop proliferating and show increased phagocytosis (Auxerx et al., 1992). These differentiated cells revert to a de-differentiated phenotype after three to four weeks of treatment (Hass et al., 1993). PMA decreases the expression of \(c\)-myc and \(c\)-myb integrin oncogenes whilst retinoic acid treatment decreases \(c\)-myc expression alone. Retinoic acid also induces differentiation in THP-1 cells, causing the acquisition of macrophage-like characteristics after treatment. The expression of the \(\beta2\)-integrin genes, CD11a and CD11b, was found to be increased after treating THP-1 with both reagents (Matikainen and Hurme, 1994). PMA treatment decreases both gene and membrane expression of CD64 (FcyRI) in THP1 cells, but only reduces membrane expression of CD32 (FcyRII). This indicates different regulatory mechanisms at work (Auxwerx et al., 1992).

Cytokines are produced by THP-1 cell lines during differentiation, maturation and the response to physiological concentrations of chemicals. THP-1 cells do not produce
detectable levels of TNF-α or IL-1β or of the mRNA coding for them (Molina et al., 1989). However, stimulation of THP-1 cells with LPS (incubated with or without IFN-γ or GM-CSF) resulted in the secretion of both TNF-α and IL-1β (Chantry et al., 1990).

Phagocytosis of latex beads or organisms such as Toxoplasma gondii has been shown to induce the release of TNF, IL-6 and IL-8 by THP-1 cells (Friedland et al., 1993). The surface expression of HLA-DR and CD54 and the production of TNF-α, IL-1β and IL-6 are induced by IFN-γ treatment. However, 1,25(OH)2D3 fails to stimulate cytokine production from THP-1 cells but does induce surface expression of CD11b and CD14 (Lozanski et al., 1992).

1.7. Immunology of HIV infection

HIV disease is divided into three phases, firstly primary infection in which 50-70% infected individuals exhibit a mononucleosis-like clinical syndrome. Secondly, an asymptomatic phase indicating a period of clinical latency and thirdly a phase during which the acquisition of opportunistic infections leads to AIDS.

The earliest response to HIV infection is antibodies against a variety of viral proteins and the production of HIV-specific cytotoxic T cells (Pantaleo et al., 1993). During the period of clinical latency and in the early stage of disease, HIV-infected individuals exhibit CD4+ T cell counts between 200 and 500 per μl and the lymphoid tissue is progressively replaced by fibrotic tissue. In the later stages of disease, the CD4+ T cell count drops below 200 per μl and the lymphoid tissue undergoes extensive fibrosis and fatty infiltration (Pantaleo et al., 1993). Thus, the patients’ immune mechanisms become impaired, since infected cells are no longer competent to respond to other pathogens (Pantaleo et al., 1994). A percentage of HIV-infected individuals designated as long-term non-progressors (LTNP) do not experience progression of HIV disease. Their CD4+ T cell count remains in the normal range (greater than 600 cells per μl) and the CD8+ T lymphocyte count is increased (between 500 and 2500 cells per μl, Salk and Salk, 1993). The continued detection of HIV-specific cytotoxicity in LTNP suggests that HIV disease is the result of virus-specific, T cell-mediated immunopathology (reviewed by Pantaleo and Fauci, 1995).

Studies using the simian immunodeficiency virus (SIV) experimental animal model showed that HIV is carried to lymphoid organs where it is processed to generate a specific immune response (Parrott and Wilkinson, 1981). SIV was detected in lymph nodes as early
as day 7 after infection, indicating that lymphoid tissues are a site for the establishment of infection and are ideal for virus replication and spreading (depending upon the state of activation of the target cells).

Antigen-specific immune responses require the aid of activated effector cells (T cells, B cells, and macrophages) which may support virus replication and release cytokines such as TNF-α, IL-6 and IL-10 that can induce virus expression. Also these responses may modulate certain components of the HIV-specific immune response which paradoxically may favour the initial establishment of HIV infection (reviewed in Pantaleo and Fauci, 1995).

Dissemination of HIV throughout the lymphoid tissue occurs once infection is established. This process precedes the development of a fully competent HIV-specific immune response (Pantaleo et al., 1994). The process of dissemination is associated with high levels of plasma viremia, p24 antigenemia, and circulating mononuclear cells that contain HIV proviral DNA (reviewed by Pantaleo and Fauci, 1995). Virus replication reaches a peak and is suddenly down-regulated. Safrit et al., (1994) have suggested that this may be caused by HIV-specific cytotoxic T lymphocytes (CTL) prior to the appearance of neutralising antibodies. This is supported by Reimann et al., (1994) whose studies using the SIV model suggested that the down-regulation of viremia indicate that HIV-specific CTL play a major role in killing virus-expressing cells thus controlling virus replication and spread. Virus-expressing cells complexed with immunoglobulin (Ig) and complement are trapped by follicular dendritic cell (FDC) in the germinal centre of lymph nodes. Koup et al., (1994) suggested that this indicated that an HIV-specific humoral immune response contributes to the downregulation of viremia through complement activation.

The lack of complete elimination of HIV by the primary immune (Fauci et al., 1991) response has led to the suggestion that important components of the immune response are not present during this critical stage of infection. However the presence of virus-specific CTL and the dramatic decrease of virus-expressing cells in lymph nodes contradicts the suggestion that the cell-mediated immune response is inadequate (Reimann et al., 1994; Safrit et al., 1994). Another possibility is that the virus is able to evade the primary immune response. Indeed, neutralising antibodies are not detected during the primary immune response to HIV that may allow the virus to exist in a latent from in a large proportion of infected cells. Despite the down-regulation of replication, some individuals experience a different clinical outcome, progressing to AIDS within 12 months of sero-conversion with
very low viremia after primary infection, and strong HIV-specific cytotoxic activity (Schnittman et al., 1989, Embretson et al., 1993).

This diversity of the clinical outcomes of infection prompted studies of the cell-mediated immune response. Particular interest was shown in changes in the T cell receptor (TCR) repertoire during primary HIV infection (Pantaleo et al., 1994). Oligonucleotides that encompass all known V\(\beta\) sequences were used to examine the TCR repertoire on peripheral blood mononuclear cells obtained at different stages of infection. Using a combination of PCR and cytofluorometry the results indicated that during the first 8 weeks following primary infection, cells expressed a restricted number of V\(\beta\) domains (Pantaleo et al., 1994).

Studies have shown that the HIV envelope and MHC class II molecules share homologous sequences. Anti-Class II antibodies had an inhibitory effect on CD4\(^+\) T lymphocyte activation which contributes to the progression of HIV disease (Golding et al., 1988, Golding et al., 1989; Pantaleo and Fauci, 1995). Another two mechanisms associated with disease progression have been suggested which involve apoptosis or the abnormal activation of programmed cell death, and a switch from a Th1 to a Th2 pattern of cytokine production. Additionally, it has been proposed that HIV may cause immunosuppression and disease by inducing anergy in CD4\(^+\) T cells following abnormal antigen presentation by other CD4\(^+\) T cells and costimulatory molecule CD80 on tissue macrophages (reviewed in Pantaleo and Fauci, 1995). A significant depletion of CD4\(^+\) T cells from HIV-infected patients caused an increase in delayed type hypersensitivity (DTH) reactions this leads to allergies (Emery and Lane, 1997).

1.8. General Biology of Immune complexes

In 1911, Von Pirquet suggested that the toxic effects of antigen-antibody interactions caused serum sickness. This was confirmed in the 1950s and 1960s with animal models of acute and chronic serum sickness. Antibodies react with antigen that is fixed in the tissue or free in the circulation. Once in the circulation, the immune complexes may fix complement and be eliminated from the circulation by the mononuclear phagocytic system. Persistence as soluble complexes in the circulation allows the immune complexes to escape mononuclear phagocyte clearance and to deposit in endothelial or vascular
structures causing an inflammatory response which could lead to immune complex disease (Spiegel, 1985).

The classical complement pathway is activated by immune complexes and helps to protect the body against the development of immune complex disease. Complement reacts with immune complexes and binds to erythrocyte CR1 thus inhibiting immune precipitation and dissolving immune aggregates; these reactions prevent the accumulation of soluble complexes in local tissues. CR1 acts as a cofactor to Factor I, which catabolises the conversion of C3b to iC3b and subsequently to C3dg (Davies et al., 1994).

The precipitation of antigen-antibody at equivalence or in antibody excess requires intact IgG molecules for Fc-Fc interactions that promote precipitation (Davies et al., 1994). Gavin et al., (1995) demonstrated in vitro that recombinant soluble FcγRII (rsFcγRII) is effective in modifying immune complex formation and delays immune precipitation in a dose-dependent manner. This effect can be inhibited by anti-FcγRII MoAb Fab' fragments. This process may inhibit immune complex-induced inflammation in the Arthus reaction in vivo (Fries et al., 1984).

The solubilisation of immune precipitates is caused by covalently binding C3b to immune complexes thus reducing the various forces that hold the aggregate together. This process is inefficient, as large amounts of complement are required (Fries et al., 1984). The transfer of immune complexes from red cells to human monocytes is CR1-dependent, as monocytes possess more binding sites. This facilitates transferring of complexes to fixed macrophages from red cells in the sinusoidal circulation of the liver and spleen (Emlen et al., 1992).

Inflammation and tissue injury with the release of autoantigens may be due to immune complexes that escape the mononuclear phagocytic system and deposit in the tissues. These autoantigens cause further tissue injury as they stimulate an autoimmune response with the formation of more immune complexes and the consequent release of more autoantigens. Mononuclear phagocytes of the liver and spleen clear immune complexes as tissue macrophages bear both Fc and complement receptors (CR3 and CR4) which allow opsonised complexes to interact with both groups of receptors. A defect in mononuclear phagocytic function, defective immune complex delivery to the mononuclear phagocytic system or low levels of erythrocyte CR1 lead to the development of disease by impaired complex clearance. In early studies, the use of IgG - and IgM-coated cells showed
that IgG-complexes were cleared in the spleen while IgM-complexes showed transient retention in the liver (Davies et al., 1994).

Formed at equivalence, antigen-antibody complexes have been shown to 1) stimulate the release of prostaglandin E2 (PGE2), TNF-α, IL-1β and the IL-1 receptor antagonist, 2) to suppress IFN-γ-induced MHC class II expression and the expression of FcγRI. Berger et al., (1996b) showed in vitro that immune complexes induce human monocytes to secrete IL-6 and IL-10 in a dose-and antigen: antibody-ratio-dependent manner. Previous studies showed IL-10 secretion is augmented in a paracrine manner by PGE2 synthesised upon stimulation by immune complexes but immune complex induced IL-10 secretion is not dependent on PGE2. Immune complex induced IL-6 secretion is increased by PGE2 but the synthesis of both is decreased by IL-10. IL-6 is involved in the growth and differentiation of B cells and increases the production of antibodies. IL-6, IL-10 and PGE2 are closely associated with Th2-like immune responses. As a regulatory factor, PGE2 induces Th2 while it acts with IL-10 to suppress the production of the Th1-stimulatory cytokines. Thus, the presence of immune complexes may result in the secretion of IL-6 and IL-10 that interferes with the development of a Th1 response and therefore inhibits cell-mediated immunity to malignancies and intracellular pathogens.

1.8.1. Immune complexes and HIV-1

Feijoo et al., (1995), and Briant et al., (1996) have showed the interaction of gp120 anti-gp120 immune complexes with PBMCs latently infected with HIV-1 in the G0/G1-phase induced the cells into the S/G2/M-phases of the cell cycle and caused them to express CD25 and HLA-DR. This activation caused provirus integration and virus production. In the presence of soluble gp120 alone, only AP-1 was indicating that cross-linking of CD4 is required for NF-κB activation.

Circulating immune complexes (CIC/gp120-anti-gp120) which occur naturally in HIV infection has anti-CD4 activity and increase HIV-1 infection (Feijoo et al., 1995). Aceituno et al., (1997) demonstrated that naturally occurring circulating immune complexes from HIV+ patients induced apoptosis in normal human CD4 lymphocytes which was accompanied by increased expression of Fas (CD95; Oyaizu et al., 1993). Recent reports noted that the cross-linking of CD4 with gp120-anti-gp120 in vitro resulted in cellular activation and induced apoptosis (Siliciano, 1996). Other studies showed that stimulation of Fas antigen
expression induced apoptosis in HIV+ patients (Katsikis et al., 1997, Wang et al., 1994). Results from Aceituno et al., (1997) showed Fas antigen expression preceding the induction of apoptosis by CIC-HIV+ mainly in asymptomatic individuals and not in AIDS patients. Interestingly it has been shown that uninfected cells undergo spontaneous apoptosis rather than infected ones. This implies that there are two different processes in destroying CD4+ cells in HIV+ patients one spontaneous and the other mediated by virus containing immune complexes.

1.9. Cytokines and HIV

1.9.1. Cytokine-mediated regulation of HIV

In vitro, infected macrophages treated with IL-4 (dose-dependent), IL-10, IL-12, IL-13 and interferon's showed a decline in HIV infection (Kornbluth et al., 1990). Interferon-gamma (IFN-γ) was most effective when added in the early stages of infection. When macrophages were treated for 5 days before infection with IL-4, they became completely resistant to HIV (Schuitemaker et al., 1992).

IL-6 and TNF-α are believed to enhance the expression of HIV in macrophages. Also IL-1, IL-12, and TGF- up regulate viral expression. These cytokines stimulate macrophage permissiveness to lymphocytotropic strains of HIV (Kinter et al., 1995, Lazdins et al., 1991).

1.9.1.1. Effect of cytokines on the growth cycle of HIV-1 in macrophages

TNF-α, IL-1β, and IFN-γ down regulate surface expression of CD4 on human macrophages. These cytokines act at the level of transcription. Acting on primary macrophages, TNF-α, and IFN-γ inhibit HIV-1 infection. TNF-α inhibits that part of the viral cycle concerned with fusion or uncoating. GM-CSF increases CD4 surface expression in primary macrophages but may not be involved in HIV-1 replication. (Herbein, 1997).

IL-2 and phytohemagglutinin A (PHA) activate T-lymphocytes allowing complete reverse transcription and production of proviral DNA, integration and active HIV replication (Zack et al., 1990). In contrast to this, although the IL-2R is present on mononuclear phagocytes, IL-2 does not cause modulation of HIV-1 replication in primary macrophages if
they are treated after exposure to the virus (Allen et al., 1990, Montaner et al., 1995). On the other hand TGF-β inhibits HIV-1 replication in primary macrophages (Poli et al., 1991) stimulates its growth if the cells are pre-treated (Lazdins et al., 1991). Also, the inhibition of initial reverse transcription by IL-10 prevents HIV-1 replication in primary macrophages which may help the virus in monocytes/macrophages to remain in a latent stage (Montagnier et al., 1994, Saville et al., 1994).

The cellular transcription factor NF-κB is bound to the inhibitory protein I-κB in the cytoplasm and is activated by TNFα which stimulates infected macrophages to produce infectious virus. A combination of phosphorylation and proteolytic degradation causes the dissociation of a p65/p50 heterodimer from I-κB followed by a translocation of NF-κB to the cell nucleus and recognition of target DNA sequences present in both cellular and viral genes. It binds to the long terminal repeat (LTR) sequence of the HIV promoter resulting in increased transcription and expression (Butera et al., 1993). TNFα stimulates HIV-1 replication by binding to the TNF-R1 but not the TNF-R2 (Butera et al., 1993). Similarly IL-1β binding to the IL-1R1 rather than the IL-1R2 stimulates HIV-1 transcription through activation of NF-κB (Osborn et al., 1989, Poli et al., 1994). IL-13 inhibits HIV-1 replication during and after viral transcription in primary human macrophages but not in peripheral blood lymphocytes (Montagnier et al., 1993). IFN-α, β, γ were found to reduce HIV-1 replication and to inhibit viral assembly and budding (Biswas et al., 1992).

1.10. AIMS OF PROJECT

The initial aims of the project were to develop a specific T cell line to examine CD80 modulation on human monocytes and the effect of HIV infection. The lack of success in establishing a specific T cell line caused a change direction.

The new aim of this project was to examine the effects of immune complexes on mononuclear phagocytes (MPs) and the influence of HIV and HIV-derived proteins on these effects. To meet these aims, various types of immune complex were used at different concentrations including complexes comprising HIV-derived antigens and specific antibodies. The effect of their presence on MPs surface antigen expression and cytokine production were examined in HIV-infected and non-infected monocytic cell lines and peripheral blood monocytes.
2.0. MATERIALS and METHODS

2.1. MATERIALS

2.1.1. Chemicals and reagents

Chemicals, reagents and their suppliers used in this study are listed in Appendix B (page169).

2.2. METHODS

2.2.1. General Tissue Culture Methods

2.2.1.1. Heat inactivated foetal calf serum

All foetal calf serum (TCS Biological Ltd, Claydon, Buckingham, UK) and human serum from pooled, male, AB plasma (Sigma Chemical Co. Ltd, Poole, UK) were heat inactivated in a water bath (56°C, 30 minutes) and aliquoted in 50ml centrifuge tubes. The sera were stored at -20°C until use.

2.2.1.2. Maintenance of cell lines

The B95-8 cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, Wiltshire, UK. These cells release high titres of transforming Epstein-Barr virus (EBV) which was used to establish continuous lymphocyte lines from human donors. The human monocytic cell line THP-1 (Tsuchiya et al., 1980) was obtained from the same place. H9 cells, Human T-lymphocytic H9 cells (Popovic et al., 1984), originally derived from the HUT78 cell line, were obtained from the MRC AIDS Directed Programme. The Mono Mac 6-cell line established from the peripheral blood of a 64-year-old male with acute monocytic leukaemia was obtained from the German cell culture collection, Munich, Germany, (Ziegler-Heitbrock et al., 1988). The cells were maintained in exponential growth phase by frequent sub-culturing into Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture medium (Sigma Chemical Co. Ltd, Poole, Dorest, UK) containing 10% foetal calf serum (TCS Biological Ltd, Claydon, Buckingham, UK) or 10% human serum from male AB
plasma (complete RPMI-10AB; Sigma Chemical Co. Ltd, Poole, UK), 200mM L-glutamine (Sigma Chemical Co. Ltd, Poole, Dorset, UK) and 10mL penicillin-streptomycin solution (10,000 units penicillin and 10mg streptomycin per ml; Sigma Chemical Co. Ltd, Poole, Dorset, UK). In long-term cultures the medium was supplemented with 80μg/ml Kanamycin (Sigma Chemical Co. Ltd, Poole, Dorset, UK). All the cell lines used were grown in suspension and were sub-cultured at 3-4 day intervals by dilution of the cells to a concentration of 2 x 10^5 /ml.

Cell lines were frozen and stored in liquid nitrogen at a concentration in excess of 1 x 10^7 /ml, using 70% RPMI-1640 medium. 20% foetal calf serum and either 10% glycerol (for THP-1 cells; BHD Chemicals Ltd, Poole, Dorset, UK) or 10% DMSO (Sigma Chemical Co. Ltd, Dorset, UK) as a sterile filtered, cryopreservant. When the cells were required, they were quickly defrosted, carefully diluted using complete medium (at 37°C) and centrifuged (200g/3 mins). The cells were resuspended in fresh complete medium and transferred to a 37°C/ 5% CO₂ humidified incubator. This procedure was designed to minimise the exposure of the cells to the toxic cyropreservatives.

2.2.1.3. Isolation of peripheral blood mononuclear cells (PBMCs)

Human peripheral blood was collected into a tube containing sufficient heparin to give a final concentration of 10U/ml (Sigma Chemical Co. Ltd, Poole, Dorset, UK). The blood was diluted using an equal volume of RPMI-1640 medium. Twelve millilitre volumes of 'Lymphoprep' (Nycomed Ltd, Sheldon, Birmingham, UK) were pipetted into an appropriate number of 50-ml Nunc centrifuge tubes (Becton Dickinson UK Ltd, Coweley, Oxford, UK). Each tube was tilted to allow the diluted blood to be carefully layered onto the 'Lymphoprep' at a 3:1 ratio respectively. The tubes were centrifuged at 200g for 30 minutes. The majority of the medium above the 'interface layer' was removed and discarded. The 'interface layer' was collected (using a sterile plastic Pasteur pipette) and twice washed using RPMI-1640 medium (200g/ 5 mins).

2.2.1.4. Culturing human monocyte-derived macrophages

Human peripheral blood mononuclear cells were isolated as described in Section 2.2.1.3 and were pipetted at 1 x 10^6/ml in 24-well plates which were incubated for 2 hours at
37°C/ 5% CO₂. The plates were washed with RPMI-1640 medium using sterile Pasture pipettes and incubated with 2ml complete RPMI-10AB medium in each well.

2.2.1.5. Determination of viable cell concentration

The Trypan Blue dye exclusion test was used to estimate the viable cell count. Equal volumes of the cell suspension and 0.2% Trypan Blue solution (2% v/v in PBS; Sigma Chemical Co. Ltd, Poole, Dorest, UK) were mixed and viable counts performed using an Improved Neubauer haemocytometer. Adherent cells were obtained by “cold-shock” (exposure to -20°C for up to 3 minutes) prior to counting using the haemocytometer.

2.3. PRODUCTION OF Epstein-Barr VIRUS (EBV) AND GENERATION OF B-CELL LINES

2.3.1. Production of Epstein-Barr Virus (EBV)

B95-8 cells at a concentration of 1 x10⁶/ml were incubated for 3 days in a humidified incubator (37°C, 5% CO₂). The cells were over 90% viable as determined by trypan blue exclusion. The cells were centrifuged for 10 minutes (200g, 4°C) and the supernatant containing EBV was filtered using a 0.20-μm filter. Aliquots (2.5 ml) were stored at -130°C liquid nitrogen.

