Innate Immune Responses in HIV-1 Infected Macrophages

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Declaration

I, Jhen Man Tsang, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

In this study M-CSF differentiated human monocyte derived macrophages were used to investigate HIV-1 interactions with macrophage innate immune responses.

Macrophages may be an important host cell for HIV-1. HIV-1 can infect and replicate within these cells without causing host cell cytopathicity unlike in T cells. Macrophages also aid in the spread of the virus and are likely to act as a viral reservoir protected from antivirals and immune responses due to the unique localisation of the virus within these cells.

Surprisingly HIV-1 infection has little effect on the steady state transcriptome of MDM and despite the role of macrophages to detect incoming pathogens; no innate immune response to HIV-1 could be detected, in contrast with other viruses tested. The lack of immune response was not due to active viral suppression and addition of exogenous IFN or activation of the innate immune response at the time of HIV-1 infection can restrict viral infection in these cells, despite HIV-1 having a full complement of accessory proteins known to counteract IFN inducible restriction factors. This HIV-1 restriction induced by IFN was long lasting, likely for the lifetime of the MDM. IFN treatment of MDM with established HIV-1 infection however only transiently suppressed viral replication. Comparisons of MDM with other cell types which do show an innate immune IFN response to HIV-1 showed that MDM have relatively low levels of TLR7 gene expression, suggesting that MDM may lack one of the PRRs for detecting HIV-1.

HIV-1 infection of MDM was found to attenuate NFκB activation in response to TLR stimulation and this attenuation could be reversed by priming the MDM with IFNγ. However this attenuation of the NFκB signal did not translate into decreased protein expression for a selection of proinflammatory cytokines examined.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AIM2	Absent in melanoma 2
ALR	Absent in melanoma 2 like receptor
ANK	Ankyrin
APOBEC3G	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
ASC	Apoptosis-associated speck-like protein containing a carboxy-terminal CARD
AT	Adenine Thymine
ATP	Adenosine triphosphate
BIV	Bovine immunodeficiency virus
BSA	Bovine serum albumin
BST-2	Bone marrow stromal antigen 2
CAEV	Caprine arthritis-encephalitis virus
CARD	Caspase activation and recruitment domains
CARDIF	CARD adapter inducing IFNβ
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CG	Cytosine Guanine
cGAMP	Cyclic-GMP-AMP
cGAS	Cyclic-GMP-AMP synthase
CLR	C-type lectin receptors
CNS	Central nervous system
CPSF6	Cleavage and polyadenylation specificity factor subunit 6
cRNA	Complementary RNA
CSF1R	Colony-stimulating factor 1 receptor
CTLD	C-type lectin-like domains
CXCL	Chemokine (C-X-C motif motif) Ligand
CXCR	Chemokine (C-X-C motif) Receptor
Cy	Cyanine dye
Cyp	Cyclophillin
DAI	DNA-dependent activator of IFN-regulatory factors
DAPI	4,6 diamidino-2-phenylindole
DAVID	Database for annotation, visualization and integrated discovery
DC	Dendritic cell

DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
ED_{50}	Effective dose for 50% of maximal activity
EDTA	Ethylenediaminetetraacetic acid
EIAV	Equine infectious anemia virus
eIF	Eukaryotic initiation factor
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
Env(s)	Envelope(s)
ERK	Extracellular regulated kinases
FACS	Fluorescence-activated cell sorting
Fas	TNF receptor superfamily, member 6
FasL	Fas Ligand
FCS	Foetal calf serum
FIV	Feline immunodeficiency virus
FOXO3a	Forkhead box protein O3a
G	Guanine
G418	Geneticin
Gal	Galactosidase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Gamma activated sequence
G-CSF	Granulocyte colony-stimulating factor
GFP	Green flourescent protein
GM-CSF	Granulocyte-macrophage stimulating factor
gp	Glycoprotein
HIN	Hemopoietic expression, interferon-inducibility, nuclear localization
HIV	Human Immunodeficiency Virus
iE-DAP	g-D-glutamyl-meso-diaminopimelic acid
IFI-16	Gamma-interferon-inducible protein 16
IFN	Interferon
IFNAR	Interferon α/β receptor
IFNGR	Interferon gamma receptor
IFNR	IFN receptor

IFNR	Interferon receptor
IKK	IκB Kinase
IL	Interleukin
IP-10	Interferon induced protein - 10
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factors
ISG	IFN stimulated gene
ISG	interferon stimulated gene
IκB	Inhibitor of NFκB
JAK	Janus protein tyrosine kinases
JNK	Jun N-terminal kinases
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MAVS	Mitochondrial antiviral-signalling protein
M-CSF	Macrophage colony-stimulating factor
MDA5	Melanoma differentiation-associated antigen 5
mDC	Monocyte derived dendritic cells
MDM	Monocyte derived macrophages
MDP	Muramyl dipeptide
MDS	Multidimensional scaling
MHC	Major histocompatability complex
miRNA	microRNA
MKP	Mitogen activated protein kinase phosphatase
MOI	Multiplicity of infection
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
Mx2	Myxovirus resistance 2
MyD88	Myeloid differentiation primary response gene 88
Nef	Negative regulatory factor
NEMO	NFκB essential modulator
NES	Nuclear export signal
NFAT	Nuclear factor of activated T-cells

NFκB	Nuclear factor kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerisation domain like receptor
NLS	Nuclear localisation signal
NOD	Nucleotide-binding oligomerisation domain
OAS	2′,5′-oligoadenylate synthetase
PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood lymphocyte
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PDL	Programmed cell death 1 ligand
PHA	Phytohaemagglutinin
PKR	Protein kinase R
PMA/TPA	Phorbol 12-myristate 13-acetate/12-O-tetradecanoylphorbol 13-acetate
PMB	Polymixin B
PRR	Pattern recognition receptor
PYHIN	Pyrin and HIN domain-containing protein family
qPCR	Quantitative polymerase chain reaction
Rev	Regulator of expression of virion proteins
RHD	Rel-homology domain
RIG-I	Retinoid acid inducible gene–1
RIP2	Receptor interacting protein 2
RLR	Retinoid acid inducible gene–1 like receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RPMI	Roswell park memorial institute
RTC	Reverse transcription complex
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAMHD1	Sterile α motif and HD domain-containing protein-1
SDS	Sodium dodecyl sulfate
SIV	Simian immunodeficiency virus
SOCS-2	Suppressor of cytokine signaling-2

ssDNA	Single stranded deoxyribonucleic acid
ssRNA	Single stranded ribonucleic acid
STAT	Signal transducers and activators of transcription
STING	Signalling through stimulator of IFN genes
SYK	Spleen tyrosine kinase
TANK	TRAF family member-associated NF-kappa-B activator) binding kinase
Tat	Trans-Activator of Transcription
TBK	TANK binding kinase
TBS	Tris-buffered saline
TD	Transactivation domain
TF	Transmitted founder
Th	T helper
TIR	Toll–IL1 receptor
TLR	Toll like receptors
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
TRIM	Tripartite motif
TYK2	Tyrosine Kinase-2
Vif	Viral infectivity factor
VISA	Virus-induced signalling adapter
Vpr	Viral protein R
Vpu	Viral protein unique
Vpx	Viral protein X
VSV	Vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1. Introduction

In 2011, the global epidemic of Human Immunodeficiency Virus (HIV)-1 infection was estimated to affect 34 million people worldwide (http://www.unaids.org, global report, 2012). Though the number of new infections are decreasing, there were still 2.5 million new infections from that year alone and 1.7 million deaths that year were attributed to acquired immune deficiency syndrome (AIDS) caused by HIV-1. (http://www.unaids.org)

1.1 HIV-1

HIV-1 belongs to the lentivirus genus of retroviridae and is usually sexually transmitted via the genitourinary mucosa. Active viral replication occurs in permissive target cells locally and within regional lymph nodes. Typically 2-4 weeks after infection, patients may experience a flu-like illness, during which antibodies to HIV-1 appear (seroconversion) and high levels of virus are present in peripheral blood. The subsequent fall in viral load is attributed to humoral (Koup, Safrit et al. 1994; Aasa-Chapman, Hayman et al. 2004) and cellular adaptive anti-viral immune responses (Hess, Altfeld et al. 2004) but these do not achieve sterilising immunity as a result of various viral evasion strategies and capacity for escape mutations (Goulder, Phillips et al. 1997; Hay, Ruhl et al. 1999; Draenert, Verrill et al. 2004; Munier and Kelleher 2007). Afterward there is an asymptomatic phase, in which the lower viral load is sustained in the peripheral blood, although high levels of viral replication can be seen in lymph nodes (Pantaleo, Graziosi et al. 1993). With time, the viral burden starts to increase again and numbers of CD4+ T cells decline. The loss of CD4+ T cells has been attributed to a number of factors: CD8+ T cell killing of HIV-1 infected CD4+ T cells, direct killing of infected T cells by HIV-1, bystander activation and apoptosis, and loss of progenitor cells through death or exhaustion (Hazenberg, Hamann et al. 2000; McCune 2001; Grossman, Meier-Schellersheim et al. 2002; Appay and Sauce 2008).

CD4+ T cell depletion is the predominant marker of immunodeficiency as patients develop AIDS, and is characterised by frequent opportunistic infections from other viruses, bacteria, fungi and protozoa (Vajpayee, Kanswal et al. 2003). Patients also have an increased frequency of neoplastic diseases caused by oncogenic viruses such as Epstein Barr virus and Kaposi's sarcoma associated herpesvirus (Boshoff and Weiss 2002), which are normally controlled by a healthy immune system.

1.1.1 Origins of HIV-1

The origins of HIV are thought to be from zoonotic transmission from non-human primates (Sharp and Hahn 2011; Hemelaar 2012). HIV-2 transmission to humans is thought to be from sooty mangabys, and although related to HIV-1 has not spread to the same extent, being mainly localised to Western Africa compared to HIV-1 which has spread worldwide. Studies have reported that HIV-2 is transmitted less efficiently compared to HIV-1 (1994; Gilbert, McKeague et al. 2003), is less pathogenic and progression to AIDS takes longer (Pepin, Morgan et al. 1991; Marlink, Kanki et al. 1994).

The origins of HIV-1 is thought to have occurred via transfer of simian immunodeficiency virus (SIV) from chimpanzees (Gao, Bailes et al. 1999), though there is some evidence to suggest that there may have also be transfer from gorillas (Van Heuverswyn, Li et al. 2006). Multiple independent zoonotic events have led to four groups of HIV-1 so far discovered in humans. The main group M (Major) is the strain responsible for the global pandemic of 34 million infected people worldwide and is thought to contribute to over 90% of HIV-1 infections. The groups, O (Outlier), N (Non M and Non O) and the newly discovered P are much less prevalent, with a handful of cases of group N and so far only 2 cases for group P (Plantier, Leoz et al. 2009; Vallari, Holzmayer et al. 2011), with both groups localised in Cameroon.

HIV-1 diversity has led to the formation of distinct subgroups based on sequence analysis of the viral genome. With group M further classified into 9 subgroups, also called clades, and associated with the letters A-D F-H and J-K. Clades E and I were originally described, but later found to be recombinant forms of other clades. Clade B is the predominant form of HIV-1 seen in Europe, the Americas, Japan, Thailand, and Australia, whereas clade C is the predominant form seen in Sub-Saharan Africa (Hemelaar 2012), where HIV-1 cases make up 69% of the global total (www.unaids.com).

1.1.2 HIV-1 Structure

HIV-1 is a roughly spherical virus of about 120nm in diameter, and like all retroviruses is comprised of an ribonucleic acid (RNA) genome which integrates into the host cell genome through a double stranded deoxyribonucleic acid (dsDNA) intermediate created by reverse transcription. The genome of HIV-1 consists of 2 copies of a 10kb positive sense single stranded (ss)RNA (Ratner, Haseltine et al. 1985; Wain-Hobson, Sonigo et al. 1985), which contains the *gag pol* and *env* genes which encode for the structural and enzymatic proteins, as well as 6 accessory genes *tat, rev, vpu, vif, nef*, and *vpr* (Morrow, Park et al. 1994). This viral genome is enclosed within a cone shaped capsid made up of the viral protein p24, and this capsid is itself surrounded by the matrix and then by the viral envelope; a lipid bilayer taken from the host cell envelope during budding of the viral particle, which contains host proteins (Aloia, Tian et al. 1993) as well as the env encoded glycoproteins (gp)41 and gp120, which determines the tropism of the virus (Shioda, Levy et al. 1991; Arrildt, Joseph et al. 2012). Packaged within the viral particle are also components from the host cell (Ott 2008), as well as the viral enzymes reverse transcriptase, protease and integrase, which are required for the virus to replicate successfully.

1.1.3 HIV-1 Lifecycle

The lifecycle of HIV-1 can be split into 2 main stages. The early stages of the HIV-1 lifecycle include binding and entry of the virion into the host cell, uncoating, reverse transcription and integration of the viral DNA into the host cell genome. The late phase consists of viral gene transcription, production of viral proteins, viral assembly and budding of mature virions from the surface of the host cell. This is summarised in Figure 1.

Figure 1 - The HIV-1 Lifecycle

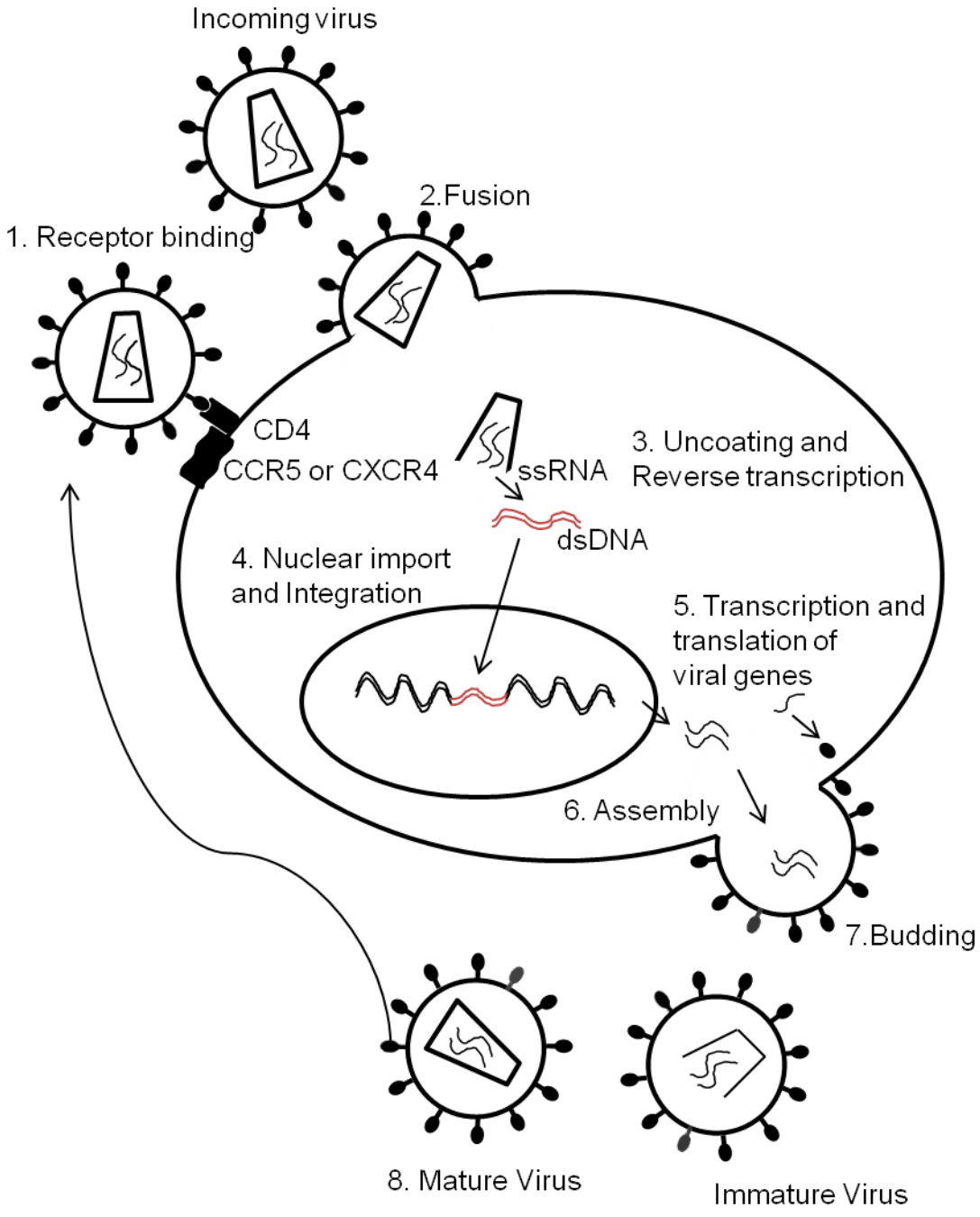


Diagram showing the major steps in the HIV-1 lifecycle

The first step of infecting the target cell occurs via binding of the viral gp120 protein to its primary receptor CD4 on the target cell (Dalgleish, Beverley et al. 1984). This restricts the tropism of HIV-1 to CD4+ T cells, monocytes, macrophages and some DC subsets like pDCs which express CD4 (Sandgren, Smed-Sorensen et al. 2013). Although CD4 negative cell types have been shown to have been infected (Hoffman, LaBranche et al. 1999; Kolchinsky, Mirzabekov et al. 1999; Marras, Bruggeman et al. 2002), CD4 independent Envs are subject to negative selection due to increased sensitivity to neutralisation by antibodies (Hoffman, LaBranche et al. 1999; Edwards, Hoffman et al. 2001; Haim, Strack et al. 2011), suggesting that these CD4 independent Envs may evolve naturally during late disease when the host is immunocomprimised.

Binding to CD4 causes a conformational change in gp120 which then enables binding to the chemokine co-receptors, principally CCR5 or CXCR4 (Zhang, Canziani et al. 1999). Binding of the co-receptor leads to a further conformational change exposing gp41 which embeds into the host cell membrane and initiates fusion of the host and viral envelopes. The fusion of the envelopes releases the HIV-1 capsid into the cytoplasm of the host cell, after which capsid is dismantled through a process called uncoating. This process is still not fully understood and there are a number of different models for the process but most of them agree that it occurs sometime after fusion and entering the cytoplasm and before nuclear entry, though recent studies suggest that the capsid may remain intact until it reaches the nuclear pore (Schaller, Ocwieja et al. 2011; Shah, Shi et al. 2013). This uncoating frees the ssRNA genome, which is bound to other proteins in the form of the reverse transcription complex (RTC). Reverse transcriptase converts the ssRNA to dsDNA, a process which is thought to begin before uncoating, which is actively transported to the nucleus where it is integrated into the host genome by the viral enzyme integrase.

Though viral gene transcription can occur before integration (Engelman, Englund et al. 1995), it is inefficient and it is only after integration that viral replication really begins.

Multiply spliced viral genes (Tat Nef and Rev) are the first genes transcribed, and critically the concentration of Rev accumulates until there is enough to stabilise production and export of full length ssRNA genomes out of the nucleus to the plasma membrane where they are incorporated into the newly formed viruses (Pollard and Malim 1998), which bud from the host cell membrane.

1.1.4 HIV-1 restriction factors

Within the virus lifecycle are multiple steps in which restriction by the host cell can occur. Once within the cell HIV-1 may encounter post entry restriction factors.

After fusion of the viral and cellular envelopes, the viral core must uncoat to free the viral genome and associated proteins required for integration and replication. TRIM5α protein from old world monkeys, such as rhesus macaques have been shown to be able to strongly restrict HIV-1 by interfering with uncoating and reverse transcription as well as targeting the incoming virus for proteasomal degradation (Munk, Brandt et al. 2002; Stremlau, Owens et al. 2004; Wu, Anderson et al. 2006). Human TRIM5α however has a much reduced capacity to restrict HIV-1 (Stremlau, Owens et al. 2004) and the same is true of the proteins from new world monkeys with the exception of owl monkeys. The HIV-1 restriction seen in owl monkey cells has been attributed to the formation of the TRIM-Cyp protein, which is a fusion of TRIM5α with Cyclophilin A (CypA) (Sayah, Sokolskaja et al. 2004). CypA in humans is known to interact with HIV-1 and is a HIV-1 cofactor involved in regulating uncoating and nuclear entry of the reverse transcribed viral DNA (Luban 1996). The formation of a human TRIM-Cyp fusion protein was shown to also be able to restrict HIV-1 (Neagu, Ziegler et al. 2009), suggesting human TRIM5α still retained its ablilty to restrict HIV-1, but that HIV-1 has evolved its capsid to avoid binding to this restriction factor. Similarly for SIV, it seems that the virus has evolved to evade the restriction in its natural host, as TRIM5α from old world monkeys are able to restrict HIV-1, but their ability to restrict SIV is much lower. A protein from the same family as TRIM5α, TRIM22 has also been shown to

have antiviral activity. This restriction has been shown to reduce viral production in infected cells via disruption of Gag localisation to the plasma membrane (Barr, Smiley et al. 2008), and more recently has also been shown to interfere with LTR driven transcription (Kajaste-Rudnitski, Marelli et al. 2011).

The last step of the virus lifecycle requires budding from the host cell, and it is at this stage that tetherin/BST-2, exerts its effects on HIV-1. This protein can tether otherwise normal HIV-1 particles to each other and the host cell envelope preventing normal viral budding and release (Neil, Zang et al. 2008; Perez-Caballero, Zang et al. 2009). Protease treatment or physical shearing has shown that these tethered HIV-1 virions released are fully infectious (Klimkait, Strebel et al. 1990; Neil, Eastman et al. 2006). HIV-1 has evolved to counteract this restriction mechanism using the accessory protein Vpu, which has been reported to rescue viral production in the presence of tetherin (Neil, Sandrin et al. 2007). Vpu has been reported to co-localise with tetherin (Neil, Zang et al. 2008) and downregulate cell surface tetherin expression by targeted degradation (Douglas, Viswanathan et al. 2009; Sato, Misawa et al. 2012). However this downregulation of tetherin expression seems to be dependent on cell type. Vpu has been reported to rescue viral production without affecting cell surface expression of tetherin in CEMx174 and H9 cells suggesting more than one mode of action (Miyagi, Andrew et al. 2009).

Similar to tetherin, apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G) restricts HIV-1 by preventing infection of subsequent cells. APOBEC3G is a host cell ssDNA cytidine deaminase (Suspene, Sommer et al. 2004) and can be incorporated into newly formed viral particles (Xu, Chertova et al. 2007; Strebel and Khan 2008). When the resulting viral particle enters a new host cell and begins reverse transcription, the packaged APOBEC3G, deaminates cytodine to uracil, leading to guanine to adenine mutations in the resulting provirus (Browne, Allers et al. 2009). Another of the HIV-1 accessory proteins has evolved to counteract this

restriction, this time the accessory protein Vif inhibits packaging of APOBEC3G into the viral particle, by causing proteosome mediated degradation of APOBEC3G (Kao, Goila-Gaur et al. 2007).

Recently two new restriction factors have been discovered. It is known that cells from the myeloid lineage are refractory to genetic manipulation with HIV-1 based lentiviral vectors (Neil, Martin et al. 2001), while SIV based lentivectors however have been able to transduce these cells (Negre, Mangeot et al. 2000). The accessory protein Vpx, which is present in HIV-2 and SIV but not found in HIV-1, was found to enable HIV-1 based vectors to transduce DCs and macrophages (Sharova, Wu et al. 2008; Berger, Goujon et al. 2009), leading to the hunt for a restriction factor, which was believed to act early in the HIV-1 lifecycle probably blocking reverse transcription. This restriction factor was discovered to be sterile α motif and HD domain-containing protein-1 (SAMHD1) (Laguette, Sobhian et al. 2011) and SAMHD1 negative cells from patients with Aicardi-Goutières syndrome, were shown to be permissive to infection with HIV-1 (Berger, Sommer et al. 2011). SAMHD1 is a triphosphohydrolase which restricts HIV-1 by hydrolysing deoxynucleotide triphosphates (dNTPs) to nucleoside and inorganic phosphate (Goldstone, Ennis-Adeniran et al. 2011), reducing the concentration and availability of nucleotides available to HIV-1 reverse transcriptase. This has been shown to particularly affect viruses with reverse transcriptases which have low affinity for dNTPs (Lahouassa, Daddacha et al. 2012). The HIV-2/SIV accessory protein Vpx counteracts this by binding to SAMHD1 and targeting it for proteosomal degradation (Hrecka, Hao et al. 2011).

The most recently discovered HIV-1 restriction factor is myxovirus resistance 2 (Mx2 also known as MxB). The Mx proteins were originally discovered in mice and Mx1 was found to be inducible by type I IFN and to a lesser extent type II IFN. Murine Mx1 localises to the nucleus and confers resistance to influenza virus (Zurcher, Pavlovic et al. 1992). The human homologues MxA/Mx1 and Mx2 likewise are inducible by type I

and type II IFNs, however unlike in mice, they localise in the cytoplasm (Aebi, Fah et al. 1989), though Mx2 was later found to have a nuclear form containing a nuclear localisation signal (NLS) (Melen, Keskinen et al. 1996; Melen and Julkunen 1997). Like the murine protein, human Mx1 can inhibit influenza virus (Pavlovic, Zurcher et al. 1990), as well as a number of other DNA and RNA viruses (Pavlovic, Zurcher et al. 1990; Habjan, Penski et al. 2009; Netherton, Simpson et al. 2009; Zhao, Pang et al. 2011; Li, Zhang et al. 2012). Until recently no antiviral activity had been found for the human Mx2 protein. Two recent publications have now reported that Mx2 is a restriction factor for HIV-1 (Goujon, Moncorge et al. 2013; Liu, Pan et al. 2013). Mx2 restriction specificity is determined by binding of viral capsid (Goujon, Moncorge et al. 2013), with capsid mutants able to escape restriction. This restriction may occur via a CypA dependent mechanism as CypA knockdown in SupT1 cells rescues HIV-1 from Mx2 restriction (Liu, Pan et al. 2013). The mechanism of restriction is thought to be via a block to viral integration (Goujon, Moncorge et al. 2013; Liu, Pan et al. 2013). One report showed that restriction by Mx2 may be due to inhibition of reverse transcription with decreased levels of 2LTR circles seen in Mx2 expressing cells (Goujon, Moncorge et al. 2013), though the other report showed that the amount of 2LTR circles were unaffected by Mx2 (Liu, Pan et al. 2013).

As well as these protein based restriction factors, anti-HIV-1 microRNAs (miRNAs) have been described. miRNAs are short RNA sequences which are involved in posttranscriptional regulation. This regulation of gene expression is by sequence specific binding to their targets, and usually results in negative regulation of gene expression (Bartel 2009), either by blocking translation, or by inducing degradation of the target mRNA. There have also been reports that miRNAs can upregulate gene expression (Place, Li et al. 2008), and one report showed both up and downregulation of gene expression, dependant on the cell cycle status (Vasudevan, Tong et al. 2007).

Comparisons of permissive and non permissive cells showed elevated levels of miRNA-28, miRNA-150, miRNA-223 and miRNA-382, in non permissive cells. Knock down of these miRNAs increased susceptibility to HIV-1 infection and expression of the miRNAs in permissive cells restricted HIV-1 infection (Wang, Ye et al. 2009).

Figure 2 summarises this showing the stage in the viral lifecycle each restriction factor acts upon and also the viral accessory proteins which counteract them.