2.3.1.1. Labelling mononuclear cells using MACS

Mononuclear cells (obtained as described in Section 2.2.1.3) were washed using 5ml ice cold PBS containing 5mM EDTA plus 1% FCS. The cells were resuspended in 80μl of ice cold buffer and 20μl of the MACS anti-CD3 microbeads (Miltenyi Biotec. Inc, Sunnyvale, USA) per 10⁷ mononuclear cells was added. After gentle mixing, the cells were incubated at 6°C in the dark for exactly 15 minutes. The cells were centrifuged (200g) once and were resuspended in 1-ml cold buffer for immediate separation using the MACS.
2.3.1.2. Separating mononuclear cells stained with magnetic beads

An A2 column fitted with a 25G needle and side attached syringe was used for the separation. The column was washed with several volumes of ice cold buffer and filled with buffer for approximately half an hour before separation of the cells. The mononuclear cell suspension (Section 2.2.1.3) was loaded at the top of the column under sterile conditions immediately after magnetic staining. The effluent cells were collected as the "non-magnetic fraction" (B-lymphocytes and monocytes) after rinsing the column several time with ice-cold buffer. The 25G needle was changed to a 22G "washing needle" and the columns flushed with ice cold buffer (the "wash fraction"). The column was removed from the magnetic field and rinsed several times with ice cold buffer to collect the "magnetic fraction" (T lymphocytes). The three fractions were centrifuged (200g; 5min); the supernatant decanted and 2.5ml complete RPMI-1640 medium added to each fraction. A viability count was performed and the B cell fraction was transferred into a 50ml Nunc centrifuge tube and 2.5ml of culture supernatant from B95-8 cells (containing EBV) was added. After incubation for 2 hours in a 37°C water bath, a further 5 ml complete RPMI-1640 medium was added, 10 ml of the cell suspension was transferred to 25 cm² vented cell culture flask and incubated at 37°C, 5% CO₂. After 3-4 days, the suspension cells were diluted to a concentration of 5 x 10⁵/ml and split between two, 25 cm² vented cell culture flasks. The B lymphocyte cells transformed by EBV (B-transformed cells) were expanded into 75cm² flasks then cryopreserved at -130°C as described in Section 2.2.1.2.

2.3.1.3. METHODS FOR MEASURING CELLULAR PROLIFERATION

A solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co. Ltd, Poole, Dorset, UK) was prepared by dissolving sufficient compound in PBS to give a final concentration of 5mg/ml. The solution was warmed to 37°C to dissolve the MTT, and passed through a 0.2-μM filter. Shortly before use, pyruvic acid (Sigma Chemical Co. Ltd, Poole, Dorset, UK) was admixed to give a final concentration of 1mM. A 50-μl volume of this solution was added to each well on plates to be assayed for cellular proliferation and incubated for 3 hours (37°C/ 5% CO₂). Dark Formazan crystals could be observed in wells contains live cells. The plates were centrifuged at 400g for 5minutes and the supernatant removed.
Fifty microlitres of DMSO was added to every well, and the contents were mixed using a multichannel pipette, to dissolve any formazan crystals. The plates were agitated on a plate shaker (Model R100, Luckhams) for 5 minutes and the optical density at 540 nm was recorded for each well using a Shimadzu, UV-1201 spectrophotometer.

2.4. LONG TERM HUMAN CELL LINE PRODUCTION

Human peripheral blood mononuclear cells stimulated with adjuvant peptide (PPD) and Bacilli Calmette-Guerin (BCG) were used in an attempt to develop antigen-specific T cell lines.

2.4.1. Preparation of Live BCG Stocks

Previously cultured BCG (1 ml) was added to 17 ml sterile middlebrook 7H9 broth plus 2 ml middlebrook OADC enrichment broth (Difco Laboratories Ltd, West Molesey, Surrey, UK) in a 25 cm²-vented tissue culture flask. The BCG culture was grown at 37°C and passaged every 7 days. A stock culture was produced by growing the organism to density, which gave an absorbence of 0.1 at 280 nm using a Shimadzu, UV-1201 spectrophotometer.

2.4.1.1. Determination of the optimal concentrations of PPD and BCG

Human mononuclear cells at 4 x 10^6/mL obtained as described in Section 2.2.1.3 were incubated at an optimum concentration determined in preliminary experiments at 8 x 10^5 cells/well with PPD (0, 10, 20, 30, 40, and 50 µg/ml) or BCG (dilution of stock solution 0, 1:2, 1:4, 1:8, 1:16, and 1:32). The plates were incubated at 37°C, 5% CO2 for 7 days and cellular proliferation assayed as described in Section 2.3.1.3.

2.4.1.2. Production of antigen specific T-cells using PPD

PBMNC were isolated as described in Section 2.2.1.3 and re-suspended in complete RPMI-1640 medium with 80 µg/ml kanamycin solution, 25 mM Hepes buffer, 200 mM L-glutamine and 10% human serum from male AB plasma (complete RPMI-10AB). The
mononuclear cells were washed with RPMI-1640 medium (200g; 10mins) suspended in complete RPMI-10AB medium and a viable count was obtained (Section 2.2.1.5). Two millilitres of mononuclear cells (2 x 10^6 /ml) were placed in each well of a 24-well microtitre plate with enough PPD suspension (Sigma Chemical Co. Ltd, Poole, Dorest, UK) suspension to give a final concentration of 10μg/ml. The plates were incubated at 37°C/ 5% CO₂ for 7 days. On day 3, one millilitre of supernatant was removed from each well and replaced with one millilitre fresh complete RPMI-10AB medium and 30U/ml of recombinant interleukin 2 (rIL-2, R&D Systems Europe Ltd, Abingdon, UK). The latter was also added every following alternate week of culture. EBV-transformed B cells from the same donor were incubated for 1 hour in mytomycin C solution (25μg/ml; Sigma Chemical Co. Ltd, Poole, Dorest, UK), washed three times in complete RPMI-10AB medium (200g; 5mins) and re-suspended. The mononuclear cells incubated with PPD for 7 days were layered on Lymphoprep, centrifuged for 30 minutes (200g), washed twice with RPMI-10AB medium (200g; 5mins) and assayed for viability (Section 2.2.1.5). Mytomycin C-treated B cells (2 x 10^6 /ml) were mixed with 1 x 10^6 /ml autologous antigen specific T-cells and 200μl of the mixed cells were aliquoted in 96-well plates that were incubated at 37°C/ 5% CO₂. On day 3, 50μl of supernatant was removed and 50μl of complete RPMI-10AB medium was added to 96-well plates without rIL-2 (added only on alternate weeks).

A spectrophotometer (Shimadzu UV-1201) was used to determine the protein concentration of BCG solutions by measuring the optical density at 280nm each time BCG was added to the cultures. The BCG dilution used was the one with an absorbance of 0.1 as determined in Section 2.4.1.1.

2.5. THE EFFECT OF CYTOKINES AND ACTIVE METABOLITES

2.5.1. Effect of cytokines and other stimulators on MM6 cells

Mono Mac 6 cells (MM6 cells) at 2 x 10^5/ml were incubated (37°C/ 5% CO₂) in 96-well round-bottomed plates for 3, 5, and 7 days. Wells were supplemented with cytokines or other stimulators to give the following final concentrations:
<table>
<thead>
<tr>
<th>PMA (ng/ml)</th>
<th>VitD3 (M)</th>
<th>RIFN-γ (U/ml)</th>
<th>rM-CSF (ng/ml)</th>
<th>rGM-CSF (ng/ml)</th>
<th>rIL-6 (ng/ml)</th>
<th>rIL-10 (ng/ml)</th>
<th>TNF-α (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{-7}$</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>$10^{-9}$</td>
<td>50</td>
<td>5</td>
<td>5</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>10</td>
<td>$10^{-5}$</td>
<td>100</td>
<td>10</td>
<td>10</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>-</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Cells were assayed as described in Section 2.3.1.3. In some assays, cellular proliferation was measured by tritiated thymidine incorporation as described in Section 2.5.1.1.

### 2.5.1.1. Detecting MM6 cells proliferation using tritiated thymide (H³-TdR)

To each well of a 96-well plate containing stimulated cells was added 10μl of RPMI-1640 medium containing 1μCi H³-TdR (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). The plates were incubated for 4hrs (37°C/ 5% CO₂) and the cells harvested cells on to fibre glass filter discs (Whatman International Ltd, Maidstone, UK) using an ILACON cell harvester. Each disc was placed in a scintillation vial (Wallac Scintillation products, Milton Keynes, UK) to dry and 4mL scintillation fluid (Wallac Scintillation Products, Milton Keynes, UK) added. The associated radioactivity was measured using a Wallac 1410 Liquid Scintillation counter.

### 2.6. DETECTION OF SURFACE ANTIGEN EXPRESSION USING FLOW CYTOMETRY

All flow cytometric analyses were performed using a Becton Dickinson FACScan (Becton Dickinson UK Ltd, Coweley, Oxford, UK) equipped with an argon ion laser (488nm; 15mW) and FACScan research software.
2.6.1. Detection of surface antigen expression

PBMNC were obtained as described in Section 2.2.1.3. Cells were resuspended at 0.5-1 x 10⁶/ml in PBS and 100μl aliquots were mixed with appropriate volumes of monoclonal antibodies in FACScan tubes. Control cells were incubated with 10-μl mouse IgG1-FITC/ mouse IgG2a-PE dual tag (Sigma Chemical CO. Ltd, Poole, Dorest, UK). Test cells were stained with CD3-PE (10μl; Sigma Chemicals CO. Ltd, Poole, Dorest, UK) and CD28-FITC (10μl; Sigma Chemicals CO. Ltd, Poole, Dorest, UK). Tubes were incubated at 4°C for 1 hr in the dark, were washed twice in PBS (200g/5minutes; 4°C) and resuspended in 0.5ml cold PBS containing 2% formaldehyde. The tubes were left on ice before being analysed by flow cytometry.

2.6.1.1. Effect of cytokines and other stimulators on surface antigen expression.

PBMNC were isolated as described in Section 2.2.1.3 and 2.2.1.4. Human peripheral blood mononuclear cells or MM6 cells were resuspended in RPMI-1640 complete medium at 2 x 10⁵/ml and incubated in 25cm² vented tissue culture flasks with the concentrations of cytokines and stimulators shown below:

<table>
<thead>
<tr>
<th></th>
<th>PMA (ng/ml)</th>
<th>VitD3 (M)</th>
<th>RIFN-γ (U/ml)</th>
<th>rM-CSF (ng/ml)</th>
<th>rGM-CSF (ng/ml)</th>
<th>RIL-6 (ng/ml)</th>
<th>rIL-10 (ng/ml)</th>
<th>RTNFα (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1E-07</td>
<td>10</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>0.05</td>
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</table>

Flasks were incubated for 3, 5, and 7 days. Human monocyte-derived macrophages were gently removed from the flask using a scraper (Marathon Laboratory Supplies, London, UK) and the cells were washed twice (200g, 5mins). MM6 cells were washed similarly. Cells were stained for surface antigens as described in Section 2.6.1. The following antibody combination were used at 10μl/mL for each antibody: -CD16-FITC/ CD80-PE (Becton Dickinson UK Ltd, Coweley, Oxford, UK); CD11c-FITC/ CD23-PE (Sigma Chemicals CO. Ltd, Poole, Dorest, UK / Becton Dickinson Ltd, Coweley, Oxford, UK); CD14-FITC/ HLA-DR-PE (Becton Dickinson UK Ltd, Coweley, Oxford, UK); mouse IgG1-FITC/ mouse IgG2a-PE dual tag and mouse IgG1-FITC/mouse IgM-R-PE dual tag (Sigma Chemicals CO. Ltd, Bodenham, Abingdon, Oxfordshire, UK).
Poole, Dorest, UK). The cells were incubated at 4°C for 1 hr in the dark, washed twice with cold PBS (200g, 5mins, 4°C), re-suspended in 0.5ml cold PBS and left on ice until analysed by flow cytometry.

2.6.1.2. Assessing viability using propidium iodide

The viability of cells treated with cytokines or other stimulators was assessed using propidium iodide. After incubation as described in Section 2.5.1.1, the cells were counted and aliquotted at approximately 0.5-1x10^6/mL in FACScan tubes. The cells were washed twice using sterile PBS at room temperature (200g, 5mins) and re-suspended in 900μl sterile PBS at room temperature. One hundred microlitres propidium iodide (400μg/ml; Sigma Chemical Co. Ltd, Poole, Dorest, UK) was added to each tube and incubated (37°C/5% CO₂) for 30minutes. The cells were analysed immediately using the FACScan.

2.7. CYTOKINE RELEASE BY IMMUNE COMPLEX STIMULATION OF HUMAN BLOOD

Whole blood was treated for various lengths of time with immune complexes and the resulting cytokine release was assayed.

2.7.1. Optimisation of keyhole limpet hemocyanin (KLH)-containing immune complexes

A doubling dilution series using 20μl of rabbit antiserum to KLH in PBS (10.05mg/ml, Organon Teknika Cappel Co. Durham, NC, USA) was carried out in a 96-round bottom plate. To each well was added 20μl of KLH suspension in RPMI-1640 (10mg/ml; Sigma Chemical Co.Ltd, Poole, Dorest, UK) and the plate was incubated at 4°C overnight. Wells were examined for the presence of precipitate and the dilution below the last well containing visible precipitate (1:8) was chosen. A sub agglutination titre of 1:32 was used also in experiments.
2.7.1.1. Effect of KLH-containing immune complexes on human blood

Human peripheral blood was collected in a 50ml syringe (Becton Dickinson UK Ltd, Coweley, Oxford, UK) containing enough endotoxin-free heparin to give a final concentration of 10U/ml (Leo Laboratories Ltd, Risborough, Buckinghamshire, UK). One-millilitre aliquots of heparinised blood were pipetted into endotoxin-free cryovials. To control tubes was added 50μl of RPMI-1640 medium. Test vials were supplemented with 50μl of either the 1:8 or the 1:32 dilution of KLH immune complexes, antibody alone or antigen alone. These cryovials were incubated for 0.5, 2, 4, 8 and 12 hours at 37°C/5% CO₂. The tubes were centrifuged (800g; 5mins) and the serum collected using endotoxin-free gilson pipette tips. The serum was immediately frozen at -20°C until analysed by ELISA for cytokine production.

2.7.1.2. Preparation of stock rabbit or goat anti-human serum and rabbit or goat serum solutions.

Under sterile and endotoxin-free conditions, two dilutions of rabbit or goat anti-human serum and rabbit or goat sera (1:50 and 1:100, Sigma Chemicals CO Ltd, Poole, Dorest, UK) were prepared in RPMI-1640. The dilution’s were prepared in endotoxin free vials and stored at 4°C for use in experiments.

2.7.1.3. Determination of the effect of in situ formed rabbit or goat antihuman Ig-human Ig complexes.

Blood was collected as described in Section 2.7.1.1. Control tubes were supplemented with 10-μl complete RPMI-1640 medium or 10μl of a 1:50 (or a 1:100) dilution of goat (or rabbit) serum. Test vials were supplemented with 10μl of a 1:50 or a 1:100 dilution of rabbit or goat anti-human immunoglobulin alone or with the same dilutions of a non-immune rabbit or goat serum. These cryovials were incubated for 0.5, 2, 4, 8, 12 and 16 hours (37°C/5% CO₂) centrifuged (800g; 5mins) and the serum collected and stored as described in Section 2.7.1.1.
2.7.1.4. Detecting immune complexes in human blood using the FACScan

Human peripheral blood was collected and prepared as described in Section 2.7.1.1. To control cryovials was added either 10μl or 50μl of RPMI-1640 medium and to test cryovials, either 10μl or 50μl of rabbit or goat anti-human serum, or rabbit or goat non-immune sera (dilutions 1:50 or 1:100). These cryovials were incubated for 30 mins (37°C/5% CO₂). To prepare a control for FACScan analysis, 100μl of the samples were incubated with 10μl of either a 1:10 or a 1:20 dilution of monoclonal mouse IgG1-FITC/ mouse IgG2a-R-PE Dual TAG (Sigma Chemicals CO. Ltd, Poole, Dorest, UK). Experimental samples were prepared by incubating 100μl of each sample with 10μl of either 1:10 or 1:20 dilution of monoclonal mouse anti-goat IgG-FITC conjugate (Sigma Chemical CO. Ltd, Poole, Dorest, UK), or monoclonal anti-rabbit-IgG FITC conjugate (Sigma Chemical CO. Ltd, Poole, Dorest, UK) as appropriate. The tubes were gently vortexed and incubated (30mins) at room temperature in the dark. Two millilitres of 1 x FACS lysing solution (Becton Dickinson UK Ltd, Coweley, Oxford, UK) was added to each tube, gently vortexed and incubated for 10 minutes at room temperature in the dark. Tubes were centrifuged for 5minutes at 200g, the supernatant decanted and 2ml sterile PBS added. Tubes were centrifuged for 5minutes at 200g, the supernatant decanted and the pellets re-suspended in Facsflow containing 1% v/v formaldehyde solution (BDH Chemicals Ltd, Poole, Dorest, UK). The samples were left at room temperature until being analysed using the FACScan.

2.7.1.5. Preparation of rHIV-1 gp120 immune complexes

Complexes containing HIV proteins were prepared in endotoxin-free cryovials as described in Section 2.7.1 using a 1:8 dilution of rabbit anti-serum to rHIV-1 MN gp120 (MCR AIDS Directed Programme, Potters Bars, Herts, UK) and a 1:8 dilution of sheep anti-serum to rHIV-1 IIIB gp120 (CHO; MCR AIDS Directed Programme, Potters Bars, Herts, UK). The antigens used were rHIV-1 MN gp120 (200μg/ml; Baculovirus; MCR AIDS Directed Programme, Potters Bars, Herts, UK) and rHIV-1 IIIB gp120 (200μg/ml; CHO; MCR AIDS Directed Programme, Potters Bars, Herts, UK).
2.7.1.6. Effect of rHIV-1 gp120 immune complexes on whole blood

Human peripheral blood was collected in a 50ml syringe containing enough endotoxin-free heparin to give a final concentration of 10U/ml and aliquoted (1ml) in endotoxin-free cryovials. Control tubes were supplemented with 50μl RPMI-1640 medium and test vials with 50μl of each of the complexes described Section 2.7.1.5. These cryovials were incubated for 8 and 12 hours (37°C/ 5% CO₂). The tubes were centrifuged (800g; 5minutes) and the serum collected with endotoxin-free gilson pipette tips. Serum was stored at -20°C until analysed for cytokine production.

2.8. EFFECT OF PREFORMED IMMUNE COMPLEXES ON HIV-1 INFECTED CELLS

2.8.1. Production and Storage of Virus Stocks

Isolates of HIV-1 RF (ADP103; chronically infected H9 cells; Popovic et al., 1984a) and HIV-1 Ba-L (ADP118; a monocytotropic strain; Gartner et al., 1986) were obtained from the MRC AIDS Directed Programme. Stocks of these HIV-1 strains were produced by growing the virus in H9, THP-1 and Mono Mac 6 cell lines. Exponentially replicating cells were counted and approximately 2 x 10⁵ cells were transferred to a centrifuged tube. The cells were pelleted by centrifugation (200g/3mins) and washed using PBS (200g/3mins). The appropriate strain of HIV was removed from storage at -70°C and quickly defrosted. A 100-μl volume of virus suspension was added to the cell pellet followed by 5ml fresh complete medium (without penicillin-streptomycin solution) and transferred to a 25cm²-vented tissue culture flask. The infected cell suspension was incubated at 37°C/ 5% CO₂ for several days. Cell suspensions were observed daily for signs of cytopathic effects and were sub-cultured at 3-4 day intervals by dilution of the cells to a concentration of 2 x 10⁵/ml. The use of KC57-FITC in Sections 2.8.1.1 determined the optimal infection of cells that were suspended and transferred to a centrifuged tube and the cells harvested by centrifugation (200g/3mins). The cells were resuspended in 10ml fresh complete medium and incubated at 37°C/ 5% CO₂ for a further 24 hours. After this time, the cells were centrifuged (200g/3mins) and 1ml aliquots of the supernatant were placed in cryovials. Vials were stored at -70°C until use.
2.8.1.1. **Optimal infection of Mono Mac 6 and THP-1 cell lines**

Mono Mac 6 and THP-1 cell lines were infected with HIV-1 RF (H9), and HIV-1 Ba-L (H9) strains according to Section 2.8.1 for 3, 5, 10, and 20 days in 25cm² vented tissue culture flasks. The infected cells were harvested (350g/5mins) and resuspended at 0.5-1 x 10⁶ viable cells/ml in FACScan tubes. Cells were washed twice with PBS (150g/3mins); resuspended in 80µl PBS and incubated with monoclonal antibodies as in Section 2.6.1.1. In addition the monoclonal antibody KC57-FITC (Coulter Immunology Co. Hialeah, FL, USA) which binds p24, the core protein of HIV-1, was used to stain cells according to the suppliers instruction (see appendix A). The tubes were incubated at 4°C for 1hr in the dark, washed twice with cold PBS (200g/3mins) and resuspended in 0.5ml cold PBS containing 1-% v/v formaldehyde. The tubes were kept at room temperature until analysed by flow cytometry.

2.8.1.2. **Surface antigen expression on human monocyte-derived macrophages**

Human peripheral blood was collected in a 50ml syringe containing endotoxin-free heparin to give a final concentration of 10U/ml blood. Peripheral blood mononuclear cells were isolated as described in Section 2.2.1.3 and 2.2.1.4. The cells were washed (200g; 10 mins) and resuspended in RPMI-10 complete medium before being incubated in 24-well plates (2ml cell suspension/well) with either 50µl of HIV-1 RF (H9), or HIV-1 Ba-L (H9). The plates were incubated for 5 days at 37°C/ 5% CO₂. Monocyte-derived macrophages were gently removed by scraping. Cells were counted and resuspended at a concentration of 0.5 x 10⁶ viable cells/ml in FACScan tubes. After washing twice with PBS (200g/3mins), the cells were resuspended in 80µl PBS and incubated with monoclonal antibodies as described in Section 2.6.1.1 The tubes were incubated at 4°C for 1hr in the dark. The cells were washed twice with cold PBS (200g/3mins/ 4°C) and resuspended in cold PBS containing 1% v/v formaldehyde. The tubes were left at room temperature until being analysed by flow cytometry.

2.8.1.3. **Preparation of HIV-infected Mono Mac 6 cells for electron microscopy**

Mono Mac 6 cells were infected with HIV-1 RF (H9), and HIV-1 Ba-L (H9) for 10 days as described in Section 2.8.1.1. The cells were washed in PBS (200g/3mins) and the cell
pellets were transferred to Nunc Eppendorf tubes (Becton Dickinson UK Ltd, Coweley, Oxford, UK). Glutaraldehyde (Grade 1, 25% v/v aqueous stock, Sigma Chemical Co. Ltd, Poole, Dorest, UK) was diluted in PBS (2.5%v/v) and 1mL volumes were used to resuspend the cell samples. The cells were immediately centrifuged (200g/3mins). Supernatants were carefully discarded, with minimum disruption of the pellets, and 1mL (w/v) of 4% glutaraldehyde buffer (made with 0.1M sodium cacodylate buffer pH7.4; Hayman Ltd Witham, Essex, UK) was added. The cells were left to fix in glutaraldehyde buffer 1 hour at room temperature with the occasional gentle mixing, then centrifuged (200g/3mins) and resuspended in PBS. At this point if the samples could not be processed further they were stored at room temperature in fixative buffer (v/v; made up of 0.2M sodium cacodylate buffer at pH7.4, distilled water, 40%-w/v formaldehyde, and 25%-w/v glutaraldehyde, Hayman Ltd Witham, Essex, UK). After fixing the cells in glutaraldehyde buffer, the cells are thoroughly washed by decanting and resuspending a number of times (3 or 4) in 0.1M sodium cacodylate buffer pH7.4 over a period of 1hour.

Post-fixation of the cells utilised a 1-hour exposure to 2% w/v osmium buffer (Sigma Chemical Co. Ltd, Poole, Dorest, UK) at room temperature in a fume cupboard. This buffer was prepared from 25mL distilled water/g of osmium tetroxide to form osmium acid then equal amount of acid was mixed with equal amount of 0.2M cacodylate buffer. Cells were thoroughly washed using 3 changes of PBS over a 1-hour period. Cells were resuspended in increasing concentrations of alcohol (25%, 50%, 75%, 90%, and absolute alcohol 96%; Hayman Ltd, Witham, Essex, UK) at 20 minutes for each periods (room temperature). Cells were centrifuged (200g/3mins) and the supernatant carefully discarded at each stage. At this point the cells can be left overnight (room temperature) in absolute alcohol if samples cannot be processed further. After alcohol treatment the samples were resuspended in v/v Propylene oxide and absolute alcohol (1:1 ratio; Hayman Ltd Witham, Essex, UK) than Propylene oxide at 20 minutes for each periods (room temperature). Cells were centrifuged (200g/3mins) and the supernatant carefully discarded at each stage.

The resin was prepared by mixing 12g Epon 812 (Taab Laboratories Equipment Ltd, Aldermaston, Berks, UK), 4.75g dodecenylsuccinic anhydride (DDSA; Taab Laboratories Equipment Ltd, Aldermaston, Berks, UK) and 8.25g (2,4,6-tri (dimethylaminomethyl) phenol) (MNA; Taab Laboratories Equipment Ltd, Aldermaston, Berks, UK) in a polycarbonate beaker using a wooden spatula. Shortly before use, 0.5g of 2,4,6-tris- (dimethylaminomethyl) (DNP-30; Taab Laboratories Equipment Ltd, Aldermaston,
Berks, UK) was mixed with the resin. A 2:1 ratio of acetone (Hayman Ltd Witham, Essex, UK) to resin was prepared and specimens were exposed to this for 60 minutes at room temperature. The cells were centrifuged (200g/3mins) and a 1:2 ratio of acetone to resin mixture was carefully added. After 60 minutes at room temperature the samples were centrifuged (200g/3mins) and resin alone was added to every sample, care being taken not to disturb the pellets. These were maintained at room temperature for approximately 4 hours, to allow the resin to penetrate. The samples were transferred to an oven, where the resin was cured by incubating at 60°C for 16 hours. Ultrathin sections were cut using an ultramicrotome and placed onto copper grids. Than each section was stained with a drop of 2% w/v uranyl acetate (20 minutes at 60°C) then washed with distilled water and a drop of 4 mg/ml lead citrate was added on each section and left at room temperature for 7 minutes. All samples were examined using a Phillips T400 transmission electron microscope.

2.9. HIV-INFECTED, HUMAN MONOCYTE-DERIVED MACROPHAGES AND IMMUNE COMPLEXES

Human monocyte-derived macrophages infected with HIV were incubated with immune complexes. Surface antigen expression was analysed by flow cytometry and supernatants were collected to detect any cytokines released.

2.9.1. Effect of Immune Complexes on HIV-Infected Human Monocyte-Derived Macrophages

Human peripheral blood was collected in a 50ml syringe containing endotoxin-free heparin (final concentration 10U/ml blood). PBMNC were stained as described in Sections 2.2.1.3 and 2.2.1.4. Cells were aliquoted (1ml) in quadruplicate in the wells of a 24-well plate. For each complex (described in Sections 2.7.1.2 and 2.7.1.5) two wells were supplemented with 50μl HIV-1 Ba-L (H9) and two with 50μl medium. Each plate was incubated for 5 days (37°C/ 5% CO₂) and the wells washed twice with RPMI-1640 medium using a sterile Pasture pipette. Wells were supplemented with 1ml complete RPMI-10 medium and either 50μl RPMI-1640 or an immune complex. Preparation was such that HIV-
infected and control cells were each incubated with and without immune complexes. Identical plates were incubated for 8hrs or 12 hours (37°C / 5% CO₂).

Supernatants from each well were collected after 8 or 12 hours in cryovials and immediately frozen at -20°C until used to detect the cytokines released by ELISA. Cells were gently scraped from each well, counted using Trypan Blue and divided between five FACScan tubes. Cells were analysed for surface antigen expression as described in Section 2.6.1.1.

2.9.1.1. Effect of sera from HIV+ve patients on HIV-infected human monocyte-derived macrophages

Human peripheral blood was collected in a 50ml syringe containing endotoxin-free heparin (final concentration 10U/ml blood). Peripheral blood mononuclear cells were collected as described in Section 2.2.1.3 and 2.2.1.4. Twenty-four well plates were prepared and treated as described in Section 2.9.1 but instead of the addition of prepared immune complexes, 50μl of HIV^+ sera was added to each well. Duplicate wells were supplemented with 50μl RPMI-1640 medium to act as control.

2.10. ANALYSIS OF CYTOKINE PRODUCTION

Supernatants from stimulated cells were analysed for the presence of IL-6, IL-10 and TNF-α using "in-house" ELISA assays.

2.10.1. Detecting cytokines using ELISA

The ELISAs performed were 'capture' assays. The first antibodies used were mouse anti-human IL-6 (4μg/ml; MAB206, Serotec Ltd, Kidlington, Oxford, UK), mouse anti-human TNF-α (4μg/ml; AB210NA, Serotec), and mouse anti-human IL-10 (8μg/ml; MCA926, Serotec Ltd, Kidlington, Oxford, UK). ELISA plates (96-well flat bottomed plates) were incubated with 50μl/well of the appropriate first antibody diluted to 4μg/ml in carbonate-bicarbonate coating buffer (0.2M; pH 9.6). The plates were incubated at 4°C overnight, washed five times with distilled water, blotted and air-dried. This washing procedure was carried out each time the plates were incubated. Plates were blocked by incubating 1% w/v
Marvel dried milk powder in PBS (50μl/well) for 30 minutes at room temperature, and drying as before. Test samples were diluted 1:4 and 1:8 in 1% w/v Marvel and 50μl were added to each well. Plates were incubated for 4 hours at room temperature, washed and dried as before and incubated with 50μl/well of the appropriate second antibody diluted in 1-% w/v Marvel. The second antibodies used were goat anti-human IL-6 (2μg/ml; AB206NA, Serotec Ltd, Kidlington, Oxford, UK), mouse anti-human TNF-α (2μg/ml; MCA747M, Serotec Ltd, Kidlington, Oxford, UK), and goat anti-human IL-10 (8μg/ml; L-5020, Sigma Chemical CO. Ltd, Poole, Dorest, UK). Plates were incubated for 4 hours at room temperature, washed and dried as before and 50μl/well of the appropriate third antibody was added at a dilution of 1:1000 in 1-% w/v Marvel. Anti-goat IgG alkaline phosphatase conjugate was used for IL-6 and IL-10 (A-2168, Sigma Chemical CO. Ltd, Poole, Dorest, UK) whilst anti-mouse IgG alkaline phosphatase conjugate was used for TNF-α (A-4312, Sigma Chemical CO. Ltd, Poole, Dorest, UK). Plates were incubated for 4 hours at room temperature, washed and dried as before and 50μl/well of p-nitrophenyl phosphate substrate (pNPP, N-1891, Sigma Chemical CO. Ltd, Poole, Dorest, UK) was added to each well at final concentration of 1mg/ml dissolved in distilled water. Plates were incubated for 1 hr in the dark at room temperature and were read at 405nm on a spectrophotometer (Labsystems, Basingstoke, Hants, UK).

2.11. STATISTICS

The statistical significance of the data was determined using appropriate analysis methods and the Statview analytical computer package. Non-parametric data was analysed using the Mann-Whitney U test. Parametric data was analysed using a factorialised, analysis of variance and Post-hoc Fisher's tests.
3.0. RESULTS

3.1. Determination of the optimal concentration of PPD and BCG for the production of T cell lines.

Cells were stimulated with PPD and BCG as described in Section 2.4.1.1 and cellular proliferation was measured using the MTT assay. The results are shown in Figure 3.1A-B. The concentrations of PPD and BCG that produced the highest optical density (O.D) with the MTT assay were found to be 10\(\mu\)g/ml (PPD) and a dilution of 0.0625 (BCG). However, these values were not greater than baseline values obtained with non-stimulated cells. Stimulation of mononuclear cells with BCG showed no significant effect on cellular proliferation measured by tritiated thymidine incorporation (Section 2.5.1.2; Figure 3.1C-D).

3.1.1. Production of an antigen-specific T-cell line

One of the initial aims of the project was to examine the effect of immune complexes on antigen presentation in HIV infection. In order to examine this, an attempt was made to establish antigen specific T cell lines using peripheral blood mononuclear cells stimulated with PPD (10\(\mu\)g/mL) or BCG (dilution at absorbance of 0.1). However, the production of antigen-specific T cells, by discontinuous stimulation with either antigen was not successful. Initial stimulation produced antigen specific T cell blasts but the cell count decreased thereafter. The use of IL-2 at 10U/mL in discontinuous stimulation did not support T-blast formation. Culturing the cells with BCG for 5 or 7 days had no effect on the discontinuous stimulation of antigen-specific T-cell lines.

The discontinuous stimulation assay was repeated 44-times for PPD and BCG. Stimulating T cells with PPD and with either 10U/mL or 30U/mL IL-2 showed an initial difference in T-cell blast numbers but the end result was the same as indicated by the example of two donors in Table 3.1.
Table 3.1. Effect of IL-2 concentration on discontinuous stimulation of human T cells.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>10U/mL</th>
<th>30U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2 \times 10^6$ cell/mL</td>
<td>$2 \times 10^6$ cell/mL</td>
</tr>
<tr>
<td>2*</td>
<td>$0.66 \times 10^5$ cell/mL</td>
<td>$1.59 \times 10^6$ cell/mL</td>
</tr>
<tr>
<td>3</td>
<td>cells too low to detect</td>
<td>$4.44 \times 10^5$ cell/mL</td>
</tr>
<tr>
<td>4*</td>
<td></td>
<td>$3.2 \times 10^5$ cell/mL</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>cells too low to detect</td>
</tr>
</tbody>
</table>

* = Stimulation of resting cells with IL-2 after they have previously been stimulated with PPD.

This indicates that after each stimulant, the cell number increased when 30U/mL IL-2 was used compared to 10U/mL.

T cells stimulated with BCG were also supplemented with 10U/mL IL-2 but different feeder cells were used and the IL-2 was added on different days as indicated by the example of two donors in Table 3.2.
Figure 3.1 A and B. Determination of the Optimal concentration of PPD and BCG by MTT assay

After incubating human mononuclear cells in 96-well plates for 7 days with PPD (A) and BCG (B) at various concentrations (37°C/5% CO₂), cellular proliferation was assayed using MTT. Formazan crystals were solubilized using DMSO and the optical density of each well measured at 540nm. The results show the mean and standard deviation with n=15.
C. Evaluation of the effect of BCG on the proliferation of mononuclear cells assessed by tritium incorporation

![Graph showing incorporation of tritiated thymidine vs BCG dilution](image)

D. Evaluation of the effect of BCG on the proliferation of T-cell blasts assessed by tritium incorporation

![Graph showing incorporation of tritiated thymidine vs BCG dilution](image)

Figure 3.1 C and D. Determination of cellular proliferation using tritiated thymidine incorporation

After incubating human mononuclear cells (C) and T-cell blasts (D) in 96-well plates for 7 days with BCG at various concentrations (37°C/5% CO₂) cellular proliferation was assayed using tritiated thymidine incorporation. Formazan crystals were solubilized using DMSO and the optical density of each well measured at 540nm. The results show the mean and standard deviation with n=15
Table 3.2. Effect of different feeder cells on discontinuous stimulation of human T cells.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Donor1</th>
<th>Donor2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 x 10^6 cell/mL</td>
<td>2 x 10^6 cell/mL</td>
</tr>
<tr>
<td>2*</td>
<td>1.56 x 10^5 cell/mL</td>
<td>3.65 x 10^5 cell/mL</td>
</tr>
<tr>
<td>3</td>
<td>0.75 x 10^5 cell/mL</td>
<td>4.75 x 10^5 cell/mL</td>
</tr>
<tr>
<td>4*</td>
<td>1.3 x 10^5 cell/mL</td>
<td>3.3 x 10^5 cell/mL</td>
</tr>
<tr>
<td>5</td>
<td>4.5 x 10^5 cell/mL</td>
<td>13.4 x 10^5 cell/mL</td>
</tr>
<tr>
<td>6*</td>
<td>5.1 x 10^5 cell/mL</td>
<td>0.55 x 10^5 cell/mL</td>
</tr>
<tr>
<td>7</td>
<td>9.0 x 10^5 cell/mL</td>
<td>cells too low to detect</td>
</tr>
<tr>
<td>8*</td>
<td>2.65 x 10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.3 x 10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>10*</td>
<td>2.8 x 10^5 cell/mL</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.3 x 10^5 cell/mL</td>
<td></td>
</tr>
</tbody>
</table>

* = Stimulation of resting cells with IL-2 after they have previously been stimulated with BCG. Feeder cells used for donor 1 were homologous, EBV transformed B cells and for donor 2 were homologous mononuclear cells.

Another technique used to produce T cell lines was to stimulate the cells with 30U/mL IL-2 on day 4 then re-stimulate them with BCG on day 7. In this experiment the cell count gradually decreased from 2 x 10^6 /mL to 1.38 x 10^6 /mL to 0.375 x 10^6 /mL respectively. A continuous technique was used in which 30U/mL IL-2 was added each day for one week but the result was similar. With all the techniques used, the same pattern was observed in that IL-2 addition give a transient increase in T-cell proliferation.
4.0. IMMUNE COMPLEXES

4.1. EFFECT OF IMMUNE COMPLEXES ON HUMAN MONOCYTES DERIVED MACROPHAGES IN VITRO

Due to the failure to obtain antigen specific T cell lines, the project was adapted to look at the effect of immune complexes on human monocytes derived macrophages in AIDS. In order to understand the role immune complexes play in AIDS study the effect of immune complexes on normal monocytes to establish normal parameters.

4.2. SURFACE ANTIGEN EXPRESSION AND ITS MODULATION BY CYTOKINES AND OTHER STIMULATORS

In order to establish the effect of immune complexes on the phenotypic and functional activities of mononuclear phagocytes, it was necessary to establish base-line cellular characteristics and how both cytokines and other stimulators may moderate these.

4.2.1. Effect of cytokines and other stimulators on MM6 cells

Mono Mac 6 cells (MM6) were incubated with cytokines and other stimulators at various concentrations to determine their effect on cell proliferation and differentiation as described in Sections (2.5.1.1, 2.5.1.2, and 2.6.1.2). Initially an MTT assay was used to measure cellular proliferation (Figures 4.1A-4.8A). Subsequently, because the readings with MTT appeared to be so low, the experiments were repeated using tritiated thymidine (Figures 4.1B-4.8B). To determine if cytokines and other stimulants were inhibiting cellular proliferation or being toxic to the cells, the experiment was repeated again using propidium iodide to detect non-viable cells as described in Section 2.5.1.1 (Figures 4.1C-4.8C). The effect of the individual treatments is described below.
4.2.1.1. Phorbol myristate acetate (PMA)

Previous work has shown that PMA induces monocytic differentiation (Ziegler-Heitbrock et al., 1994). Cells cultured with PMA showed a significant decrease in respiration and therefore proliferation at all concentrations tested, with a profound effect at 10ng/mL, 30ng/mL, and 50ng/mL on days 5 and 7 (Fishers post hoc test: p< 0.0001; Figure 4.1A). Similar results were obtained using tritiated thymidine to assess cellular proliferation (Figure 4.1B, Fishers post hoc test: p< 0.0001). Analysis using propidium iodide showed a significant rise in the number of damaged cells at the lower concentrations of PMA (Figure 4.1C, Fishers post hoc test: p<0.0001). However, at concentrations of 30ng/mL and 50ng/mL, PMA did not appear to cause cell death.

These results suggest that at 30 and 50ng/mL, PMA inhibited the proliferation of MM6 cells by promoting their differentiation into more mature cells that lack the capacity for proliferation. It also suggests that PMA, even at low concentration is toxic for rapidly proliferating cells.

4.2.1.2. 1,25 dihydroxyvitamin D3 (VitD3)

VitD3 can control the growth and differentiation of several cell types (Oberg et al., 1993). Using the MTT assay, cells cultured with VitD3 appeared to show a significant increase in respiration and therefore proliferation at a concentration of 10^{-7}M/mL after 3 days in culture (Figure 4.2A). Other concentrations of Vit D3 showed no effect. In contrast to this, when proliferation was measured using the tritiated thymidine assay, VitD3 significantly inhibited cellular proliferation at all concentrations tested (Figure 4.2B; Fisher post hoc test: p<0.0001). This inhibition appeared to be due in part to a decrease in cell viability since propidium iodide incorporation was significantly greater in treated cells than controls (Figure 4.2C; Fisher post hoc test: p<0.0001).

Thus, these results suggest that VitD may inhibit cellular proliferation through inducing differentiation but that it may also be toxic to MM6 cells at the concentrations tested. Again it appears that it is most toxic in rapidly proliferating cells.
A. Evaluation of the effect of PMA on the proliferation of MM6 cells using the MTT assay

B. Evaluation of the effect of PMA in the proliferation of MM6 cells assessed by tritium incorporation after day 3

Figure 4.1 A and B. The effect of PMA on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
Figure 4.1 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO2) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
A. Evaluation the effect of VitD3 on the proliferation of MM6 cells using the MTT assay

![Graph showing Optical Density at 540nm (arbitrary units) vs. Concentration of VitD3 (M/mL) for Day3, Day5, and Day7.]

B. Evaluation of the effect of VitD3 on the proliferation of MM6 cells assessed by tritium incorporation after day 3

![Graph showing Incorporation of tritiated thymidine (counts per min) vs. Concentration VitD3 (M/mL) for Day3, Day5, and Day7.]

Figure 4.2 A and B. The effect of VitD3 on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
C. Evaluation of the effect of VitD3 on the viability of MM6 cells using PI after day 3

![Graph showing the effect of VitD3 on cell viability]

Figure 4.2 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using a propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
4.2.1.3. Interferon gamma (IFN-\(\gamma\))

IFN\(\gamma\) induces the differentiation of cells in vitro such that they express characteristics of mature monocytes (Ziegler-Heitbrock et al., 1994). IFN-\(\gamma\) appeared to have no effect on cellular proliferation when assayed using the MTT assay (Figure 4.3A). However, the incorporation of tritiated thymidine was significantly inhibited by the presence of IFN-\(\gamma\) at all concentrations tested (Figure 4.3B, Fisher post hoc test: \(p<0.0001\)). This may be due in part to a toxic effect of IFN-\(\gamma\) on MM6 cells since propidium iodide incorporation was significantly increased at all concentrations tested (Figure 4.3C; Fishers post hoc test: \(p<0.0001\)).

These results suggest that IFN-\(\gamma\) inhibit cellular proliferation by causing damage to the rapidly proliferating cells.

4.2.1.4. Monocyte colony stimulating factor (M-CSF)

M-CSF has been shown to support the differentiation and survival of cells (Vincent et al., 1992). MM6 cells cultured with M-CSF showed no significant difference in their growth characteristics when assayed using MTT (Figure 4.4A). However, when cell proliferation was assessed by tritium incorporation, M-CSF showed a significant inhibitory effect on cellular proliferation at all concentrations tested (Figure 4.4B; Fishers post hoc test: 1ng/mL, 5ng/mL, \(p<0.0001\); 10ng/mL, \(p=0.0005\); 20ng/mL, \(p=0.0008\); 30ng/mL, \(p=0.0062\)). Propidium iodide incorporation was significantly increased by M-CSF treatment at all concentrations tested (Figure 4.4C, Fishers post hoc test: \(p<0.0001\)).

These results suggest that M-CSF cause a decrease in cellular proliferation that may be due to toxic effects on the rapidly proliferating cells.

4.2.1.5. Granulocyte-monocyte colony stimulating factor (GM-CSF)

GM-CSF supports the differentiation and survival of cells (Vincent et al., 1992). MM6 cells cultured with GM-CSF showed significant difference in their growth characteristics at day 5 when assayed using MTT except at the 10ng/mL concentration (Figure 4.5A; Fishers post hoc test: 1ng/mL, \(p=0.0268\); 5ng/mL, \(p=0.0079\); 20ng/mL, \(p=0.0001\); 30ng/mL, \(p<.0001\)). However, when cellular proliferation was assessed by tritium incorporation, GM-CSF showed an effect on cellular proliferation although it’s not inhibiting proliferation at 1ng/mL,
A. Evaluation of the effect of rIFN-γ on the proliferation of MM6 cells using the MTT assay

B. Evaluation of the effect of rIFN-γ on the proliferation of MM6 cells assessed by tritium incorporation after day 3

Figure 4.3 A and B. The effect of IFN-γ on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
Figure 4.3 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO2) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
Figure 4.4 A and B. The effect of M-CSF on the proliferation of MM6 cells
MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
Figure 4.4 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
and 5ng/mL (Figure 4.5B; Fishers post hoc test: 1ng/mL, p=0.0158; 5ng/mL, p=0.0169). Propidium iodide incorporation was significantly increased by GM-CSF treatment at all concentrations tested (Figure 4.5C, Fishers post hoc test: p<0.0001).