Figure 2 - HIV-1 restriction factors and viral countermeasures

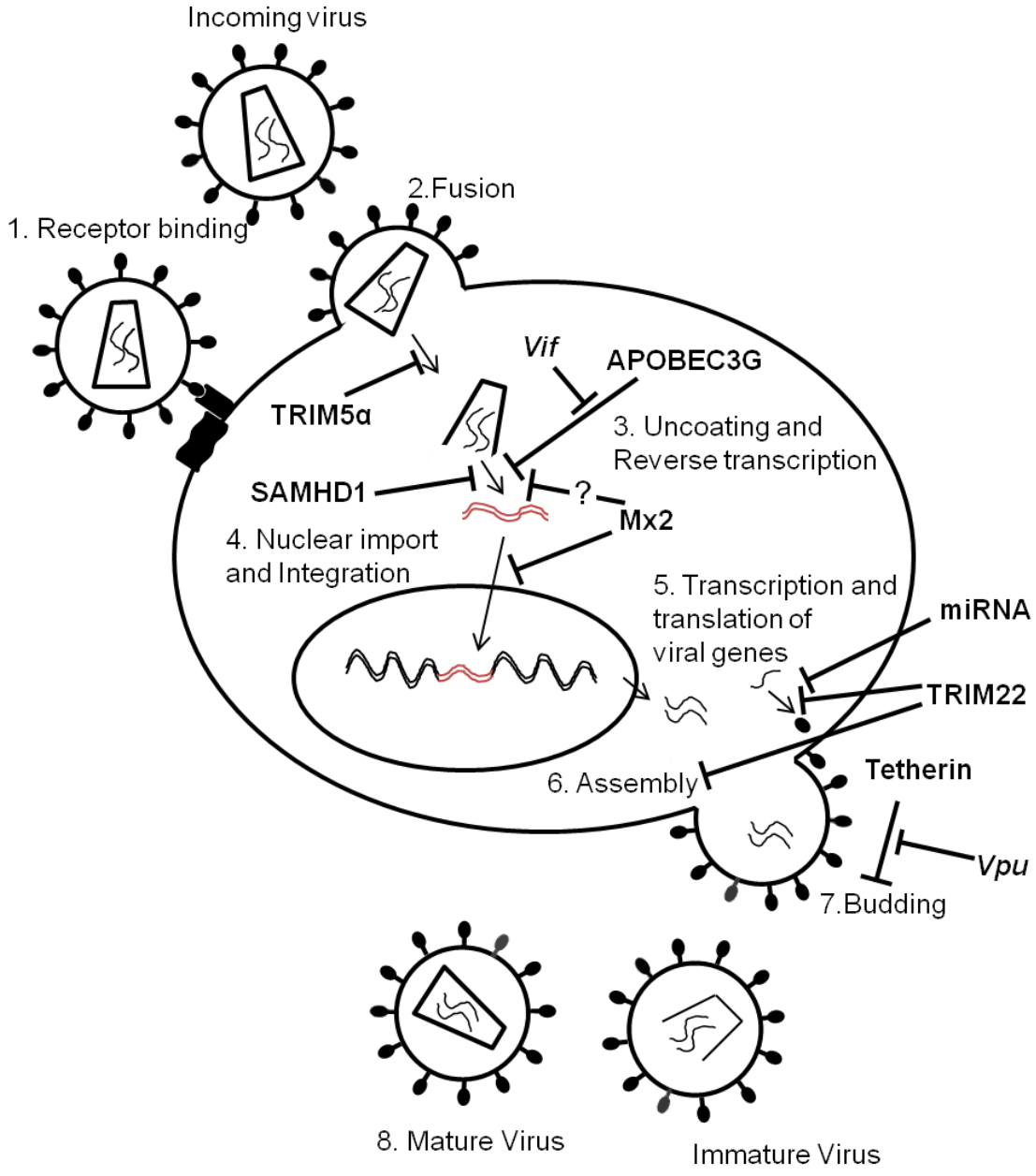


Diagram detailing a number of HIV-1 restriction factors, where they act in the virus lifecycle and viral accessory proteins which counteract them.

1.2 Macrophages

HIV-1 infection and depletion of CD4+ T cells is critical to the development of AIDS. Unlike other retroviruses, the lentivirus family are able to effectively infect cells which are not dividing such as macrophages, and these cells are thought to be another major target cell for HIV-1 infection.

Macrophages are tissue resident mononuclear phagocytes which are present throughout the body albeit given different names depending on their location (Table 1).

Table 1 - Different names of macrophages distributed throughout the human body.

Name of cell	Location
Adipose tissue macrophages	Adipose tissue
Alveolar macrophages (Dust cells)	Lungs
Epithelioid cells	Granulomas
Giant cells	Connective tissue
Histiocytes	Connective tissue
Hofbauer cell	Placenta
Kupffer cells	Liver
Microglia	Brain
Osteoclasts	Bone
Peritoneal macrophages	Peritoneal cavity
Sinusoidal lining cells	Spleen

Table showing the different names given to macrophage populations depending on their localisation within the human body

Macrophages were originally thought to derive from circulating blood monocytes which migrate out of the blood stream in response to chemical signals, such as cytokines released during infection, inflammation and tissue damage. It was originally thought that macrophages were terminally differentiated cells derived from monocytes alone, but evidence from mice show that some of the tissue resident macrophages are generated by self renewal of cells already present in the tissues. Some of these macrophage populations have been shown to have been laid down during embryogenesis, and replenish themselves independently of circulating blood monocytes (Yona, Kim et al. 2013). It is likely that the self renewal of these cell populations are from local precursors (Merad, Manz et al. 2002; Ajami, Bennett et al. 2007), though macrophages have also been shown to be able to proliferate under certain conditions (Jenkins, Ruckerl et al. 2011), and the ability of different cells to transdifferentiate into macrophages has also been described (Sasmono, Ehrnsperger et al. 2007; Rapino, Robles et al. 2013).

Monocytes are commonly defined by their CD14 expression and make up around 10% of the total peripheral blood mononuclear cells (PBMCs). They were originally thought to be a homogeneous population of cells, but it was later found that subsets of cells existed within the CD14+ monocyte population which stained positive for the cell surface marker CD16. The classical CD14+CD16- monocytes make up the majority of the population, whilst the CD14+CD16+ make up the remainder and account for around 10% of the total monocytes in a healthy individual, though the levels of CD16+ cells can be transiently increased by exercise (Steppich, Dayyani et al. 2000). Since then further subgroups have been described. The CD16+ can be divided into two groups by the amount of CD14 expression. The population of cells expressing low levels of CD14 have a reduced ability to phagocytose and are proinflammatory releasing tumour necrosis factor (TNF)-α in response to bacterial lipopolysaccharide (LPS) compared to the anti-inflammatory interleukin (IL)-10 secreting CD14 high

expressing population of CD16+ monocytes (Skrzeczynska-Moncznik, Bzowska et al. 2008). A CD56+ population of monocytic cells showed increased ability to induce T cell proliferation to specific antigens, and although cytokine production to stimulation was similar to that of conventional CD14+ CD56- monocytes, they also produced IL-6 and IL-1β (Sconocchia, Keyvanfar et al. 2005). More recently the CD16+ population has been further subdivided on the basis of CCR2 expression (Shantsila, Wrigley et al. 2011). These different monocyte subsets have been shown to express different levels of cell surface markers, have different phagocytic capacity and differ in cytokine secretion to stimulation (Ziegler-Heitbrock 2007; Serbina, Cherny et al. 2009; Settles, Etzrodt et al. 2011). It appears that at least some of the subsets of cells may be more differentiated forms of the conventional monocytes rather than a separate lineage of cells (Ancuta, Liu et al. 2009). Studies have been performed looking at the relationship between different monocyte subsets and DCs and whether their functions differ depending on the subset of cells differentiated from. However most of these studies were performed in mice. One study reported that in humans, the different monocyte subtypes broadly responded in the same way with a few differences and that DCs differentiated from the different monocyte subsets had similar phenotypes, expressing DC markers and responding to stimulation in similar ways, though the amounts of some cytokines released in response to stimulation differed (Sanchez-Torres, Garcia-Romo et al. 2001).

Monocytes are thought to be the principal precursor for the production of many macrophage and DC populations (Gordon and Taylor 2005). Macrophage colony-stimulating factor (M-CSF) is an essential cytokine for monocyte development and is a survival factor for all cells of the mononuclear phagocyte family, which includes monocytes DCs and macrophages and their precursors (Erickson-Miller, Brennan et al. 1990; Langstein and Schwarz 1999). For monocytes in culture, the presence of M-CSF in serum and the M-CSF produced by the monocytes themselves (Lee,

Kaushansky et al. 1990) is enough to differentiate them into macrophages, though this process is much more efficient when additional M-CSF is added, and is the most common method of generating macrophages from peripheral blood monocytes in vitro (Hume 2006).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has also been shown to generate macrophages, however it is more commonly used to generate immature DCs in vitro, in addition to IL-4, and unlike M-CSF which is expressed constitutively, GM-CSF is expressed as part of the inflammatory response (Campbell, van Nieuwenhuijze et al. 2011). However GM-CSF has been shown to be required for required for maturation of alveolar macrophages (Bonfield, Raychaudhuri et al. 2003; Carey and Trapnell 2010) and MDM differentiated with GM-CSF have been reported to be most like alveolar macrophages and have similar cell surface markers (Akagawa, Komuro et al. 2006). These observations suggest that GM-CSF may also be involved in the differentiation of some macrophage subsets.

Macrophages are a heterogeneous population due to their different tissue locations as well as the array of functions they perform. In mice they are classified under 2 broad groups M1, classically activated macrophages and M2, alternatively activated macrophages.

IFNγ and bacterial products such as LPS generate classically activated, M1, macrophages, which are characterised by pro-inflammatory gene expression, antimicrobial killing of intracellular pathogens, increased complement mediated phagocytosis, increased antigen presentation, and are associated with the Th1 adaptive immune response (Murray, Rubin et al. 1983; Nathan, Murray et al. 1983; Dalton, Pitts-Meek et al. 1993; Drevets, Leenen et al. 1996; Verreck, de Boer et al. 2004). They are usually classified by high IL-12, high IL-23 and low IL-10 expression, as well as their ability to produce reactive oxygen or nitrogen intermediates ROI/RNI

and proinflammatory cytokines such as CXCL-10 also known as interferon induced protein (IP)-10 (Gordon and Taylor 2005; Mantovani 2006).

Alternatively activated, M2, macrophages are a broad group of macrophages, encompassing macrophages activated with the non classical stimuli IL-4 and IL-13 as well as immune complexes, IL-10, and glucocorticoid or secosteroid hormones (Stein, Keshav et al. 1992; Goerdt and Orfanos 1999; Gordon 2003). M2 macrophages have a phenotype of low IL-12, low IL-23 and high IL-10 expression, and are generally anti-inflammatory in nature and are associated with Th2 responses (Verreck, de Boer et al. 2004). M2 macrophages are involved in immunoregulation and tissue remodelling and show upregulation of non opsonic receptors such as mannose, scavenger and C-type lectin receptors (Stein, Keshav et al. 1992; Mantovani, Sica et al. 2004), upregulation of major histocompatability complex (MHC) class II (de Waal Malefyt, Figdor et al. 1993) and increased endocytosis (Montaner, da Silva et al. 1999). As well as expressing high levels of IL-10, M2 macrophages have decreased pro-inflammatory cytokine expression and increased expression of IL-1 receptor agonist (Dinarello 1991; Scotton, Martinez et al. 2005).

1.2.1 Macrophage Functions

Macrophages perform a variety of functions. One of the earliest roles for macrophages is seen in tissue remodelling during embryogenesis, and can be seen during development of the limb buds (Henson and Hume 2006). Macrophages were named for their ability to internalise large particles or phagocytose, and during embryogenesis macrophages are seen to congregate in the interdigital spaces of the developing limb bud, there they are involved in the phagocytosis and clearing of cells undergoing programmed cell death (Hopkinson-Woolley, Hughes et al. 1994).

Macrophages continue this role later in life where they are also involved in removing damaged tissues by phagocytosis of apoptotic cells, aiding wound healing and tissue

repair (Hopkinson-Woolley, Hughes et al. 1994; Henson and Hume 2006; Richardson, Slanchev et al. 2013).

1.2.2 Macrophage innate immune responses

The other major role for macrophages is in the immune system. Macrophages can be thought of as sentinel cells and are located throughout the tissues of the body. Macrophages can employ a variety of responses upon encountering a pathogen.

The innate immune system refers to host recognition of microbial pathogens by germ-line encoded receptors that, unlike immunoglobulin and T cell receptors, do not undergo gene rearrangement and therefore do not structurally 'adapt'. Innate immune receptors show specificity for molecular motifs found on microbial organisms but not commonly found or exposed in the mammalian host. These motifs are known as pathogen associated molecular patterns (PAMPs), and are detected by the innate immune pattern recognition receptors (PRRs).

Macrophages can directly kill pathogens that are encountered via phagocytosis. Pathogens such as bacteria can be recognised by macrophages either directly via cell surface PRRs such as the mannose receptor or indirectly if the bacterium has been opsonised, coated with either complement or antibodies (Aderem and Underhill 1999). This recognition of the pathogen by the phagocytosis receptors, leads to actin remodelling and the enveloping of the pathogen by the plasma membrane of the macrophage, leading to the internalisation of the pathogen in a vacuole called the phagosome (Aderem and Underhill 1999). After internalisation, phagosome maturation occurs where the phagosome acidifies due to fusing with endosomes and finally with the lysosome, forming the phagolysosome where the hydrolytic enzymes from the lysosome digests the phagocytosed pathogen.

Macrophages are also involved in the inflammatory response and secrete a variety of cytokines and chemokines which can attract and activate other immune cells to the site

of infection. They are also an important bridge to the adaptive immune response and can recruit and activate the adaptive immune system against incoming pathogens by presenting antigen to T cells via MHC, activating the adaptive immune response (Carrasco and Batista 2007; Junt, Moseman et al. 2007; Barrio, Abes et al. 2012; Bayer, Varani et al. 2013).

1.2.3 Role of IFN Gamma in Macrophage Function

IFNγ is a key modulator of macrophage function. This cytokine is principally produced by activated CD4+ Th1 type cells and natural killer (NK) cells. Stimulation of macrophages by IFNγ has many effects (Schroder, Hertzog et al. 2004), including upregulation of both the class I antigen presentation pathway as well as the class II pathway which is involved in activation of CD4+ T cells and also induction of an antiviral state which includes the upregulation of retroviral restriction factors. Importantly many macrophage effector functions are modulated by IFNγ. In mice exposure to IFNγ, has been reported to upregulate phagocytosis (Perri, Shabani et al. 2008) and antimicrobial activities via production of reactive oxygen intermediates (Green, Nacy et al. 1991; Gordon, Jack et al. 2005). IFNγ also primes mouse macrophages, making them more sensitive to TLR stimuli (Jurkovich, Mileski et al. 1991) and pathogens such as Leishmania (Green, Crawford et al. 1990), and enhances innate immune responses (Totemeyer, Sheppard et al. 2006). These effects are mainly restricted to mice, though upregulated phagocytosis and killing of intracellular pathogens have been reported in human macrophages (Gordon, Jack et al. 2005)

Another effect of macrophage stimulation with IFNγ is the induction of autophagy (Gutierrez, Master et al. 2004). Autophagy is a cellular process for clearing intracellular debris and organelles, and also for recycling of nonessential cellular components in times of starvation. A double membrane is created around the cytoplasmic components and they are targeted for degradation by lysosomes. As well as for

recycling damaged organelles and recycling of components during starvation autophagy is also important in clearing intracellular pathogens such as replicating viruses or bacteria which have managed to escape into the cytoplasm of the macrophage (Rich, Burkett et al. 2003; Amano, Nakagawa et al. 2006; Levine and Kroemer 2008).

1.3 Pattern recognition receptors

Cell associated PRRs are diverse, and include membrane bound receptors such as transmembrane Toll like receptors (TLRs) and C-type lectin receptors (CLR), and cytosolic receptors including retinoid acid inducible gene–1 (RIG-1/I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) as well as the newer cytosolic DNA sensors, with each receptor specifically recognising certain types of PAMPS (Table 2- page 42). Microbial activation of these receptors induces an intracellular signalling cascade that leads to activation of transcription factors or other effectors that regulate defence responses. The physiological significance of innate immune cellular activation pathways has been confirmed in numerous experimental models with targeted genetic deletion of individual PRRs or components of downstream signalling pathways. In general these are associated with increased susceptibility to selected infections and frequently impaired adaptive immune responses (Qureshi and Medzhitov 2003; Diebold, Kaisho et al. 2004; Drennan, Nicolle et al. 2004; Tabeta, Georgel et al. 2004; Honda, Yanai et al. 2005; Hawn, Smith et al. 2006; Hoshino, Sugiyama et al. 2006; Yarovinsky, Hieny et al. 2008). Similar findings have been reported in rare human examples of genetic deficiency of PRRs or innate immune signalling components (Medvedev, Lentschat et al. 2003; Picard, Puel et al. 2003), and genetic polymorphisms in PRRs are increasingly reported to correlate with clinical infectious diseases (Agnese, Calvano et al. 2002; Bochud, Hawn et al. 2003; Bochud, Chien et al. 2008; Davila, Hibberd et al. 2008; Leoratti, Farias et al. 2008)

1.3.1 Toll Like Receptors

Macrophages express many PRRs and the most studied of these are the TLRs which were originally discovered as toll receptors in Drosophila. TLRs are transmembrane proteins comprised of leucine rich repeats (LRR) which are responsible for the recognition of specific PAMPs and intracellular Toll–IL1 receptor (TIR) domains responsible for interaction with adaptor molecules and downstream signal transduction.

TLRs can be split into two groups based on location, the cell surface, plasma membrane expressed TLRs 1,2,4,5 and 6 which recognise molecular patterns found on the surface of the pathogen including microbial structures such as LPS and flagellin. The second group are the endosomal TLRs 3,7,8 and 9 which recognise microbial RNA and DNA (Takeuchi and Akira 2010). There is also a 10th TLR in humans however the function and ligand for TLR10 is still as yet unknown, though based on pull down experiments where TLR10 was found to heterodimerise with TLRs 1 and 2 and the computational 3D structure of these dimmers, there is speculation it could possibly be a bacterial lipopeptide with a structure similar to Pam3CSK4 (Govindaraj, Manavalan et al. 2010).

Though TLRs recognise a diverse set of PAMPS and have differing cellular localisations (summarised in Table 2) they signal via common signalling pathways either through the Myeloid Differentiation primary response gene 88 (MyD88) dependant pathway, which as the name suggests, signals via the adaptor protein MyD88 and the MyD88 independent pathway which is mediated by the TIR-domain-containing adapter-inducing interferon-β (TRIF) protein (Takeda and Akira 2004).

The MyD88 dependent pathway is utilised by all TLRs with the exception of TLR3. Upon stimulation of the TLR with its ligand, MyD88 recruits IL-1 receptor-associated kinase (IRAK) members to the TLR complex via the death domains which are present on both proteins. IRAK is then phosphorylated leading to activation of nuclear factor

kappa B (NFκB) and the mitogen activated protein kinase (MAPK) pathway and finally to inflammatory cytokine expression (Takeda and Akira 2004; Kawai and Akira 2010) The MyD88 independent pathway is utilised by TLR3 and TLR4 and signals via TRIF which via also leads to NFκB and MAPK kinase activation and additionally activates the transcription factor interferon regulatory factor (IRF)3 which leads to type I IFN expression. For unknown reasons though all the other TLRs function using just one signalling pathway, TLR 4 seems to require both (Hoebe, Du et al. 2003; Yamamoto, Sato et al. 2003).

Table 2 - PRRs cellular localisation and ligands.

Pattern recognition receptor	Localization	Ligand(s)	Ligand origin
Toll-like receptors			
TLR1/2	Plasma	Triacyl lipopeprides	Bacteria
TLR2	Plasma membrane	PGN, porins, lipoarabinomannan HA protein tGPI-mucin, Zymosan	Bacteria, Viruses, Protozoa
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma	LPS	Bacteria
TLR5	Plasma	Flagellin	Bacteria
TLR6/2	Plasma	Diacyl lipoprotein, lipoteichoic acid	Bacteria, Viruses
TLR7	Endolysosome	ssRNA	Bacteria, Viruses
TLR8	Endolysosome	ssRNA	Bacteria, Viruses
TLR9	Endolysosome	CpG DNA, dsDNA, Malaria hemozoin	Bacteria, Viruses, Protozoa
TLR10	Endolysosome	Unknown	Unknown
Scavenger receptors			
SR-A	Plasma	LPS, LTA, CpG DNA, proteins	Bacteria
MARCO	Plasma	LPS, proteins	Bacteria
CD36	Plasma	Diacylated lipopeptide	Bacteria
LOX-1	Plasma	Protein	Bacteria
SREC	Plasma	Protein	Bacteria

Table 2 continued

PRR	Localization	Ligand(s)	Ligand origin
C-type lectins			
DC-SIGN	Plasma membrane	LPS, ManLAM, CPS, CTL	Bacteria, Virus, Protozoa
Mannose receptor	Plasma membrane	LPS, CPS, ManLAM	Bacteria, Virus, Fungi, Protozoa
Dectin-1	Plasma membrane	β-Glucan, mycobacterial ligand	Fungi
Dectin-2	Plasma membrane	β-Glucan, high mannose structures	Fungi
MINCLE	Plasma	SAP130	Fungi
NOD-like receptors			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
NLRP1	Cytoplasm	MDP, Anthrax lethal toxin	Bacteria
NLRP3	Cytoplasm	RNA, LPS, LTA, MDP Viral RNA, Crystaline structures, ATP	Bacteria,Viruses, Protozoa, Fungi
NLRC4	Cytoplasm	Flagellin	Bacteria
Naip5	Cytoplasm	Flagellin	Bacteria
DNA sensors			
IFI16	Cytoplasm	dsDNA	Virus
DAI	Cytoplasm	dsDNA	Virus
AIM2	Cytoplasm	dsDNA	Virus
DDX41	Cytoplasm	dsDNA	Bacteria ,Virus
DHX9	Cytoplasm	CpG-B	Bacteria, Virus, Fungi
DHX36	Cytoplasm	CpG-A	Bacteria, Virus, Fungi
cGAS	Cytoplasm	dsDNA	Virus

Table 2 continued

PRR	Localization	Ligand(s)	Ligand origin
RIG-like receptors			
RIG-I	Cytoplasm	Short dsRNA, 5′ triphosphate dsRNA	RNA viruses, DNA viruses
MDA5	Cytoplasm	Long dsRNA	RNA viruses
LGP2	Cytoplasm	RNA	RNA viruses
Other receptors			
CD14	Plasma membrane	Peptidoglycan, LTA, LPS, mannuronic acid	Bacteria
CR3	Plasma membrane	Oligosaccharides, microbial protein β-Glucans	Bacteria, Fungi
TREM2/DAP12	Plasma	LPS, microbial molecules	Bacteria, Fungi
TREM1/DAP12	Plasma	Unknown	Bacteria

A summary the types of PRRs found on mammalian cells, their localisation and ligands. Adapted from (Kumar, Kawai et al. 2011; Pluddemann, Mukhopadhyay et al. 2011)

1.3.2 C-Type Lectin Receptors

This family of receptors is characterised by their binding of carbohydrates via C-type lectin-like domains (CTLD). These receptors are important in immunity against fungi, and binding of fungal PAMPS to Dectin 1, Dectin 2 and CLEC4E/ macrophage-inducible C-type lectin (MINCLE) leads to signalling via the spleen tyrosine kinase (SYK), resulting in the activation of MAPK, nuclear factor of activated T-cells (NFAT) and NFκB (Gross, Gewies et al. 2006; Goodridge, Simmons et al. 2007; Strasser, Neumann et al. 2012).

1.3.3 RIG-I Like Receptors

There are 3 RIG-I like receptors in humans, and these DExD/H box helicases are called RIG-I, melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2).

RIG-I like receptors are comprised of a regulatory C-terminal domain, a central helicase domain responsible for ATP and nucleic acid binding and two tandem N-terminal caspase activation and recruitment domains (CARD), which are absent in LGP2. Overexpression of the tandem CARD domains has been reported to be able to signal independently of the rest of the RIG-I like receptor, (Yoneyama, Kikuchi et al. 2005) and the fact that the full length RIG-I has little activity in the absence of stimulation with nucleic acids suggested that there is a autorepression within the receptor (Saito, Hirai et al. 2007). Because of this the LGP2 receptor was thought to be a negative regulator of RIG-I and MDA5, however more recent studies have shown that it can act to positively regulate RIG-I and MDA-5 responses (Satoh, Kato et al. 2010).

Despite their structural similarity RIG-I and MDA5 recognise different subsets of viruses. RIG-I recognises short RNA (25bp-1kb) with optimal activation by blunt ended ssRNAs containing a uncapped 5’ triphosphate (Takahasi, Yoneyama et al. 2008; Lu,

Xu et al. 2010) such as those found on ssRNA viral genomes (Weber, Gawanbacht et al. 2013). In human cells the 5' triphosphate found in mRNA is commonly capped preventing self activation of innate immune responses. Additionally RNA containing a 3' overhang inhibits signalling through RIG-I, preventing self RNA products processed by the Dicer machinery from triggering innate immune responses (Marques, Devosse et al. 2006).

The ligands for MDA5 are less well characterised. It is thought to recognise longer dsRNA (>2kb) (Hornung, Ellegast et al. 2006; Pichlmair, Schulz et al. 2006; Kato, Takeuchi et al. 2008). Though more recent work reports that higher order RNA structures and not just long strands of dsRNA is the motif detected by MDA-5 (Pichlmair, Schulz et al. 2009)

Detection of nucleic acids by RIG-I and MDA5 along with the binding of adenosine triphosphate (ATP) in RIG-I leads to unfolding of the protein which exposes the CARD and helicase domains allowing for ologomerisation and the binding of the protein IFNβ promoter stimulator 1 (IPS-1) (also known as mitochondrial antiviral signalling protein (MAVS), virus induced signalling adapter (VISA), and CARD adapter inducing IFNβ (CARDIF)) which is located on the outer mitochondiral membrane (Kawai, Takahashi et al. 2005; Meylan, Curran et al. 2005; Seth, Sun et al. 2005; Xu, Wang et al. 2005). IPS-1 binding and activation by the RLRs leads to activation of the transcription factors NFκB, IRF3 and IRF7 and the induction IFN-β and the antiviral response (Yoneyama, Kikuchi et al. 2004)

1.3.4 NOD Like Receptors

The NLRs are a large family of cytosolic receptors, and the functions of many are currently poorly understood, with 23 members having been discovered in humans (Kanneganti, Lamkanfi et al. 2007). These receptors are characterised by a C-terminal LRRs which are responsible for the recognition of microbial structures, a central NOD,

and a variable N-terminal domain which is usually contains a protein binding region which is then responsible for downstream signalling (Kanneganti, Lamkanfi et al. 2007).

NOD1 and NOD2 contain CARDs and recognise components of bacterial cell walls, peptidoglycans. NOD1 recognises g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and NOD2 recognises muramyl dipeptide (MDP), binding of which leads to recruitment of receptor interacting protein 2 (RIP2) via the CARD domain, and subsequently NFκB activation (Girardin, Boneca et al. 2003; Yang, Yin et al. 2007)

A number of NLRs including NLRP1, NLRP3, and NLRC4 are also involved in the formation of the inflamasome. The NLRs have been described to form inflamasome complexes via self oligomerisation via their nucleoptide binding NACHT domains and recruitment of pro-caspase 1 and the adaptor protein ASC (Schroder and Tschopp 2010; Davis, Wen et al. 2011). Formation of the complex activates caspase 1 which can protelytically cleave pro-IL-1β and pro-IL-18 into the inflammatory cytokines IL-1β and IL-18 (Martinon, Burns et al. 2002)

1.3.5 DNA sensors

The most recently discovered set of PRR are the cytosolic DNA sensors. The first one to be described was DNA-dependent activator of IFN-regulatory factors (DAI) (Takaoka, Wang et al. 2007) and binding of dsDNA to DAI was shown to activate innate immune responses by signalling through stimulator of IFN genes (STING) and TANK (TRAF family member-associated NF-kappa-B activator) binding kinase (TBK)-1 leading to activation of IRF3 and NFκB and subsequent IFN-β production (Takaoka and Taniguchi 2008). However knockout studies of DAI showed little effect on IFN-β production in mouse embryonic fibroblasts stimulated with poly(dA:dT) (Wang, Choi et al. 2008) and mice lacking DAI also showed normal responses in response to DNA virus infection compared to TBK-1 and STING knockout mice (Ishii, Kawagoe et al. 2008), suggesting redundancy in DNA sensing.

This led to the discovery of further cytosolic DNA sensors. The pyrin and HIN domain-containing (PYHIN) family of proteins, also known as the absent in melanoma 2 (AIM2) like receptors (ALRs), are comprised of a pyrin domain and a hemopoietic expression, interferon-inducibility, nuclear localization (HIN) domain. There are 4 members of the PYHIN family in humans, two of which have been shown to be involved in DNA sensing. AIM2 binds to dsDNA via its C-terminal oligonucleotide/oligosaccharide-binding domain, and via its pyrin domain interacts with ASC to activate caspase-1 and the inflammasome as well as NFκB activation (Hornung, Ablasser et al. 2009). The other PYHIN member which has been shown to recognise dsDNA is gamma interferon inducible protein 16 (IFI-16). IFI-16 was shown to recognise dsDNA motifs from vaccinia virus and HSV-1, with downstream signalling shown to be mediated by STING (Unterholzner, Keating et al. 2010) which appears to be a critical adaptor protein for NFκB and IRF3 activation by cytosolic DNA sensors similar to MyD88 and TRIF for TLRs.