These results suggest that GM-CSF cause cellular differentiation but that the decrease in proliferation may be due to toxicity.

4.2.1.6. Interleukin-6 (IL-6)

This multifunctional cytokine is synthesised by many different cells after appropriate stimulation. It exerts multiple functions during the immune response including hematopoiesis, neural differentiation and the acute phase reaction (Schoester et al., 1994). MM6 cells cultured with IL-6 showed a slight increase in proliferation at day 3 and a decrease by day 5 when assessed by MTT (except at 1ng/mL and 5ng/mL). This suggest that IL-6 promotes growth and differentiation of these cells (Figure 4.6A; Fishers post hoc test: 0.05ng/mL, 0.1ng/mL, p<0.0001; 0.5ng/mL, p=0.0003). However, when cell proliferation was assessed by tritium incorporation, IL-6 showed no significant effect since the proliferation of the non-treated cells was similar to that of the treated cells (Figure 4.6B). Propidium iodide incorporation was significantly increased by IL-6 treatment at all concentrations tested (Figure 4.6C; Fishers post hoc test: 0.05ng/mL, 0.1ng/mL, 1ng/mL, 5ng/mL, p<. 0001; 0.5ng/mL, p=0.0002).

These results suggest that IL-6 may have had an effect on cellular proliferation (as evidenced by the increase in the MTT assay) but that the inability to detect this as with tritium incorporation. This may have been due to the toxic effect of IL-6 as evidenced by the increase in cell death.

4.2.1.7. Interleukin-10 (IL-10)

This cytokine is secreted by the Th0 and Th1 subsets of CD4+ and the Ts0 and Ts1 subsets of CD8+ cells, activated B lymphomas, Epstein-Barr virus-transformed Burkitt's lymphoma lines, monocytes/macrophages and keratinocytes. IL-10 has pleotropic effects on different cell types (Spittler et al., 1995). MM6 cells cultured with IL-10 showed no overall difference in proliferation when assayed using MTT (Figure 4.7A). However, when cell proliferation was assessed by tritium incorporation, IL-10 showed a significant effect on
A. Evaluation of the effect of GM-CSF on the proliferation of MM6 cells using the MTT assay

B. Evaluation of the effect of GM-CSF on the proliferation of MM6 cells assessed by tritium incorporation after day 3

Figure 4.5 A and B. The effect of GM-CSF on the proliferation of MM6 cells
MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.

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C. Evaluation of the effect of GM-CSF on the viability of MM6 cells using PI after day 3

Figure 4.5 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
A. Evaluation of the effect of IL-6 on the proliferation of MM6 cells using the MTT assay

B. Evaluation of the effect of IL-6 on the proliferation of MM6 cells assessed by tritium incorporation after day 3

Figure 4.6 A and B. The effect of IL-6 on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
C. Evaluation of the effect of IL-6 on the viability of MM6 cells using PI after day 3

Figure 4.6 C. Effect of propidium iodide on MM6 cell viability
MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
cell proliferation (Figure 4.7B; Fishers post hoc test: 0.05ng/mL, p=0.04; 0.1ng/mL, p=0.0048; 0.5ng/mL, p=0.0013; 1ng/mL, 5ng/mL, p<.0001). Propidium iodide incorporation was significantly increased by IL-10 treatment at all concentrations tested (Figure 4.7C, Fishers post hoc test: p<.0001).

These results suggest that IL-10 causes a decrease in cellular proliferation that may be in part due to induced differentiation but may also be due to cellular damaged induced by the presence in culture of IL-10.

4.2.1.8. Tumour necrosis factor-α (TNF-α)

This cytokine is secreted by activated monocytes/macrophages and exerts anti-proliferative effects and leads to differentiation. MM6 cells cultured with TNF-α showed a significant decrease in proliferation using the MTT assay at day 5 except at 0.1ng/mL and 0.5ng/mL (Figure 4.8A; Fishers post hoc test: 0.05ng/mL, 5ng/mL, p<.0001; 1ng/mL, p=0.0466). When cell proliferation was assessed by tritium incorporation, TNF-α showed a tendency to inhibit cellular proliferation which reached significance at 5ng/mL (Figure 4.8B, Fishers post hoc test: 5ng/mL, p<.0001). Propidium iodide incorporation was significantly increased by TNF-α treatment at all concentrations tested (Figure 4.8C; Fishers post hoc test: 0.05ng/mL, 0.1ng/mL, 0.5ng/mL, p<.0001; 5ng/mL, p<.0002).

These results suggest that TNF-α may cause differentiation and a decrease cellular proliferation but some of the decrease may be due to the toxic effects of this cytokine. Toxicity is less at dose that gives maximal inhibition of proliferation.

4.2.2. Effect of cytokines and other stimulators on surface antigen expression on human macrophages and MM6 cells

To determine if cytokines or other stimulants cause a change in surface antigen expression associated with cellular proliferation or differentiation the following markers were examined CD80, CD23, HLA-DR, CD11c, CD14 and CD16.
Figure 4.7 A and B. The effect of IL-10 on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
C. Evaluation of the effect of IL-10 on the viability of MM6 cells using PI after day 3

Figure 4.7 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
Figure 4.8 A and B. The effect of TNF-α on the proliferation of MM6 cells

MM6 cells were incubated for 3, 5 and 7 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell proliferation was assessed using an MTT assay (A); tritiated thymidine incorporation (B). Results are expressed as the mean and standard deviation with n=15.
Figure 4.8 C. Effect of propidium iodide on MM6 cell viability

MM6 cells were incubated for 3 days (37°C/5% CO₂) in the presence of a range of cytokines and other stimulators and their effect on cell viability was assessed using an propidium iodide assay (C). Results are expressed as the mean and standard deviation with n=15.
4.2.2.1. CD16

CD16 is expressed at very low levels on monocytes but its expression is greater on fully differentiated macrophages. Only 2% (+1.8) of control peripheral blood monocytes was not as marked with MM6 cells which show a higher constitutive expression of CD16 than cultured, control, peripheral blood monocytes (5.2+/-.0 2% versus 2.1+/-.1.8% Figure 4.9B). This expression was particularly significantly increased in cells treated with 0.05ng/mL TNFα (p<0.0001); 30ng/mL GM-CSF (p=0.0002), 10^-7M Vit D3 (p=0.0007), 1ng/mL IL-10 (p=0.0041), 0.5ng/mL IL-6 (p=0.0099), 1ng/mL M-CSF (p=0.0316), and over the whole incubation period. Expression reached a maximum at day 5 with these stimulators and thereafter declined.

4.2.2.2. CD80

CD80 is expressed at very low levels on monocytes but its expression is greater on fully stimulated differentiated macrophages. Only 3.28% (+1.21) of control peripheral blood monocytes incubated for three days exhibited CD80 (Figure 4.10A). This expression was significantly inhibited by PMA on blood monocytes and was significantly increased by IL-6 (17.77 +/- 0.5), IL-10 (19.58 +/- 0.95) and 0.05mg/mL TNFα (11.05 +/- 0.64). The effect was most obvious at day 3, levels of expression falling by days 5 and 7 (Figure 4.10A, Fishers post hoc test: 0.05ng/mL IL-6, 1ng/mL IL-10 and 0.05ng/mL TNF-α, p<.0001).

The effect of the various cytokines and chemicals was not as marked with MM6 cells which show a lower constitutive expression of CD80 than cultured control peripheral blood monocytes (0.53 +/- 0.03 versus 3.28 +/-1.21; Figure 4.10B). This expression was particularly significantly increased in cells treated with 1ng/mL GM-CSF (p<0.0001); 0.05ng/mL IL-6 (p<0.0001); 1ng/mL IL-10 (p<0.0001); 0.05ng/mL TNF-α (p<0.0001) over the whole incubation period. Expression reached a maximum at day 5 for IL-10 and TNFα (and thereafter declined) but reached a maximum for IL-6 at day 7.

4.2.2.3. CD11c

CD11c is expressed at very high levels on monocytes but its expression is greater on fully differentiated macrophages. It is also up regulated by inflammatory mediators (Kishimoto et
Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-CD16 monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing CD16 with n=5.
Figure 4.10 A and B. The effect of cytokines and stimulators on surface antigen expression

Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-CD80 monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing CD80 with n=5.
Control peripheral blood monocytes incubated for three days showed a high level of CD11c expression (72.12±14.65%, Figure 4.11A). PMA, Vit D3 and IFNγ, an effect that persisted throughout the experiment significantly inhibited this expression at day 3. After 5 days in culture, IL-6, IL-10 and TNF-α had also significantly reduced CD11c expression. By contrast, MM6 cells which show a lower constitutive expression of CD11c than cultured control peripheral blood monocytes (9.86 +/- 0.27% versus 71.12 +/-14.65%; Figure 4.11B) showed a significant increase in CD11c after 3 days treatment with 10ng/ml PMA (p<0.0001) which slowly declined over the incubation period. Additionally, IL-6 induced a significant increase in CD11c expression after 5 days in culture that rapidly declined thereafter. Unstimulated cells did not show a significant change despite the fact that over 7 days in culture monocytes on the characteristics of macrophages.

4.2.2.4. CD23

CD23 is expressed by a large variety of hematopoietic cells and is implicated in the initiation and development of parasitic and allergic reaction and in the differentiation of cells of the myelomonocytic lineage (Dugas et al., 1996). CD23 is expressed at low levels on monocytes but its expression is greater on fully differentiated macrophages. Only 4.49% (+4.65) of control peripheral blood monocytes incubated for three days exhibited CD23 (Figure 4.12A). This expression was significantly increased particularly by 10U/ml IFN-γ (21.0 +/- 3.16%) and 30ng/mL GM-CSF (43.42 +/- 1.11). The effect was most obvious at day 3, decreasing with increasing length in culture (Fishers post hoc test: 10U/mL IFN-γ, p<0.0001, 30ng/mL GM-CSF, p<0.0001). GM-CSF at 1ng/mL dramatically increased CD23 expression at day 7 (19.66 +/- 0.64, Figure 4.12A, p<0.0001).

The effect of the various cytokines and chemicals on MM6 cell was not as marked as with peripheral blood mononuclear cells which showed a lower constitutive expression of CD23 than MM6 cells (4.49 +/-0.58% versus 7.22 +/- 0.43%; Figure 4.12 A and B). CD23 on MM6 cells was particularly significantly increased in cells treated with 1ng/mL M-CSF (p<0.0001) and 30ng/mL GM-CSF (p<0.0001) over the whole incubation period. Expression, reached a maximum at day 5 for 10U/mL IFN-γ (p<0.0001), 1ng/mL M-CSF (p<0.0001), 30ng/mL M-CSF (p<0.0001), and 1ng/mL GM-CSF (p<0.0001) then declined thereafter to day 7. IL-6, IL-10 and TNF-α all inhibited CD23 expression at days 3 and 5. At
day 7, expression in IL-10 treated cells was enhanced (Fishers post hoc test for change in IL-10 expression over the course of the experiment; p<0.0001).

4.2.2.5. CD14

The CD14 antigen is expressed on monocytes and macrophages and was originally identified as a myeloid differentiation antigen also involved in triggering the activation of myelomonocytic cells (Bosco et al., 1997). The mean percentage of cells expressing CD14 is higher for fully differentiated macrophages than for monocytes. CD14 was found to be expressed on 88.56 % (±6.87) control peripheral blood cells at (Figure 4.13A). This expression significantly reduced by all cytokines and stimulants tested with the exception of M-CSF at a concentration of 1ng/mL. Especially potent were PMA (6.03+0.59%), 10^-7M/mL VitD3 (60.88 +/- 2.16%), IFN-γ (38.18±5.5%), 30ng/mL GM-CSF (28.22 +/- 0.66%), 30ng/mL M-CSF (43.73+/- 2.37%), and TNF-α (31.89 +/- 2.78%) all with p<0.0001 value. The effect of PMA was similar throughout the course of the experiment. The effect of those cytokines inducing differentiation e.g. IFN-γ, GM-CSF30 and TNF-α was maximal at day seven of culture.

Again the effect of the various cytokines and chemicals on MM6 cells was not as marked. These cells show a lower constitutive expression of CD14 than cultured control peripheral blood monocytes (12.33 +/- 0.52% versus 88.56 +/-6.87%, Figure 4.13B). This expression was particularly significantly increased in cells treated with 10ng/mL PMA (p<0.0001) and 10^-7M/ml VitD3 (p<0.0001) for three and five days. At day 3, the remaining cytokines showed a slight inhibitory effect on CD14 expression. However, by day five, significant increases were caused by 0.05ng/ml IL-6 (p<0.0001), 1ng/ml IL-10 (p<0.0001), 0.05ng/ml TNF-α (p<0.0001), which returned to normal by day 7.

4.2.2.6. HLA-DR

Figure 4.14A and B shows a high level of HLA-DR expression on fully differentiated macrophages and on the mature monocyte phenotype, MM6 cells. As many as 96.28±2.61% of control peripheral blood monocytes incubated for three days exhibited HLA-DR (Figure 4.14A). This expression was significantly decreased after 3 days in culture with all cytokines and stimulants tested (with the exception of the lower concentrations
Figure 4.11 A and B. The effect of cytokines and stimulators on surface antigen expression

Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-CD11c monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing CD11c with n=5.
Figure 4.12 A and B. The effect of cytokines and stimulators on surface antigen expression

Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-CD23 monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing CD23 with n=5.
Figure 4.13 A and B. The effect of cytokines and stimulators on surface antigen expression

Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-CD14 monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing CD14 with n=5.
Figure 4.14 A and B. The effect of cytokines and stimulators on surface antigen expression

Human peripheral blood monocytes (obtained by separation over lymphoprep and adherence to plastic) and MM6 cells were incubated for 3, 5, and 7 days (37°C/5% CO₂) with a range of cytokines and stimulators. Cells were recovered and were stained with an anti-HLA-DR monoclonal antibody and analysed by flow cytometry. Results are expressed as the mean % cells (± standard deviation) expressing HLA-DR with n=5.
M-CSF and GM-CSF). This effect reached a maximum at day 7 for IFN\(\gamma\) and 30ng/mL GM-CSF. The effect of the other stimulants either remained constant or diminished with longer culture periods.

The effect of the various cytokines and chemicals was not as marked on MM6 cells which showed a lower constitutive expression of HLA-DR than cultured control peripheral blood monocytes (65.62 +/- 0.48% versus 96.28 +/- 2.61%; Figure 4.14B). This expression was decreased after 3 days incubation with PMA, (38.46 +/- 1.49), VitD3 (48.54 +/- 1.37), 0.05ng/mL IL-6 (37.19 +/- 0.47%), 1ng/mL IL-10 (35.02 +/- 0.64%), 0.05ng/mL TNF-\(\alpha\) (39.17 +/- 1.60%) the effect remaining constant throughout the period of the experiment. The remaining stimulants caused a significant decrease in HLA-DR expression after 5 days in culture that became less evident by day 7 (Figure 4.14, Fishers post hoc test for the time-related changes in antigen expression: 10ng/mL PMA, 10^{-7}M/mL VitD3, 10U/mL IFN-\(\gamma\), 1ng/mL M-CSF, 30ng/mL M-CSF, 1ng/mL GM-CSF, 30ng/mL GM-CSF, 0.05ng/mL IL-6, 1ng/mL IL-10, 0.05ng/mL TNF-\(\alpha\), p<0.0001).
5.0. IMMUNE COMPLEX-STIMULATED RELEASE OF CYTOKINES FROM HUMAN BLOOD

5.1. KLH-stimulated cytokine release

Human blood was incubated with immune complexes containing KLH for various lengths of time. Supernatants were analysed for the presence of IL-6, IL-10, and TNF-α cytokines as described in Sections (2.7.1 and 2.7.1.1). Cells were incubated with immune complexes (made with higher (Ab1) and lower (Ab2) concentrations of antibody) or with antigen or antibody alone as controls.

5.1.1. IL-6

Unfortunately, both the antigen alone and the highest concentration of antibody alone stimulated IL-6 production (Figure 5.1A; Fishers post hoc test (5% level): p=0.0015 and p<0.0001 respectively). However, the lower concentration of antibody alone did not significantly induce IL-6 production. The presence of the immune complexes significantly increased the production of IL-6 when compared to non-stimulated controls. The Ab2Ag immune complex was the only complex to reached true significance since it caused a time-dependent increase in IL-6 which was significantly greater than that produced by the Ag or Ab alone (Table 5.1 shows the Fishers post hoc analysis). The effect was apparent at 4 hours but increased with time.

Table 5.1: Fishers post hoc analysis at 5% level for IL-6

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Figure 5.1A. The effect of Keyhole Limpet Hemocyanin on the IL-6 release in whole blood

Whole blood was incubated with a complex comprising Keyhole Limpet Hemocyanin (KLH) and rabbit anti-KLH for 0.5, 2, 4, 8, and 12 hours (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for IL-6 (A). Using an ELISA. Ab1=1:4 dilution of rabbit anti-KLH; Ab2=1:8 dilution of rabbit KLH; Ab1Ag or Ab2Ag= immune complexes constituted with 1:4 or 1:8 dilution of anti-KLH (n=5).
Figure 5.1B. The effect of Keyhole Limpet Hemocyanin on the IL-10 release in whole blood

Whole blood was incubated with a complex comprising Keyhole Limpet Hemocyanin (KLH) and rabbit anti-KLH for 0.5, 2, 4, 8, and 12 hours (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for IL-10 (B). Using an ELISA. Ab1=1:4 dilution of rabbit anti-KLH; Ab2=1:8 dilution of rabbit KLH; Ab1Ag or Ab2Ag= immune complexes constituted with 1:4 or 1:8 dilution of KLH (n=5).
Figure 5.1 C. The effect of Keyhole Limpet Hemocyanin on the TNF-α release in whole blood

Whole blood was incubated with a complex comprising Keyhole Limpet Hemocyanin (KLH) and rabbit anti-KLH for 0.5, 2, 4, 8, and 12 hours (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for TNF-α (C). Using an ELISA. Ab1=1:4 dilution of rabbit anti-KLH; Ab2=1:8 dilution of rabbit KLH; Ab1Ag or Ab2Ag= immune complexes constituted with 1:4 or 1:8 dilution of KLH (n=5).
5.1.2. IL-10

Due to the large standard deviations and the variation in IL-10 detection over time, no significant effect of the immune complexes on IL-10 production was detected (Figure 5.1B).

5.1.3. TNF-α

Both the Ab1Ag and the Ab2Ag immune complexes stimulated significant release of TNFα compared to non-stimulated cells (Figure 5.1C, Fishers post hoc test: \( p<0.0001 \) and \( p=0.0002 \) respectively). However, Ab1 and Ag alone also stimulated TNFα release which was significantly higher than in control cells (\( p=0.015; p<0.0001 \)) suggesting that the TNFα release seen with the immune complexes may not be complex-dependent.

5.2. Rabbit or goat anti-human IgG immune complex-stimulated cytokine release from whole blood.

Human blood was incubated for various times with rabbit or goat anti-human IgG or with rabbit or goat serum (as controls) to detect if the complexes formed \textit{in situ} stimulated the release of IL-6, IL-10 or TNF-α as described in Sections 2.7.1.2 and 2.7.1.3.

5.2.1. IL-6

Despite repetition on several occasions, the release of IL-6 by non-stimulated cells was always greater than any of the treated cells (Figure 5.2). This IL-6 production was switched off almost completely in the presence of immune complexes formed \textit{in situ} with both the higher and the lower concentrations of rabbit anti-human IgG (Table 5.2 shows the Fishers post hoc test (5% level): \( p<0.0001 \)). Rabbit serum at lower concentrations inhibited IL-6 production (Fishers post hoc test (5% level): \( p<0.0001 \)). IL-6 production was inhibited by goat anti-human IgG and goat serum at both concentrations over an 8-hour incubation period (Fishers post hoc test (5% level): \( p<0.0001 \)). RS1 caused the release of IL-6 at higher concentrations than the control at 4hrs whilst GS1 did not have the same effect until after 12 hours in culture.
Figure 5.2 A and B. The effect of rabbit and goat anti-human IgG on release of IL-6 from whole blood

Whole blood was incubated for 0.5, 2, 4, 8, 12, and 16 hours (37°C/5% CO₂) to form complexes in the blood using rabbit anti-human IgG (Fc specific; A) and goat anti-human IgG (Fc specific; B). The supernatant was analysed using ELISA. Two concentrations of anti-serum were used i.e.: 1:50(HR1 or HG1) and 1:100(HR2 or HG2). As a control, normal rabbit or goat serum (10μL) was added to the blood. Again two concentrations were used 1:50(RS1 or GS1) and 1:100(RS2 or GS2, n=5)
Table 5.2: Fishers post hoc analysis at 5% level for IL-6

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</table>

5.2.2. IL-10

Despite repetition on several occasions, the release of IL-10 by non-stimulated cells was always high, and in many cases higher than that of the treated cells (Figure 5.3). This IL-10 production was reduced in the presence of either rabbit or goat serum, the presence of the immune complexes formed in situ showing a trend towards reversing this inhibition, particularly after 8 hours incubation. IL-10 was significantly inhibited by rabbit serum at both concentrations (Table 5.3 shows the Fishers post hoc test (5% level): p=0.0093; p=0.0398) and rabbit anti-human IgG reversed this effect.

After 30 minutes incubation, all control cultures showed high levels of IL-10 production. This was decreased in the presence of either goat or rabbit serum but this effect was reversed by the presence of in situ formed complexes.
Figure 5.3 A and B. The effect of rabbit and goat anti-human IgG on release of IL-10 from whole blood

Whole blood was incubated for 0.5, 2, 4, 8, 12, and 16 hours (37°C/5% CO₂) to form complexes in the blood using rabbit anti-human IgG (Fc specific; A) and goat anti-human IgG (Fc specific; B). The supernatant was analysed using ELISA. Two concentrations of anti-serum were used i.e.: 1:50(HR1 or HG1) and 1:100(HR2 or HG2). As a control, normal rabbit or goat serum (10μL) was added to the blood. Again two concentrations were used 1:50(RS1 or GS1) and 1:100(RS2 or GS2, n=5).
Table 5.3: Fishers post hoc analysis at 5% level for IL-10

<table>
<thead>
<tr>
<th>Concentrations</th>
<th>Mean Diff</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C, HR1</td>
<td>27.607</td>
<td>0.2116</td>
</tr>
<tr>
<td>C, RS1</td>
<td>57.795</td>
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<td>HR1, RS1</td>
<td>30.188</td>
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<td>C, HR2</td>
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<tr>
<td>C, RS2</td>
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<tr>
<td>HR2, RS2</td>
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<tr>
<td>C, HG1</td>
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<tr>
<td>C, GS1</td>
<td>114.709</td>
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<tr>
<td>HG1, GS1</td>
<td>36.156</td>
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<tr>
<td>C, HG2</td>
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<tr>
<td>C, GS2</td>
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</tr>
<tr>
<td>HG2, GS2</td>
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<td>0.3824</td>
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5.2.3. TNF-α

Despite repetition on several occasions, the release of TNF-α by non-stimulated cells was not significantly different to the treated cells (Figure 5.4). Fisher’s post hoc analysis showed that over the time course examined there were significant differences in TNFα production between the control cells and those treated with either serum or in situ formed immune complexes. However, owing to the large standard deviations, none of the individual observations reached significance. However, the trend was for an increase in TNFα production with increasing time in culture.
Figure 5.4 A and B. The effect of rabbit and goat anti-human IgG on release of TNF-α from whole blood

Whole blood was incubated for 0.5, 2, 4, 8, 12, and 16 hours (37°C/5% CO₂) to form complexes in the blood using rabbit anti-human IgG (Fc specific; A) and goat anti-human IgG (Fc specific; B). The supernatant was analysed using ELISA. Two concentrations of anti-serum were used i.e.: 1:50(HR1 or HG1) and 1:100(HR2 or HG2). As a control, normal rabbit or goat serum (10μL) was added to the blood. Again two concentrations were used 1:50(RS1 or GS1) and 1:100(RS2 or GS2; n=5).
Table 5.4: Fishers post hoc analysis at 5% level for TNF-α

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<thead>
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<th>Concentrations</th>
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<td>C, RS1</td>
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</tr>
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</table>

5.3. Identification of the cells binding rabbit or goat anti-human IgG containing immune complexes in whole blood using flow cytometry

Whole blood was incubated with various dilutions of either rabbit or goat anti-human IgG as test samples and either rabbit or goat sera as controls. Cells were assayed by flow cytometry in an attempt to identify to which cell type the complexes were binding. As described in Section 2.7.1.4.

5.3.1. Analysis of adherence of complexes to lymphocytes

The results of the analysis are shown in Figure 5.5. Generally, the goat serum and the goat complexes bound to lymphocytes more effectively than the rabbit complexes or rabbit serum (Fishers post hoc test (5% level): p<0.0001). Additionally, the complexes (both rabbit and goat) bound to more lymphocytes than the serum alone (p<0.0001). The effect seemed to be more pronounced using a 1/20 dilution of the fluorescent second antibody.
Figure 5.5. Detecting rabbit or goat anti-human IgG on lymphocytes in whole blood

Rabbit or goat anti-human IgG and rabbit or goat serum were incubated for 0.5 hour (37°C/5% CO₂) in whole blood to form complexes. The rabbit or goat anti-human IgG (Fc specific) was added to whole blood (10μL/mL) at 1:50 and 1:100 dilution (HR1 or HG1 and HR2 or HG2). The rabbit or goat serum (10μL/mL) was added at 1:50 and 1:100 dilution (RS1 or GS1 and RS2 or GS2) respectively. The whole blood was then incubated with either 1:10 or 1:20 dilution of fluorescent antibody (10mins) and the red blood cells were lysed with 1x FACS lysing solution for 0.5 hour. Cells were analysed using a FACScan (n=5, 10,000 events).
(p<0.0001) with the goat immune complexes binding most cells at a 1 in 5000 dilution and the rabbit immune complexes showing optimal binding between the 1/1250 and 1/2500 dilutions.

5.3.2. Analysis of adherence of complexes to Monocytes

Adherence of immune complexes to human monocytes was most obvious using the 1/20 dilution of the fluorescent antibody (Figure 5.6, p<0.0001). At a dilution of 1/2500 there was a clear difference between the effect of the serum and the effect of the immune complexes on the monocyte, the complexes showing a significantly higher level of binding than the serum Alone (HRvRS: p<0.0001; HG v GS: p=0.0004) The higher level of binding with the rabbit complexes and serum might be expected since rabbit antibody will bind to human Fc receptors with greater ease than goat antibody.

5.3.3. Analysis of adherence of complexes to Neutrophils

The binding of HR and HG immune complexes to neutrophils was more evident using the 1/10 dilution of the FITC labelled second antibody (Figure 4.7; p<0.0001). At this dilution, a significant difference was seen between HR and RS at the 1/10000 and the 1/1250 dilution of immune complexes. However, the most dramatic effect was observed with HG versus GS at a dilution of 1/2500 (p<0.0001).

5.4. rHIV-1 gp120-containing immune complexes release of cytokines

Human blood was incubated for various lengths of time with the following pre-formed immune complexes: rabbit anti-serum to rHIV-1 MN gp120 and rHIV-1 MN gp120; sheep anti-serum to rHIV IIIB gp120 (CHO) and rHIV-1 IIIB gp120 (CHO). The presence of the cytokines IL-6, IL-10, and TNF-α in the supernatant of these cultures was assayed as described in Sections (2.7.1.5 and 2.7.1.6).
Detection of rabbit and goat anti-human IgG complexes on monocytes in whole blood using a 1/10 dilution of FITC conjugated antibody

Detection of rabbit and goat anti-human IgG complexes on monocytes in whole blood using a 1/20 dilution of FITC conjugated antibody

Figure 5.6. Detecting rabbit or goat anti-human IgG on monocytes in whole blood

Rabbit or goat anti-human IgG and rabbit or goat serum were incubated for 0.5 hour (37°C/5% CO₂) in whole blood to form complexes. The rabbit or goat anti-human IgG (Fc specific) was added to whole blood (10μL/mL) at 1:50 and 1:100 dilution (HR1 or HG1 and HR2 or HG2). The rabbit or goat serum (10μL/mL) was added at 1:50 and 1:100 dilution (RS1 or GS1 and RS2 or GS2) respectively. The whole blood was then incubated with either 1:10 or 1:20 dilution of fluorescent antibody (10mins) and the red blood cells were lysed with 1x FACS lysis solution for 0.5 hour. Cells were analysed using a FACScan (n=5, 10,000 events).
Detection of rabbit and goat anti-human IgG complexes on neutrophils on whole blood using a 1/10 dilution of FITC conjugated antibody

Detection of rabbit and goat anti-human IgG complexes on neutrophils on whole blood using a 1/20 dilution of FITC conjugated antibody

Figure 5.7. Detecting rabbit or goat anti-human IgG on neutrophils in whole blood
Rabbit or goat anti-human IgG and rabbit or goat serum were incubated for 0.5 hour (37°C/5% CO₂) in whole blood to form complexes. The rabbit or goat anti-human IgG (Fc specific) was added to whole blood (10µL/mL) at 1:50 and 1:100 dilution (HR1 or HG1 and HR2 or HG2). The rabbit or goat serum (10µL/mL) was added at 1:50 and 1:100 dilution (RS1 or GS1 and RS2 or GS2) respectively. The whole blood was then incubated with either 1:10 or 1:20 dilution of fluorescent antibody (10mins) and the red blood cells were lysed with 1x FACS lysing solution for 0.5 hour. Cells were analysed using a FACScan (n=5, 10,000 events).
5.4.1. Stimulation of IL-6 by HIV protein containing immune complexes in whole blood

Neither MN nor CHO antigens affected the constitutive release of IL-6 by whole blood. (Figures 5.8 A and B). By contrast, immune complexes containing these antigen significantly enhanced the release of IL-6 by whole blood (MN: p=0.005; CHO: p<0.0001). The increase was evident after 8 hours culture with the CHO-containing immune complexes but was only significant at 12 hours with the MN containing immune complexes.

5.4.2. Stimulation of IL-10 by HIV protein containing immune complexes in whole blood

Although relatively high levels of IL-10 were detected in the presence of either immune complex, similar levels were detected in untreated cells. Thus, there was no significant effect overall of the immune complexes on IL-10 release by whole blood (Figure 5.9 A and B)

5.4.3. Stimulation of TNF-α by HIV protein containing immune complexes in whole blood

Neither immune complex had a significant effect on the release of TNF-α by whole blood (Figure 5.10 A and B). However, this may be due to the fact that the supernatants were only analysed after 8 and 12 hours incubation. The slope of the graphs is negative suggests that TNF-α is possibly released earlier and may be switched off by the production of IL-6.
Figure 5.8 A and B. The released of IL-6 from complexes incubated with whole blood

The complexes rHIV-1 MN gp120 anti-HIV-1 gp120 (A) or rHIV-1 IIIB gp120 CHO anti-HIV-1 IIIB gp120 CHO (B) were incubated in whole blood for 8hrs and 12hrs (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for IL-6 using an ELISA.

Untreated=no complex added; AbAg=immune complex formed with a 1:4 dilution of antigen; Ag=antigen used at 1:4 dilution (n=5).
Figure 5.9 A and B. The released of IL-10 from complexes incubated with whole blood

The complexes rHIV-1 MN gp120 anti-HIV-1 gp120 (A) or rHIV-1 IIIB gp120 CHO anti-HIV-1 IIIB gp120 CHO (B) were incubated in whole blood for 8hrs and 12hrs (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for IL-10 using an ELISA.

Untreated=no complex added; AbAg=immune complex formed with a 1:4 dilution of antigen; Ag=antigen used at 1:4 dilution (n=5).
Figure 5.10 A and B. The released of TNF-α from complexes incubated with whole blood

The complexes rHIV-1 MN gp120 anti-HIV-1 gp120 (A) or rHIV-1 IIIB gp120 CHO anti-HIV-1 IIIB gp120 CHO (B) were incubated in whole blood for 8hrs and 12hrs (37°C/5% CO₂). After incubation the supernatant was harvested and analysed for TNF-α using an ELISA. Untreated=no complex added; AbAg=immune complex formed with a 1:4 dilution of antigen; Ag=antigen used at 1:4 dilution (n=5).
6.0. INVESTIGATION OF HIV-1 INFECTED CELLS
INCUBATED WITH PREFORMED IMMUNE COMPLEXES

6.1. HIV-infection of MM6 cells

In order to determine microscopically whether or not MM6 cells were being
productively infected with HIV-1_{Ba-L} (H9) and HIV-1_{RF} (H9), cells were incubated for ten days
with the virus and prepared as described in Sections 2.8.1.1 and 2.8.1.3. Figures 6.1 -6.3 show that both the Ba-L and RF strains of HIV have infected MM6 cells. A small number of
virus particles were detected in vacuoles as shown in Figures 6.2-6.3 suggesting a low level
of virus production. The level of p24 detected by KC57 monoclonal antibody showed a 50%
increase from day3 to day 5 but from day 5 the level of p24 remained the same until day 20
(Figures 6.5 and 6.6). Microscopically, virus particles could not be observed in infected cells
until day 10 of culture and the cells appeared similar to controls (Figure 6.1). In Figure 6.1, a
non-infected cell shows a normal nucleus with typical cytoplasmic inclusions. However, in
Figures 6.2A and B and 6.3 numerous large mitochondria can be seen as well as diverse
vesicles and large vacuoles containing virus and what appears to be the break down of the
nucleolus (Figure 6.3).

6.2. Surface antigen expression on HIV-1 infected of MM6 and THP-1
cell lines

An initial study was performed using the MM6 and THP-1 cell lines which were
incubated for 10 days with HIV-1_{Ba-L} (grown in H9; 200μl) and HIV-1_{RF} (grown in THP-1;
100μl) as described in Sections 2.8.1 and 2.8.1.1. Surface antigen expression on the cell
lines was analysed using flow cytometry as described in Section 2.8.1.1.

6.2.1. CD16

Less than 10% of MM6 cells and about 10% of THP1 cells expressed the CD16
(FcγRIII) antigen. When infected with HIV of either strain, both cell lines showed a
Figure 6.1. A transmission electron micrograph of a control MM6 cell

MM6 cells were incubated for 10 days (37°C/5% CO₂) then they were fixed using 2.5% glutaraldehyde, post-fixed with 4% glutaraldehyde buffer, and dehydrated through an ethanol series. Samples were embedded in Epon 812 resin and ultrathin sections were cut and stained using lead citrate and uranyl acetate. Examination by transmission electron microscopy indicated that cells contained a relatively large, lobate nucleus (N) that often contained a prominent nucleolus (NE). A thin layer of heterochromatin (H) typically surrounded the nucleus. Few vesicles such as lysosomes (L) and mitochondria (M) could be identified in the cytoplasm of most cells, and endoplasmic reticulum (ER) was sparse (x 6 000).
Figure 6.2 A. A transmission electron micrograph of a MM6 cell infected with HIV-1 Ba-L.

MM6 cells were incubated with HIV-1 Ba-L for 10 days (37°C/5% CO₂) and exponentially replicating cells were fixed using 2.5% glutaraldehyde, post-fixed with 4% glutaraldehyde buffer, and dehydrated through an ethanol series. Samples were embedded in Epon 812 resin and ultrathin sections were cut and stained using lead citrate and uranyl acetate. Examination by transmission electron microscopy indicated that cells contained many relatively large, vacuoles (VO), lysosomes (L), mitochondria (M), endoplasmic reticulum (ER) and virus (V) could be identified in the cytoplasm of cells shown (A x 36 000).
MM6 cells were incubated with HIV-1 Ba-L for 10 days (37°C/5% CO₂) and exponentially replicating cells were fixed using 2.5% glutaraldehyde, post-fixed with 4% glutaraldehyde buffer, and dehydrated through an ethanol series. Samples were embedded in Epon 812 resin and ultrathin sections were cut and stained using lead citrate and uranyl acetate. Examination by transmission electron microscopy indicated that cells contained many relatively large, vacuoles (VO), lysosomes (L), mitochondria (M), Golgi vesicles (G), endoplasmic reticulum (ER) and virus (V) could be identified in the cytoplasm of cells shown (x 36 000).
MM6 cells were incubated with HIV-1 \textsubscript{RF} for 10 days (37°C/5% CO\textsubscript{2}) exponentially replicating cells were fixed using 2.5% glutaraldehyde, post-fixed with 4% glutaraldehyde buffer, and dehydrated through an ethanol series. Samples were embedded in Epon 812 resin and ultrathin sections were cut and stained using lead citrate and uranyl acetate. Examination by transmission electron microscopy indicated that cells contained many large, vacuoles (VO), lysosome vesicles (L), mitochondria (M), endoplasmic reticulum (ER), virus (V) and nuclei material (NM) could be identified in the cytoplasm of cell shown (x \textsubscript{6000}).
significant increase in the expression of this antigen (Figure 6.4). However, the HIV-1$_{Ba-L}$
had a more profound effect than the RF strain (Fishers post hoc test (5% level): $p<0.0001$).

6.2.2. CD80

Less than 10% of MM6 cells and fewer than 5% of THP1 cells expressed the T cell
co-stimulatory antigen CD80. When infected with HIV (Ba-L), both cell lines showed a
significant increase in the expression of this antigen (Figure 6.4). A similar effect was seen
with the RF strain but the effect was greater on THP-1 cells than on MM6 cells (Fishers post
hoc test (5% level): $p<0.0001$).

6.2.3. CD11c

Approximately 10% of both MM6 and THP1 cells expressed the CD11c antigen
constitutively (Figure 6.4). When infected with either strain of HIV, both cell lines showed a
highly significant increase ($p<0.0001$) in the expression of this antigen (Figure 6.4).
However, the effect on THP1 cells was greater than that on MM6 cells (Fishers post hoc
test (5% level): $p<0.0001$).

6.2.4. CD23

Approximately 15% and 8% respectively for both MM6 and THP1 cells expressed the CD23 antigen
constitutively (Figure 6.4). When infected with either strain of HIV, both cell
lines showed a highly significant increase ($p<0.0001$) in the expression of this antigen
(Figure 6.4).

6.2.5. CD14

Approximately 52% MM6 cells and 2% THP1 cells expressed the CD14 antigen
constitutively (Figure 6.4). Infection with either strain of HIV had a highly significant effect on
both cell lines (Fishers post hoc test (5% level): $p<0.0001$). However, the effect on THP1
cells was to increase CD14 expression whilst that on MM6 cells was the opposite.
Figure 6.4. Antigens expression of HIV infected MM6 and THP-1 cells

MM6 and THP-1 cells were incubated for 10 days with HIV-1_{Ba-L} (H9; 200μL) and HIV-1_{RF} (THP-1; 100μL) then incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The cells were analysed using a FACSscan (n=5, 10,000 events).
6.2.6. HLA-DR

Approximately 40% and 5% respectively for both MM6 and THP1 cells expressed the HLA-DR antigen constitutively (Figure 6.4). When infected with either strain of HIV, both cell lines showed a highly significant increase (p<0.0001) in the expression of this antigen (Figure 6.4). However, the Ba-L strain on THP1 cells was greater than that on MM6 cells (Fishers post hoc test (5% level): p<0.0001).

6.2.7. KC57

Approximately 5% and 2% to MM6 and THP1 cells respectively were positive for p24 antigen due to non-specific binding of the antibody (Figure 6.4). When infected with either strain of HIV, both cell lines showed a significant increase in the expression of this antigen, which was highly significant for THP1 cells (p<0.0001; Figure 6.4).

6.3. Kinetics of HIV-1 induced alteration in cell surface antigen expression on MM6 and THP-1 cell lines

Having established that HIV has an effect on the expression of a range of surface antigens, the time after infection at which this change occurs was examined. The MM6 and THP-1 cell lines were incubated for 3, 5, 10, and 20 days with either HIV-1\textsubscript{Ba-L} (H9; 100µL) or HIV-1\textsubscript{RF} (H9; 100µL) as described in Sections 2.8.1 and 2.8.1.1. Surface antigen expression on cell lines was analysed using flow cytometry.

6.3.1. CD16

At day three, infection with the Ba-L strain eliminated the expression of CD16 on MM6 cells (Figure 6.5-6.6). However, a very high proportion of cells were expressing CD16 by day 5, which remained high at day 10 but decreased by day 20. A similar time dependent effect was observed with the RF strain. That an increased percentage of cells expressed the antigen at day 3 compared to control cells.
Figure 6.5. Antigens on HIV infected MM6 cells

MM6 and THP-1 cells were incubated for 3 and 5 days with HIV-1\textsubscript{Ba-L} (H9; 100\mu L) and HIV-1\textsubscript{RF} (H9; 100\mu L) then incubated with a range of monoclonal antibodies for one hour in the dark (4\degree C). The cells were analysed using a FACSscan (n=5, 10,000 events).
Figure 6.6. Antigens on HIV infected MM6 cells

MM6 and THP-1 cells were incubated for 10 and 20 days with HIV-1 Ba-L (H9; 100μL) and HIV-1 RF (H9; 100μL) then incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The cells were analysed using a FACScan (n=5, 10,000 events).
In THP1 cells, both virus strains showed a similar time dependent effect on CD16 expression but the increase observed on day 3 was significantly greater with the RF strain than with the Ba-L strain (Figure 6.7-6.8).

6.3.2. CD80

CD80 expression was virtually eliminated by infection with either strain in MM6 cells. This expression recovered, reaching control levels by day 10 and being significantly increased by day 20 (Figure 6.5-6.6).

Very few THP1 cells naturally express CD80. At day 3, HIV infection did not affect this expression (Figure 6.7-6.8). At day 5, an increased percentage of control and test cells exhibited CD80 but this did not reach significance. By day 10, a small but significant proportion of RF infected cells expressed CD80 that increased by day 20. The Ba-L strain also caused an increase by day 10 that reached significance by day 20.

6.3.3. CD11c

Infection of MM6 cells with either strain of HIV severely reduced the percentage of cells expressing CD11c (Figure 6.5-6.6). This effect was reversed by day 5, with both strains showing a significant increase in the percentage of cells expressing CD11c (the effect being most profound with Ba-L). The effect was maximal around day 10 with the percentage of cells expressing CD11c, decreasing by day 20.

In THP1 cells, the percentage expressing CD11c was significantly increased by day 3 in culture (Figure 6.7-6.8). This reached a maximum around day 5 which was sustained until day 10 with the RF strain but which started to decline by day 10 with the Ba-L strain. Both strains showed a continued decrease by day 20, again the effect being more profound with the Ba-L strain.

6.3.4. CD23

Infection of MM6 cells with either strain of HIV severely reduced the percentage of cells expressing CD23 (Figure 6.5-6.6). Although by day 5, CD23 could be detected on virally infected cells and this proportion increased with time in culture, the control cell
Figure 6.7. Antigens expression of HIV infected THP-1 cells

THP-1 cells were incubated for 3 and 5 days with HIV-1<sub>Ba-L</sub> (H9; 100μL) and HIV-1<sub>RF</sub> (H9; 100μL) then incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The cells were analysed using a FACScan (n=5, 10,000 events).
Figure 6.8. Antigens expression of HIV infected THP-1 cells

THP-1 cells were incubated for 10 and 20 days with HIV-1_{Ba-L} (H9; 100\muL) and HIV-1_{RF} (H9; 100\muL) then incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The cells were analysed using a FACScan (n=5, 10,000 events).
expression of CD23 also increased. This means that the level of expression of this antigen in virally infected cells remained significantly lower than in control cells throughout the experiment.

The percentage of THP1 cells expressing CD23 was significantly higher on those infected with HIV than on control cells after 3 days in culture (Figure 6.7-6.8). Expression reached a maximum by day 10 that was sustained throughout the experiment. The RF strain was most effective at inducing CD23 expression particularly from day 10 onward.

6.3.5. CD14

Infection of MM6 cells with either strain of HIV dramatically decreased the percentage of cells expressing CD14 during the early stages of culture (Figure 6.5-6.6). However, by day 20 of culture, virus-infected cell expression of CD14 had recovered considerably.

The percentage of THP1 cells expressing CD14 was significantly increased in virus infected cells after only 3 days in culture (Figure 6.7-6.8). This effect reached a maximum around day 5 and was much more pronounced with the RF strain.