RNA polymerase III was found to mediate innate immune responses to AT rich dsDNA via transcription into dsRNA containing a 5'-triphosphate moiety which is detected by RIG-I leading to subsequent signalling and IFN production as described earlier (Ablasser, Bauernfeind et al. 2009; Chiu, Macmillan et al. 2009)

In primary human mDCs, the DEXDc helicase DDX41 has been shown to bind double stranded poly(dG:dC) DNA, Listeriolysin DNA and DNA from vaccinia virus via its DEADc (Asp-Glu-Ala-Asp) domain (Zhang, Yuan et al. 2011). Recognition of DNA activates NFκB, MAPK and IRF3 and induces IFN-β production, via signalling though STING (Zhang, Yuan et al. 2011). In human pDCs 2 other helicases have been found with specificity for dsDNA. The DExD/H box helicases DHX9 and DHX36, recognise the dsDNA oligodeoxynucleotides CpG-B and CpG-A respectively. CpG-A is characterised by a pallindromic CG backbone which has been partially phosphorothioated (non bridging oxygen molecules replaced with sulphur) and the

presence of a 3' poly G tail which are not present in CpG-B. CpG-B additionally contains a fully phosphorothioated backbone. CpG recognition by DHX9 leads to NF-κB activation, TNFα and IL-6 production and DHX36 activation leads to IRF7 nuclear translocation and the production of IFNα. Both helicases are dependent on signalling via the adaptor protein MyD88 suggesting a synergy between TLR9 for detection of incoming virus with DHX9 and DHX36 detecting replicating viruses in pDCs.(Kim, Pazhoor et al. 2010).

The most recent addition to the cytosolic DNA sensor family is cyclic-GMP-AMP synthase (cGAS). Binding dsDNA induces the production of cyclic-GMP-AMP (cGAMP) from ATP and GTP (Ablasser, Goldeck et al. 2013). This cyclic dinucleotide binds to and activates STING (Ablasser, Goldeck et al. 2013; Civril, Deimling et al. 2013; Xiao and Fitzgerald 2013) leading to the dimerisation and activation of IRF3 which induces expression of IFNβ (Sun, Wu et al. 2013).

1.4 PRR activation and downstream signalling events.

Despite the variety of PRRs, their localisation within the cell as well as the range of different PAMPS recognised, binding of PAMPs to the PRRs usually leads to signal transduction via common adaptor molecules such as MyD88, TRIF, STING and TBK-1 and then downstream activation of MAPK, IRF, and NFκB transcription factors which are important in innate immune responses.

1.4.1 NFκB Signalling

The NFκB family of transcription factors is comprised of 5 members, They include RelA (p65), NFκB1 (p50; p105), NFκB2 (p52; p100), c-Rel and RelB (Li and Verma 2002). The DNA binding subunits of NFκB1 and NFκB2, p50 and p52, are formed by post-translational cleavage of larger p105 and p100 precursors. All the NFκB family members contain a structurally conserved 300 amino acid N-terminal Rel-homology domain (RHD), which is responsible for dimerisation, nuclear-localization and DNA

binding. These transcription factors exist as homo or heterodimers, with homodimers of p50 and p52 acting as transcriptional repressors (Plaksin, Baeuerle et al. 1993; Brown, Linhoff et al. 1994) due to their lack of transactivation domain (TD) compared to the other NFκB proteins (Li and Verma 2002).

The transcriptional activity of the NFκB dimers is controlled by their association with the inhibitors of NFκB (IκB) proteins, which contain ankyrin (ANK) repeats, and block the nuclear localisation sites of NFκB thus sequestering it within the cytoplasm (Huxford, Huang et al. 1998).

The most abundant component of the NFκB family is the RelA/ p50 heterodimer, in which p50 principally functions as a regulatory element interacting with IκBα (Jacobs and Harrison 1998). This complex shuttles between nucleus and cytoplasm but in unstimulated cells shows relative cytoplasmic sequestration. This is due to the fact that binding of IκBα to the RelA/p50 dimer only blocks the nuclear localisation signal (NLS) on one of the NFκB subunits which allows NFκB to shuttle into the nucleus. However IκBα also contains a nuclear export signal (NES) and because nuclear export by IκBα is more efficient than nuclear import, though there is constant shuttling to and from the nucleus, the overall effect is cytoplasmic sequestration of NFκB (Birbach, Gold et al. 2002).

NFκB activation and nuclear translocation is induced by a wide variety of signals including cell stress, cytokines such as TNFα as well as many of the PRRs described earlier. The activation of the classical/cannonical NFκB pathway involves the RelA/p50 heterodimer. An essential part of NFκB activation is phosphorylation of IκB by the IκB Kinase (IKK) complex. The IKK complex is comprised of IKKα, IKKβ and the regulatory subunit IKKγ also known as NFκB essential modulator (NEMO) which has no kinase activity. Innate immune activation leads to activation of the IKK complex and in the classical NFκB pathway IKKβ phosphorylates IKBα which leads to ubiquitination and

finally proteasome targeted degradation, thus freeing the RelA/p50 dimer from the IκB complex and allowing translocation into the nucleus where ReL A potent transcription factor activity inducing gene expression (Karin and Ben-Neriah 2000; Schreiber, Jenner et al. 2006).

This transcriptional activation is controlled by a negative feedback loop, by which RelA/p50 binds NFκB binding sites on the IκBα gene promoter and inducing expression of the inhibitor which binds RelA/p50 causing translocation back into the cytoplasm (Brown, Park et al. 1993; Sun, Ganchi et al. 1993).

In the alternative/non-canonical NFκB pathway, IKKα homodimers phosphorylate the p100 subunit of the p100/RelB dimer (Senftleben, Cao et al. 2001; Dejardin, Droin et al. 2002), subsequent ubiquitination and proteosomal degradation of the inhibitory portion of p100, releasing the N-terminal p52 subuinit which contains the RHD allowing for nuclear translocation of p52/RelB dimers (Xiao, Harhaj et al. 2001). Unlike the classical NFκB pathway, the alternative pathway is only activated by a limited range of stimuli, mainly of the TNF family, but not by TNFα itself. Aditionally unlike classical NFκB signalling which is quick and transient in response to stimulation, signalling through the alternative pathway is slow and persistant (Sun 2011). The functions of this alternative NFκB pathway are less well known, though alternative NFκB signalling is the predominant form of NFκB signalling seen in secondary lymphoid organs such as the spleen and thymus (Weih, Carrasco et al. 1994) and it may be involved in the formation of these organs as well as B cell maturation (Senftleben, Cao et al. 2001).

1.4.2 IRF Signalling

IRF3 and IRF7 are known as the master regulators of IFN. As described earlier, innate immune stimulation via PRRs can lead to phosphorylation and activation of these transcription factors. Phopshorylation of IRF3 allows it to either homodimerise or heterodimerise with IRF7, which unlike IRF3, is only expressed at low levels in most

cell types. This differential expression of IRFs means that the majority of early signalling events is via IRF3 homodimers, which can potently stimulate IFNβ production but not IFNα, (Sato, Tanaka et al. 1998; Yoneyama, Suhara et al. 1998). Activation of the IFN receptor (IFNR) by secreted IFNβ, results in an autocrine feedback loop that leads to a positive regulation of IFN production. Binding of IFNβ to the IFNR causes phosphorylation and homodimerisation of signal transducers and activators of transcription (STAT)1 and STAT2. The dimeric STAT proteins are able to translocate to the nucleus and induce expression of IRF7, which under continued PRR signalling is phosphorylated forming IRF7 homodimers which strongly induce expression of both IFNα and IFNβ.(Marie, Durbin et al. 1998; Sato, Hata et al. 1998).

1.4.3 MAPK Signalling

The MAPK signalling pathway mediates cellular responses to a wide variety of extracellular signals, such as growth factors, cytokines, stress and innate immune stimulation. Activation of MAPK signalling can lead to a wide range of cellular responses including regulation of gene expression, cell growth, mobility and differentiation. The most studied of these pathways are the extracellular regulated kinases (ERK)1/2, Jun N-terminal kinases (JNK1/2/3) and p38 MAPK (α,β,γ and δ). These signalling pathways are comprised of a cascade of 3 MAPKs, which sequentially phosporylate the downstream MAPK, and are termed MAPK Kinase Kinase (MAPKKK / MAP3K), MAPK Kinase (MAPKK / MAP2K) and finally MAPK (Krishna and Narang 2008). Signalling via the MAPK pathway often occurs via signal transduction through small G protein receptors on the cell surface, resulting in the subsequent phosphorylation and activation the MAPKKK. MAPKKK phosphorylation activates the Ser/Thr Kinase activity which allows for phosphorylation of MAPKK on serine and threonine residues. MAPKK have dual kinase specificity and phosphorylates MAPK on both threonine and tyrosine residues within the activation loop of the kinase domain,

and once activated MAPK phosphorylate their targets on serine or threonine (Krishna and Narang 2008).

1.4.4 The immune response after PRR activation

Microbial detection by PRRs and the downstream signalling events activating the MAPKs and transcription factors such as NFκB, and IRFs leads to a number of cellular responses. PRR activation can activate the cells antimicrobial mechanisms such as phagocytosis and intracellular killing (Gallego, Golenbock et al. 2011) as well as inducing the activation and release of pro inflammatory cytokines. Additionally PRR activation also leads to alteration in transcription of a number of genes involved in defence against pathogens. For example LPS stimulation of macrophages causes transcriptional regulation of over 400 genes (Sharif, Bolshakov et al. 2007) including increased expression of TNFα, IL-1, granulocyte colony-stimulating factor (G-CSF), GM-CSF, M-CSF and IL-8 to. These cytokines stimulate bone marrow production of granulocytes, recruitment of lymphocytes, neutrophills, and monocytes, differentiate the newly recruited monocytes as well as enhancing the functions of macrophages (Guha and Mackman 2001).

Activation of PRRs leading to the production of IFN is important in the control of viral infections. The secreted IFN leads to autocrine and paracrine activation of the IFNR on nearby cells. Signalling via the IFNR leads to transcription of a number of IFN sensitive genes and the induction of a general antiviral state. This antiviral state is partly due to the IFN induced expression of PKR and 2′,5′-Oligoadenylate Synthetase (OAS) which interferes with viral replication. PKR phosphorylates the translation initiation factor eIF-2α leading to general decrease in protein systhesis (Samuel 2001). IFN also induces expression of OAS and ribonuclease (RNase) L. OAS activates RNase L leading to degradation of RNA within the cell further limiting protein synthesis (Samuel 2001). Furthermore, viral restriction factors are expressed in response to IFN signalling including the HIV-1 restriction factors described earlier.

1.5 Role of Macrophages in HIV-1 Infection

Interestingly, despite their importance in the immune system lentiviruses seem to have specifically targeted macrophages as their host cell and lentiviral tropism can be seen in different host species (Rich, Chen et al. 1992; Embretson, Zupancic et al. 1993; Collins, Patterson et al. 2000; Greenhead, Hayes et al. 2000; Cummins, Guarner et al. 2007). A common feature of lentiviruses is their ability to infect cells of the monocyte/macrophage lineage, though HIV-1 and some other lentiviruses can also infect other cell types such as CD4+ T cells (Table 3).

Table 3 - Lentiviruses and cellular tropism in different species

Table adapted from Lentiviruses and Macrophages: Molecular and Cellular Interactions (2010)

Lentivirus	Host	Tropism	Disease
Maedi-Visna	Sheep	Monocytes/Macrophages	Wasting/Neurological
EIAV	Horses	Monocytes/Macrophages, DCs	Aneamia
CAEV	Goats	Monocytes/Macrophages	Arthritis/Encephalomyelitis
BIV	Cattle	Monocytes/Macrophages CD4+ and CD8+ Tcells	Immunodeficiency
FIV	Cats	Monocytes/Macrophages CD4+ and CD8+ Tcells B cells	Immunodeficiency
SIV	Monkeys	Monocytes/Macrophages, DCs CD4+ Tcells	Immunodeficiency
HIV-2	Humans	Monocytes/Macrophages, DCs CD4+ Tcells	Immunodeficiency
HIV-1	Humans	Monocytes/Macrophages, DCs CD4+ Tcells	Immunodeficiency

Table summarising the different lentiviruses their hosts, cellular tropism and diseases caused.

HIV-1 infection of macrophages is attributed to macrophage expression of the chemokine receptor CCR5, which is commonly used for cellular entry (Keele, Giorgi et al. 2008). HIV-1 infects target cells primarily by interactions between the viral gp120 envelope protein with CD4 and chemokine receptors, most commonly CCR5 or CXCR4. It was widely thought that macrophages are the initial target of HIV-1 during early primary infection and that they provide permissive cells for HIV-1 at the site of entry (Shen, Richter et al. 2009), given their prevalence in mucosa (Lee, Starkey et al. 1985). Many studies have observed that the majority of the HIV virions seen in primary infections are CCR5/macrophage (R5/M)-tropic (Schuitemaker, Koot et al. 1992), even though both R5 and CXCR4 /T cell (X4/T)-tropic viruses can be transmitted (Delwart, Mullins et al. 1998). Dual tropic X4/R5 strains of HIV-1 are able to utilize both co-receptors and are thought to be a transitional step from R5 to X4 viruses (Coetzer, Cilliers et al. 2007), which infect primarily CD4+ T-lymphocytes (Schuitemaker, Koot et al. 1992; Blaak, van't Wout et al. 2000; Nishimura, Brown et al. 2005). Though recently studies have shown that co-receptor usage does not always correlate to cell tropism (Goodenow and Collman 2006), the majority of viruses utilising CCR5 are macrophage tropic. CCR5 co-receptor usage is seen in almost all primary HIV-1 isolates, and people with 2 defective copies of the CCR5 gene are much more resistant to HIV-1 (Hill and Littman 1996; Huang, Paxton et al. 1996; Samson, Libert et al. 1996; Rana, Besson et al. 1997). Recently a patient who had been infected with HIV-1 10 years earlier had undergone a bone marrow transplant to treat newly diagnosed myeloid leukaemia. This patient received CD34+ stem cells from an individual who was homozygous for the defective CCR5 gene, and remarkably remained undetectable for HIV-1 after discontinuation of HAART for over 20 months (Hutter, Nowak et al. 2009).

Though activated T cells as well as macrophages express CCR5, a study looking at transmission of HIV-1 in the female genital tract found that the majority of HIV-1 infected cells in the cervical subepithelial mucosa were macrophages (Greenhead,

Hayes et al. 2000). A second study also showed that vaginal macrophages express CD4, CCR5 and CXCR4 are permissive to HIV-1 infection with HIV-1 entering the macrophages within 30 minutes of exposure, and that they support HIV-1 replication (Shen, Richter et al. 2009). These studies further support the theory that macrophages are an important early part of the lifecycle of HIV-1.

However this theory is now being challenged by the elucidation of transmitted founder (TF) viruses. Mathematical modelling and single genome analysis were used to identify the likely TF virus in a number of cases (Salazar-Gonzalez, Bailes et al. 2008), and though the viral dependence on the CCR5 co-receptor was confirmed (Keele, Giorgi et al. 2008), cDNA clones of these TF viruses were found to replicate less efficiently in macrophages compared to in T cells (Salazar-Gonzalez, Salazar et al. 2009; Ochsenbauer, Edmonds et al. 2012) and this has been determined to be a function of the Env (Ochsenbauer, Edmonds et al. 2012).

As well as being a potential target for infection at the point of entry, macrophages contribute to the spread of HIV-1 in other important ways. HIV-1 is naturally transmitted through bodily fluids (Delwart, Mullins et al. 1998), and macrophages are thought to be carriers of HIV in these fluids (Quayle, Xu et al. 1997; Coombs, Reichelderfer et al. 2003), helping deliver HIV to the new host. Spread of HIV-1 through the body is also aided by infected macrophages, they can transmit the virus to other permissive cells as well as by presenting virus to CD4+ T cells (Groot, Welsch et al. 2008). Cell to cell transmission of virus by macrophages was reported to be more efficient than infection by cell free virus (Carr, Hocking et al. 1999). This transfer of HIV-1 from macrophages to T cells is thought to occur through a viriological synapse (Groot, Welsch et al. 2008). Similar transfer of HIV-1 products has been observed to CD4- cells, and the transfer of Nef to B cells has been reported to inhibit class switching (Xu, Santini et al. 2009) and may be involved in the impaired B cell function seen in AIDS (Lane, Masur et al. 1983).

HIV-1 infection can lead to a loss of CD4+ T cells through direct infection by the virus. HIV-1 infected macrophages can also deplete CD4+ T cells. Apoptosis of bystander uninfected CD4+ T is induced by cell to cell contact with HIV-1 infected macrophages and is thought to be mediated by FasL and TNF (Badley, McElhinny et al. 1996; Abbate, Dianzani et al. 2000; Zhang, Li et al. 2001).

Monocytes are thought to be involved in allowing HIV-1 to access the central nervous system (CNS) because of their ability to cross the blood brain barrier (Fischer-Smith, Bell et al. 2008) after which they differentiate into macrophages. The other theory is that HIV-1 gains access to the brain when the blood brain barrier breaks down (Toborek, Lee et al. 2005). Regardless of the route of entry, the majority of HIV-1 infected cells in the brain are macrophages (Koenig, Gendelman et al. 1986). HIV-1 infection of macrophages in the brain is thought to be an important factor leading to HIV-1 associated dementia due to the neurotoxic effects of glutamate production by these cells (Tian, Erdmann et al. 2008).

Macrophages may be the natural host cell for HIV-1, as unlike in T cells where HIV replication leads to the death of the infected T cell (Petit, Arnoult et al. 2002), HIV-1 infects and replicates within macrophages with little cytopathic effect (Noursadeghi, Tsang et al. 2009). Macrophages are thought to act as a reservoir for HIV, and in vitro have been shown to survive for long periods of time after infection while secreting new virions (Miyagi, Schwartzkopff et al. 2008). Macrophage tropic viruses also predominate in viral escape after anti-retroviral therapy. HIV-1 can be found in circulating monocytes and macrophages, when it can no longer be detected in T cells (Igarashi, Brown et al. 2001; Brown, Zhang et al. 2006; Cassol, Alfano et al. 2006). These recovered virions show evolutionary mutations, but no mutations related to resistance to the antiviral drugs (Crowe and Sonza 2000). This could be due to the fact that higher concentrations of antiviral drugs are required in macrophages compared to CD4+ T cells (Aquaro, Bagnarelli et al. 2002). There is also the possibility of a

reservoir of infected cells in an immunopriviliged site which has reduced access to the antivirals such as the CNS (Aquaro, Svicher et al. 2006). Until recently, HIV-1 was thought to bud into intracellular endosomes within macrophages. It has now been discovered that this compartment is in fact directly communicating with the plasma membrane, suggesting that it may arise from a complex invagination of the cell membrane (Deneka, Pelchen-Matthews et al. 2007). The localisation of HIV-1 in this compartment may help the virus evade neutralising antibody responses (Koppensteiner, Banning et al. 2012)

Alveolar macrophages have also been shown to be permissive to infection by HIV-1 (Ieong, Reardon et al. 2000; Rice, Connor et al. 2002) and evidence of HIV-1 infected cells have been shown in biopsies of HIV-1 infected patients (Hoshino, Nakata et al. 2002). Although alveolar macrophages themselves have been shown to be permissive to HIV-1 infection in vivo, they have not been detected in all patients and are present in low copy numbers in these cells (Nakata, Weiden et al. 1995). A second study failed to detect viral p24 in alveolar macrophages from HIV-1 infected individuals, but HIV-1 was detected in nearly all alveolar macrophages after culturing in the presence of GM-CSF and TNFα (Lebargy, Branellec et al. 1994). These studies suggest that though alveolar macrophages are infected by HIV-1, the lungs may not be a site of active viral replication.

Overall these findings strongly suggest an important role for macrophages in HIV-1 infection and disease progression, they provide permissive cells at the site of entry, help disseminate virus throughout the body, act as a reservoir of long lived virus protected from the immune system inside specialised compartments within the macrophage and macrophages can also access immune privileged sites such as the CNS, where antiviral drugs may have difficulty entering in the effective doses required for activity in macrophages. As a result there is substantial interest in the study of HIV-1 interactions with macrophages.

1.6 Innate Immune Recognition of HIV-1

Macrophages express many PRRs and demonstrate potent innate immune responses (Nau, Richmond et al. 2002; Ferrero, Biswas et al. 2003; Pietila, Veckman et al. 2005). Importantly, this includes PRRs that recognise viral PAMPs such as dsRNA (eg. TLR3 or RIG-I) and ssRNA (TLR8). The capacity of macrophages to mount an innate immune response to incoming HIV-1 has not been assessed systematically. Uridine-rich oligonucleotides derived from HIV-1 to model potential PAMPs have been shown to induce innate immune activation of murine macrophages, human PBMCs and the human myeloid leukemia cell line THP-1 primed with IFNγ (Meier, Alter et al. 2007; Gantier, Tong et al. 2008), but there are no reports of such responses in primary human macrophages. In addition, stimulation of macrophages with recombinant gp120, a component of the viral envelope, activates the classical NFκB pathway and the MAPK pathway, both of which play an important role in innate immune cellular activation (Lee, Liu et al. 2003; Lee, Tomkowicz et al. 2005; Fantuzzi, Spadaro et al. 2008). These events may be initiated through interaction with CCR5, but the physiological significance of this model is not clear since its natural ligand, the chemokine CCL4, does not induce such events (Fantuzzi, Spadaro et al. 2008). Interestingly it appears that TRIM5α may be a PRR for retroviral capsids. Binding of TRIM5α to viral capsids has been reported to activate NFκB signalling and upregulate downstream inflammatory gene expression (Pertel, Hausmann et al. 2011).

Innate immune cellular activation of human macrophages is known to induce genome-wide transcriptional changes that can be demonstrated by microarray transcriptional profiling (Nau, Richmond et al. 2002). These include inflammatory mediators, type I IFNs and IFN stimulated genes (ISGs), components of immunological intracellular signaling pathways and genes involved in cell-cycle regulation. Reports that recombinant gp120 stimulation or HIV-1 infection of monocyte-derived macrophages (MDM) may induce innate immune regulated gene expression support

the hypothesis that HIV-1 infection in macrophages does invoke an innate immune response in macrophages (Borghi, Fantuzzi et al. 1995; Lee, Liu et al. 2003; Woelk, Ottones et al. 2004), but these findings are not consistent. Both induction and deficiency of type I IFN responses to HIV-1 infection in macrophages have been reported in the early literature (Gendelman, Narayan et al. 1985; Szebeni, Dieffenbach et al. 1991; Gessani, Puddu et al. 1994; Francis, Fan et al. 1996; Gessani, Borghi et al. 1997). More recently proteomic analysis of HIV-1 infected macrophage culture supernatants did not identify IFNs or ISGs (Ciborowski, Kadiu et al. 2007), and in further transcriptional profiling experiments a lack of IFN responses was noted despite widespread changes to other immune response related gene expression (Brown, Kohler et al. 2008).

pDCs are thought to be the major producer of type I IFN in response to viral infections. Though they too express a number of PRRs able to recognise various viruses, it appears that they preferentially utilise TLR7 and TLR9 rather than the intracellular RLRs (Kato, Sato et al. 2005). HIV-1 also triggers type I IFN production in these cells primarily in response to ssRNA stimulation of TLR7 (Beignon, McKenna et al. 2005).

1.7 HIV-1 infection of Macrophages and effects on innate immune responses

The existing literature suggests that HIV-1 infection of macrophages may contribute to immunodeficiency independently of CD4+ T cell depletion. HIV-1 mediated inhibition of complement and Fc-receptor phagocytosis (Biggs, Hewish et al. 1995; Kedzierska, Ellery et al. 2002; Azzam, Kedzierska et al. 2006) and attenuated intracellular killing of Toxoplasma gondii (Biggs, Hewish et al. 1995) suggest that the role of macrophages as host defence effector cells may be compromised. Importantly, macrophages also function as sentinel cells of innate immunity involved in recognition of microbial pathogens that leads to both activation and regulation of host immune responses to wide ranging microbial pathogens (Nau, Richmond et al. 2002). Innate immune stimulation of MDM and related monocyte derived dendritic cells from HIV-1 infected subjects have frequently been reported to show attenuated or altered host cell responses (Howell, Groveman et al. 1997; Tachado, Zhang et al. 2005; Martinson, Roman-Gonzalez et al. 2007), but these observations are unlikely to represent direct effects since the extent of HIV-1 infection of monocytes in vivo, is estimated to be very low (Spear, Ou et al. 1990; Naif, Li et al. 1998; Sonza, Mutimer et al. 2001). The same may be true of reports of deficient innate immune responses in alveolar macrophages from HIV-1 infected subjects (Ieong, Reardon et al. 2000; Koziel, Li et al. 2000; Gordon, Jarman et al. 2005; Nicol, Mathys et al. 2008). Nonetheless two additional lines of evidence support the hypothesis that HIV-1 inhibits innate immune responses in macrophages. Firstly, the latently infected myeloid leukaemia cell line, U1, in which HIV-1 replication can be activated by PMA-induced differentiation into a macrophage-like adherent phenotype, shows reduced inflammatory cytokine production compared with the parental non-infected U937 cell line (Tachado, Zhang et al. 2005; Nicol, Mathys et al. 2008). This effect has been attributed to upregulation of mitogen activated protein kinase phosphatase (MKP)-1 by the HIV-1 accessory protein

Nef, and consequent inhibition of the innate immune signalling cascade through ERK1/2 (Tachado, Zhang et al. 2005). Secondly, in a variety of models, other interactions between HIV accessory proteins and components of the innate immune signalling pathway have also been reported to inhibit the function of the NFκB family of transcription factors (Akari, Bour et al. 2001; Bour, Perrin et al. 2001; Muthumani, Choo et al. 2006). The NFκB family of heterodimeric transcription factors are a major target for innate immune receptor mediated signalling in general (Kawai and Akira 2007). Inhibition of this pathway by microbial pathogens is increasingly evident (Kravchenko, Kaufmann et al. 2008). More recently HIV-1 has been shown to interfere with IRF3 signalling, by targeting IRF3 for lysosomal degradation via the accessory protein Vpu (Doehle, Chang et al. 2012; Doehle, Chang et al. 2012). Although another report shows that Vpu does not affect IRF3, but the reduction in type I IFN expression may be due to inhibition of NFκB signalling (Hotter, Kirchhoff et al. 2013).

The effect of HIV-1 infection of macrophages on cellular responses to IFNγ stimulation has received little attention in recent years. Before the introduction of highly active antiretroviral therapy, there was some interest in the potential for IFNγ treatment to compensate for CD4+ T cell depletion. These reports suggested normal IFNγ responses in monocyte derived macrophages from HIV-1 infected patients (Murray, Gellene et al. 1985; Murray, Scavuzzo et al. 1987), but as discussed earlier few monocytes are likely to have harboured viral infection in these studies. That IFNγ stimulation of HIV-1 infected macrophages does elicit a response is illustrated by consequent inhibition of viral replication (Meylan, Guatelli et al. 1993; Creery, Weiss et al. 2004), but there have been no comprehensive studies on the effect of HIV-1 on the broader range of cellular responses to IFNγ. In this respect altered responses may contribute to immunodeficiency or dysregualted immune activation and hence immunopathogenesis of HIV-1 related disease.

1.8 Aims

Macrophages fulfil important roles in the host immune system. They are sentinel cells of innate immunity and as a result of cellular activation, can induce and regulate a broad range of immunological responses. In addition macrophages are effector cells of host defence against microbial pathogens. In this respect, they are specialised in their ability to phagocytose microbial organisms and kill intracellular pathogens, particularly in response to the cytokine IFNγ in the context of Th cell mediated immunity (Gordon, Jack et al. 2005). It is evident that IFN priming of host cells can inhibit HIV-1 infection and that a number of key HIV-1 restriction factors are inducible by type I and type II IFNs (Asaoka, Ikeda et al. 2005; Argyris, Acheampong et al. 2007; Neil, Zang et al. 2008). Therefore it is critically important to define the nature of macrophage innate immune responses to HIV-1 and the physiological significance of such responses on viral infection.

The focus of this thesis is to investigate the host pathogen interactions with respect to the innate immune functions of macrophages, specifically the following questions:

- Does HIV-1 trigger innate immune responses in MDM?
- What are the consequences of IFN responses for HIV-1 infection and replication in macrophages?
- Does HIV-1 infection of macrophages impair innate immune responses to other pathogens?
- Does HIV-1 infection affect IFNγ priming/activation of macrophages?

I address these questions with an ex vivo model, using MDM cultures inoculated with sucrose purified CCR5 tropic HIV-1 to investigate the effect of HIV-1 infection on activation of selected innate immune signalling pathways and downstream immune responses in MDM. As well as innate immune responses to the virus itself I study the effect HIV-1 infection has on macrophage innate immune responses to co-infecting

pathogens and stimulation by IFNγ. Importantly, innate immune signalling in macrophages induces complex and wide-ranging transcriptional responses (Nau, Richmond et al. 2002). Therefore, in addition to testing the hypothesis that HIV-1 inhibits innate immune signalling in a more physiological macrophage model, the assessment of effects has been extended on downstream immune response genes using whole genome transcriptional profiling.