6.3.6. HLA-DR

Infection of MM6 cells with either strain of HIV severely reduced the percentage of cells expressing HLA-DR (Figure 6.5-6.6). This effect was maximal after 3 days in culture with expression only returning to near normal levels by day 20 of culture.

In THP1 cells, HLA-DR could not be detected in non-infected cells but a small percentage of cells in infected cultures showed expression of this antigen (Figure 6.7-6.8). Expression reached a maximum around day 10 and was particularly evident with the RF strain.

6.3.7. KC57

Infection of MM6 cells with either strain of HIV dramatically increased percentage of cells expressing p24 antigens (Figure 6.5-6.6). After only 3 days in culture, approximately 50% of MM6 cells were positive for p24. By day 5, this had reached almost 100%. Both strains seemed equally effective.
By contrast, a p24 antigen (Figure 6.7-6.8) was not detected in THP1 cells until day 5 when 100% of cells were positive. Again, both virus strains were equally effective in infecting the cells.

6.4. Effect of immune complexes on HIV-1 infected monocyte-derived macrophage surface antigen expression.

Human monocyte-derived macrophages were infected with HIV-1$_{Ba-L}$(H9) for 5 days and subsequently incubated with either rabbit, goat or rHIV-1 IIIB gp120 (CHO) containing immune complexes as described in Section 2.9.1.

Immune complexes containing human IgG and either rabbit or goat IgG were formed by incubating rabbit and goat anti-human IgG (50μl/ml of 1/50 dilution) in human blood for 30 minutes. The blood was centrifuged (800g; 5mins) and the serum (which should contain the rabbit and goat complexes) collected and used as described in Section 2.9.1. The surface antigen expression was examined using flow cytometry. In general, owing to the large standard deviations recorded, there was no apparent effect of immune complexes on surface antigen expression in virus infected cells at the times examined (Figures 6.9 - 6.11).

6.5. Effect of immune complexes on cytokine release by HIV-1 infected monocyte-derived macrophages.

Human monocyte-derived macrophages were infected with HIV-1$_{Ba-L}$(H9) for 5 days and subsequently incubated with rHIV-1 IIIB gp120 (CHO), rabbit and goat complexes as described in Section 2.9.1.

6.5.1. rHIV-1 IIIB gp120 (CHO) containing complexes

The presence in culture for 8 hours of HIV protein-containing immune complexes led to a slight but significant increase in IL-6 secretion. This effect was lost in HIV infected cells (Figure 6.9 and Figure 6.12). By 12 hours culture, this effect of the immune complexes was no longer evident but non-infected cells showed much higher release of IL-6 than infected cells (Figure 6.9; Fishers post hoc: p<0.0001).
Detection of surface antigen expression on monocyte-derived macrophages infected with HIV-1 Ba-L and incubated with rHIV-1 IIIB gp120(CHO) complex for 8hrs

Detection of surface antigen expression on monocyte-derived macrophages infected with HIV-1 Ba-L and incubated with rHIV-1 IIIB gp120(CHO) complex for 12hrs

Figure 6.9. The expression of surface antigen on monocyte-derived macrophages incubated with rHIV-1 IIIB gp120 (CHO) complex

Monocyte-derived macrophages were infected with HIV-1Ba-L (H9; 100μL) for 5 days, 50μL RPMI-1640 medium was added to control and 50μL rHIV-1 IIIB gp120 (CHO) complex to all test wells. Plates were incubated for 8hrs, and 12hrs (37°C/5% CO₂) and the cells incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The surface antigen expression was analysed using the FACScan. Controls cells were treated with FITC/PE dual labelled monoclonal antibodies isotype controls. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. All results were gated using these (n=5, 10,000 events).
Detection of surface antigen expression on monocyte-derived macrophages infected with HIV-1Ba-L and incubated with rabbit anti-human IgG complex for 8hrs

Detection of surface antigen expression on monocyte-derived macrophages infected with HIV-1Ba-L and incubated with rabbit anti-human IgG complex for 12hrs

Figure 6.10. The expression of surface antigen on monocyte-derived macrophages incubated with rabbit anti-human complex

Monocyte-derived macrophages were infected with HIV-1Ba-L (H9; 100µL) for 5 days, 50µL RPMI-1640 medium was added to control and 50-µL rabbit anti-human complex to all test wells. Plates were incubated for 8hrs, and 12hrs (37°C/5% CO₂) and the cells incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The surface antigen expression was analysed using the FACScan. Controls cells were treated with FITC/PE dual labelled monoclonal antibodies isotype controls. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. All results were gated using these (n=5, 10,000 events).
Figure 6.11. The expression of surface antigen on monocyte-derived macrophages incubated with goat anti-human complex

Monocyte-derived macrophages were infected with HIV-1 Ba-L (H9; 100μL) for 5 days, 50μL RPMI-1640 medium was added to control and 50-μL goat anti-human complex to all test wells. Plates were incubated for 8hrs, and 12hrs (37°C/5% CO₂) and the cells incubated with a range of monoclonal antibodies for one hour in the dark (4°C). The surface antigen expression was analysed using the FACScan. Controls cells were treated with FITC/PE dual labelled monoclonal antibodies isotype controls. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. All results were gated using these (n=5, 10,000 events).
Figure 6.12. The detection of cytokines from HIV infected monocyte-derived macrophage incubated with rHIV-1 IIIB gp120 (CHO) complex

Monocyte-derived macrophages were infected with HIV-1 Ba-L (H9, 100μL) for 5 days. 50μL RPMI-1640 medium was added to control and 50μL rHIV-1 IIIB gp120 (CHO) complex to all test samples which were and incubated for 8hrs and 12hrs (37°C/5% CO2). The release of IL-6, IL-10, and TNF-α was detected in the supernatant using ELISA. Cell treatment:
M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex (n=7).
After eight hours in culture, the presence of HIV-protein containing immune complexes significantly increases the release of IL-10 by both infected and non-infected cells (Figure 6.12; Fishers post hoc: p<0.0001). This effect remains evident at 12 hours (Figure 6.12, Fishers post hoc: p<0.0001).

Little TNF-α was detected after incubation with HIV-protein containing immune complexes for 8 hours (Figure 6.12). After 12 hours incubation, TNFα production is greater from infected cells than non-infected but the presence of immune complexes seems to inhibit this release (Figure 6.12).

6.5.2. Rabbit immune complexes

In general, rabbit immune complexes had no significant effect on cytokine release from monocyte-derived macrophages incubated for 5 days with or without HIV (Figure 6.10 and Figure 6.13). After incubation for 8 hours, IL-10 could be detected in all cultures at the same level (Figure 6.13). IL-6 and TNFα could barely be detected. After 12 hours however, a significant increase in IL-6 release by non-infected cells (compared with 8 hours) could be detected (Figure 6.13). Also, the level of IL-6 present in the supernatant of HIV infected, HIV complex-containing cultures were greater than that of cultures containing HIV alone. No difference in TNFα levels was observed.

6.5.3. Goat immune complexes

In general, goat immune complexes had no significant effect on cytokine release from monocyte-derived macrophages incubated for 5 days with or without HIV (Figures 6.11 and Figure 6.14).


Human monocyte-derived macrophages were infected with HIV-1Ba-L (H9) for 5 days and subsequently incubated with HIV+ sera as described in Section 2.9.1.1. Surface antigen expression was assayed using flow cytometry. Owing to the large standard deviations
Detection of cytokines released from monocyte-derived macrophages infected with HIV-1 Ba-L and incubated with rabbit anti-human IgG complexes 8hrs

![Graph showing cytokine concentrations](image)

Factors

Detection of cytokines release from monocyte-derived macrophage infected with HIV-1 Ba-L and incubated with rabbit anti-human IgG complexes for 12hrs

![Graph showing cytokine concentrations](image)

Factors

Figure 6.13. The detection of cytokines from HIV infected monocyte-derived macrophage incubated with rabbit anti-human IgG complex

Monocyte-derived macrophages were infected with HIV-1Ba-L (H9; 100μL) for 5 days then RPMI-1640 medium (50μL) was added to control wells and 50μL rabbit anti-human IgG complex to all test wells and the plates were incubated for 8hrs and 12hrs (37°C/5% CO₂). The release of IL-6, IL-10, and TNF-α from supernatant was detected using an ELISA. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. Results represent the mean and standard deviation with n=7.
Figure 6.14. The detection of cytokines from HIV infected monocyte-derived macrophage incubated with goat anti-human IgG complex

Monocyte-derived macrophages were infected with HIV-1Ba-L (H9; 100μL) for 5 days then RPMI-1640 medium (50μL) was added to control wells and 50μL goat anti-human IgG complex to all test wells and the plates were incubated for 8hrs and 12hrs (37°C/5% CO₂). The release of IL-6, IL-10, and TNF-α from supernatant was detected using an ELISA. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. Results represent the mean and standard deviation with n=7.
Figure 6.15. Surface antigen expression on HIV infected monocyte-derived macrophage incubated with HIV-sera
Monocyte-derived macrophages were infected with HIV-1 Ba-L (H9; 100μL) for 5 days, 50μL RPMI-1640 medium was added to control and 50μL HIV-sera to all test wells. Plates were incubated for 8 hrs, and 12 hrs (37°C/5% CO₂) and the cells incubated with a range of monoclonal antibodies for one hour in dark (4°C). The surface antigen expression was analysed using the FACScan. Control cells were treated with FITC/PE dual labelled monoclonal antibodies isotype controls. Cell treatment: M=control uninfected; MC=with complex; MI=HIV-infected; MIC=HIV-infected with complex. All results were gated at 2500 events (n=5).
observed, it was not possible to identify any effect of HIV serum on surface antigen expression (Figure 6.15).

6.7. Effect of serum from HIV+ individuals on cytokine production by HIV-1 infected monocyte-derived macrophages.

Human monocyte-derived macrophages were infected with HIV-1_{Ba-L} (H9) for 5 days and subsequently with HIV-sera as described in Section 2.9.1.1. The supernatants were analysed using ELISA. After 8 hours, IL-6 and TNFα could not be detected in the supernatants from either infected or non-infected cells (Figure 6.16). By contrast, IL-10 was clearly detectable in all cultures.

After 12 hours, IL-6 could be detected in all cultures (Figure 6.16). Whilst the level produced by non-infected cells was the same regardless of the presence of HIV serum, in HIV infected cells the presence of serum appeared to increase IL-6 production (although this just failed to reach significance). IL-10 was present in all cultures at significantly higher levels than after 8 hours. The presence of HIV+ sera in the HIV infected cultures appeared to cause a significant increase in IL-10 production.

TNFα production was detected in control, non-treated cells that disappeared in serum-treated cells. No TNFα production was detected in HIV infected cells.
Detection of cytokine release from monocyte-derived macrophages infected with HIV-1 Ba-L and incubated with HIV-sera for 8hrs

![Graph showing cytokine levels](image1)

Factors: M, MC, MI, MIC

IL-6, IL-10, TNF-alpha

Detection of cytokine released from monocyte-derived macrophages infected with HIV-1 Ba-L and incubated with HIV-sera for 12hrs

![Graph showing cytokine levels](image2)

Factors: M, MC, MI, MIC

IL-6, IL-10, TNF-alpha

Figure 6.16. Detecting cytokines released from HIV-infected monocyte-derived macrophage incubated with HIV+ sera

Monocyte-derived macrophages were infected with HIV-1 Ba-L (H9; 100μL) for 5days, 50μL RPMI-1640 medium was added to control and 50μL HIV+sera to all test wells. Plates were incubated for 8hrs, and 12hrs (37°C/5% CO₂). The supernatant was analysed for IL-6, IL-10, and TNF-α using the appropriate ELISA (n=5).
7.0. DISCUSSION

7.1. Introduction

The original aim of this project was to investigate the role of CD80 in HIV infection. It was based on the observations that patients with full-blown AIDS are unable to develop secondary immune responses. It is known that antigen presentation without appropriate costimulation through CD80 leads to anergy and failure to produce memory cells. It was thought possible that failure to produce appropriate cytokines (due to a tendency to mount a TH2-type response) may prevent appropriate CD80 expression in HIV-infected individuals.

To this end, an attempt was made to establish antigen specific T cell lines to be used in examine antigen presentation. Also, the effect of cytokines on monocyte surface antigen was investigated in order to understand their role in modulating the functional characteristics of these cells.

7.1.1. Development of antigen specific T cell lines

The classical pathway used by other researchers for setting up antigen specific T cells lines, did not succeed in the present study as it resulted in a limited production of T cell blasts. Several techniques were used to obtain these antigen specific T cells, which ended with the same disappointing results. Discontinuous and continuous stimulation protocols used were with PPD or BCG and with various concentration of IL-2. No definitive effect was observed. Therefore, a different technique was tried using PBMNC as feeder cells that were incubated with 30U/ml IL-2 to stimulate T cell blasts. Unfortunately, the cells died which were possibly due to apoptosis or bacterial infection.

Other researchers have been able to establish antigen specific T cell lines using PPD and live BCG (Pancholi et al., 1992; Fazal et al., 1995). There are several possible explanations why antigen specific T cell lines were not obtained. Firstly, the initial stimulation was not strong enough to give adequate endogenous IL-2 production. Secondly, not enough IL-2 was present during resting phase. Thirdly, the concentration of antigen was not high enough to stimulate antigen specific T cell proliferation. The presence of antigen presenting cells (APC) is vital for IL-2 production (Lombardi et al., 1994; Tormey et al., 1997) but feeder cells such as the EBV B-transformed cells used in this study may not produce IL-2 although they may act as presenting cells. Sander et al., (1995) studied the
production of Th1 and Th2 cells in response to live BCG and detected that stimulating peripheral blood mononuclear cells with BCG results in the production of fourteen cytokines including IL-2. It was found that 2% v/v IL-2 was detected from day 2 to day 10. Other studies indicated those IL-2 peaks first at days 4-5 and again at days 7-8 allowing restimulating of T cell clones and up-regulating B7 expression. This suggested that enough IL-2 was present to stimulate an initial antigen-specific reaction (Sansom and Hall, 1993; Wyss-Coray et al., 1993; Sander et al., 1995).

There is a possibility that stimulated cytotoxic T cells could inhibit a BCG specific response by destroying infected antigen presenting cells. Turner and Dockrell, (1996) showed that PBMC incubated with live BCG for 7-days activated CD8+ T cells which showed cytolytic activity against target cells infected with live mycobacterium. It is known from previous work by Rook et al., (1985) that BCG grows poorly in RPMI 1640 medium and also is inhibited by human serum at concentrations of >5% v/v but this contrast to the current study which indicates no effect on the cells responding to live BCG.

The use of IL-2 concentrations greater then 10U/ml during the resting phase improved T cell proliferation as shown in Section 3.1. The study by Cohen et al., (1996) demonstrated that incubating human PBMC with IL-2 (with or without IL-4) yielded predominantly Th1-type clones. These Th1 clones induced proliferation of both naïve CD45RBhi and memory CD45RBloCD4+ T cells which are B7 dependent and the addition of IL-2 (20-100U/ml) to the culture prevented the hyporesponsiveness of B7- transfected cells (Ding and Shevach, 1994).

The concentrations of PPD or live BCG in the current study were similar to those used by others. However, no dose effect was observed (Figure 3.1,A and B). This maybe due to the induction of high or low dose although this seems unlikely. The response may not be antigen-dependent but may be due to defective antigen presentation (Fidler et al., 1996; Khalili et al., 1997,). Other antigens that are strong stimulators have been used to produce T cell lines. Stimulators such as Phaseolus vulgaris agglutinin (PHA), tetanus toxoid (TT), Concanavalin A (Con A), and mouse anti-human CD3 monoclonal antibody (OKT3, Keystone et al., 1993; LI et al., 1995; Thomssen et al., 1995; Fazal et al., 1995; Cohen et al., 1996). In response to Con A, activated T cells show increased expression of CD80 (B7-1) and CD86 (B7-2) molecules. The blocking of both molecules completely suppresses proliferation and IL-2 production. These molecules individually play a different role in proliferation and cytokine production as anti-CD86 Fab partially inhibited the Con A
stimulated response whereas anti-CD80 Fab had no effect. Therefore, CD86 is required in part by Con A while other stimulators require both CD80 and CD86 (Perrin et al., 1997).

One important possible difference between the present study and work done by others is the choice of donors. Other studies used subjects who were BCG-vaccinated. In the present study, the immune status of the individual was unknown. Although the cells from individual donors were stimulated by PPD and BCG the response was not a constant gradual increase in T cell numbers (Section 3.1) as shown by others. One other possible reason for the lack of T cell line establishment is that the environment was not compatible.

7.2. Cellular differentiation and proliferation

Human T cells subsets, T helper 1 (Th1) and Th2 cells produce cytokines originally described from mouse CD4+ T-cell clones (Mosmann et al., 1986; Cherwinski et al., 1987; Del Prete et al., 1991). The subsets produce different cytokines with Th1 cells producing interleukin 2 (IL-2), interferon γ (IFN-γ) and tumour necrosis factor α (TNF-α) and Th2 cells producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Both subsets secrete IL-3, tumour necrosis factor α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and members of the chemokine (CK) families. Th0 is a precursor cell that expresses both patterns of cytokines (Sad and Mosmann, 1994). Th2-type cytokines encourage antibody production and are associated with strong antibody and allergic responses (Mosmann and Coffman, 1989). Antibodies against CD80 or CD86 on APCs inhibit the development of Th1 and Th2 responses both in vitro and in vivo which influence T-cell differentiation (Thompson, 1995). The differentiation of Th1 cells is stimulated by moderate doses of antigen, whereas Th2 cells are stimulated by a wider range of concentrations and a higher dose of antigen may switch the immune response from Th1 to Th2 (Fitch et al., 1993; Hosken et al., 1995).

To understand the effect of Th1 and Th2 cytokines on monocyte-derived macrophages (MDM) and the immature monocyctic cell line, Mono Mac 6 (MM6) cells were incubated in vitro with cytokines and chemical stimulants to examine differentiation, proliferation and surface antigen expression. The chemical stimulants PMA and Vit D3 both cause the differentiation of MDM and MM6 cells. PMA is highly potent, especially at higher concentrations. Differentiation, evidenced by the inhibition of cellular proliferation, was caused by both chemical stimulants but Vit D3 may have been toxic at the concentrations
tested. IFN-γ, a Th1-type cytokine, induced the differentiation of MM6 cells thus inhibiting cellular proliferation. This may have been partly due to a toxic effect in this study. The synthesis of IFN-γ is known to be enhanced by IL-2, IL-12 and IL-4 whereas IL-10 inhibits Th1 cytokine synthesis (Fiorentino et al., 1989; Seder and Paul, 1994; Trinchieri et al., 1997).

Smith et al., (1998) showed that M-CSF (at higher concentrations than those used in the current study) induced monocytes to differentiate into macrophages resulting in down-regulation of IL-12 which is important for Th1 cell differentiation. The current study showed that M-CSF caused a decrease in MM6 cellular proliferation that may in part be due to toxicity but may be due to M-CSF inducing cellular differentiation toward a mature phenotype. GM-CSF, a Th2-type cytokine caused cellular differentiation and the decrease in proliferation at the higher concentrations used. Eischen et al., (1991) showed that 5ng/ml GM-CSF caused monocytes to differentiated into macrophages and promoted better cell survival in a dose dependent manner. The Th2-type cytokines IL-6, IL-10 and TNF-α caused differentiation and decreased cellular proliferation but this was not obvious due to the low growth rate found in this study. These results demonstrated that both Th1- and Th2-type cytokines and chemical stimulants used caused the differentiation of MDM and MM6 cells. Most of the stimulants used appeared to increase cell death as well as inhibit cellular proliferation. This may be due to deactivation of these monocytic cell lines initiating apoptosis (Heidenreich et al., 1997).

7.2.1. Surface antigen expression on differentiated cells

7.2.1.1. CD16

An increase in CD16 receptor expression has been shown to be associated with monocyte differentiation. This surface antigen was increased by all cytokines and stimulators used but particularly by IL-6, IL-10 and TNF-α (Section 4.2.2.1). The effect of these cytokines was obvious at day 3 and levels decreased on days 5 and 7. Allavena et al., (1998) showed that incubating human monocytes for 3 days with IL-10 (20ng/ml) induced high levels of CD16 but this expression declined thereafter. This was also observed by the use of a lower concentration of IL-10 (1ng/ml) in present study. Incubating human monocytes with IFN-γ and GM-CSF using different units of concentration, from the one used
in present study, showed a dose dependent decrease in CD16 expression (Olikowsky et al., 1997; Kruger et al., 1996).

These results suggest that MM6 cells are less responsive to the effects of the stimulants used than peripheral blood monocytes. The later time of maximum expression suggests that the immature MM6 cells may have needed to differentiate before expression could be enhanced and that this may have depleted levels of stimulant resulting in a lower level of expression of the surface antigen. Or that MM6, being a transformed cell line, may not be able to express normal levels of the antigen. This indicates that CD16 expression is influenced in particular by Th2-type cytokines and is dose dependent.

7.2.1.2. CD80

CD80, a costimulatory molecule is present on antigen presenting cells (Lenschow et al., 1996). This surface antigen is expressed at very low levels on monocytes but its expression is greater on stimulated, fully differentiated macrophages. Expression on blood monocytes was strongly inhibited by PMA (p<0.0001) and significantly increased at day 3 by the Th2-type cytokines IL-6 (p<0.0001 day 3 v day 7) and IL-10 and by TNF-α (Section 4.2.2.2) but the level decreased on days 5 and 7. Other have shown that in resting CD14+ monocytes high concentrations of IL-6 (50ng/ml), TNF-α(40ng/ml) and GM-CSF (50ng/ml) did not alter CD80 expression (Creery et al., 1996). In the current study low concentration IL-6, and TNF-α, increased CD80 expression but GM-CSF was unaltered. Other, have show that IL-10 moderately increased CD80 expression as observed in this current study (Creery et al., 1996).