I also investigate effects of IFN stimulation on macrophages, how it affects HIV-1 infection and replication in these cells and also what effect IFN has on macrophages with established HIV-1 infection.

Chapter 2. Materials and Methods

2.1 Cell Culture Media

2.1.1 MDM Media

Primary human cells were all cultured in RPMI 16400 with L-glutamine (GIBCO) supplemented with 5% heat inactivated pooled type AB male human serum (Sigma Aldrich).

2.1.2 MDM differentiation media

The standard MDM differentiation media is RPMI 16400 with L-glutamine supplemented with 5% heat inactivated autologous serum and 20ng/ml M-CSF (R&D Systems).

Alternatively differentiated macrophages were differentiated by either replacing the M-CSF with 100ng/ml GM-CSF (A gift from Schering-Plough Research Institute, Kenilworth, NJ) or RPMI with autologous serum alone.

2.1.3 Cell line media

293T and RAMOS cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% Foetal Calf Serum (FCS) (Biosera), 2 mM L-glutamine (Gibco), and 100U/ml penicillin/streptomycin (Invitrogen).

NP2 cells were cultured in DMEM supplemented with 5% FCS, 1µg/ml puromycin (Sigma Aldrich) and 100 µg/ml geneticin (G418) (Sigma Aldrich).

2.2 Primary Human Cells

The study was approved by the joint University College London/University College London Hospitals National Health Service Trust Human Research Ethics Committee and written informed consent was obtained from all participants.

2.2.1 Purification of PBMCs from peripheral venous blood

Peripheral venous blood was obtained from healthy volunteers. 100ml was collected in heparinised syringes for purification of PBMCs and a further 20ml in a non heparinised syringe to harvest serum. The 20ml of unheparinised blood was transferred to vacutainer serum collection tubes (Beckton Dickinson) which were centrifuged at 300rpm for 15 minutes. The serum layer on the top was transferred to a 15ml falcon and heat inactivated in a waterbath at 56'C for 30 minutes before addition to cell culture medium at a 5% concentration.

PBMCs were obtained by density-gradient centrifugation of heparinised peripheral blood, taken from healthy volunteers, through Lymphoprep (Axis-Shield) following the manufacturer's instructions. Briefly, the heparinised blood was mixed with 50ml of phosphate buffered saline (PBS) solution at a 2:1 ratio with before carefully layering 30ml of the diluted blood mixture on top of 15ml of room temperature Lymphoprep in a 50ml falcon tube. The falcons were centrifuged at 800g for 20 minutes and the cloudy interface layer containing the PBMCs was carefully transferred to new 50ml falcons avoiding the Lymphoprep. PBS was added to top up the tubes before pelleting by centrifugation at 400g for 5 minutes. The PBMC pellets were pooled into a single falcon tube and washed further three times in PBS to remove any residual lymphoprep again being pelleted by centrifuging at 400g for five minutes. The PBMCs were then resuspended at $10x10^6$ cells/ml in RPMI supplemented with 5% human serum which had been heat inactivated by heating to 56'C for 30 minutes in a waterbath.

2.2.2 Purification of monocytes and differentiation into macrophages

Monocytes were purified from the PBMC mixture by plastic adhesion. The PBMCs were seeded at a density of $2x10^6$cells/cm^2 onto tissue culture plastic plates or flasks (TPP). For confocal microscopy nuclear translocation experiments, cells were either seeded onto sterile round glass coverslips (VWR) which were then inserted into the wells of a 24 well plate, or onto optically clear 96 well plates (PerkinElmer). After

seeding, the cells were placed in an incubator for one hour at 37°C and 5%CO_2. During this time the monocytes adhere to the plastic/glass and non adherent cells were removed by gentle washing with PBS, the remaining adherent cells which are mainly monocytes were cultured in RPMI media containing 5% autologous donor serum supplemented with 20ng/ml M-CSF. Alternatively differentiated MDM were generated by replacing the M-CSF with 100ng/ml GM-CSF or in RPMI and serum without additional cytokines. After three days in culture, the media was changed to RPMI media with 5% autologous serum and cultured for a further three days. At this stage the cells were considered to be MDM and all further culture and stimulations were done in MDM media.

Light microscopy and flow cytometric analysis was used to follow the differentiation of monocytes to MDM. Adherent cells were detached by incubating at 37ºC for five minutes in non enzymatic cell dissociation solution (Sigma) before staining for CD14 ((HB246) IgG2b supernatant (American Type Tissue Collection)) and CCR5 (monoclonal rat anti-human CCR5 obtained from Drs. J. McKeating and C. Shotton, via the Centralised Facility for AIDS Reagents (Repository Reference ARP3214.1) (http://www.nibsc.ac.uk/spotlight/aidsreagent/)).

2.2.3 Purification of monocytes from peripheral human blood

For experiments where monocytes were used (Figures 28 and 29), these were obtained by purification from PBMCs using the Miltenyi magnetic bead sorting system. PBMCs were purified from peripheral blood as described before. The PBMCs were then washed three times in ice cold magnetic activated cell sorting (MACS) buffer (PBS with 0.5% bovine serum albumin (BSA) and 2mM EDTA) counted and resuspended in ice cold MACS buffer at a concentration of 80µl/10^7 cells. Monocytes were positively selected for by addition of 20µl CD14 microbeads (Miltenyi) per 10^7 cells. The beads and cells were mixed and then incubated in the fridge (4'C) for 15 minutes, before washing in MACS buffer (2ml/10^7 cells) before centrifugation at 300g for 10 minutes.

The cell pellet was then resuspended in 500µl MACS buffer. This cell suspension was then passed through a 0.4µm mesh to obtain a single cell suspension which was loaded onto a LS column which had been prewashed with 3ml of MACS buffer, and placed in a magnetic separator which had been chilled in the freezer (-20'C). After the initial cell suspension had flowed through the column, 3x 3ml of MACS buffer was passed through the column to flush out the remaining unbound cells. To elute the monocytes, the column was removed from the magnet and 5ml MACS buffer was passed through the column under pressure. The final elution of CD14+ monocytes were counted and then washed, centrifuged and resuspended in MDM medium at 10^6cells/ml for use in experiments.

2.2.4 Purification of pDCs from peripheral human blood

Plasmacytoid dendritic cells (pDCs) were isolated from PBMCs using the Miltenyi magnetic bead sorting system. The PBMCs were treated in the same manner as for purification of monocytes, except they were resuspended in ice cold MACS buffer at a concentration of 400µl/10^8 cells. Alternatively PBMCs which had already been depleted of CD14+ cells were used. The pDCs were negatively selected for by addition of 100µl pDC Biotin-Antibody cocktail (Miltenyi) per 10^8 cells. The cell/antibody mixture was incubated in the fridge for 10 minutes. The cells were washed two times with 10ml MACS buffer before being resuspended in MACS buffer at a concentration of 400ul/10^8 cells. Anti-Biotin beads were added at a concentration of 100µl/10^8 cells and the mixture incubated in the fridge for a further 15 minutes. The cells were then washed with 10ml of MACS buffer before being resuspended in 500µl of buffer, strained through a 0.4µm mesh and passed through a LS column. The purified pDCs pass through the column and the other cell types remain bound to the magnetic column. The pDCs are washed and resuspended at 10^6cells/ml of MDM media for use in experiments. The pDC negative fraction was eluted by removing the

column from the magnet and passing 5ml of MACS buffer through the column under pressure.

2.2.5 Lymphocytes

Lymphocytes were obtained from the non adherent cells after monocyte adhesion to plastic. The CD14 negative and pDC negative fractions after magnetic cell separation were also used as sources for lymphocytes.

2.3 Cell Lines

2.3.1 293T cells

293T cells are a cell line thought to originate from human embryonic kidney cells, and they express the SV40 large T antigen (Graham, Smiley et al. 1977). These cell lines are easy to transfect and express high levels of inserted transgenes. Cells were passaged approximately 1:3 using trypsin/EDTA (Gibco) every 2 days or 1:5 every 3 days.

2.3.2 NP2 cells

NP2 cells are a glioma cell line stably transduced to express the HIV-1 coreceptors CD4 and CXCR4 (NP2-X4) or CD4 and CCR5 (NP2-R5) (Soda, Shimizu et al. 1999). These cells were used to determine the titre of HIV-1 stocks depending on the envelope of the particular strain titred. NP2 cells were cultured in DMEM supplemented with 5% FCS, 100µg/ml G418 and 1µg/ml puromycin to maintain selection for the HIV-1 co-receptors. The cells were split 1:10 once a week, and are seeded 24hours before use.

2.4 HIV-1 strains and HIV-1 based lentiviral vectors

The HIV-1 strains used were either generated by transfection of full length molecular clones of HIV-1 or by propagation of infectious virus stocks in peripheral blood lymphocytes (PBLs) and MDM. Yu2 (Li, Kappes et al. 1991), NL4-3 (Adachi,

Gendelman et al. 1986) and 89.6 (Collman, Balliet et al. 1992) strains were produced by transfecting 293T cells with full length infectious clones using Fugene 6 (Roche). Supernatant from transfected cell cultures containing the virus was collected 72 hours post transfection. BaL, JR-CSF, ADA-M and MN25-2 (a primary isolate) strains were propagated in PBLs obtained from non adherent cells in the MDM preparations.

2.4.1 Virus Production in 293T cells

HIV-1 viruses for which there were full length molecular clones and lentiviral vectors were generated by transfection of 293T cells. $2x10^6$ 293T cells in 10ml of DMEM containing 10% FCS were seeded into a T75 flask the night before transfection. The next day media was refreshed 2 hours prior to transfection. Plasmid DNA transfection was performed using Fugene 6. Virus containing supernatant from the transfected cells were collected 48-72 hours post transfection.

2.4.2 Production of HIV-1 in PBL and MDM

For viruses for which a molecular clone was not available, these were propagated in either PBLs and/or MDM depending on the tropism of the virus. PBLs were cultured in RPMI supplemented with 20% FCS and 0.5 µg/ml of phytohaemagglutinin (PHA) (Sigma) for three days to activate the T cells. The activated cells were inoculated with HIV-1 at a MOI 1, and were cultured in RPMI supplemented with 20% FCS and 20U/ml IL-2 (Peprotech). Supernatant from these cultures were collected weekly, when additional PHA activated T cells were added to the culture to replenish T cell numbers and was further incubated with RPMI supplemented with 20% FCS and 20U/ml IL-2. CCR5 utilising strains of HIV-1, such as BaL used in these experiments were also passaged/produced in MDM. MDM were inoculated with the PBL passaged BaL at a MOI1 and supernatant from infected MDMs was collected every 6-7 days.

2.4.3 Ultracentrifugation of HIV-1

All HIV-1 culture supernatants were filtered through a 45μm filter (Millipore) to remove large cellular debris, before ultracentrifugation at 23,000 RPM for 2 hours through a 25% sucrose cushion to remove soluble contaminants. The viral pellet was then resupended in MDM media and stored in aliquots in liquid nitrogen.

2.4.4 HIV-1 titration

Viral stocks were titrated on NP2 cells. NP2 cells were seeded 24 hours earlier to allow for attachment to the tissue culture plastic as well as for expression of the receptors required for HIV-1 infection. Serial log dilutions of the virus were added to NP2 cell lines expressing the receptors required for HIV-1 entry (either CD4 and CCR5 for R5 tropic viruses or CD4 and CXCR4 for X4 tropic viruses). Viral titre was calculated by staining the NP2 cells for intracellular gag 72 hours after infection, and are calculated based on number of infected foci per ml. X4/R5 dual tropic viruses were able to infect both cell lines and the titre used was dependent on the target cell I wanted to infect.

2.4.5 HIV-1 based lentiviral vectors

HIV-1 based vectors pseudotyped with vesicular stomatitis virus (VSV)-G envelope were generated by transfection of 293T cells with 3 plasmids: p8.91 (gag-pol), pMD-G (VSV-G envelope) and pHRSIN-CSGW encoding GFP. Supernatant was collected, and sucrose purified as with the other HIV-1 viruses. Titre of VSV-G pseudotyped vectors was determined by flow cytometric analysis of infected 293T. Serial dilutions of virus were used to infect the cells and GFP expression was analysed by FACS 48-72 hours later. Ideally dilutions of virus generating approximately 10-15% GFP positive cells were used to ensure infection was in the linear range, and used to calculate viral titres.

2.5. Quantification of reverse transcriptase activity

For viruses lacking a reporter gene and that could not be titrated by gag staining, the amount of reverse transcriptase in the viral sample was measured using the reverse transcriptase assay colorimetric kit (Roche). Approximately 10^5 IU correlates to 1ng of reverse transcriptase activity in this assay.

2.6 HIV-1 infection of MDM

HIV-1 infected MDM were generated by inoculating MDM with HIV-1 at an MOI of three (based off the titre obtained on NP2 cells) for three hours. Cells were then washed to remove cell free HIV-1 and fresh media was added to the cells. Seven days after infection cells were assessed for HIV-1 infection status by intracellular p24 staining. Only homogeneously infected cultures were used for the experiments.

2.7 Intracellular p24 and gag staining

Cells were fixed with ice cold methanol:acetone (1:1) for five minutes, then incubated with mouse anti HIV-1 p24 monoclonal antibodies (E365/ 366, NIBSC, UK) for one hour at room temperature followed by anti-mouse immunoglobulin (Ig) antibody conjugated to β-galactosidase (gal) (Southern Biotechnology Associates) for one hour at room temperature. Detection of gag/p24 was visualised by addition of 0.5mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) diluted in PBS containing 3 mmol/l potassium ferricyanide, 3 mmol/l potassium ferrocyanide and 1 mmol/L magnesium chloride, and incubation overnight at 37°C. Infected cells are stained blue and are counted by microscopy to calculate virus titres in focus forming units /ml.

2.8 p24 ELISA

Supernatant from HIV-1 infected cells were collected at various times after infection and p24 was quantified by ELISA against recombinant protein standards (Kit version 5, from AIDS Vaccine Programme, National Cancer Institute- Fredrick).

2.9 Cell culture viability testing (MTT assay)

Relative viability of HIV infected and uninfected MDM cultures was compared by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cell culture supernatants were removed and replaced with 1 mg/ml MTT (Sigma-Aldrich) in serum-free RPMI media (1ml/10^6 cells) and incubated for three hours at 37°C. This was then replaced with dimethyl sulfoxide (Sigma-Aldrich) to permeabilise cells in a plate shaker for five minutes (room temperature). Relative formazan concentration was quantified by spectrophotometry (OD_{540nm}) within 0.1-1.8 linear range.

2.10 Innate immune and cytokine stimulation of MDM

Poly I:C, ultrapure lipopolysaccharide from E. coli O111:B4, CL075 (3M002), gardiquimod, ssRNA40, zymozan, loxoribine, MDP, CpG (all purchased from Invivogen) and Pam3CSK4 (Axis-Shield) were used as minimal innate immune stimuli. Recombinant human IFNβ was obtained from Merck Serono and recombinant human IFNγ was obtained from Peprotech.

2.11 Confocal Microscopy: NFκB and IRF3/7 Nuclear translocation assay

MDMs seeded onto glass coverslips were fixed with a 3.7% paraformaldehyde solution for 10 minutes at room temperature, washed with tris-buffered saline (TBS) and permeabilised with 0.2% triton-X 100 for 10 minutes at room temperature. Permeabilised cells were blocked with TBS containing 10% goat serum for 30 minutes, then incubated with the primary antibody (rabbit anti human p65 subunit of RelA (sc-372) or rabbit anti human IRF3 (sc-9082) both from Santa Cruz Biotechnology) at 1/50 dilution in blocking buffer and incubated overnight at 4°C. Unbound primary antibody was washed off with TBS and the secondary antibody (Goat anti-Rabbit AF-633, Invitrogen A21072) was added at 1/500 in blocking buffer at room temperature for one hour in the dark. Coverslips were washed in TBS then nuclei were counterstained with

4,6 diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) at 2µg/ml in TBS for five minutes before mounting onto glass slides using Vectashield hard set solution (Vector).

Imaging was done using a Leica SP2 confocal microscope. Sub saturating fluorescence images were captured using a pin hole of one Airy (114.5 µm), scan speed of 400 Hz and four frame averaging. At least five high power fields were taken of each coverslip, with fields selected solely on density of cells to get a good image from looking through the DAPI channel. Images were analysed using the software Metamorph v7.17 (Molecular devices) and results expressed as the ratio between nuclear and cytoplasmic staining intensities of individual cells, and proportion of cells demonstrating positive co-localisation of DAPI/ RelA staining (correlation coefficient >0.5) as markers of NFκB nuclear translocation.

2.12 RNA extraction

RNA from cell cultures were extracted using the Qiagen RNAeasy spin columns, following the manufacturer's instructions. The RNA quality and quantity was analysed using the Nanodrop ND-1000.

2.13 DNA Microarrays for Transcriptional profiling

RNA was extracted as above, and was additionally checked electophoretically using the RNA 6000 nanochip (Agilent) and Agilent 2100 bioanalyser to check for RNA integrity. 500ng of sample RNA was firstly used to generate cDNA and then cRNA labelled with Cy5 using the Agilent Low RNA Input Linear Amplification Kit. The labelled cRNA was extracted using Qiagen RNAeasy spin columns and cRNA concentration and labelling intensity was analysed on the Nanodrop ND-1000. Labelled cRNA was hybridized onto Agilent 4x44k whole human genome array along with a standard reference (universal human reference RNA mix, Stratagene) labelled in Cy3. Arrays were scanned using Agilent's dual laser microarray scanner G2565BA. Data was extracted using Agilent's Feature Extraction software (v9.5.1). Data was log2

transformed and then median normalised. Duplicate probes and genes without refseq accession numbers were discarded. MultiExperiment Viewer v4.0 (Saeed, Sharov et al. 2003) was used to compare gene expression values by a paired T-test, and genes with 2 fold or greater changes were further analysed by DAVID (Database for Annotation, Visualization and Integrated Discovery), a bioinformatics database which clusters genes according to their function (Dennis, Sherman et al. 2003). Multidimensional scaling (MDS) was used to compare similarity/dissimilarity of expression data using selected gene lists, and generates a 2/3 dimensional representation, with larger distance between samples representing more differences in expression in the gene list selected.

Minimum information about a microarray experiment (MIAME) compliant microarray data is accessible from the ArrayExpress database at www.ebi.ac.uk/arrayexpress under the accession numbers E-TABM-942, E-TABM-1157, E-MEXP-1904 and E-MEXP-2032.

2.14 PCRs

RNA was treated with DNAseI (Turbo DNA free, Ambion) to remove genomic DNA contamination. Purified RNA samples were used to generate cDNA using a cDNA Synthesis Kit (Bioline). Semi-quantitative PCRs were performed by serial dilutions of cDNA using primers spanning spliced HIV-1 transcripts, which detect actively replicating virus. β-actin was used as a control to show equal loading of cDNA. Taqman probe based qPCRs were performed on cDNA samples for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), STAT1, CCL2, IRF7, TRIM5α, IFNβ, IP-10 TLR7 and TLR9. Primers and probes were designed to cross intron/exon boundaries where possible. Sequences of all the probes and primers used are in Table 4

Table 4 - List of PCR primers and probes

Primer	Sequence
β actin (forward)	AGCCTCGCCTTTGCCGA
β actin (reverse)	CTGGTGCCTGGGGCG
SK38	CCACCTATCCCAGTAGGAGAAATC
SK39	CCTTTGGTCCTTGTCTTATGTCC
P659	GACTCATCAAGTTTCTCTATCAAA
413MOD	AGTCTCTCAAGCGGTGGT
TLR7 (forward)	TTTACCTGGATGGAAACCAGCTA
TLR7 (reverse)	TCAAGGCTGAGAAGCTGTAAGCTA
TLR7 (probe)	AGAGATACCGCAGGGCCTCCCG
TLR9 (forward)	GGACCTCTGGTACTGCTTCCA
TLR9 (reverse)	AAGCTCGTTGTACACCCAGTCT
TLR9 (probe)	ACGATGCCTTCGTGGTCTTCGACAAA
GAPDH (forward)	GGCTGAGAACGGGAAGCTT
GAPDH (reverse)	AGGGATCTCGCTCCTGGAA
GAPDH (probe)	TCATCAATGGAAATCCCATCACCA
IFNβ (forward)	GAGCTACAACTTGCTTGGATTCC
IFNβ (reverse)	CAAGCCTCCCATTCAATTGC
IFNβ (probe)	ACAAAGAAGCAGCAATTTTCAGTGTCAGAAGCT
IP10 (forward)	TGAAATTATTCCTGCAAGCCAATT
IP10 (reverse)	CAGACATCTCTTCTCACCCTTCTTT
IP10 (probe)	TGTCCACGTGTTGAGATCATTGCTACAATG
STAT1 (forward)	GTACCTGGCACAGTGGTTAGAAAA
STAT1 (reverse)	CGGATGGTGGCAAATGAAAC
STAT1 (probe)	CAAGACTGGGAGCACGCTGCCAA

Table 4 continued

Primer	Sequence
IRF7 (forward)	AGAGTCTTCTTCCAAGAGCTGGT
IRF7 (reverse)	ACAGCCAGGGTTCCAGCTT
IRF7 (probe)	TGGCTCCCCACGCTATACCAT
TRIM5α (forward)	CGCTACTGGGTTGATGTGACAG
TRIM5α (reverse)	CCCTCGTGCCCCATATATTATCTG
TRIM5α (probe)	CTGAAGATAAGAGACAAGTGAGCTCTCCGAAAC
CCL2 (forward)	GCTGTGATCTTCAAGACCATTGTG
CCL2 (reverse)	TGGAATCCTGAACCCACTTCTG
CCL2 (probe)	CCAAGGAGATCTGTGCTGACCCCAA

List of primers and probes used for standard and qPCR.

Standard PCR for spliced HIV-1 transcripts was performed using primers for a spliced HIV-1 *tat*, *rev*, n*ef* product and *β actin*. Control samples without RT confirmed the absence of genomic DNA contamination for these products. PCR products were run on a 1% agarose gel alongside a molecular marker (HyperladderI, Bioline) Samples were visualised under UV and relative amounts of HIV-1 transcript were assessed semi-quantitatively by densitometry and limiting dilution PCR, normalised to *β actin* levels.

qPCR reactions were carried out by first generating cDNA using the qScript cDNA supermix (Quanta Biosciences). cDNA samples were then analysed for gene content using Platinum qPCR mastermix (Invitrogen), with 7.5µM of the primers and probe shown in table 4. Gene copies per sample were calculated using a standard curve generated from plasmid DNA containing gene of interest and normalised to GAPDH copy number.

2.13 Western immunoblotting analysis of HIV-1 gag expression and innate immune signalling

Cell lysates from MDM cultures were collected directly into SDS sample buffer (62.5 mM Tris HCl, pH 6.8, 10% Glycerol, 2% SDS, 0.01% bromphenol blue and 5% 2-mercaptoethanol), sonicated and heated to 100°C for five minutes before polyacrylamide gel electrophoresis (NuPage 4-12% gradient gels (Invitrogen)) and transfer on to Amersham Hi-bond membranes (GE healthcare). Membranes were blocked for one hour in 5% milk powder in TBS with 0.05% Tween-20 (Sigma-Aldrich) and then immunoblotted sequentially with primary antibody overnight (4°C), biotin-conjugated secondary antibody for two hours (room temperature) and horseradish peroxidase-conjugated streptavidin for one hour (room temperature), all prepared in TBS/Tween with 1% milk powder. Membranes were washed with TBS/Tween after each step. Immunostains were developed with Amersham ECL™

reagent (GE healthcare) and visualized on Amersham Hyperfim™ ECL (GE healthcare). Mouse anti HIV-1 gag (p24) antibody (E365/ 366, NIBSC, UK), rabbit anti IκBα (Cell Signalling Technology), rabbit anti phosphorylated Erk1/2 (clone 197G2, Cell Signalling Technology), rabbit anti phosphorylated p38 MAP kinase (Cell Signalling Technology), rabbit anti phosphorylated STAT1 (Y701 and S727, Cell Signalling Technology) and rabbit anti actin (Sigma-Aldrich) were used as primary antibodies. Biotin conjugated sheep anti mouse IgG and sheep anti rabbit IgG (Dako) were used as secondary antibodies. HRP conjugated streptavidin was obtained from R&D systems.

2.15 Cytokine measurements

The concentration of cytokines in MDM culture supernatants was quantified using a cytometric bead array inflammation kit for TNFα, IL-1, IL-6, IL-8, IL-10 and IL-12p70 (Becton Dickinson) using the FACSArray Bioanalyzer System (Becton Dickinson), according to manufacturer's instructions.

Chapter 3. HIV-1 infection and replication in M-CSF differentiated monocyte derived macrophages.

3.1 Introduction

Macrophages are thought to play an important role in providing permissive cells at sites of entry into a new host, aiding in establishment of infection. HIV-1 infected macrophages can be seen in vivo. Alveolar macrophages from HIV-1 infected patients have been reported to be infected with the virus (Hoshino, Nakata et al. 2002). Macrophages from the vagina have also been reported to be permissive to HIV-1. Macrophages in the vagina mucosa are readily infected with HIV-1 in vaginal explants, with over 90% of HIV-1 infected cells staining positive for macrophage markers (Greenhead, Hayes et al. 2000). Additionally macrophages purified from vaginal tissues by positive selection have also been reported to be permissive to HIV-1 (Shen, Richter et al. 2009).

There are obvious limitations to obtaining macrophages directly from human subjects. Therefore studying the effects HIV-1 infection has on macrophages has been performed using a variety of macrophage models.

There are several monocytic cell lines used to model macrophages in HIV-1 infection, including; Mono Mac 1, Mono Mac 6 and HL-60 as well as the more commonly used U937 and Thp-1 cell lines which can be induced into a more differentiated, macrophage like phenotype (Cassol, Alfano et al. 2006).

The Mono Mac 1 and 6 cell lines were derived from a patient with monoblastic leukemia (Ziegler-Heitbrock, Thiel et al. 1988). These cells resemble mature blood monocytes (Ziegler-Heitbrock, Thiel et al. 1988; Steube, Teepe et al. 1997). However these cells are very heterogeneous and unlike blood monocytes express low levels of CD14, with as few as 10% of the Mono Mac 6 cells expressing any detectable levels of

CD14 at all (Ziegler-Heitbrock, Thiel et al. 1988; Valentin, Nilsson et al. 1994). More importantly these cell lines are heterogeneous with regards to expression of the HIV-1 co-receptors for HIV-1 entry; CCR5, CXCR4 and particularly CD4 (Valentin, Nilsson et al. 1994; Genois, Robichaud et al. 2000). Although the Mono Mac 1 cells express the HIV-1 co-receptor CCR5 which is required for infection by R5 utilising virus, CD4 expression on these cells is variable, with only around 60% of expressing CD4 (Genois, Robichaud et al. 2000). In contrast to the Mono Mac 1 cell line, the Mono Mac 6 cell line cannot be infected by R5 utilising viruses as they do not express CCR5. Mono Mac 6 cells do express CXCR4 and there have been reports of both positive (L'Age-Stehr, Niedrig et al. 1990) and negative (Valentin, Nilsson et al. 1994) infection with X4 utilising viruses. As with the Mono Mac 1 cell line, these cells have variable CD4 expression with fewer than 10% of these cells expressing CD4 (Ziegler-Heitbrock, Thiel et al. 1988; Valentin, Nilsson et al. 1994). The lack of uniform CD4 expression on these cell lines could lead to only a proportion of cells being infected by HIV-1 and thus may be expected to give non representative data on the effects of HIV-1 infection when looking at whole culture responses.

The HL-60 cell line was derived from a patient with acute myeloid leukaemia (Birnie 1988). These cells are promyelocytic and can be differentiated into granulocyte like cells (Birnie 1988) as well as macrophage like cells (Rovera, Santoli et al. 1979; Murao, Gemmell et al. 1983; Birnie 1988) depending on the reagents used. When undifferentiated these cells express CD4 and CXCR4, making them susceptible to infection by X4 utilising HIV-1 (DiFronzo, Pise-Masison et al. 1997). Despite expressing the receptors required for infection, low levels of HIV-1 replication were seen when these cells were infected with the X4 utilising virus NL4-3 (Pise-Masison and Holland 1995). HIV-1 replication was also reported to cause cell death in this cell line, however an increase in the percentage of infection could be seen in the surviving cells (Pise-Masison and Holland 1995). A chronically infected subclone of HL-60

(Butera, Perez et al. 1991) enables easy comparisons of the effects of HIV-1 on cellular responses, however a major disadvantage of the HL-60 cell line is the fact that it is genetically unstable and loses myelod cell lineage markers with time in culture (Leglise, Dent et al. 1988).

The Thp-1 cell line was derived from a patient with acute monocytic leukaemia (Tsuchiya, Yamabe et al. 1980). These cells are monocytic and can be induced to resemble macrophage like cells by stimulation with vitamin D3 or more commonly with PMA (Matikainen and Hurme 1994; Keuper, Dzyakanchuk et al. 2011; Chang, Chan et al. 2012; Arcangeletti, Germini et al. 2013). Differentiation of these cells into a macrophage like phenotype unlike in the other cells previously mentioned is not terminal and they dedifferentiate in culture (Tsuchiya, Yamabe et al. 1980). As with the HL-60 cell line a HIV-1 chronically infected subclone also exists (Mikovits, Raziuddin et al. 1990). Another similarity to the HL-60 cell line is the fact that Thp-1 cells are also unstable and lose CD4 expression. One study reported that around 80% of Thp-1 cells were CD4+ and this percentage decreases with time in culture (Konopka and Duzgunes 2002). Another issue with this cell line is different susceptibilities to HIV-1 infection. Undifferentiated cells have been reported to be permissive to X4 utilising virus, but upon differentiation become resistant to infection (Kitano, Baldwin et al. 1990). There have also been both positive (Honda, Rogers et al. 1998) and negative (Meylan, Spina et al. 1993) reports of infection with R5 utilising HIV-1.

The U937 cell line are pro-myelocytic cells derived from a patient with histiocytic lymphoma and exhibit monocytic properties (Sundstrom and Nilsson 1976). U937 cells are commonly differentiated using PMA to resemble macrophage like cells (Hass, Bartels et al. 1989). Vitamin D3 has also been used to differentiate these cells into macrophage like cells (Kim, Abraham et al. 1991; Rots, Iavarone et al. 1999) although others have also used vitamin D3 to generate cells used to model monocytes (Baek, Haas et al. 2009). Similar to Thp-1 cells, when undifferentiated this cell line can be

infected with X4 utilising virus (Zhou and Spector 2008) though this is dependent on the particular subclone (Moriuchi, Moriuchi et al. 1998). U937 cells are separated into plus or minus groups depending on the ability of HIV-1 to infect and replicate within these cells (Franzoso, Biswas et al. 1994; Moriuchi, Moriuchi et al. 1997; Biswas, Mengozzi et al. 1998). Differentiation of these cells with vitamin A induces expression of CCR5, but only the plus clones and not the minus clones become permissive to R5 virus infection (Moriuchi, Moriuchi et al. 1998). Additionally plus clones have been reported to lack the IFNGR2 subunit of the IFNγ receptor, (Bovolenta, Lorini et al. 1999) and HIV-1 has been shown to be able to replicate in these cells in the presence of IFNγ (Bovolenta, Lorini et al. 1999). As discussed earlier in section 1.2.3 IFNγ is a key modulator of macrophage functions and the lack of a functional receptor could limit the use of this cell line in when examining innate immune responses regulated by IFNγ.

As well as the limitations of the cell lines already mentioned including the variation found in different subclones (Garotta, Thelen et al. 1991; van den Berg, Williams et al. 1994), a shared concern of using transformed cell lines for investigating immune responses is the role of NFκB in cancer. Increased NFκB activity is associated with transformation of cells (Rayet and Gelinas 1999; Karin, Cao et al. 2002; Sethi, Sung et al. 2008; Shostak and Chariot 2011) and as such assessment of innate immune activation in cell lines may be confounded.

One attractive alternative due to the ability to genetically manipulate these cells (Mandegar, Moralli et al. 2011) are embryonic stem cells, which can be induced to undergo haemopoiesis and produce monocytes (Brook, Cowley et al. 2010) which can then be differentiated into macrophages.

An alternative to using cell lines is to use macrophages derived from primary human monocytes. These will likely provide a more physiological cell type in order to assess the impact of HIV-1 infection on innate immune responses, compared to in transformed

cells where the activity of the major innate immune transcription factor NFκB may be altered. As discussed earlier in section 1.2, different cytokines can be used for differentiation of MDM. The most commonly used cytokine is M-CSF (Hume 2006), though GM-CSF is occasionally used as well (Akagawa, Komuro et al. 2006). M-CSF generates macrophages which are reported to most resemble tissue macrophages compared to MDM differentiated with GM-CSF which are reported to similar to alveolar macrophages (Akagawa, Komuro et al. 2006). Additionally, M-CSF is the major cytokine required for macrophage differentiation. Mice which lack a functional M-CSF gene have significantly reduced numbers of macrophages, with a complete absence of macrophages found in some tissues such as the bone marrow (Wiktor-Jedrzejczak, Ratajczak et al. 1992). A similar phenotype is seen in CSF1R knockout mice (Dai, Ryan et al. 2002). Importantly M-CSF differentiated macrophages have been reported to be more permissive to infection by HIV-1 (Bergamini, Perno et al. 1994) compared to GM-CSF differentiated macrophages which are resistant to infection. M-CSF antagonists have been reported to inhibit HIV-1 replication in MDM (Kutza, Crim et al. 2000). The resistance of GM-CSF differentiated MDM to HIV-1 infection is likely due to decreased expression of the HIV-1 co-receptors CCR5 and CXCR4, as GM-CSF stimulation has been reported to rapidly reduce mRNA levels of the two HIV-1 co-receptors (Di Marzio, Tse et al. 1998). Additionally GM-CSF stimulation has been reported to inhibit HIV-1 replication in MDM (Kedzierska, Maerz et al. 2000).

There are a number of disadvantages to using primary cells compared to cell lines. As well as being more time consuming to obtain and culture, the major disadvantage of using primary cells is the genetic variability found between donors. (For example, CCR5Δ32 heterozygosity in patients makes them more resistant to HIV-1 (Buseyne, Janvier et al. 1998; Chalmet, Van Wanzeele et al. 2008)). One option would be to use stem cell lines for generating monocytes to differentiate into macrophages (Brook,

Cowley et al. 2010), with the benefit that they are also able to be genetically manipulated (Lufino, Popplestone et al. 2011).

3.2 Objectives

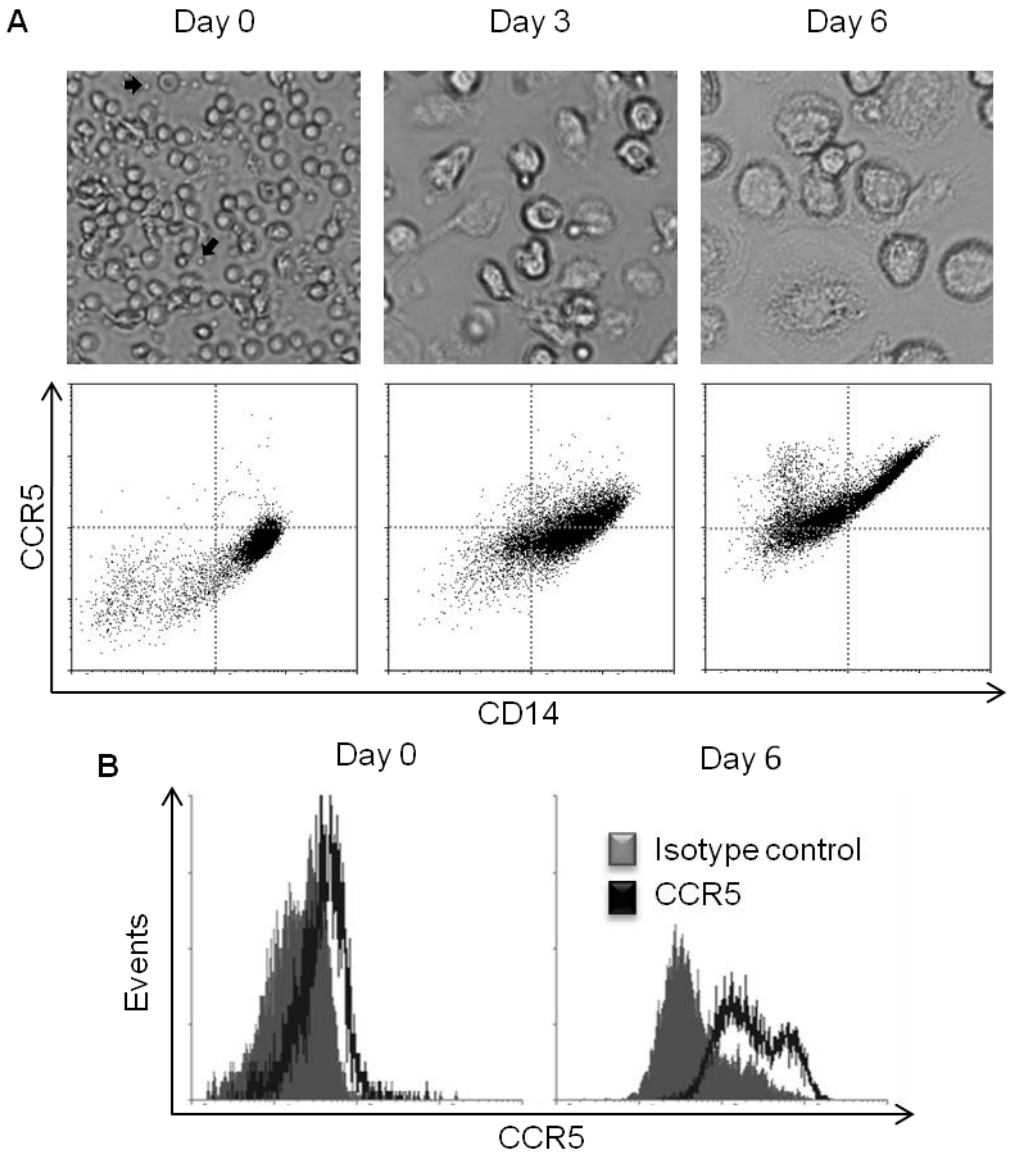
- Adopt and characterise the model for HIV-1 infection of M-CSF differentiated MDM
- Assess the effect of HIV-1 infection and replication in macrophages on the host cell survival and steady state transcriptome.

3.3 Results

3.3.1 Generation of Macrophages from Peripheral Blood Monocytes

The macrophages used in this study were generated from monocytes obtained from the peripheral blood of healthy volunteers and differentiated in the presence of the cytokine M-CSF. Adhesion to plastic was used as the method to purify out monocytes from the PBMC mixture. Though initially a substantial amount of lymphocyte contamination can be present in the culture (Figure 3A – Day 0), after three days in culture and additional washes the culture contains over 99% CD14+ cells (Figure 3A – Day 3). At this point, the monocytes have started to differentiate, cell surface expression of CD14 decreases, the cells become more granular, and increase in size with the cells beginning to flatten out on the tissue culture plate. After six days in culture, the cells are classed as MDM and have a large flattened morphology (Figure 3A – Day 6). Cell surface expression of CD14 expression is further decreased while cell surface expression of the HIV-1 co-receptor CCR5 increases (Figure 3B), which may increase permissibility to infection with R5 utilising virus (Naif, Li et al. 1998).

Figure 3 - CD14 and CCR5 expression on peripheral blood monocytes as they differentiate into macrophages.



(A) Day 0, one hour after plating PBMCs on tissue culture plastic non adherent cells were washed off and M-CSF added to the culture. The majority of the cells are monocytes expressing CD14 as seen in the dot plot, with contamination by smaller cells, which are mainly lymphocytes (Black arrows), (smaller, CD14 negative cells), which can also be seen in the light microscopy images. Day 3, These contaminating cells are removed when the cells are washed and fresh media without M-CSF is added. Day 6, After six days in culture the monocytes have differentiated into macrophages and are showing reduced levels of CD14 expression compared to the parental monocytes. **(B)** The expression of the chemokine receptor CCR5 is increased in day 6 cells compared to freshly isolated monocytes.

3.3.2 HIV-1 infection of MDM

Previous studies have reported that M-CSF differentiated MDM are permissive to infection by HIV-1. In order to investigate the effects HIV-1 infection has on innate immune responses in macrophages, a model of established infection in M-CSF differentiated MDM was adopted. MDM were inoculated for three hours at a multiplicity of infection (MOI) of three (HIV-1 virus was titrated on permissive NP2 cells. Using this readout, three infectious particles per MDM were added to the cell culture) , with either a well characterised CCR5 using and macrophage tropic laboratory propagated strain, HIV-1 BaL, or a CXCR4 using T cell tropic full length clone, NL4-3. Productive viral infection was then assessed by detection of nascent viral transcripts and p24 capsid protein/p24 precursor Gag protein.

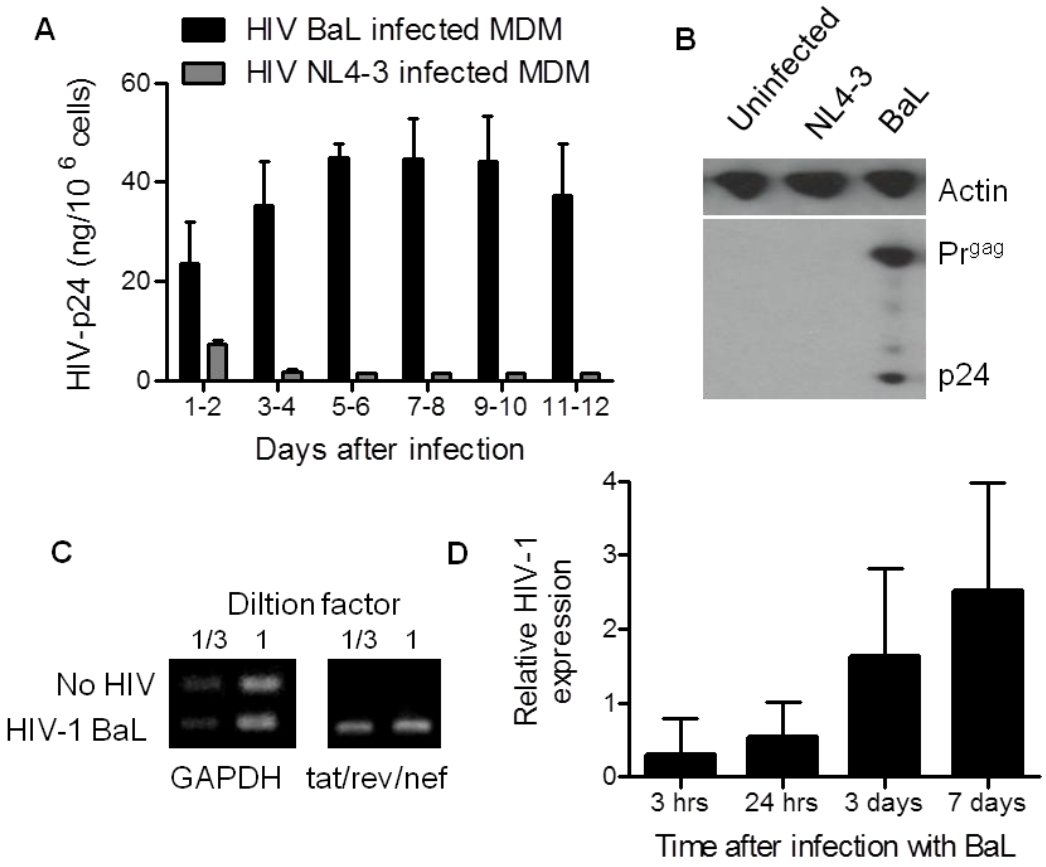
Infection and replication was confirmed by analysis of HIV-1 transcripts by RT-PCR using the P659 and 413MOD primers (Figure 4C). These primers only detect the spliced transcripts of Tat, Rev, and Nef which are produced during viral gene expression, and not the genome from mature virions (Brussel and Sonigo 2004). Semi-quantitative assessment of viral gene expression was obtained by densitometry and normalisation to GAPDH expression to give a measurement for relative gene expression (Figure 4D). Spliced HIV-1 transcripts were detected in MDM cultures inoculated with HIV-1 BaL in which viral gene expression increased with time. In contrast, no nascent viral transcripts were detected in MDM inoculated with the NL43 strain. Viral replication in MDM infected with HIV-1 BaL was further confirmed by analysis of both cell free p24 and cell associated gag. Cell associated gag products were analysed by Western blotting. Seven days after exposure to HIV-1, MDM cell lysates were harvested and probed for capsid proteins. Gag was detected in MDM infected with HIV-1 BaL but not the NL4-3 strain (Figure 4B). The antibody used to probe for p24 also detected the p24 precursor, (Pr)55^{gag}, protein, which also demonstrates active viral replication in MDM as this precursor is processed to form the

mature virion (Murakami 2012). Supernatant from HIV-1 inoculated MDM were collected every 48 hours and analysed for the presence of p24 by ELISA (Figure 4A). The levels of p24 present in the supernatant increased with time, correlating with the gene expression data. Maximal p24 levels plateau at around six days post infection for HIV-1 BaL infected cells. p24 is also initially detected in the supernatant of NL4-3 inoculated cells; however the levels of p24 drop very rapidly and is below the detection limit of the assay after the second time point.

For this study I wanted to adopt a model of uniformly infected MDM to avoid any potential bystander cell effects. Therefore the infection status of the cell culture was assessed. After allowing infection to establish for seven days, MDM were fixed and stained for intracellular gag proteins (Figure 5A). Seven days after infection, the MDM culture was uniformly infected by HIV-1 BaL. Gag staining was evident in very few MDM exposed to the NL4-3 strain of HIV-1, correlating with the earlier assessments where no gag was detected by western blotting and little p24 was found in cell supernatants.

Infection of MDM by HIV-1 did not cause gross cytopathic effects when visualised by light microscopy (Figure 5A) in contrast to the studies which report HIV-1 attenuation of Akt-1 activity leading to decreased phosphorylation of the transcription factor forkhead box protein O3a (FOXO3a), increased nuclear translocation and induction of apoptosis (Cui, Huang et al. 2008). The mitochondrial function of the MDM was assessed as a surrogate for cell viability (Ricci, Munoz-Pinedo et al. 2004) using the MTT assay. No significant differences in mitochondrial function were detected between uninfected MDM and MDM which had been infected with HIV-1 for seven days (Figure 5B). RNA integrity can also be used as a marker for cell death as ribosomal RNA is degraded during apoptosis (Del Prete, Robles et al. 2002). Lack of cytopathic effect was also confirmed by analysing the RNA integrity of MDM infected with HIV-1 BaL (Figure 5C and D).

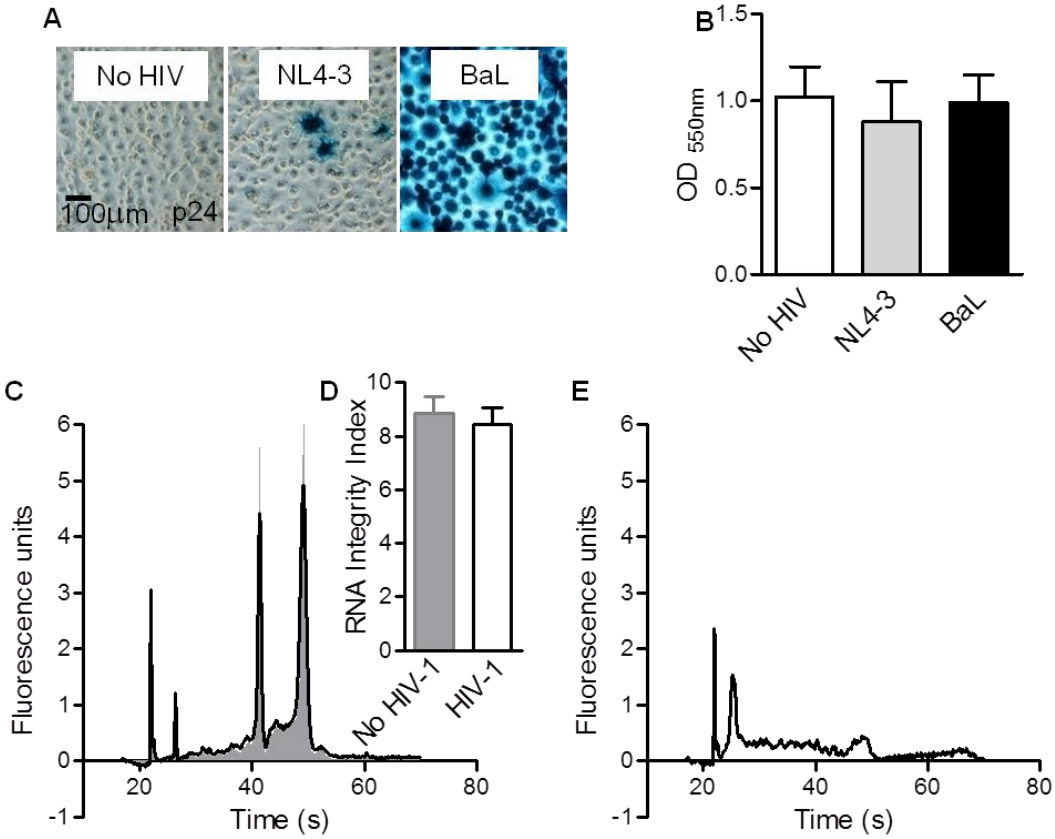
Figure 4 - Productive infection of MDM by a R5 tropic strain of HIV-1



(A) MDM were inoculated with either BaL or NL4-3 strains of HIV-1 at MOI3. The media was changed and supernatants were harvested every 48 hours and analysed for HIV-1 p24 content. Low levels of p24 can be seen in the NL4-3 inocculated MDM at early time points is likely due to contamination by the initial inocculum. **(B)** Western blotting was used to probe for the presence of HIV-1 p24 in MDM infected with HIV-1 (The primary antibody also detected the uncleaved 55kDa gag precursor). **(C)** RNA was harvested from MDM infected with BaL at various time points after infection and converted into cDNA. PCR was performed on serial dilutions of the cDNA to measure the relative levels of spliced HIV-1 genes (*tat/rev/nef*). **(D)** The amount of viral gene expression was quantified by densitometry and relative gene expression is shown as a relative value to GAPDH.

The bars in A and D represent the mean ± SD of three separate experiments. B and C are representative images of at least three experiments.

Figure 5 - MDM Infection by HIV-1 BaL is not cytopathic



(A) Light microscopy images of MDM stained for intracellular HIV-1 p24 (Dark cells) seven days after infection. A few HIV-1 positive cells can be seen in the culture inoculated with the X4 tropic strain NL4-3, compared to a completely infected culture with the R5 tropic strain BaL. (Representative images for >3 experiments with the exception of the NL4-3 image which was selected to show HIV-1 infected cells, and is not representative of the proportion of infected cells in the culture). Infection with HIV-1 is not cytopathic in these cells as a monolayer of cells is still visible. **(B)** No cytopathic effect can be attributed to HIV-1 infection of MDM when analysed by MTT assay looking at mitochondria function. (bars represent the mean ± SD of five separate experiments) **(C)** Representative RNA electrophoretograms showing the 2 major ribosomal RNA species (18S and 28S) for MDM with (black outlined) or without HIV-1 infection (grey filled) and **(D)** the integrity scores analysed in a RNA bioanlyser show similar integrity values (bars represent the mean ± SD of six separate experiments). **(E)** An example of degraded RNA extracted from alveolar macrophages

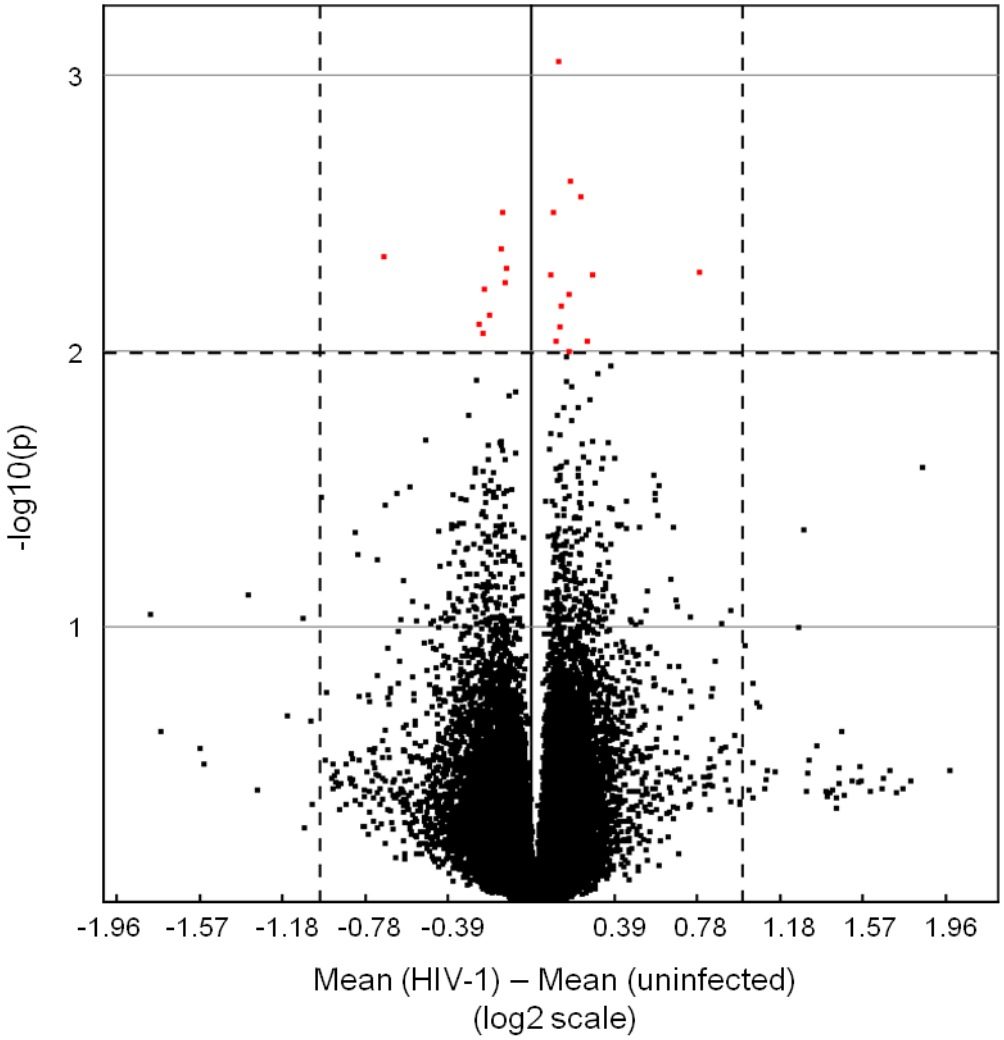
3.3.3 Steady state transcriptome in MDM infected with HIV-1

Though cytopathic effects of HIV-1 infection on MDM were not detected in this model other studies have reported that HIV-1 causes cytopathicity in MDM (Cui, Huang et al. 2008) as well as inducing a pro-apoptotic state in which apoptosis is more readily induced by other stimuli such as TNF-related apoptosis-inducing ligand (TRAIL) (Huang, Erdmann et al. 2006). In order to look for any HIV-1 effects on MDM, the steady state transcriptome of MDM which had been infected by HIV-1 for seven days was analysed using whole genome cDNA microarrays. MDM infected with HIV-1 showed no modifications to the expression of genes involved in apoptosis. Indeed, HIV-1 infection in this model appears not to exert major effects on the steady state transcriptome of MDM despite the high levels of viral replication in these cells. When comparing MDM infected with HIV-1 for seven days to uninfected MDM I could detect few significant changes in gene expression attributed to HIV-1 (Figure 6). This volcano plot shows that the majority of genes are as a whole not significantly changed upon HIV-1 infection. Of the 41,000 probes on the microarray, only 13 of which are significantly altered and these show less than two fold up or downregulation compared to uninfected cells. Of these 13 genes only two have associated genes of known function; IL-21R ($p=0.0087$) and aldehyde dehydrogenase 8 family, member A1 ($p=0.0021$) which are upregulated in HIV-1 infected cells by 1.72fold and 1.02fold respectively.

Hierarchical clustering of these samples shows that the greatest differences between them were due to donor variability. HIV-1 infected samples paired together with uninfected samples from the same donor and away from samples from other donors (Figure 7). The lack of effect on the MDM transcriptome is likely masked by the donor variation; therefore paired analysis was performed, comparing differences between uninfected and HIV-1 infected cells from the same donor (Figure 8). When the samples are paired, some transcriptional changes can be seen in HIV-1 infected cells

with the majority of transcriptional changes only up or downregulating gene expression by a factor of 1.5. However compared to transcriptional changes induced by stimulation with 10ng/ml LPS, representing a typical PAMP known to induce a wide range of transcriptional changes, HIV-1 infection has little effect, both when looking at the number as well as the magnitude of changes (Figure 9A). Functional annotation clustering analysis of the gene expression changes attributed to HIV-1 infection show that the three main groups of genes affected are involved in responses to external stimuli, carbohydrate binding and the cell cycle (Figure 9B). The cluster of enrichment scores for these groups is relatively low when compared with the three most enriched groups when stimulated with LPS. LPS stimulation of MDM gives scores of 7 – 9 for the three most enriched groups compared to scores of 1.4 - 2.2 in cells infected with HIV-1 for seven days.