MM6 cells showed a lower expression of CD80 than peripheral blood monocytes, which was significantly increased by TNF-α and the Th2-type cytokines GM-CSF, IL-6, and IL-10. The expression reached a maximum at day 5 for IL-10 and TNF-α but reached a maximum for IL-6 at day 7. These results indicate that MM6 cells require a greater exposure time compared to peripheral blood monocytes suggesting that the immature MM6 cells may need to differentiate before expression be enhanced. This may have depleted the level of the stimulators resulting in lower levels of expression of the surface antigen. The results suggest that a range of cytokines may affect CD80 expression on monocytes but that the effect is not as great on the immature MM6 cells.
7.2.1.3. CD11c

CD11c/CD18 is an adhesion molecule, which allows contact between cells and between cells and the matrix (Dustin and Springer, 1991). This surface antigen is expressed at high levels on monocytes but its expression is greater on fully differentiated macrophages. PMA, VitD3 and IFN-γ were found to inhibit the high constitutive expression of CD11c after 3 days incubation. Krugluger et al., (1997) demonstrated that blood monocytes incubating for 3 days with IL-6, IL-10, TNF-α and Vit D3 resulted in decreased CD11c expression. In the current study, which used different concentrations of IL-6, IL-10, and TNF-α to the previous study, a decrease in CD11c expression was seen on day 5 and with Vit D3 on day 3. It seems that CD11c expression is affected only moderately by stimulants (with the exception of PMA) on MDM and that both Th1- and Th2-type cytokines are able to maintain this expression.

PMA, which is potent in inducing differentiation, rapidly increased expression of CD11c on MM6 cells (p<0.0001). IL-6 showed a similar pattern to PMA but maximum expression of CD11c appeared at day 5 and then declined. Vit D3 and GM-CSF30 respectively increased and decreased CD11c on MM6 cells with maximum expression on day 3 (p<0.0047 and p<0.0173) declining thereafter. However the effect of TNF-α and IL-10 on CD11c expression was maximal at day 5 (p<0.0001 for TNF-α and IFN-γ; p<0.0129 for IL-10).

7.2.1.4. CD23

CD23 is expressed at low levels on monocytes but its expression is greater on fully differentiated macrophages. In the present study, peripheral blood monocytes expressed low levels of CD23 which was increased by IFN-γ (p<0.0001), and GM-CSF (p<0.0001) by day 3 and decreased thereafter. Alderson et al., (1992) detected an increase in CD23 expression at early as 24 to 48 hours after incubating human monocytes with GM-CSF (10-30ng/ml). This slightly declined at 72 and 96 hours as observed in current study. The low concentration of GM-CSF (1ng/ml; p<0.0001) dramatically increased CD23 on day 7 as observed in the present study. IL-6 and IL-10 (p<0.0001) caused an increase in CD23 expression. Other studies which used different concentrations did not show this increase after 3 days (Willheim et al., 1991, Spittler et al., 1995). TNF-α also causes an increase that like IL-6 and IL-10 declined by days 5 and 7.
MM6 cells expressed low levels of CD23. Its expression, which was increased by M-CSF, GM-CSF and IFN-γ (p<0.0001), reached a maximum at day 5 then declined. IL-6, IL-10 and TNF-α (p<0.0001) all inhibited CD23 expression on days 3 and 5 in the present study. Other researchers showed that THP-1, MM6, and U937 cells incubated for 3 days with different concentrations of IL-6, IFN-γ, and TNF-α increased CD23 expression. (Gessl et al., 1993). Also, IL-10 augmented CD23 expression on U937 cells in a concentration-and time-dependent manner (Alderson et al., 1992). Thus, the present results for MM6 cells do not agree closely with previously published results but this may be due to different concentrations used or due to non-standardisation between flow cytometric analyses. The results in the present study suggest that the expression of CD23 on MM6 cells and peripheral blood monocytes be regulated in a similar manner.

7.2.1.5. CD14

CD14 is expressed at high levels on monocytes but its expression is greater on fully differentiated macrophages. CD14 expression was high on control peripheral blood cells. This expression was decreased by all cytokines and stimulators used with the exception of the lower M-CSF dose (Section 4.2.2.5). Others have shown that incubating human monocytes with higher concentrations of IL-10 and different units for M-CSF and GM-CSF caused an increase in CD14 expression (Buelens et al., 1997, Chapuis et al., 1997). The effect of IL-10 was dose dependent and was best added at initiation of the culture, as addition on day 3 was less stimulatory. If IL-10 was added at day 6 it did not increase CD14 expression (Allavena et al., 1998).

MM6 cells showed a lower expression of CD14 than peripheral blood monocytes. PMA and Vit D3 increased CD14 on days 3 and 5. At day 3, the remaining cytokines showed a slight decrease effect on CD14 expression. On day 5 significant increases were caused by IL-6, IL-10 and TNF-α which returned to normal by day 7. These results suggest that a high proportion of MM6 cells can be induced to express CD14 when treated with cytokines or stimulants that are associated with the induction of differentiation. Weber et al., (1993) demonstrated that TNF-α increased phagocytosis and induced CD14 expression on MM6 cells, in part supporting the current study.
The results indicate that all the stimulants used increased CD14 expression on MM6 cells. On MDM, VitD3, GM-CSF and M-CSF were more effective in affecting CD14 expression.

7.2.1.6. HLA-DR

HLA-DR was expressed on a large proportion of peripheral blood monocytes after incubation for 3 days. This expression was significantly decreased after 3 days in culture with all cytokines and stimulants tested (with the exception of the lower concentrations M-CSF and GM-CSF). This effect reached a maximum at day 7 for IFN-γ, and GM-CSF (higher concentration). The effect of the other stimulants either remained constant or diminished with longer culture periods. By contrast, Tormey et al., (1997) have shown that IFN-γ (at higher concentrations than that used in the present study), IL-6, and TNF-α (used at different units/ml) enhanced HLA-DR expression. The same study by Tormey et al., (1997) showed VitD3 (10⁻⁸ M/ml; lower than the concentration used in the present study) downregulated HLA-DR expression. Human monocytes in the presence of IL-10 (10ng/ml and 20ng/ml) have been shown to inhibit antigen presentation by lowering the level of MHC class II as observed in the present study (Thomssen et al., 1995; Allavena et al., 1998). The effect of IL-10 was dose dependent and was best added at the initiation of the culture, as addition on day 3 was less inhibitory. If IL-10 was added at day 6, only a modest reduction of MHC classII was observed (Allavena et al., 1998).

MM6 cells express lower levels of HLA-DR then control peripheral bloods monocytes but overall the chemical stimulants and cytokines inhibited the expression of HLA-DR. A decrease in expression was noted at day 3 for PMA, IL-10, and TNF-α, the effect remained constant throughout the period of experiment. The other stimulants caused a significant reduction after day 5 with little change thereafter. These results suggest that MM6 cells and peripheral blood monocytes respond in a similar manner to the cytokines and stimulants used with respect to HLA-DR expression. This response is to both Th1-type and Th2-type cytokines.
7.3. The effect of immune complexes on whole blood

The inability to develop antigen specific T cell lines necessitated a change in direction for the project. Clinical observation demonstrated the presence of immune complexes in terminal AIDS patients and others have shown (in vitro) that immune complexes may affect cytokine secretion (Trial et al., 1995; Manetti et al., 1996). It was shown that the cytokines TNF-α, IL-10 and IL-6 were affected by immune complexes have been the cytokines shown in the present study to be most active in affecting monocyte activation and differentiation. The role of monocyte activation in HIV replication is still uncertain but immune complexes in vivo may stimulate the release of cytokines that alter MNP activation leading to enhanced HIV replication and exacerbation of disease. To this end, the present study was modified to examine the effect of immune complexes on mononuclear phagocytes and to determine if HIV infection, or complexes containing HIV proteins, has an effect on the outcome.

7.3.1. KLH-stimulated cytokine release

Mixing the antigen with rabbit anti-KLH serum at sub-agglutinating concentrations made keyhole limpet hemocyanin containing immune complexes. Complexes formed in antigen excess stimulated high levels of IL-6 release in whole blood which reached significance after 12 hours. At 30 minutes after stimulation, the Ab2Ag complex stimulated the highest levels of IL-10 but this failed to reach significance due to the high levels of IL-10 induced by the untreated cells and by anti-KLH alone. This early production of IL-10 may favour IL-6 production that switches off TNFα production, preventing a significant rise in this cytokine. A study carried out by Berger et al (1996b), showed that the incubation of human monocytes in vitro with immune complexes formed at equivalence with tetanus toxoid and polyclonal anti-tetanus toxoid antiserum induced the release of IL-6, and IL-10 in a Ab: Ag ratio-dependent manner. The synthesis of IL-10 affected the immune complex-induced secretion of TNF-α since IL-10 suppresses the production of the Th1-type cytokines IL-2, IL-12 and IFN-γ. These results, together with those of the present study suggest that immune complex formed at equivalent or in Ag excess cause a bias towards a Th2-type response. The results of the present study were complicated by the use of whole blood which is considered to be physiologically more relevant. KLH was used since it is considered to be a "neo" antigen. However, it has been shown recently that sera from non-immunised donors
contain natural antibodies (IgM and IgG) which bind KLH. These natural antibodies form immune complexes (IC) with KLH antigen, activate the classical complement pathway and affect cytokine secretion (Thornton et al., 1996). This may explain the cytokine responses to antigen alone seen in the present study.

7.3.2. Rabbit or goat anti-human IgG immune complex stimulated cytokine release from whole blood.

Although fluctuations in IL-10, IL-6, and TNF-α could be detected in the supernatants, the cultures containing goat or rabbit immune complexes did not show a difference to those control cultures containing goat or rabbit serum alone. Differences were greater with rabbit serum compared to goat serum this may be due to rabbit antibody binding to FcγR1 receptors. Laufer et al., (1995) showed that IgA and IgG immune complexes incubated with monocyte-derived macrophages for 24 hours increased levels of the complement protein C3 which enhanced the production of TNF-α. Also, it has been shown that tetanus toxoid complexes incubated with human monocytes in vitro inhibit IL12 secretion via TNF-α-induced IL-10 and prostaglandin synthesis. This indicates a Th1 to Th2 shift (Berger et al., 1997). The production of cytokines appears to depend on the antigen/antibody ratio. Tetanus toxoid antigen alone induced a typical Th1-like cytokine release with high levels of IL-12 and IFN-γ. Immune complexes formed at equivalence induced a marked secretion of IL-6 and IL-10 therefore inhibiting IL-2 and IFN-γ secretion. This indicates that these immune complexes occur during the course of chronic infectious disease with a Th1 to Th2 switch (Berger et al., 1996a).

In the present study it was not practically possible to measure the Ab:Ag ratio in the complexes used, information which would have allowed a more in-depth discussion.

7.3.2.1. Identification of the cells binding rabbit or goat anti-human IgG human blood using flow cytometry

Whole blood was incubated with various dilutions of either rabbit or goat anti-human IgG as test samples and either rabbit or goat sera as controls. Cells were assayed by flow cytometry in an attempt to identify to which cell type the complexes were binding.
Generally, the goat serum and goat complexes bond to lymphocytes more effectively than the rabbit complexes or rabbit serum. This is unusual since rabbit antibodies are known to bind to human FcR more effectively than goat antibodies. This suggests that the human Ig in goat complexes was binding to the FcR or that the goat antibody had a greater affinity for human Ig, with the resulting complexes binding to cellular FcR and complement more effectively than those containing rabbit antibodies. Maximum binding levels for the complexes were 18% of lymphocytes, 25% of monocytes and 20% of neutrophiles. This may explain some of the differences observed in immune complex induced cytokine secretion between the present study and others. The goat and rabbit immune complexes were formed in plasma that contained complement. Also, engagement of different FcR and CR or different cells by immune complex leads to a variety of sequelae (Ross and Medof, 1985; Masuda and Roos, 1993; Ohkuro et al., 1995).

In particular, Voice and Lachmann, (1997) demonstrated that immune complexes formed from four IgG subclasses varied in size and binding specificity. The immune complex bound to human neutrophils and induced specific granule release via Fcγ (CD16, CD32) and complement (CR1 and CR3) receptors. The combination of receptors used varying with the isotype of the IgG in the complex. Release of granule contents would have a range of effects of other cells in whole blood and would influence cytokine release.

7.3.3. rHIV-1 gp120-containing immune complexes release of cytokines

Human blood was incubated for various lengths of time with the following pre-formed immune complexes: rabbit anti-serum to rHIV-1 MN gp120 and rHIV-1 MN gp120; sheep anti-serum to rHIV IIIB gp120 (CHO) and rHIV-1 IIIB gp120 (CHO). Neither MN nor CHO antigens affected the constitutive release of IL-6 by whole blood. By contrast, immune complexes containing this antigen significantly enhanced the release of IL-6 by whole blood (MN). The increase was evident after 8 hours culture with the CHO-containing immune complexes but was only significant at 12 hours with the MN containing immune complexes. The release of IL-6 seemed to reduce the release of TNFα. No significant effect of the presence of either immune complex was seen on the release of IL-10 or TNF-α by whole blood. However, this may be due to the fact that the supernatants were only analysed after 8 and 12 hours incubation. IL-10 release probably occurred much earlier and it is possible
that TNFα was released earlier due to the negative value of the slope of the graph obtained.

Others have shown an effect of HIV proteins on cytokine release. The incubation of PBMC with HIV-1 transmembrane glycoprotein gp41 induced an increase in IL-10 production within 3 hours and a reduction of IL-2 and IFN-γ. This reaction was also observed when recombinant HIV-1 gp120 was used (Barcova et al., 1998, Koutsonikolis et al., 1997). HIV-1 gp120 molecule (FLgp120) and its fragments (rp120cd and rp120) incubated with human monocytes caused an upregulation of CD14 and CD44. The rp120cd peptide significantly increased the expression of CD16 and the TNF-receptor type II. However, rp120 downregulated HLA-DR, CD64, IFN-γ receptor and induced IL-10 production. These types of changes are seen in blood of AIDS patients (Zembala et al., 1997).

7.4. HIV-1 infected MM6 cells and surface antigen a pilot study

This pilot study indicated that both strains of HIV were more effective in infecting THP-1 cells than MM6 cells. However, both cell lines showed significant changes in the expression of the surface antigens examined as a result of virus infection. This change in expression was similar for both cell lines with the exception of the CD14 and HLA-DR antigens which were down-regulated by virus infection in MM6 cells and up-regulated by infection in THP1 cells.

7.4.1. Kinetics of HIV-1 induced alteration in cell surface antigen expression on MM6 and THP-1 cell lines

MM6 and THP-1 cell lines were infected for 3, 5, 10, and 20 days with either HIV-1_{Ba-L}(H9; 100μl) or HIV-1_{RF}(H9; 100μl) to detect any changes in morphology and surface antigen expression. When examined microscopically, MM6 cells were productively infected with HIV-1_{Ba-L}(H9) and HIV-1_{RF}(H9), and showed a small number of virus particles in vacuoles at day 10 but not at day 5. By day 20, many cells were dead and no virus was visible.
7.4.1.1. *Surface antigen expression on HIV-1* \(_{ \text{Ba-L} } \) infected MM6 cells

Nottet et al., (1993) showed that HIV-1-infected monocyte-derived macrophages and non-infected macrophages express the same level of Fcγ and complement receptors. The present study concurs with these results for CD16 (Figure 6.9). However, in MM6 cells, 5 days after HIV-1 infection, cells expressed higher levels of CD16 than non-infected cells (Figure 6.5). Others have shown that HIV-1 infection did not alter receptor function as internalisation and killing of opsonized Escherichia coli (E. coli) was just as effective in HIV-infected and non-infected macrophages (Nottet et al., 1993).

In the present study, infection by Ba-L and RF strains of HIV caused an increase in the % of cells expressing CD23, a receptor for IgE. This may be indicative of a Th2 switch in HIV-infected patients that enhances IgE production in the early, intermediate or advanced phases of infection (Manetti et al., 1996). Also, CD14, HLA-DR and CD80 expression increased indicating that MM6 cells were maturing and that antigen presentation was not impaired by HIV as demonstrated by Nottet et al., (1993). CD80 expression increased at day 5 but reached a maximum at day 10 for both strains of the virus. Although, the expression of CD23, CD14, CD80, and HLA-DR was below the level of controls this continued to day 20. However, both virus strains caused an increase in CD16 and CD11c, (a cell adhesion molecule that allows the transfer of virus to adjacent cells) which reached a maximum at day 10. This suggests that the virus regulated the biological function of MM6 cells without impairing their ability to present antigen.

Thieblemont et al., (1995) demonstrated that circulating peripheral blood monocytes of HIV-infected individuals exhibit an abnormal level of CD16 and HLA-DR antigens compared to seronegative individuals. Two subpopulations of CD14 were detected in HIV-infected individuals expressing at CD14\(_{\text{low}}\)CD16\(_{\text{high}}\) and CD14\(_{\text{high}}\)CD16\(_{\text{low}}\). The CD14\(_{\text{low}}\)CD16\(_{\text{high}}\) subpopulation comprised 40% of the monocyte population in AIDS patients but less than 5% of the total in HIV seronegative individuals. Also, HIV-infected individuals exhibited low levels of soluble circulating CD14 but high levels of CD16, the latter decreasing in AIDS patients. It was noted that CD14\(_{\text{low}}\)CD16\(_{\text{high}}\) population produced higher levels of TNF-α, and IL-1α than CD14\(_{\text{high}}\)CD16\(_{\text{low}}\) from AIDS patients. One possible reason for the emergence of the CD14\(_{\text{low}}\)CD16\(_{\text{high}}\) population may be due to a Th2 switch as it is known that IL-1, IL-4, IL-6, IL10, TNF-α, IFN-γ, TGF-β, and GM-CSF modulate the expression of CD14 and CD16 (Thieblemont et al., 1995).
Another early study by Thieblemont et al., (1995) demonstrated that CD80 is expressed at lower levels on monocytes from HIV-1 infected patients than from controls, an observation which concurs with the present study. A study by Montaner et al., (1994) showed that by treating cells with IL-10 before infection produces lower levels of virus with those treated after. This probably explains the results of Orlikowsky et al., (1996) that demonstrated HIV-dependent modulation of antigen expression. The study treated HIV-infected macrophages with IL-10 and IFN-γ. The cytokines alone or together prevented an increase in CD80. CD16 expression was increased with IL-10 but decreased by IFN-γ (Orlikowsky et al., 1996). The results of Orlikowsky et al., (1996) also showed that non-HIV infected MDM treated with IFN-γ showed decreased CD80 and CD16 expression this implies that another cytokine is required to induce expression. The present study support Orlikowsky et al., (1996) findings as non-HIV infected MDM treated with IL-10 showed both antigen receptors increasing after 3 days (Figure 4.9A-4.10A).

7.4.2. Effect of immune complexes on surface antigen expression on HIV-1 infected monocyte-derived macrophages.

Human monocyte-derived macrophages were infected with HIV-1 Be-L (H9) for 5 days and subsequently incubated with either rabbit, goat or rHIV-1 IIIB gp120 (CHO) containing immune complexes. Owing to the large standard deviations recorded, there was no apparent effect of immune complexes on surface antigen expression in virus infected cells at the times examined.

7.4.2.1. Effect of immune complexes on cytokine release by HIV-1 infected monocyte-derived macrophages.

It has been shown that monocytes infected with HIV-1 show increased production of many cytokines although production of IL-12 (a Th1-type cytokine which induces IFN-γ production) is inhibited (Chehimi et al., 1994, Manetti et al., 1993). HIV-infected monocytes produce increased levels of IL-6 in serum which may contribute to polyclonal B-cell activation (Breen et al., 1990, Trentin et al., 1992). HIV-infection in monocytes also induced high levels of intracellular TNF-α (Thieblemont et al., 1995) and IL-10 both in vitro and in vivo which inhibited antigen-dependent T-cell responses (Manetti et al., 1996; Del Prete et
The present study detects cytokines such as IL-6, IL-10, and TNF-α released in vitro from HIV-infected MDM incubated with immune complexes (Figure 6.12-6.14).

7.4.3. rHIV-1 IIIB gp120 (CHO) containing complexes

MDM incubated with HIV containing (CHO) complexes showed a significantly increase in IL-10 secretion compared to control cells. This was evident at both 8 and 12 hours and the levels were not affected by HIV infection of the cells. At 8 hours, the complexes failed to stimulate significant levels of IL-6. However, by 12 hours of culture, the level of IL-6 had significantly increased (compared to 8 hrs) in the presence of immune complexes. This increase was lost in HIV infected cells. Levels of TNF-α were barely detectable at 8 hours. After 12 hrs of culture, TNF-α was detected in the supernatant infected cells; this level being significantly reduced by the presence of immune complexes.

These results suggest that early production of IL-10 inhibit the production of IL-6. However, over a longer period, the reduced rate of production of IL-10 allows IL-6 production. Indeed, it has been shown that the production of IL-6 by HIV protein-stimulated THP-1 cells could be increased by adding a neutralising anti-IL-10 antibody to the culture (Finnegan et al., 1996). Also, Sutterwala et al., (1998) demonstrated that genetically modified mice lacking the FcRy chain failed to produce IL-10 in response to stimulation with immune complexes.

In the present study, it was shown the release of TNF-α in HIV infected cells. It has been shown that such release inhibits IL-6 production providing the appropriate conditions for virus proliferation (Finnegan et al., 1996). Interestingly, the presence of HIV-CHO complexes appeared to inhibit TNF-α production by infected cells suggesting that the complexes may prevent viral replication. However, such effects may depend on the size and concentration of the complexes that could not be determined in the present study due to the limited availability of reagents.

7.4.3.1. Rabbit and goat immune complexes on HIV-infected monocyte-derived macrophages

Rabbit and Goat antibody containing complexes had no effect on IL-10, IL-6 or TNF-α production by control or HIV-infected MDM. This contrasts with the effect of HIV-CHO complexes. This difference may be due to the size or concentration of the respective complexes.
complexes but may also indicate that effects observed with the HIV-CHO complexes may be due wholly or in part to the antigen as suggested by the work of Takeshita et al., (1995). This could not be verified in the present study due to the limited amount of virus antigen available from the MRC repository.

7.5. Effect of serum from HIV+ individuals on surface antigen expression on HIV-1 infected monocyte-derived macrophages.

Human monocyte-derived macrophages were infected with HIV-1Ba-L (H9) for 5 days and subsequently incubated with HIV+ sera. Surface antigen expression was assayed using flow cytometry. Owing to the large standard deviations observed, it was not possible to identify any effect of HIV serum on surface antigen expression. This may have been due to the length of the incubation period that was required for collection of supernatants. Previous experiments in this study demonstrated that changes were usually maximal after 3 or 5 days depending on the cell type used.