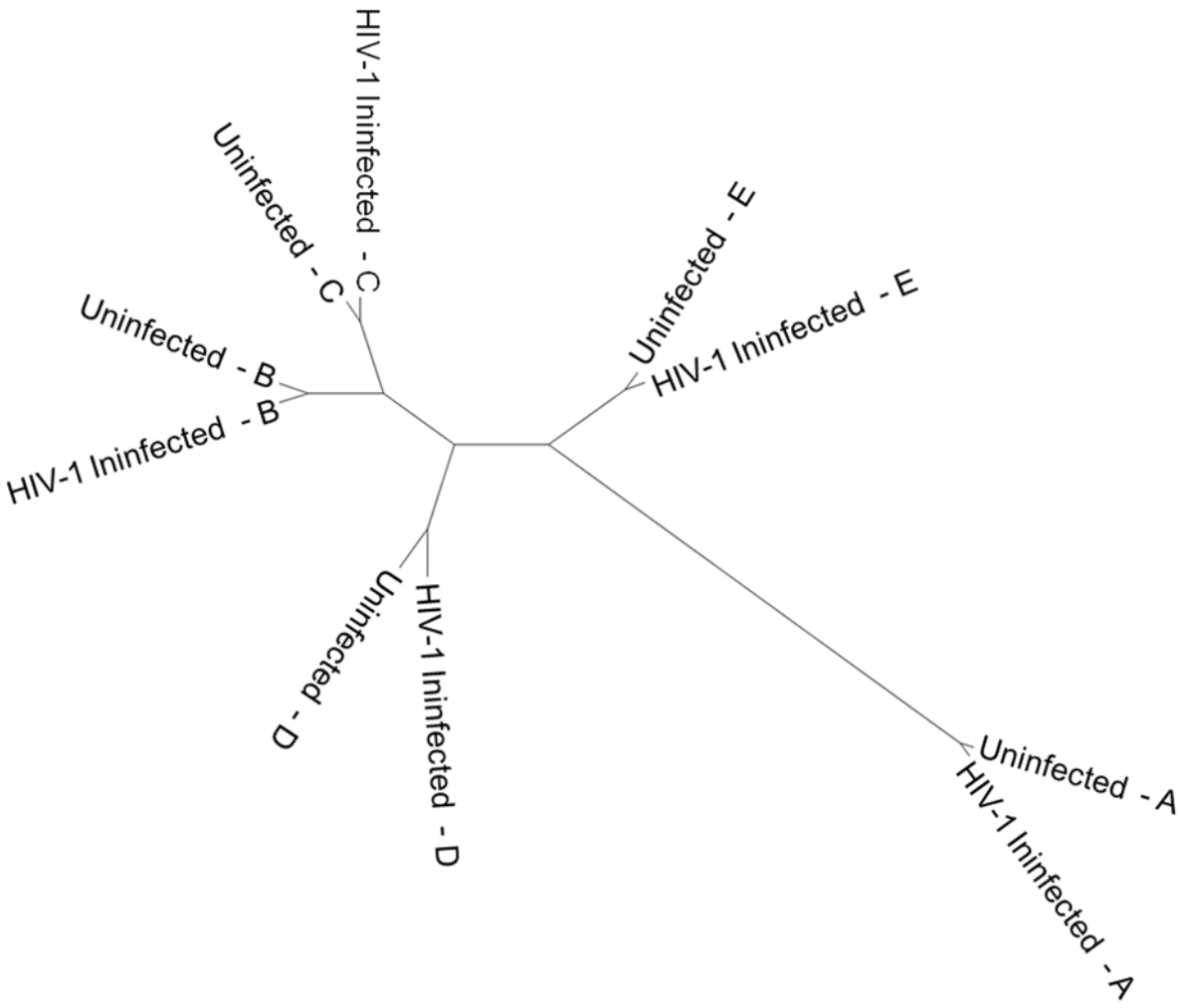
Figure 6 - No significant changes in the steady state transcriptome of HIV-1 infected MDM compared to uninfected MDM



Microarray analysis of whole genome transcription of uninfected and HIV-1 infected MDM. This volcano plot was generated in multi experiment viewer (T-Test p=0.01) and shows steady state transcriptional changes of HIV-1 infected MDM compared to uninfected MDM 7 days after infection. Each dot represents one of 41000 probes on the microarray. Vertical dashed lines represent 2 fold gene expression changes. The red dots above the horizontal dashed line each respresent a single significantly up or downregulated probe.

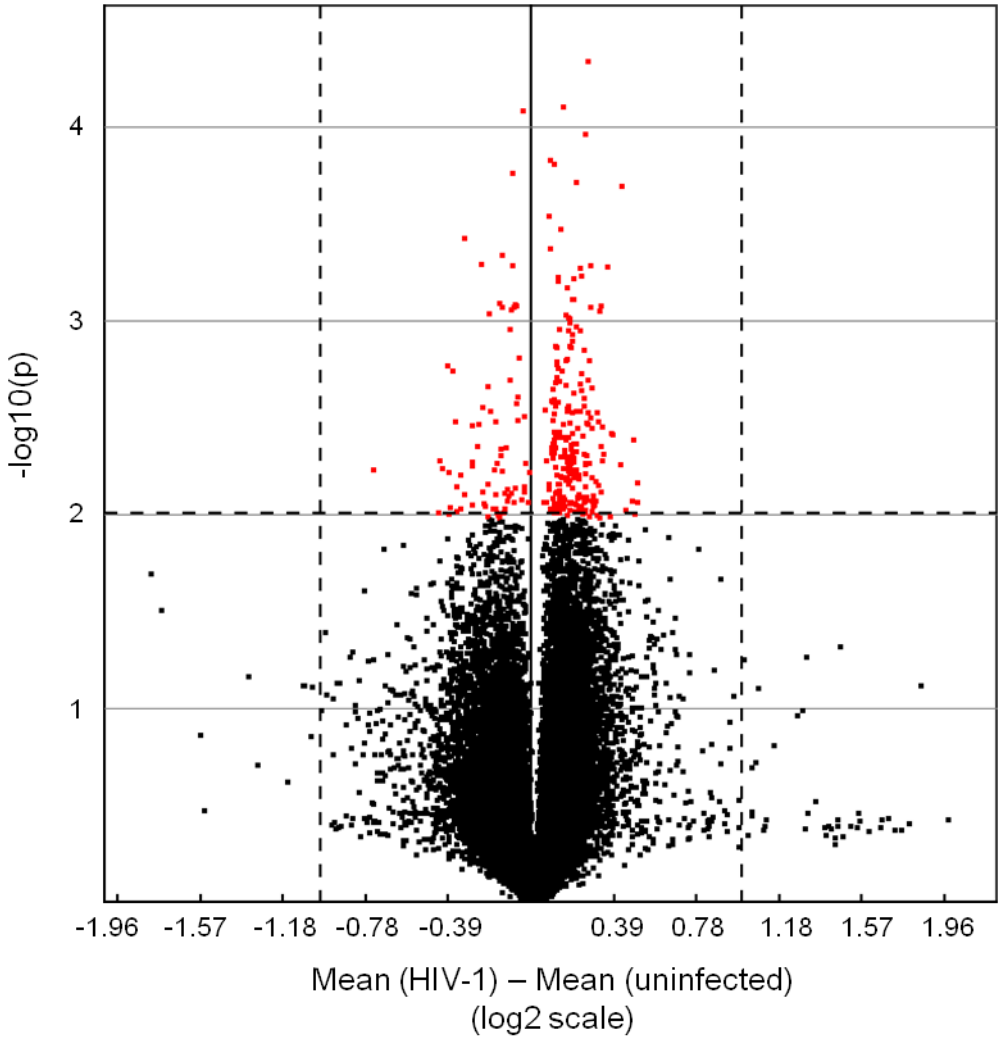
(-log10(p) value of two corresponds to p=0.01).

Figure 7 - Hierarchical clustering of uninfected and HIV-1 infected samples from different donors.



Heirarchical clustering of gene expression in MDM which had or had not been infected with HIV-1 BaL for seven days. Samples from the same donors cluster together showing more similarity between donor samples than from HIV-1 infection status.

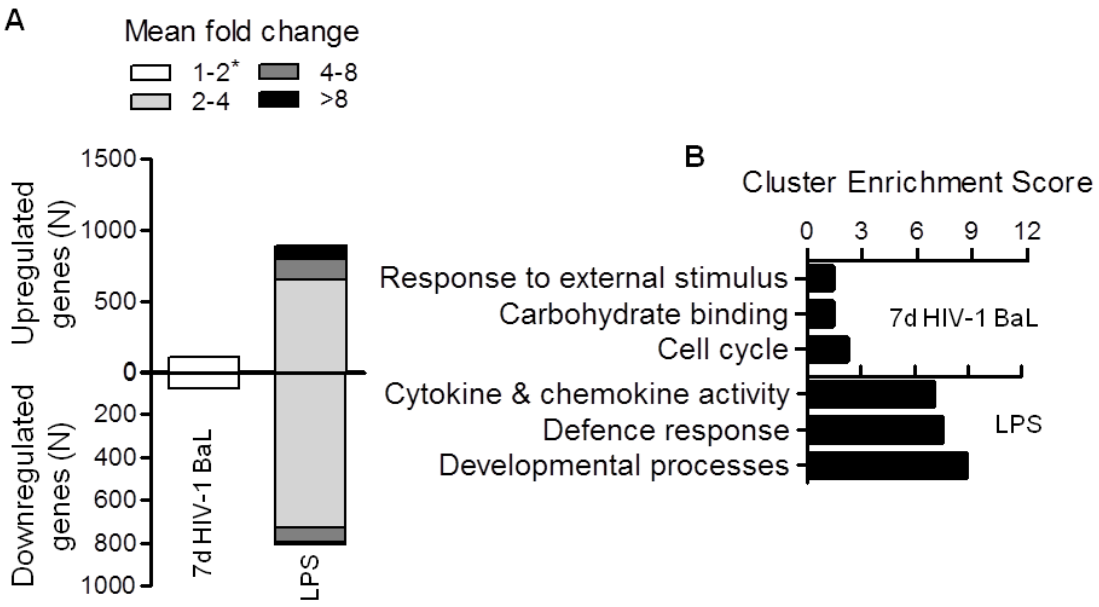
Figure 8 - Paired analysis of gene expression changes in MDM infected with HIV-1 for 7 days.



Microarray analysis of whole genome transcription of uninfected and HIV-1 infected MDM. This volcano plot was generated in multi experiment viewer (T-Test p=0.01) and shows steady state transcriptional changes of HIV-1 infected MDM compared to uninfected MDM 7 days after infection when uninfected and HIV-1 infected samples are paired by donor. Each dot represents one of 41000 probes on the microarray. Vertical dashed lines represent 2 fold gene expression changes. The red dots above the horizontal dashed line each respresent a single significantly up or downregulated probe.

(-log10(p) value of two corresponds to p=0.01).

Figure 9 - Analysis of transcriptional changes in MDM when paired by donor.



(A) Significant gene expression changes (P<0.05, t-test) detected by whole genome microarray transcriptional profiling in HIV-1 BaL infected MDM seven days after infection appear quantitatively modest compared with 4 h stimulation lipopolysaccharide (10 ng/ml). Data are derived from three separate microarray experiments for each stimulus compared with eight unstimulated MDM microarrays. 1-2 fold changes in gene expression is shown only for the 7d HIV-1 samples. **(B)** Functional annotation clustering analysis for significant gene expression differences identified by microarray transcriptional profiling in MDM infected with HIV-1 or stimulated for 4 h with LPS (10 ng/ml)

3.4 Discussion

Macrophages are sentinel cells of the innate immune system and may be involved in HIV-1 infection at several levels, by providing permissive cells at site of infection, contributing to spread of virus to T cells and by providing a long-lived viral reservoir. Many studies investigating the effects of HIV-1 in macrophages utilise cell lines due to their relative ease of culture. However as discussed earlier there are fundamental disadvantages of using cell lines due to variability in sub clones which can dramatically affect interactions with HIV-1. Also transformed cell lines have potentially altered NFκB signalling, which is an important part of many innate immune signalling pathways and may therefore be expected to confound host-pathogen interactions which I aim to study.

In this study, M-CSF differentiated MDM were used to investigate HIV-1 effects on macrophages. Purification of monocytes from PBMCs was performed by adhesion to tissue culture plastic. Though the adhesion to plastic method is a cheap and easy way of purifying monocytes from PBMCs, it can lead to considerable contamination with other cell types particularly lymphocytes (Figure 3 – Day 0. CD14 negative cells). However this contamination, can be reduced by more vigorous washing and is also less of an issue when the monocytes are kept and differentiated into macrophages in culture as the lymphocyte contamination is removed with later wash steps (Figure 3- Day 3). CD14 positive selection using the Miltenyi magnetic bead system is an alternative method of purifying monocytes from peripheral blood, and can give a higher purity of monocytes at the early stages of culture. The downside of this method of monocyte isolation is that it is much more expensive and time consuming.

By day six the cells are deemed to be MDM and in line with the literature show decreased levels of CD14 (Spottl, Hausmann et al. 2001; Daigneault, Preston et al. 2010), though by this parameter the cells in the culture may be somewhat heterogeneous (Figure 3 –Day 6) and may represent MDM at differing stages of

differentiation/maturity. Despite this heterogeneity all the cells in the MDM in the culture were infected with the R5 utilising, macrophage tropic strain of HIV-1 BaL seven days after inoculation with the virus. It is likely that this models a spreading infection within the culture, but the kinetics of this was not studied. HIV-1 BaL infection of M-CSF differentiated MDM was productive and was demonstrated by detection of viral transcripts as well as intracellular gag and p24 present in the supernatant from newly synthesised viral particles. In fact viable virus could be collected from supernatants of BaL infected MDM weeks after infection. Though the NL4-3 strain of HIV-1 does not appear to infect MDM (or very rarely) as seen by detection of viral transcripts and intracellular gag products, p24 was detected in the supernatant. p24 in the supernatant is likely due to the virus in the initial inoculum which had not been removed in the initial washes, as p24 levels in NL4-3 inoculated MDM supernatants decrease at later time points.

Unlike in some studies I found no evidence of cytopathic effect in MDM infected with HIV-1. No gross cytopathic effects were visible by light microscopy, and there was no significant change in mitochondrial function or ribosomal RNA integrity both of which can be used as markers for cell death (Del Prete, Robles et al. 2002; Ricci, Munoz-Pinedo et al. 2004). Furthermore using whole genome transcriptional microarrays I could not detect any transcriptional changes which could make them more susceptible to stimulation induced apoptosis. The lack of apoptosis found in this model may be due to a number of factors. The particular virus used could have an effect as one study reports that addition of wild type Nef protected MDM from HIV-1 induced apoptosis (Olivetta and Federico 2006). Different methods of generating MDM could also alter susceptibilities to apoptosis. An earlier publication reported TRAIL induced apoptosis in HIV-1 infected MDM (Lum, Pilon et al. 2001). However in this case the MDM were obtained by culturing monocytes for 6 days in media containing only serum without additional cytokines (GM-CSF or M-CSF), before infection with HIV-1 for 10 days

followed by stimulation with TRAIL. Additionally GM-CSF stimulation the day before infection with HIV-1 appeared to increase the level of apoptosis. Another study reported lack of apoptosis in HIV-1 infected MDM (Zhang, Li et al. 2002), with HIV-1 infection inducing expression of the anti-apoptotic gene BCL-2. The range of different results seen could be due to different culture conditions, methods of generating MDM or differences in the virus used, or a combination of the three.

Surprisingly microarray analysis of the steady state transcriptome also revealed that HIV-1 infection of MDM makes relatively few changes to gene expression. When analysed as a group there was remarkably little difference between uninfected and HIV-1 infected MDM with any differences masked by donor variability. Only 13 probes out of 41,000 were significantly different between HIV-1 infected and uninfected cells. Of these 13 probes only 2 of which have known genes associated with them; IL21R which is on average is upregulated by 1.7 fold and aldehyde dehydrogenase 8 family, member A1 which on average is upregulated by only 1.02 fold, which is likely not biologically significant. IL21R is important for the proliferation and differentiation of T, B and NK cells. Patients with mutations in the IL21R gene have been reported to have primary immunodeficiency with defects in T cell cytokine production, B cell proliferation and immunoglobulin class switching and NK cell cytotoxicity (Kotlarz, Zietara et al. 2013). The function of IL21/IL21R in macrophages is less clear, though one recent study reports the induction of phagocytosis by IL21 in GM-CSF differentiated MDM (Vallieres and Girard 2013).

The infection and conversion of the host cell into a virus producing factory could be expected to cause gene expression changes related to immune responses or other alterations to aid viral production. Donor variability was found to be greater than changes induced by HIV-1 infection and when the samples were analysed paired by donor, small changes in gene transcription was evident in HIV-1 infected cells but these changes were small both in terms of the number of genes affected as well as the

magnitude of the up or downregulation. This is highlighted when comparing gene expression profiles of MDM exposed to HIV-1 or LPS. MDM stimulated with LPS, exhibited over 10 times more genes which were significantly altered by between two to over eight fold changes compared to the number of genes altered by HIV-1 infection which showed less than two fold changes in expression.

Functional annotation clustering analysis of significant gene expression changes due to HIV-1 infection revealed that the three most enhanced clusters of genes were related to biological functions involved in responses to external stimuli, carbohydrate binding and the cell cycle. However these changes are modest both in number and magnitude compared to when MDM were stimulated with LPS suggesting that HIV-1 infection of macrophages does not cause major changes to the transcriptome.

It may be interesting to follow the differentiation of these MDM to a later time point to assess if the culture remains heterogeneous or if the differences seen in the cells at day six are due to incomplete differentiation/maturation. Likewise the progress of infection of the MDM culture could have been followed more closely rather than a single snapshot taken seven days after infection.

3.5 Conclusions

Here I show that M-CSF differentiated MDM generated from the peripheral blood from healthy volunteers are not permissive to infection by an X4 tropic virus, NL4-3, but are permissive to infection by the R5/macrophage tropic virus BaL. This infection is productive with active viral replication beginning as early as 24 hours after infection and peaking at around 7 days post infection at which point the entire culture is infected with HIV-1. HIV-1 infection and viral replication occurs without any observable cytopathic effect to the MDM. Additionally microarray analysis of the MDM steady state transcriptome shows limited changes attributed to HIV-1 infection without concerted changes to genes involved in apoptosis. There was also no sign of transcriptional changes due to innate immune responses to the virus itself but this could be due to suppression of the immune response at this time point where the virus has established itself and is actively replicating. With the advances in proteomics, analysis of the proteome may reveal post transcriptional differences between uninfected and HIV-1 infected MDM.

The long lifespan of macrophages combined with lack of cytopathicity caused by viral infection as well as the localisation of HIV-1 in the invaginated plasma membrane, where virions are protected from neutralising antibodies, supports the theory that macrophages may be a reservoir for HIV-1. Additionally this localisation may be important in aiding the cell to cell spread of HIV-1 by macrophages.

Chapter 4. Macrophage Innate immune responses, IFN and HIV-1

4.1. Introduction

In the previous chapter I showed that productive HIV-1 infection of MDM made remarkably little impact on the steady state transcriptome of the host cell. MDM express a variety of PRRs and are able to detect and respond to a wide variety of different pathogens, including RNA viruses such as influenza virus (Cheung, Poon et al. 2002; Cheung, Poon et al. 2005), sindbis virus (Assuncao-Miranda, Bozza et al. 2010) hepatitis C virus (Radkowski, Bednarska et al. 2004) and coronavirus (Cheung, Poon et al. 2005). HIV-1 derived ssRNA has been shown to activate other cell types as discussed earlier (Section 1.6), and I expected it to activate MDM immune responses as well.

As well as ssRNA other components of the HIV-1 virion may also trigger immune responses as is the case for varicella-zoster virus which is detected by TLR2 (Wang, Kurt-Jones et al. 2005) and similarly the glycoproteins of HSV-1 have been reported to activate immune responses via both TLR2 dependant (Leoni, Gianni et al. 2012; Cai, Li et al. 2013) and independent (Reske, Pollara et al. 2008) pathways. Innate immune activation has been reported in MDM exposed to gp120, (Gessani, Borghi et al. 1997; Cheung, Ravyn et al. 2008; Fantuzzi, Spadaro et al. 2008) the surface glycoprotein of the HIV-1 envelope, as well as whole virions (HIV-1 BaL strain) denatured with 2,2'-dithiodipyridine (Fantuzzi, Spadaro et al. 2008). Various other studies have also shown infection of MDM with full length replicative HIV-1 leading to innate immune cellular activation (Borghi, Fantuzzi et al. 1995; Canque, Rosenzwajg et al. 1996; Brown, Kohler et al. 2008). The PRR that might mediate innate immune responses to HIV-1 infection in MDM has not been established, although attention has focussed on TLR7,

TLR8 and the RIG-1–like family of receptors as the principal mammalian sensors for ssRNA described to date.

As previously described (section 1.4), the canonical cellular response to innate immune activation by viruses is a signalling cascade that leads to activation of key transcriptional factors and consequently changes to regulation of gene expression dominated by type I IFN and IFN-stimulated genes. Importantly, many of the HIV-1 restriction factors described to date are IFN-inducible (Cobos Jimenez, Booiman et al. 2012), but the consequence of innate immune activation of macrophages on HIV-1 itself has not been tested.

In this chapter, I specifically assessed whether HIV-1 infection of MDM in the model adopted in this thesis led to activation of the NFκB and IRF3 transcription factors, and prototypic inflammatory or IFN-associated transcriptional responses, with a view to developing further insights into the innate immune sensors for HIV-1 in this model and the consequences of cellular activation on HIV-1 infection.

4.2 Objectives

- Assess innate immune activation of MDM by HIV-1 at the level of transcriptional factor activation, IFN responses and IFN-stimulated gene expression.
- Make novel assessments of the PRR for HIV-1 in MDM
- Assess the effects innate immune activation and IFN stimulation have on HIV-1 infection and replication in MDM

4.3. Results

4.3.1 Development of a confocal assay to measure nuclear translocation and activation.

NFκB is part of a family of transcription factors which is activated by a wide range of innate immune stimuli. Activation of immune responses in U937 cells with either LPS or a number of bacterial pathogens showed that many of the genes which have increased expression contain NFκB binding motifs in their promoter regions (Schreiber, Jenner et al. 2006).

The main transcriptional transactivator of the classical NFκB signalling pathway is RelA, which translocates to the nucleus as a result of a kinase signalling cascade that leads to protesosomal degradation of the inhibitor IκBα, as described in section 1.4.1. By looking at the activation state of NFκB I can deduce if a cell has been activated after stimulation with a PAMP. The transcription factor IRF3 which is important for antiviral IFN responses, like NFκB also translocates to the nucleus upon activation.

Detection of NFκB can be done in a number of ways. The most common ways used to detect activation of the NFκB pathway is to collect cell culture nuclear extracts for use in the Electrophoretic Mobility Shift Assay (EMSA), or by Western blotting. However the sensitivity of these assays requires large numbers of cells which frequently limits its use when working with primary cells.

Alternatively NFκB reporter plasmids could be used, but primary macrophages are difficult to transfect. In addition, transfection of DNA into these cells may trigger IFN responses that confound the experiment.

In order to measure innate immune activation in our MDM model I developed a confocal microscopy based assay (Noursadeghi, Tsang et al. 2008), which can quantify activation of endogenous transcription factors which translocate to the nucleus upon activation/stimulation of the immune response.

Using confocal microscopy I can take images of cells and visualise the localisation of NFκB Rel A. Using the software ImageJ I can define our regions of interest by using masks. This was done by applying a median filter (3×3 pixel radius) to remove background noise and to approximate the distribution of staining intensity to a median value (Figure 10B). A threshold value was then set to include all fluorescence data above background and this was used to generate a binary mask (Figure 10C). For the nuclear compartment this was done by looking at the DAPI staining. The same was done with the NFκB RelA staining which would select for the cells in the image. The cytoplasmic compartment was then defined by subtracting the nuclear mask from the NFκB mask. Using these masks the staining intensities of both the nuclear and cytoplasmic compartments of the NFκB RelA image was analysed. This was done by analysing the number and intensity of staining for individual pixels within the image. After normalisation for number of pixels the frequency intensity graphs can be used to assess NFκB activation status by comparing the relative staining of nuclear and cytoplasmic compartments. Quantification of NFκB activation was done by analysing the area under the curve for each compartment and looking at the ratio of nuclear to cytoplasmic staining.

I later automated this image analysis using the software Metamorph which is able to detect nuclei by looking for round structures in the image. The cytoplasmic compartment was defined as a band of user defined width around the perimeter of the nucleus. Finally towards the end of this work I also used the Opera High Content Screening System from PerkinElmer which was able to do the same analysis in a 96well plate format this was used for Figure 16 and Figure 17.

4.3.1.1 Analysis of NFκB Activation on LPS stimulated MDM

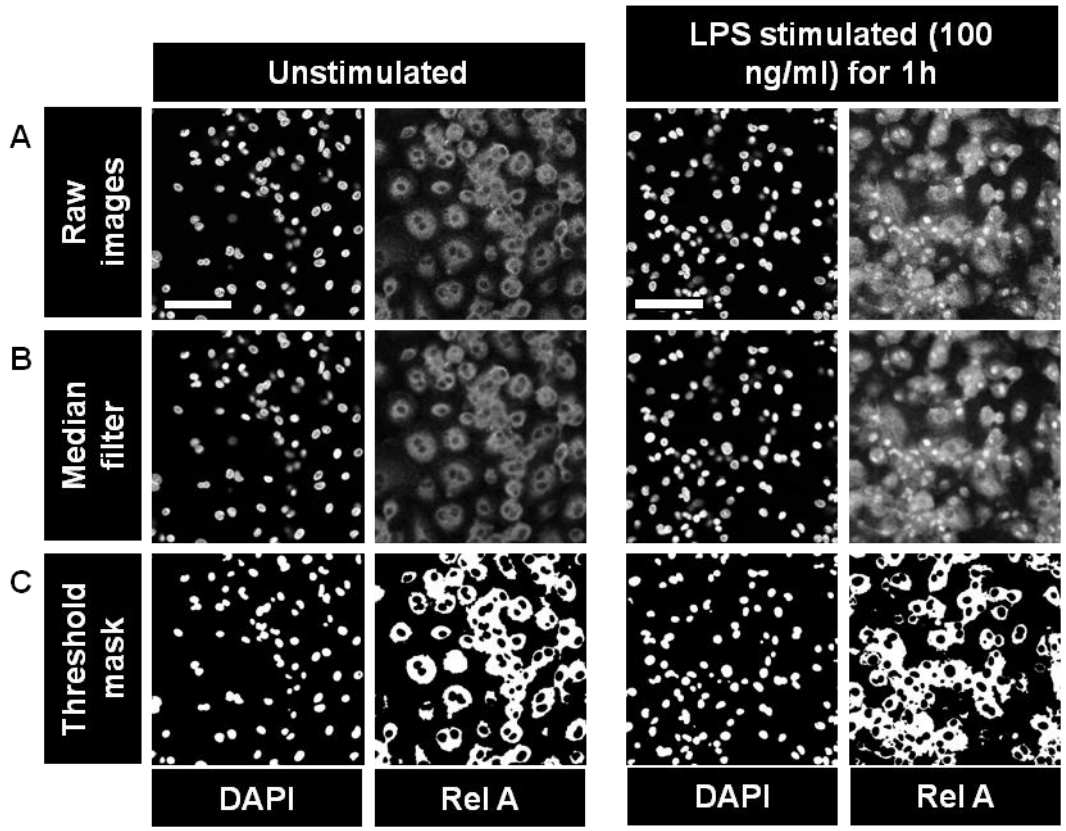
In order to develop and test the NFκB nuclear translocation assay, I first tested MDM responses to TLR4 or TLR2 stimulation with specific ligands. MDM were stimulated with 100ng/ml of LPS (to stimulate TLR4) for one hour before being fixed and stained

for NFκB RelA. Image J software was then used to create masks to define the nuclear compartments (DAPI stained), and the cytoplasmic compartments (NFκB staining subtracting the DAPI stained areas) (Figure 10). With these masks the relative staining intensity of NFκB was analysed for the cytoplasmic and nuclear compartments (Figure 11).

I tested the assay over a time course of stimulation with LPS (Figure 12) and a dose response to LPS (Figure 13). The relative staining intensities can be converted into nuclear: cytoplasmic ratios for NFκB staining intensity, allowing for easy comparison of NFκB activation status between samples (Figure 12B and Figure 13B).

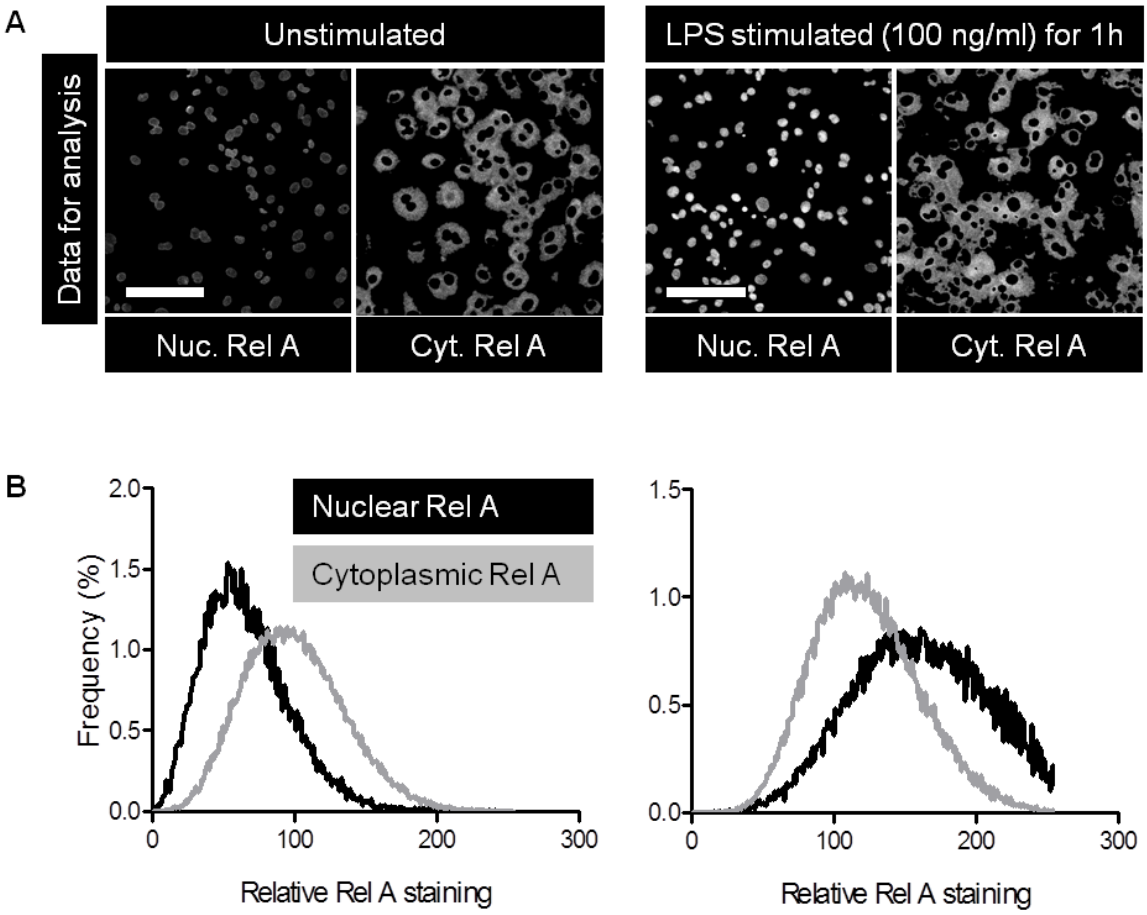
Polymixin B (PMB), a polypeptide that binds and neutralises the bioactive lipid A component of LPS. PMB completely abrogated NFκB nuclear translocation in response to LPS specifically, and had no effect on stimulation with the TLR1/2 ligand, Pam3CSK4 (Figure 14), confirming that this assay is specifically measuring transcription factor activation in response to a specific TLR pathway.

Figure 10 - Creation of binary masks for the cytoplasmic and nuclear compartments.



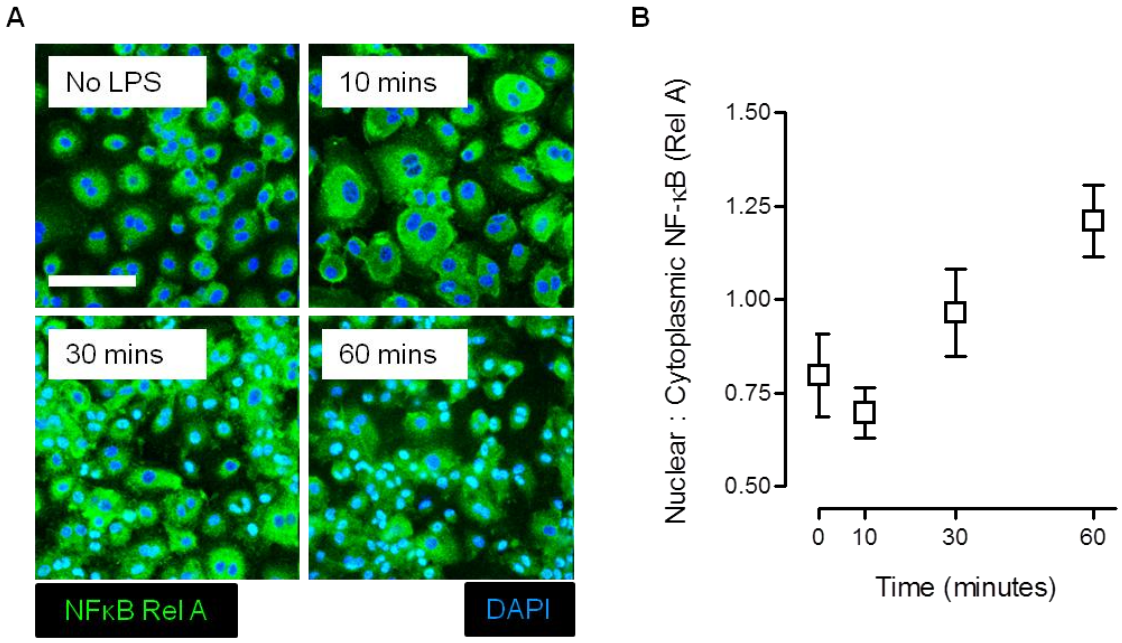
8-bit image files of DAPI and Rel A staining are shown for unstimulated and LPS stimulated macrophages (**A**). Bar represents 100 μm. The sequential processing of these images using ImageJ software is shown to produce binary masks of nuclear and cytoplasmic regions of interest. A median filter (3×3 pixels) is applied to approximate staining to a median value and remove noise (**B**) followed by automatic thresholding to generate a binary image. The nuclear compartment is defined by the DAPI mask and the cytoplasmic compartment by subtracting the DAPI mask from the Rel A mask (**C**).

Figure 11 - ImageJ analysis of NFκB staining in MDM



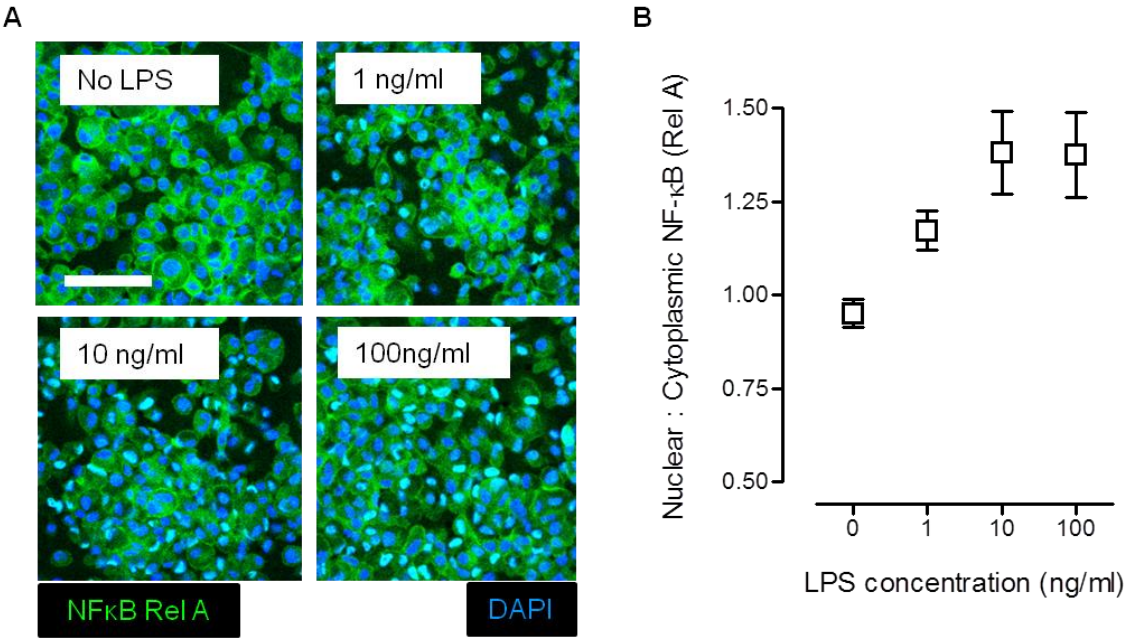
Nuclear and cytoplasmic regions of interest are applied to the original NFκB Rel A image files to extract Rel A immunofluorescence data for each region in unstimulated and LPS stimulated macrophages **(A)**. Bar represents 100 μm. Histograms of the frequency distribution of fluorescence intensity show increased nuclear:cytoplasmic Rel A staining in LPS stimulated cells compared to unstimulated cells **(B)**.

Figure 12 - ImageJ analysis of NFκB staining in MDM stimulated with LPS over a time course.



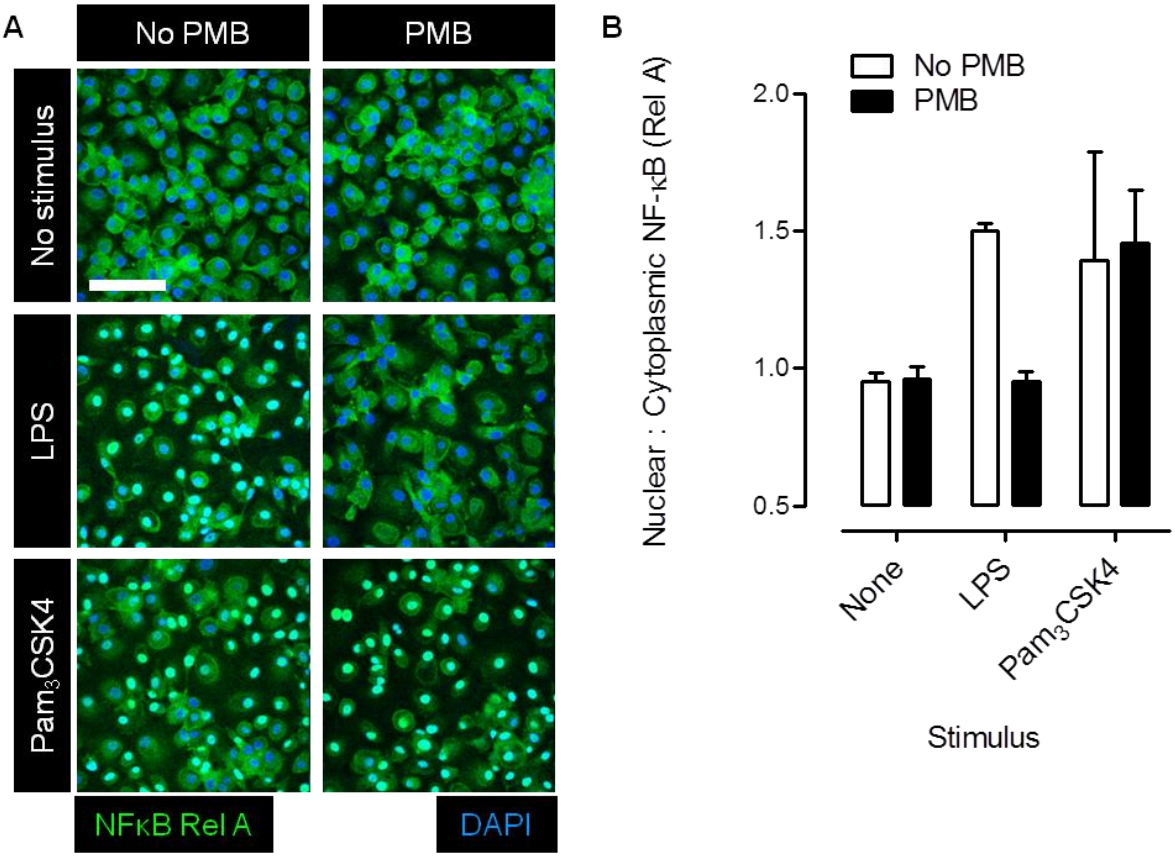
Immunofluorescence staining of Rel A **(A)** and quantification of nuclear:cytoplasmic ratios of Rel A staining **(B)** in a time course study of NFκB nuclear translocation in LPS (100 ng/ml) stimulated macrophages. Data points represent mean±standard deviation from analysis of 5 separate high power field images.

Figure 13 - ImageJ analysis of NFκB staining in MDM stimulated with a dose response to LPS.



Immunofluorescence staining of Rel A **(A)** and quantification of nuclear:cytoplasmic ratios of Rel A staining **(B)** in a dose response study of NFκB nuclear translocation in LPS (1 h) stimulated macrophages. Data points represent mean±standard deviation from analysis of 5 separate high power field images.

Figure 14 - Validation of NFκB activation measurements using PMB and a second TLR stimuli.

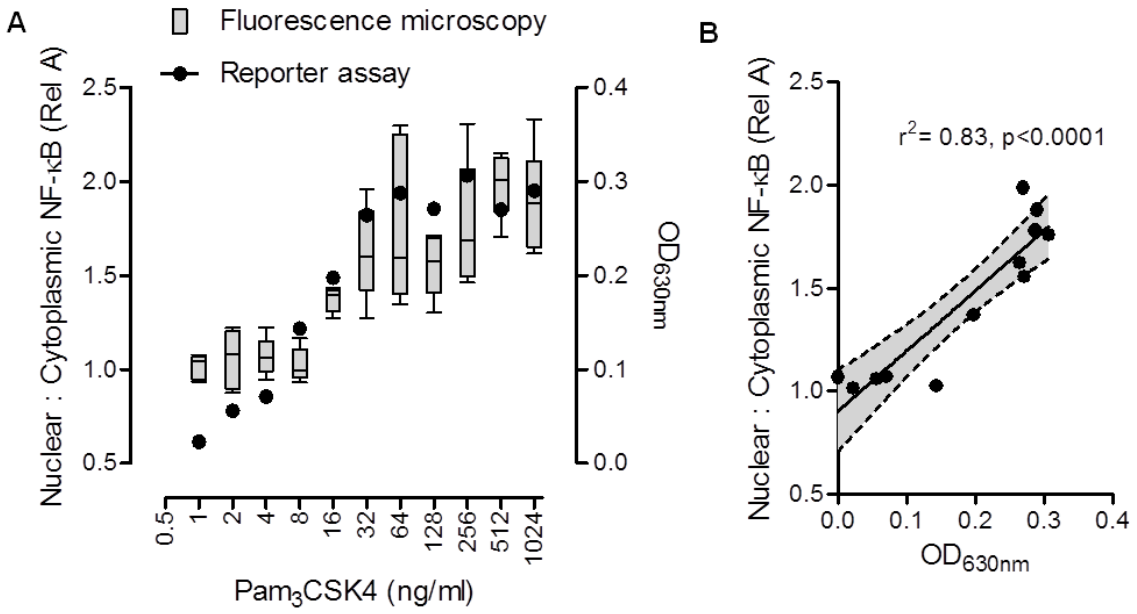


Immunofluorescence staining of Rel A **(A)** and quantification of nuclear:cytoplasmic ratios of Rel A staining **(B)** in unstimulated macrophages and after 1 h stimulation with 100 ng/ml LPS or 100 ng/ml Pam3CSK4, with and without 10 µg/ml polymyxin B (PMB). Bar represents 100 µm. Data points represent mean±standard deviation from analysis of 5 separate high power field images.

4.3.1.2 Comparison of the confocal assay with a commercial NFκB reporter assay

I evaluated the quantitation provided by this assay by comparing it to a commercial NFκB reporter gene assay using the 293 cell line transfected with TLR2 and a secreted alkaline phosphatase NFκB reporter gene construct (Invivogen). Transfected cells were seeded on to glass coverslips as described in the methods and materials and allowed to adhere for 48 hours. They were then stimulated with Pam3CSK4 (dose range 0–2 μg/ml) diluted in the manufacturer's detection media. Cells were fixed and stained for immunofluorescence staining of NFκB one hour after stimulation. Duplicate wells were allowed to incubate for six hours to allow the reporter gene and substrate reaction to take place. Cell culture supernatants were then harvested to quantify the colourimetric reaction spectrophotometrically at 630 nm. Quantitative comparison of NFκB activation using the reporter gene expression assay and NFκB nuclear translocation by confocal microscopy showed statistically significant correlation (Figure 15).

Figure 15 - Comparison between confocal microscopy assay and a commercial NFκB reporter assay.



Comparison of NFκB nuclear translocation by immunofluorescence imaging (nuclear:cytoplasmic Rel A staining) and NFκB activation by reporter gene assay (OD630) in TLR2 transfected HEK- 293 cells stimulated with Pam3CSK4 **(A)**, shows significant correlation by linear regression analysis **(B)**. Box and whisker plots represent median, SD and range of data from analysis of 5 separate high power field images. Reporter assay data points represent mean of duplicate spectrophotometric measurements. Shaded area of linear regression analysis shows 95% confidence interval.

4.3.2 Do MDM mount innate immune responses to HIV-1?

4.3.2.1 TLR stimulation of MDM and activation of NFκB and IRF3/7

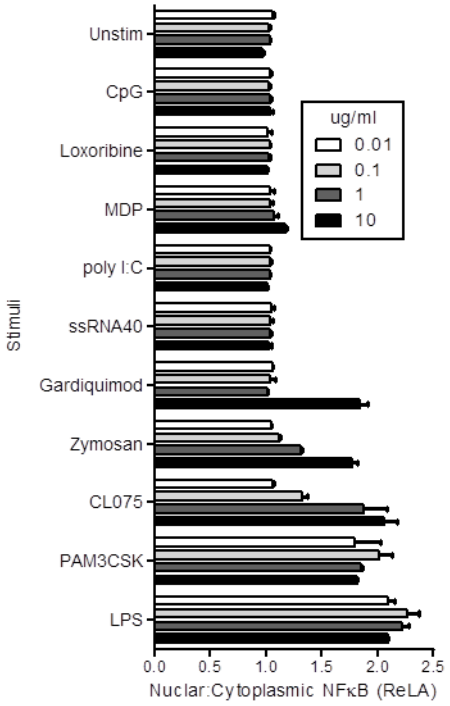
Using the assay just described, I were able to screen a number of stimuli on the MDM to test the functionality of some of the innate immune PRR pathways. MDM were stimulated with a panel of minimal stimuli at various doses ranging from 10ng/ml up to 10µg/ml for one hour before fixing and staining for NFκB (Figure 16) or IRF3 (Figure 17) to look for generic innate immune activation inflammatory and activation of transcription factors particularly associated with anti-viral IFN responses respectively.

I found that MDM responded in a dose dependent manner to zymosan (TLR2/Dectin-1 Ligand) and CL075 (TLR 7/8 Ligand) at the dose ranges tested. LPS (TLR4 Ligand) dose responsiveness was demonstrated in previous experiments using a lower dose range (Figure 13). Stimulation of the MDM with Pam3CSK4 (TLR1/2 Ligand) induced maximal nuclear translocation of the transcription factors at the lowest dose tested of 10ng/ml. MDM also show a response to gardiquimod (TLR7 Ligand, though it can also activate TLR8 at high concentrations) but only at the highest concentration tested of 10ug/ml. No activation of NFκB or IRF3 was seen in response to poly I:C (TLR3 Ligand), loxoribine (TLR7 Ligand) or ssRNA40 (TLR7/8 Ligand), a synthetic oligonucleotide, derived from HIV-1 sequences.

The ability of a range of TLR agonists to activate and translocate the transcription factors indicates that the TLR signalling pathways in these MDM are functional.

Figure 16 - NFκB activation in MDM stimulated with minimal TLR ligands.

A



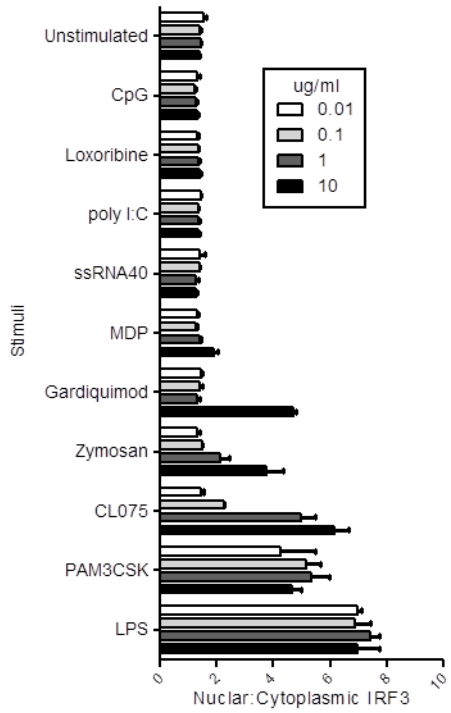
B

	2 way ANOVA p values	
Stimuli	Stimuli	Concentration
CpG	NS	NS
Loxoribine	NS	NS
MDP	NS	NS
Poly I:C	NS	NS
ssRNA40	NS	NS
Gardiquimod	p<0.001	p<0.0001
Zymosan	p<0.0005	p<0.0001
CL075	p<0.0005	p<0.0001
PAM3CSK	p<0.001	NS
LPS	p<0.0005	NS

MDM were stimulated with different TLR ligands for 1 hour at doses from 10ng/ml to 10ug/ml. MDM were then fixed and stained for either NFκB RelA. Nuclear:cytoplasmic ratios are shown for duplicate wells with each data point an average of 9 random high power field images. Statistical analysis was performed using 2 way ANOVA. * represent statistically significant differences between stimuli compared to unstimulated cells and + represent statistically significant differences between different doses of the same stimuli.

Figure 17 - IRF3 activation in MDM stimulated with minimal TLR ligands.

A



B

	2 way ANOVA p values	
Stimuli	Stimuli	Concentration
CpG	NS	NS
Loxoribine	NS	NS
MDP	NS	NS
Poly I:C	NS	NS
ssRNA40	NS	NS
Gardiquimod	p<0.0005	p<0.0001
Zymosan	p<0.001	p<0.001
CL075	p<0.0005	p<0.0001
PAM3CSK	p<0.001	NS
LPS	p<0.0005	NS

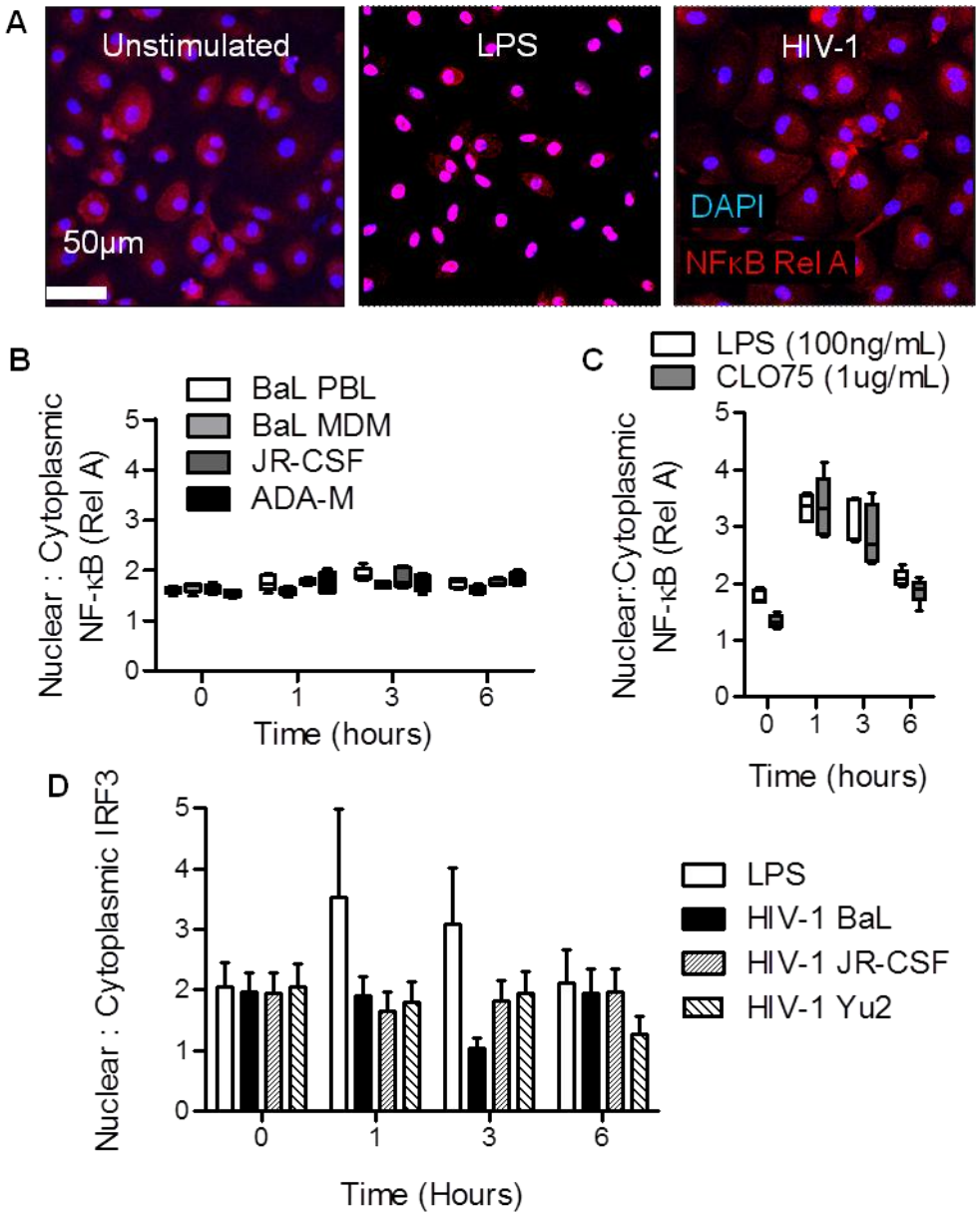
MDM were stimulated with different TLR ligands for one hour at doses from 10ng/ml to 10ug/ml. MDM were then fixed and stained for either IRF3. Nuclear:cytoplasmic ratios are shown for duplicate wells with each data point an average of 9 random high power field images. Statistical analysis was performed using 2 way ANOVA. * represent statistically significant differences between stimuli compared to unstimulated cells and + represent statistically significant differences between different doses of the same stimuli.

4.3.2.2 Measuring NFκB and IRF activation in HIV-1 stimulated MDM

After demonstrating that innate immune signalling pathways were functional to a range of stimuli tested, I tested the ability of MDM to recognise and respond to incoming HIV-1 virions.

To test if HIV-1 activates the innate immune response in our model, MDM were exposed to HIV-1 at a MOI of three for one, three or six hours and nuclear translocation of NFκB RelA and IRF3 was measured. Unstimulated cells have diffuse staining of NFκB throughout the cell, while in LPS stimulated cells the staining is mostly nuclear, with HIV-1 stimulated cells showing an unstimulated profile (Figure 18A). For a variety of HIV-1 strains tested, nuclear translocation of NFκB could not be seen (Figure 18B). In contrast, stimulation with the minimal TLR ligands LPS or CL075, did stimulate nuclear translocation of NFκB (Figure 18C). Similar results were seen when looking at IRF3 activation (Figure 18D).