7.5.1. Effect of serum from HIV+ individuals on cytokine production by HIV-1 infected monocyte-derived macrophages.

Human monocyte-derived macrophages were infected with HIV-1Ba-L (H9) for 5 days and subsequently with HIV-sera. After 8 hours, IL-6 and TNF-α could not be detected in the supernatants from either infected or non-infected cells. By contrast, IL-10 was clearly detectable in all cultures. After 12 hours, IL-6 could be detected in all cultures. Whilst the level produced by non-infected cells was the same regardless of the presence of HIV serum, in HIV-infected cells, the presence of serum appeared to increase IL-6 production (although this just failed to reach significance). Also after 12 hrs IL-10 was present in all cultures at significantly higher levels than at 8 hours. The presence of HIV+ sera in the HIV infected cultures appeared to cause a significant increase in IL-10 production.

TNFα production was detected in control, non-treated cells that disappeared in serum-treated cells. No TNFα production was detected in HIV infected cells. These results suggest a possible Th1 to Th2 switch since IL-6 was produced later in the response. However, the presence of immune complexes in the sera could not be determined due to the limited volume available. Also, clinical data could not be divulged.
7.6. Conclusion

The development of antigen-specific T cell lines in order to investigate the effect of immune complexes on macrophage function was not successful. Others have achieved such lines but the choice of stimulator cells or donors in the current study may have affected the outcome. Clearly future experiments would include the use of alternative sources of stimulator cells, selection of donors by skin reactivity to a Mantoux test and a wider range of antigen concentrations.

The role of immune complexes in disease is clearly a complex one which may depend on their concentration, the type of antibody involved, the antibody: antigen ratio within the complex, the involvement of complement, the site of formation, the cells involved and other physiological and pathological complications. In HIV infection, complexes containing HIV proteins have been demonstrated in the circulation of patients in later clinical stages of the disease. However, little is known about the effect of immune complexes on monocytes (one of the main sites of infection in HIV). Alterations in surface antigen expression as a direct or indirect result of binding immune complexes may affect monocyte circulation and migration as well as promoting inflammation and localised tissue damage. Thus, this study set out to examine the effect of immune complexes on monocyte surface antigen expression and cytokine secretion. Also, to determine the effect of those cytokines on monocyte surface antigen expression and to investigate how this is altered by HIV infection and specifically by immune complexes comprising HIV derived proteins.

To this end, monocytes and the cell line MM6 were examined for the effect of a wide range of cytokines on surface antigen expression (cellular proliferation being used as a measure of whether or not the cytokines were having the expected physiological effect on the cells). This study demonstrated that PMA, Vit D3, IFN-γ, M-CSF, GM-CSF, IL-6, IL-10, and TNF-α stimulate the differentiation of MM6 cells. IL-6, IL-10, and TNF-α increased CD16α for MDM with lower levels of expression on MM6 cells. The costimulatory molecule CD80 was expressed at lower levels on MDM than MM6 cells. The costimulatory molecule IL-6, TNF-α, and GM-CSF increased its expression whilst PMA strongly inhibited it. MM6 cells CD80 expression was increased by IL-6, IL-10, TNF-α, and GM-CSF. The expression of the adhesion CD11c expression on MDM was inhibited by all stimulants. The same was true for CD11c expression on MM6 cells except for IL-6 and PMA which increased CD11c. CD23
expression on MDM was increased by IFN-γ, GM-CSF, IL-6, and IL-10 while MM6 cells were induced to express CD23 by IFN-γ, M-CSF, GM-CSF. However, IL-6, IL-10, and TNF-α inhibited CD23 expression on MM6 cells CD14 expression was reduced by all stimulants on MDM while PMA, Vit D3, IL-6, IL-10, and TNF-α caused an increase in its expression on MM6 cells. The expression of the antigen-presenting molecule HLA-DR was inhibited by all stimulants for both MDM and MM6 cells.

When cells were incubated with immune complexes containing the naive antigen KLH, they were found to produce high levels of IL-6 in whole blood and low levels of TNF-α (with IL-10 failing to reach significance after 30 minutes) suggesting a Th2 type response. When whole blood was incubated with rabbit or goat anti-human IgG or rabbit or goat serum, rabbit complexes played an important part in stimulating and inhibiting cytokine release. IL-10 was detected at 30 minutes, which may have been inhibited by IL-6, production that reached a maximum between 4 and 8 hours. However, TNF-α reached significant levels between 8 and 16 hours. Goat complexes had a fluctuating effect on the release of cytokines. If these results are compared, whole blood incubated with KLH or rabbit anti-human IgG both produce IL-10 as early as 30 minutes and produce similar levels of IL-6. However, KLH containing complexes do not stimulate the release of any TNF-α. By contrast, rabbit complexes stimulate the release of significant levels of TNF-α between 8 and 16 hours. This suggests that the various complexes may show different affinities for FCRs and/or complement leading to distinct cytokine secretion patterns.

The incubation of MDM for 3 days with IL-6, IL-10, TNF-α increased the expression of CD16 and CD80 but decreased the expression of the surface antigens CD11c, CD23, CD14, and HLA-DR. All surface antigens decreased at 5 and 7 days. When such cells were incubated with immune complexes containing rabbit or goat antibodies no significant effect on surface antigen expression was observed, probably due to high standard deviations. Thus it was not possible to determine whether or not the cytokines released due to the presence of immune complexes affected the surface antigen expression and hence function of the peripheral blood mononuclear cells. When similar experiments were carried out with immune complexes containing HIV proteins, cells in whole blood released significant levels of IL-6 at 8 hours and 12 hours (for CHO- and MN-containing immune complexes respectively). IL-10 and TNF-α were not detected.

When MDM were incubated with HIV protein containing immune complexes (CHO) a slight but significant release of IL-6 was observed compared to that in the supernatants of
MDM alone. This IL-6 release was not affected by HIV infection of MDM. IL-10 production by MDM was significantly stimulated by the presence of immune complexes but again this production was not affected by HIV infection. By contrast, TNF-α production by HIV infected MDM was significantly greater than by non-infected cells (evident after 12 hrs). However, the presence of HIV-protein containing immune complexes, reduced TNF-α production by infected cells. This suggests that the presence of circulating immune complexes in HIV infected individuals promotes a greater Th2 type response than in non-infected persons due to the effect of HIV infection on TNF-α production. The affect of HIV-sera on surface antigen expression was not clear due to the high standard deviations. However, HIV-sera did stimulate the release of cytokines. IL-6 was not detected at 8 hours but at 12 hours for non-infected and infected cells. IL-10 was detected at 8 and 12 hours but TNF-α was not detected at all. These results suggest that these sera from HIV infected individuals may contain immune complexes or HIV protein promoting a Th2-type response.

HIV infection seemed to increase the expression of CD16 and CD80 reducing the expression of other antigens as indicated by HIV-infection of MM6 cells. Unfortunately, significant changes in the expression of surface antigens on HIV-infected MDM could not be detected due to high standard deviations. However, the high release of IL-10 by immune complexes indicates a Th2-type response. A previous study has demonstrated that IL-10 and TNF-α are required for HIV replication but by blocking IL-10 production using monoclonal antibodies and increasing TNF-α production, HIV replication may be reduced. Thus, the presence of immune complexes, which promote IL-10 and reduces TNF-α, may stimulate HIV replication and progression to full blown AIDS. Thus, the manipulation of cytokine levels or blocking of Fc and complement receptors may prove beneficial in the treatment of advanced cases.

7.6.1. Future work

Clearly further work needs to be performed comparing the effects of HIV-protein complexes and non-HIV-protein complexes, to determine the effect of HIV immune complexes on disease progression. Of particular importance would be to determine the effect of immune complex size and concentration and to clearly define the kinetics of the reaction. Also, of importance would be to determine the role of complement in these
phenomena observed in the current study and to investigate the intracellular signalling pathways involved.
APPENDIX A: STAINING CELLS WITH KC57 ANTIBODY

Preparation of 20μg/ml lysolecithin in 1% paraformaldehyde

Ten grams of paraformaldehyde were dissolved in 100ml 1N NaOH and made up to 800ml with PBS. The solution was titrated to pH 7.2 +/-0.1 using 4N HCl then made up to one litre with PBS. Ten milligram of lysolecithin was dissolved in 500ml 1% paraformaldehyde solution, mixed and stored at 2-8°C.

Preparation of 0.1% NP-40

A 0.5 ml aliquot of Nonidet P-40 was added to 499.5 ml PBS mixed and stored at 2-8°C.

Preparation of cells for stain with KC57 antibody

Cells (10⁶) was centrifuged (350g/3mins) and fixed by adding (1ml) 20-μg/ml lysolecithin in 1-% paraformaldehyde, gently vortexing and incubating for two minutes at room temperature. The cells were centrifuged (350g/5mins; 4°C), the supernatant decanted and 2ml cold absolute methanol (100%; -10 to -20°C) was added. The cells were incubated on ice for 15 minutes then centrifuged (350g/5mins, 4°C) and supernatant decanted. Cells were incubated for 5minutes with 1ml of 0.1% NP-40 (2-8°C) and kept on ice. After centrifugation (350g/5mins; 4°C) supernatant was decanted and 5μl KC57-FITC with 195μl PBS was added to the cells and incubated for 15 minutes at room temperature. The cells were centrifuged (350g/5mins, 4°C) in 1ml PBS, supernatant decanted and 1ml PBS added. Cells were analysed using the FACScan.
APPENDIX B: SUPPLIERS OF CHEMICALS AND APPARATUS

Abbott Laboratories Limited, Queenborough, Kent, ME11 5EL, U.K.
Butterfly needle (21-G)

Amersham International plc, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, U.K.
[Methyl-3H] Thymidine: *PC= TRK 120 B353, specific activity 925 Gbq/mmol, and 25Ci/mmol.

Anachem Ltd, 20 Charles street, Luton, BEDS LU2 0EB, U.K.
Treff, Tips (200µl).

Becton Dickinson UK Limited, Between Towns Road, Coweley, Oxford, Oxon, OX4 3LY, U.K.
Anti-human CD80 PE conjugate: PC= 340294, containing gelatin and 0.1% azide;
Anti-human HLA-DR phycoerythrin conjugate: PC= 7367, containing gelatine and 0.1% azide;
Anti-human Leu-20 (CD23) FITC conjugate: PC= 7797, containing gelatine and 0.1% azide;
Anti-human Leu-11c (CD16) phycoerythrin conjugate: PC= 7617, containing gelatine and 0.1% azide;
Anti-human Leu-M3 (CD14) FITC conjugate: PC= 7493, containing gelatine and 0.1% azide;
Becton Dickinson FACScan; equipped with an argon ion laser (488nm; 15mW);
Goat anti-mouse IgG FITC conjugated: PC= 349031;
Nunc centrifuge tubes, 50ml Blue Max polypropylene tubes, Falcon: PC= 2070;
Nunc Eppendorf tubes;
Nunc 24-microwell plates;
Syringe 50ml.

Beckman Instruments, (U.K.) Ltd, Oakley Court, Kings Mead, 1 Business Park, High Wycombe, Bucks HP11 1JU, U.K.
Centrifugation.

*PC= Product Code
**BHD Chemicals Limited**, Broom Road, Poole, Dorset, U.K.
Formaldehyde solution (40% w/v), (HCHO), analytical grade, BDH: PC= 10113, M.wt. 30.03; Sodium carbonate; Sodium bicarbonate; Sodium hypochlorite solution (14% available chlorine): PC= 30169.

**Bibby Sterilin Limited**, Tilling Drive, Staffordshire, ST15 0SA, U.K.
Pasture pipettes (5ml); Pipettes (10mL); Universal tubes (20mL).


**Coulter Immunology**, 440 Coulter Way, Hialeah, FL 33010, USA.
Anti-KC57-FITC: PC= PN 6604665, for detection of intracellular p24 antigen.

**Difco Laboratories Limited**, P.O. Box. 14B, Central Avenue, West Molesey, Surrey KT8 2SE, U.K.
Middlebrook 7H9: PC= 0713-17-9; Middlebrook OADC enrichment: PC= 0722-64-0.

**Dynatech Laboratories Limited**, Daux Road, Billingshurst, Sussex, MA781, U.K.
Immulon 4: PC= 011-010-3855, ELISA microtiter immunoassay plate flat bottom.

**European Collection of Animal Cell Cultures (ECACC)**, Division of Biologics, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, SP4 0JG, U.K.
B95-8 cell line; THP-1.

**Fisons plc**, FDA Laboratory Supplies, Bishop Meadow Road, Loughborough, Leicestershire, LE11 0RJ, U.K.
Absolute methanol: PC= M/4000/17; Acetone, (CH₃COCH₃), Analytical grade: PC= A/0600/17; M.wt. 58.08; Glycerol (CH₂OH.CHOH.CH₂OH): PC= G/0650/17; M.wt. 92.10.
Hayman Limited 70 Eastways Industrial Park, Witham, Essex, CM8 3YE, and U.K.
Absolute alcohol 100, \((C_2H_5OH)\) (ethyl alcohol).

ICN (Flow) Biomedicals Limited, Eagle House, Peregrine Business Park, Gomm Road,
High Wycombe, Bucks, HP13 7DL, U.K.
Hepes buffer: PC= 16-884-46; 1Molar solution; M. wt. 238.5mg/mL; RPMI 1640 medium,
phenol red-free: PC= 73-128-54, with sodium bicarbonate and without L-glutamine, sterile
filtered, endotoxin tested.

ILACON Ltd, Gilbert House, River Walk, Tonbridge, Kent TN9 1DT, U.K.
ILACON cell harvester.

Immunotech, The Binding Site Limited, Birmingham Research Park, 97 Vincent Drive
Edgbaston, B15 2SQ, Birmingham, U.K.
Anti-human CD28 FITC conjugated: PC= 1236.

Leo Laboratories Limited, Prince Risborough, Bucks, U.K.
Heparin (Mucous): PC= 0043/0086; 5,000 units/mL, endotoxin free.

L.I.P. (Equipment and Services) Ltd, 111 Dockfield Road, Shipley, West Yorkshire, BD17
7AS, U.K.
Micropipette Tips (1mL).

Life Sciences International (UK) Ltd, Unit 5, The Ringway Centre, Edison Road,
Basingstoke, Hants, RG21 2YH, UK.
Labsystems, Spectrophotometer; Luckham, Plate shaker, Model R100; Pyrgogen/
endotoxin frees 2-200µl: PC= TP62; Pyrgogen/ endotoxin frees 200-1000µl: PC= TP65.

Kartell Spa (Agent Kartell Plastics (U.K.) Ltd), Via Della 20082, Noviglio, 1-20082 Milan,
Italy.
UV Grade Cuvettes: PC= D01939.
Marathon Laboratory Supplies, Unit 6, 55-57 Park Royal Road, London, NW10 7JJ, FACScan tubes: PC= 2054; 96-microwell plates: PC= F3077; Cell Scrapers: PC= F3086; Tissue culture flasks (25 cm²): PC= F3108; Tissue culture flasks (75 cm²): PC= F3111.

MCR AIDS Directed Programme, National Institute for Biological Standards & Control, Blanche Lane, South Mimms, Potters Bar, Herts EN6 3QG, U.K. Rabbit anti-serum to rHIV-1 MN gp120; Sheep anti-serum to rHIV-1 IIIB gp120 (CHO). AGMED INC, rHIV-1 IIIB gp120 (CHO); rHIV-1 MN gp120 (Baculovirus).

Miltenyi Biotec Inc, 1250 Oakmead Park, Suite 210, Sunnyvale. CA 94088-3599, USA, Magnetic cell sorter (MACS); Monoclonal anti human CD3 (Leu™-4): PC= 456-01.

Nalgene Company, A/Subsidiary of sybron corporation, Rochester, New York, 14602-0365 USA. Cryovial (1.8mL).

Harris Scientific, 618 Western Avenue, Park Royal, London, W3 0TE, U.K. New Improved Neubauer haemocytometer slide: PC= H12-144.

Nycomed (UK) Limited, 2111 Coventry Road, Sheldon, BIRMINGHAM, B26 1BR, UK. Lymphoprep (Bouyant density 1.077 +/- 0.001 g/mL (20°C): Osmolarity 280 +/- 15 mOsm).

Organon Teknika Corporation, Cappel Research Products, 100 Akzo Avenue, DURHAM, NC 27704, USA. Rabbit anti-serum to Keyhole Limpet Hemocyanin: PC= 55966.

Phillips
T400 transmission electron microscope.

R&D Systems Europe Limited, 4-10 The Quadrant, Barton Lane, Abingdon, OX14 3YS, U.K.
Interleukin 2 (rIL-2): PC= 202-IL-010: specific activity >2.0-4.0 x 10^6 units/mg.

Roche Products Limited, Diagnostic Department, P.O. Box. 8, Welwyn Garden City, Hertfordshire, AL7 3AY, U.K.
1,α 25-dihydroxycholecalciferol (VitD3), a gift: M. wt. 416.65.

Unipath Limited (Oxoid), Basingstoke, Hants, U.K.
Phosphate buffered saline (Dulbecco ‘A’) tablets: PC= Br14a, pH7.3.

Sartorius, Longmead Business Centre, Blenheim Road, Epsom, Epsom, Surrey KT19 9QN.
Filtered 0.20-μm, 16534K.

Serotec Limited, 22 Bankside, Station approach, Kidlington, Oxford OX5 1JE, U.K.
Goat anti-human IL-6: PC= AB206NA; Interleukin 6 (rIL-6, 100ng/μL): PC= PHP045;
Interleukin 6 (rIL-6): PC= MAB206; Interleukin 10 (rIL-10, 0.1mg/mL): PC= PHP047;
Mouse anti-human interleukin 10 (IL-10): PC= MCA926; Mouse anti-human tumour necrosis factor alpha (TNF-α): PC= AB210NA; Mouse anti-human TNF-α: MCA747M.

Shimadzu Corporation, Howe, V.A, & Co Ltd, U.K.
Spectrophotometer, UV-1201.

Sigma Chemical Company Limited, Fancy Road, Poole, Dorest, BH17 7BR, U.K.
Adjuvant peptide, (PPD) N-Acetylmuramyl-L-D-isoglutamine: PC= A-9519, M. wt. 492.5;
Anti-human CD3 PE conjugated: PC= P5810; Anti-human CD8 PE conjugated: PC= P5560;
Anti-human CD11c FITC conjugated: PC= F5773; Anti-human CD45/CD14 DUAL-TAG FITC/PE conjugated: PC= F-8527; Anti-human IgG (Fc specific) developed in goat: PC= I-8885; Anti-human IgG (Fc specific) developed in rabbit: PC= I-9135; Anti-goat IgG alkaline phosphatase conjugate: PC= A-2168; Anti-mouse IgG alkaline phosphatase conjugate: PC= A-4312, gelatine and 0.1% azide; Anti-Rabbit IgG: PC= R2004; developed in goat;
Centrifugation 2-15; Centrifugation 3K-10; Dimethyl sulphoxide (DMSO): PC= D-5879; M. wt. 78.13; Ethylenediamine tetraacetic acid (EDTA) (dipotassium salt): PC= ED2P,
M.wt.368.4; Etoxate Kit: PC= 210-A1; L-glutamine, 200mM solution: PC= G7513; sterile filtered and endotoxin tested; Glutaraldehyde, (Pentane-1, -dial) Grade 1: 25% aqueous solution: PC= G582; Goat anti-human IL-10: PC= I-5020; Goat Serum: PC= G-9023; Granulocyte macrophage colony stimulation factor (rGM-CSF): PC= G-0532; Guinea pig complement serum: PC= S-1639; Hemocyanin from Keyhole Limpet: PC= H-7017; Heparin, sodium salt, and Grade 1-a from porcine intestinal mucosa: PC= H-3149; Human serum from male AB plasma: PC= H-4522; Kanamycin Solution: PC= K0129; L-a- Lysophosphatidyl choline: PC= L-4129; Macrophage colony stimulation factor (rM-CSF): PC= M-9687; Mitomycin C: PC= M-0503; Monoclonal anti-human CD14 PE conjugate: PC= P-5435; Monoclonal anti-goat IgG-FITC conjugate: PC= F-4891; Monoclonal anti-rabbit-IgG (γ-chain specific) FITC conjugate: PC= F-4151; MouselgG1/MouselgG2a DUAL-TAG FITC/PE conjugated: PC= F0528; MouselgG1/MouselgM DUAL-TAG FITC/PE conjugated: PC= F0653; Nonidet P-40 (NP-40): PC= N-3516; MTT (C_{18}H_{16}N_{5}SBr) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide: PC= M-2128; M. wt. 414.3; Osmium tetroxide (OsO_{4}, Osmic acid): PC= 05500; M. wt. 254.2; Paraformaldehyde: PC= P6148; Penicillin-streptomycin solution: PC= P0781; Phorbol 12-myristate 13-acetate (PMA): PC= P-8139; M. wt. 616.8; pNPP Substrate tablet set: PC= N-1891; Pyruvic acid (C_{3}H_{4}O_{3}), (α-ketopropionic acid): PG= P-1656; M. wt. 88.06; Rabbit Serum: PC= R-9133; RPMI 1640 medium: PC= R0883; with sodium bicarbonate and without L-glutamine, sterile filtered, endotoxin tested; Trypan Blue (C_{34}H_{24}N_{6}O_{14}S_{4}Na_{4}): PC= T8154; 0.4% solution prepared in 0.81% sodium chloride +0.06% potassium phosphate dibasic, M. wt. 960.8.

Taab Laboratories Equipment Ltd, 3 Minerva House, Calleva Park, Aldermaston, Berks, RG7 8NA, UK.

Dodecenyl succinic anhydride (DDSA): PC= D027; M.wt. 266.38; Epon /Taab 812 resin: PC= T028; M.wt. 306; 2,4,6-tri (dimethylaminomethyl) phenol, (DMP-30): PC= D032, M. wt. 265.40.

TCS Biologicals Limited, BOTOLPH CLAYDON, Buckingham, MK18 2LR, U.K.

Foetal calf serum: PC= CF006.

Wallac Scintillation products, Milton Keynes, Wallac UK.

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Optiphase 'safe': SC/1025/21, scintillation fluid; Wallac 1410 Liquid Scintillation counters;
Scintillation vials: PC= 10200.

Whatman International Ltd, Maidstone.
Fibreglass filter mats: PC= 1827842; Grade 934-AH.
9.0. REFERENCES


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