Figure 18 - NFκB and IRF3 activation in MDM after stimulation with HIV-1



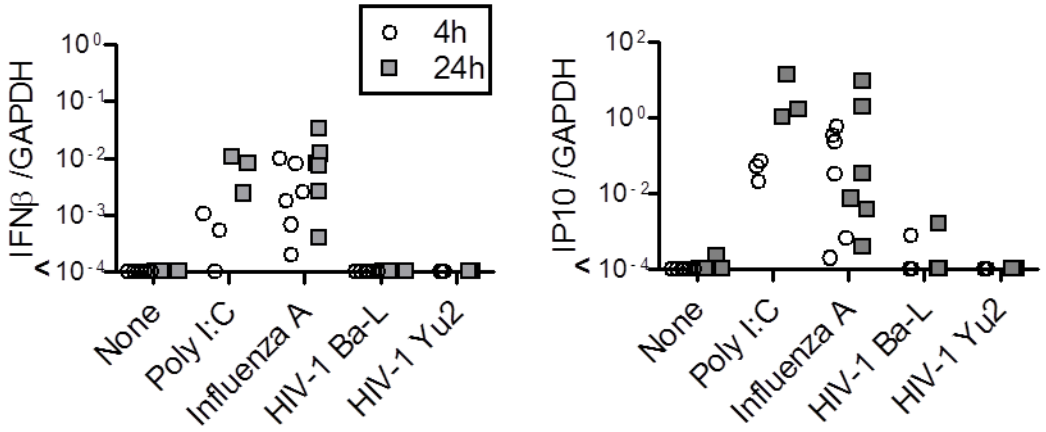
(A) Representative confocal microscopy images MDM, unstimulated or stimulated with 10ng/ml of LPS or HIV-1 BaL at a MOI of 3 for 1 hour. Nuclei are stained with DAPI (Blue) and NFκB RelA/p65 (Red). **(B)** No activation of NFκB was detected in MDM stimulated with a range of HIV-1 strains (MOI3). **(C)** The TLR 4 and 7/8 stimuli, LPS (10ng/ml) and CL075 (1µg/ml) induces significant ($P<0.0001$,ANOVA) nuclear translocation of NFκB. **(D)** No nuclear translocation of IRF3 was detected in response to a number of HIV-1 strains in comparison to LPS. Error bars represent mean±SD of 5 separate high power fields

4.3.2.3 Measurement of innate immune inflammatory and IFN responses in MDM stimulated with HIV-1

Although activation of NFκB or IRF3 was not seen in MDM in response to HIV-1, the virus may still induce an immune response. This assay may not be sensitive enough, or it is possible that HIV-1 can induce a response independently of NFκB and IRF3, as poly I:C (a model for dsRNA) has been reported to generate an IFN response without NFκB and IRF3 activation (Reimer, Brcic et al. 2008). To see whether or not HIV-1 infection induced an IFN response, RT-qPCR was performed on RNA extracted from uninfected and HIV-1 infected MDM looking for expression of IFNβ which is produced early in the IFN response (as discussed in section 1.4.2). As well as being one of the early type I IFN genes expressed, there is only one isoform of IFNβ, unlike IFNα which has 13. In order to assess the presence of any functional IFN activity as a result of HIV-1 infection, I also measured expression levels of the interferon stimulated gene (ISG), IP-10 (CXCL10), which represents one of the most sensitive ISGs and is rapidly expressed in response to both type I and type II IFNs (Ohmori and Hamilton 1995; Tebo, Kim et al. 1998; Gasperini, Marchi et al. 1999; Padovan, Spagnoli et al. 2002; Lee, Kim et al. 2009).

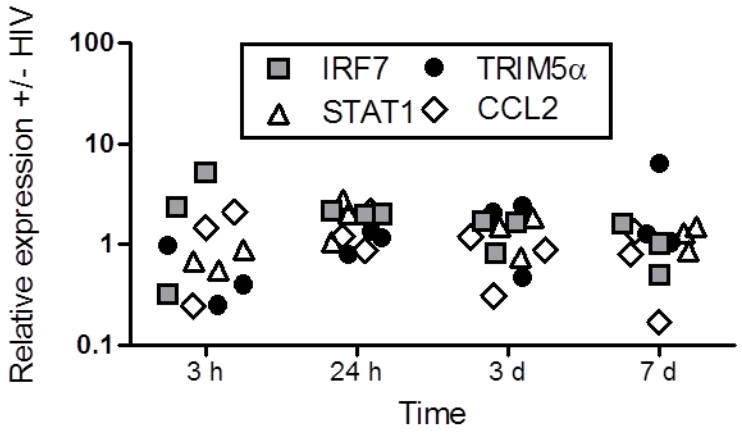
In contrast to stimulation with poly I:C and influenza, no IFNβ (Figure 19A) or IP-10 (Figure 19B) gene expression could be seen in response to HIV-1 at either 4 hours or 24 hours after infection. The lack of innate immune responses was further confirmed by qPCR to measure expression of other candidate ISGs (IRF7, STAT1 and TRIM5α) or inflammatory response genes (CCL2) over a longer time course (Figure 20). No significant differences in gene expression due to HIV-1 infection were seen at all time points tested.

Figure 19 - IFN-B and IP-10 expression in MDM in response to stimulation.



Stimulation of MDM with poly I:C (10mg/ml) or challenge with influenza A (MOI 3) induces significantly increased gene expression of IFNβ and IP10, at both early (4h) and late (24h) time points, as quantified by qPCR ($P<0.01$, Wilcoxon sign-ranked test of paired samples). Neither macrophagetropic HIV-1 strains induced detectable expression of either of these genes. Gene expression was normalised to GAPDH. Each data point represents a separate donor/experiment.

Figure 20 - Relative expression of inflammatory genes (and the IFN sensitive gene TRIM5α) over time after infection with HIV-1

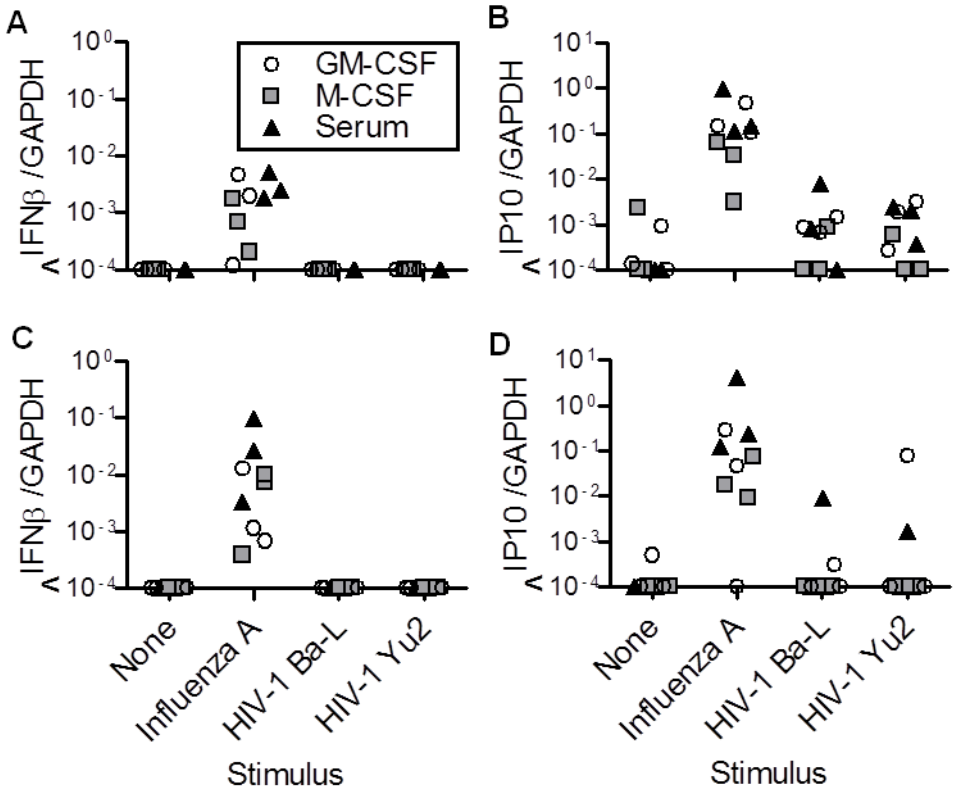


MDM were challenged with HIV-1 (MOI3) for the time course shown. At the relevant time post challenge RT-qPCR was performed to look for expression of a range of inflammatory or IFN sensitive gene in response to stimulation with HIV-1. Data shown is from 3 seperate donors. After normalisation to GAPDH gene expression is shown as a ratio and expressed as a ratio of gene expression in HIV-1 infected MDM compared to uninfected MDM.

4.3.2.4 IFN responses to HIV-1 infection of alternatively differentiated macrophages.

Macrophages can be differentiated from peripheral blood monocytes by a variety of methods. The literature reports that M-CSF differentiated macrophages are less pro-inflammatory compared to macrophages differentiated with GM-CSF. GM-CSF differentiated cells have been shown to secrete TNFα and IL-12p40 in response to LPS or mycobacterial stimulation whereas M-CSF differentiated cells secrete the anti-inflammatory cytokine IL-10 (Verreck, de Boer et al. 2004; Lacey, Achuthan et al. 2012). To test whether or not the lack of response to HIV-1 is due to how the MDM were differentiated, IFNβ and IP-10 gene expression were measured in response to HIV-1 infection in MDM generated using GM-CSF or autologous serum alone. The results of this experiment show that M-CSF differentiated macrophages respond in a similar manner to macrophages differentiated by GM-CSF or in serum alone, in response to influenza A, but still did not respond to HIV-1 (Figure 21).

Figure 21 - IFN gene expression in differentially differentiated MDM in response to HIV-1.



Relative expression of IFNβ or IP-10 gene expression (to GAPDH) were measured 4 hours **(A-B)** and 24 hours post stimulation **(C-D)** (with the stimuli shown below) in MDM differentiated by autologus serum alone (Triangles), M-CSF (Squares) or GM-CSF (Circles). Neither macrophage tropic strain of HIV-1 induced expression of IFNβ and IP-10, in contrast with Influenza A. The method of differentiating MDM did not affect the magnitude of the response to the HIV-1 viruses

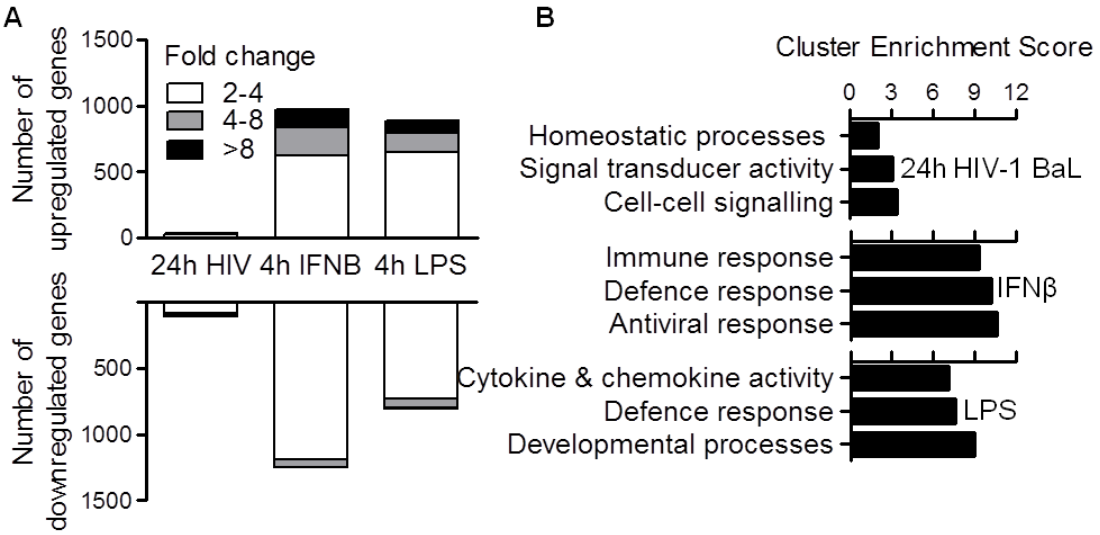
Data is from three donors with each of the donors contributing to all three MDM types.

4.3.2.5 Genome-wide innate immune responses during HIV-1 infection of MDM

The innate immune response encompasses many different pathways and activates many genes, which can be dependent on both cell type as well as stimuli (Huang, Liu et al. 2001; Chaussabel, Semnani et al. 2003). Therefore looking at a limited selection of transcription factors and genes, may fail to detect an innate immune response that bypasses the markers I have decided to measure. To test the global effect HIV-1 has on MDM, whole genome transcriptional profiling was performed 24 hours post infection, in addition to the analysis of MDM infected for seven days reported in chapter 3. HIV-1 infection of macrophages did induce some modest transcriptional changes, but these were only evident when pairing uninfected and infected cells from the same donor (Figure 22A), as donor variability masked the differences. These differences are also small when compared to those seen in LPS or IFNβ stimulated macrophages, which showed considerably greater number and magnitude of gene expression changes. In order to assess the biological significance of these apparently modest changes, functional clustering analysis of gene expression changes was performed using gene ontology associations (which group the genes according to their function). LPS and IFNβ stimulated gene expression changes in MDM showed highly significant enrichment of functionally related groups of genes with cytokine and chemokine activity, and genes involved in immune, defence and antiviral responses. These data clearly show the ability of MDM to exhibit co-ordinated inflammatory and anti-viral host defence responses, in marked contrast to MDM infected with HIV-1 which showed no such enrichment for host defence gene expression Figure 22B) suggesting that in this model, HIV-1 does not induce an innate immune response in macrophages. In addition multidimensional scaling was used to assess the relative similarity/dissimilarity of expression profiles for selected gene sets. In both IFNβ-regulated and LPS-regulated gene sets, HIV-1 infected MDM cluster together with uninfected/unstimulated MDM (Figure 23). Collectively, these bioinformatic approaches unequivocally illustrate the

lack of inflammatory or IFN-associated transcriptional responses in MDM when infected with HIV-1.

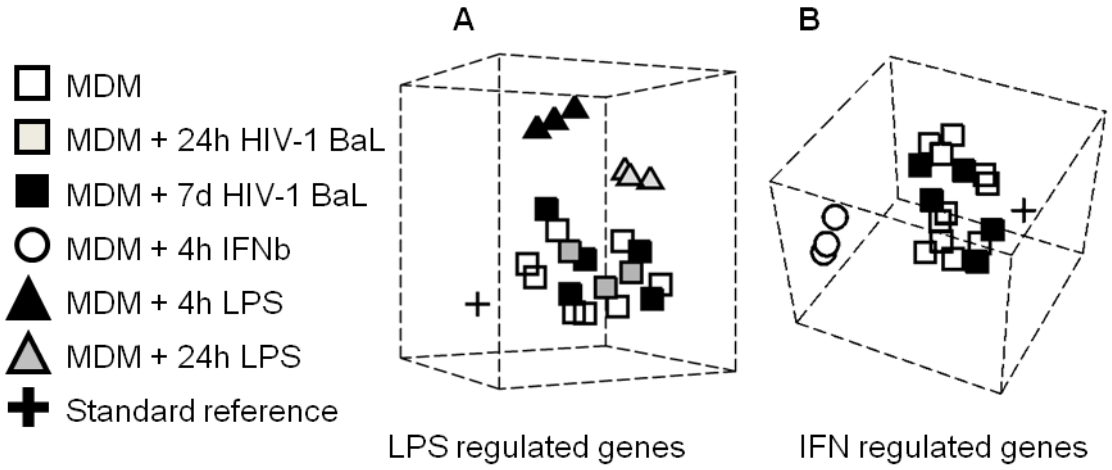
Figure 22 - Up/Downregulation of gene expression in stimulated MDM



Whole genome transcriptional microarrays were performed on MDM stimulated with HIV-1 (MOI3), IFNβ (500IU/ml - 2 ng/ml) or LPS (10ng/ml) and changes in gene espression compared with unstimulated MDM. **(A)** Analysis of transcriptional responses in HIV-1 infected MDM showed minor effects attributed to HIV-1 infection, and only when uninfected and infected cells from the same donor were paired, but both the number of genes and the magnitude of the response were several logs smaller than the response seen to LPS and IFNβ. **(B)** Functional annotation clustering analysis for significant gene expression differences identified by microarray transcriptional profiling in MDM infected with HIV-1 or stimulated for four hours with LPS or IFNβ

Data are derived from three separate microarray experiments for each stimulus compared with eight unstimulated MDM.

Figure 23 - Multidimensional scaling used to generate 3D representation of relative similarity/dissimilarity of MDM after stimulation.

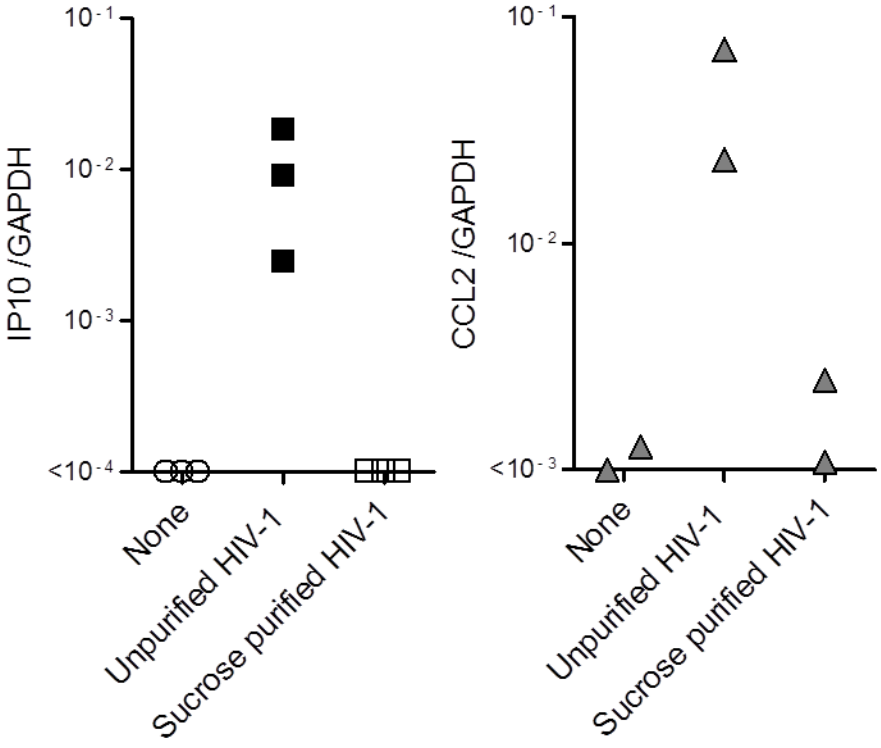


Images were generated by multidimensional scaling (MDS) analysis looking exclusively at genes whose transcriptional profiles where altered when MDM were stimulated with either LPS for 4 hours **(A)** or IFNB **(B)**. Looking at LPS stimulated genes uninfected and infected MDM cluster together, and the 4h and 24hr LPS stimulated clutering seperately, but away from unstimulated/HIV infected MDM **(A)**. Looking at IFNB stimulated genes, again HIV-1 infected MDM cluster with uninfected MDM **(B)**.

4.3.2.8 Impurities in HIV-1 preparations can lead to innate immune activation

Contrary to other published data, which do show MDM innate immune responses to HIV-1 I failed to detect any evidence of either inflammatory or IFN gene expression. When investigating what differences there are between our experiments and those already published, it was noticed in the previous reports that HIV-1 virus had been obtained by harvesting cell culture supernatants from producer cells, typically PBMC, without any effort to minimise contamination of immunoreactive molecules released by the producer cells. For the previous experiments, all HIV-1 preparations were concentrated and purified by ultracentrifugation through sucrose. I therefore tested what would happen if unpurified virus was used. The same stock of viral supernatant was used for stimulation of the cells and the only difference was the ultracentrifugation step through sucrose was omitted. These viral stocks were also titrated at the same time in order to better normalise for viral content added to the cells. When MDM were stimulated with the purified virus no detectable innate immune responses were observed as before, but when the unpurified virus was added, MDM expressed both IP-10 and CCL2 in response to HIV-1 (Figure 24)

Figure 24 - Non sucrose purified HIV-1 preparations do generate innate immune responses in MDM.



MDM were challenged with HIV-1 (MOI3), which had or had not undergone sucrose purification. After 24 hours RT-qPCR was performed to look at the relative expression of IP-10 or CCL2 in response to stimulation. Expression of both IP10 and CCL2 was evident in MDM stimulated with unpurified HIV-1 but not virus which had undergone purification.

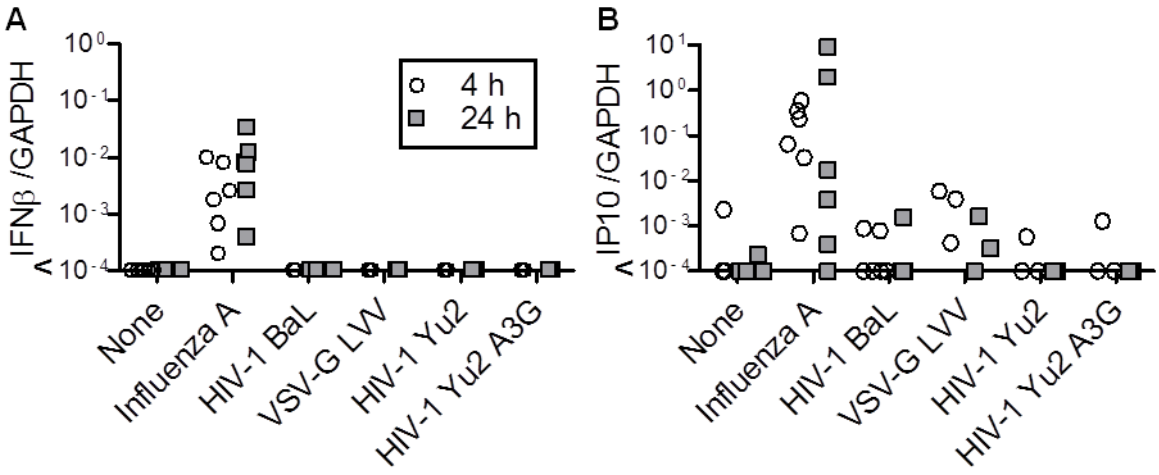
4.3.2.6 Why does HIV-1 not activate the innate immune response in MDM?

HIV-1 contains amongst other things ssRNA which is a potential ligand for TLR7 and 8. The fact that MDM can respond to the agonists gardiquimod (Stimulates TLR7, but can stimulate TLR8 at high concentrations) and CL075 (Stimulates TLR7 and TLR8, though higher concentrations are required for TLR7 stimulation) as well as influenza virus suggests that the TLR pathways for detecting viruses is functional in these cells. I first considered the possibility that HIV-1 did not invoke an innate immune response because its RNA genome was reverse transcribed rapidly on entry into MDM and therefore not accessible by RNA sensors. In order to test this hypothesis, I used a Vif deficient virus, which incorporates human APOBEC3G into the virion leading to deamination of the viral RNA and inhibits reverse transcription (Bishop, Verma et al. 2008). This Vif deficient virus, produced in cells which have been transfected with a plasmid expressing APOBEC3G (HIV-1 Yu2 A3G), also did not induce an immune response (Figure 25). It is also possible that one or more of the HIV-1 accessory proteins could be suppressing the response to the viral RNA. HIV-1 infection has been reported to inhibit innate immune responses via degradation of endogenous IRF3 (Doehle, Hladik et al. 2009) by the accessory proteins Vif and Vpr (Okumura, Alce et al. 2008). To test this hypothesis a minimal HIV-1 based lentiviral vector (Zufferey, Nagy et al. 1997) (produced using the packaging plasmid pCMVΔR8.91 and pHRSIN-CSGW which expresses GFP) pseudotyped with the VSV-G envelope was used (VSVG-LVV). The produced virus does not contain the accessory proteins of HIV-1 but does contain ssRNA as well as the gag-pol products which form the viral particle and the enzymes required for reverse transcription and integration. This virus also did not induce expression of either IFNβ or IP-10 mRNAs (Figure 25). These results suggest that in this model, HIV-1 is not actively suppressing the response and that availability of the RNA is not the factor preventing detection.

4.3.2.7 Is IFN priming of MDM required for detection of HIV-1?

Type I IFN priming has been reported to enable immune responses to corona viruses (Kuri, Zhang et al. 2009) and enhance immune responses to influenza (Phipps-Yonas, Seto et al. 2008). I therefore tested the hypothesis that IFN priming of MDM may upregulate PRRs that increase the capacity of the cell to detect HIV-1. Analysis of the genome wide transcriptional responses to IFNβ revealed upregulation of number of PRRs in MDM including TLR8 and the RLRs (Figure 26A). Importantly IFNβ gene expression is not induced by itself, so qPCR was used to test if IFNβ primed macrophages can produce IFNβ in response to HIV-1. The increased induction of IFNβ message in response to polyI:C is consistent with the priming effect of upregulating the expression of TLR3 and RIG-I, however HIV-1 still did not induce an IFN response in these experiments (Figure 26B).

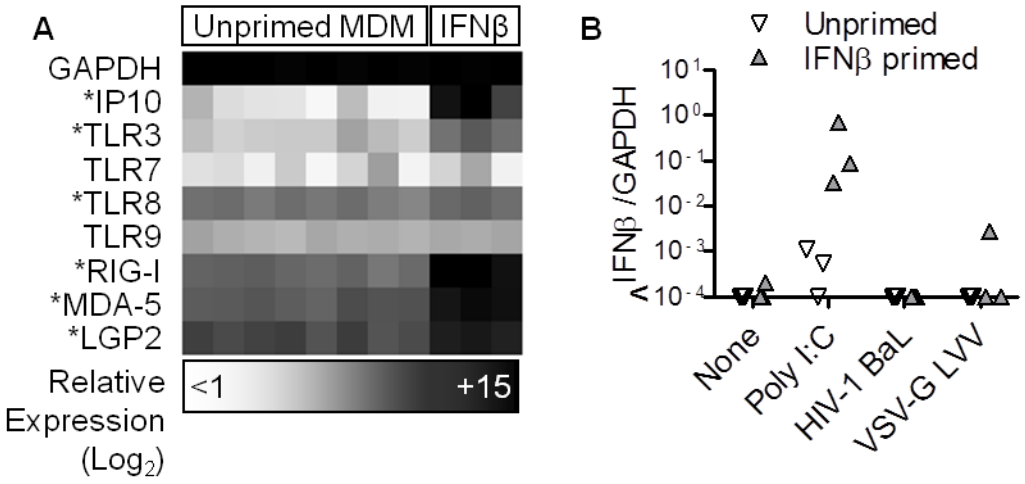
Figure 25 - IFN gene expression in MDM exposed to Influenza A and a variety of HIV-1 virions.



MDM were stimulated with Influenza A or a range of HIV-1 virions for 4 and 24 hours. RT-qPCR was performed to look at the relative expression of IFNβ **(A)** or IP-10 **(B)** (to GAPDH) in response to the stimuli. HIV-1 BaL as well as minimal lentiviral vectors (VSV-G LVV) and HIV-1 which had incorporated APOBEC3G into the virion (HIV-1 Yu2 A3G) did not induce expression of IFNβ or IP-10 in contrast to Influenza.

Data points are from separate donors/experiments.

Figure 26 - IFN Priming of MDM upregulates expression of some PRR does not enable MDM innate immune responses to HIV-1



MDM were primed with (500IU/ml) IFNβ for 24hours. **(A)** Whole genome transcriptional microarray analysis of eight unstimulated and three IFN primed MDM shows upregulaton of a set of PRRs in response to IFNβ (marked with an *). The IFN responsive gene IP-10, was included as a positive control to show priming had took place. **(B)** MDM were stimulated with a range of stimuli after priming with IFNβ, and relative expression of IFNB was measured. (Data is from three separate donors). Priming did not enable MDM to express IFNβ in response to HIV-1, in comparison to Poly I:C stimulation where priming enhanced the response.

4.3.4 What is the PRR for HIV-1?

4.3.4.1 pDCs and monocytes innate immune responses to HIV-1

I have shown that macrophages do not mount innate immune IFN responses to HIV-1. Consequently, they are not likely to be the source of type I IFN production reported during primary HIV viraemia (Stacey, Norris et al. 2009). It is therefore of interest to see what the differences are between MDM and cells which are able to mount IFN responses to HIV-1. By comparing the expression of PRRs between the cell types I may be able to deduce why MDM do not respond to HIV-1.

I sought to interrogate the expression of candidate PRRs and IFN responses to HIV-1 in defined PBMC populations. Of these populations, pDCs are thought to be the major producer of type I IFNs in response to HIV-1. In addition, pDCs (Meier, Chang et al. 2009) and monocytes (Simmons, Scully et al. 2013) have both been reported to generate type I IFN responses upon challenge with HIV-1, therefore I focussed on these cells particularly.

The Miltenyi MACS system was used to separate monocytes and pDCs from the PBMC mixture, before testing the abilities of each cell type to respond to HIV-1 infection by producing IFN. This is summarised in Figure 27.

As expected pDCs were able to mount an IFN response when stimulated with the control ligand CpG as well as HIV-1 (Figure 28). Monocytes were also able to mount an IFN response to HIV-1 despite the lack of response seen in MDM (Figure 28).

Figure 27 - Isolation of different cell types from PBMCs

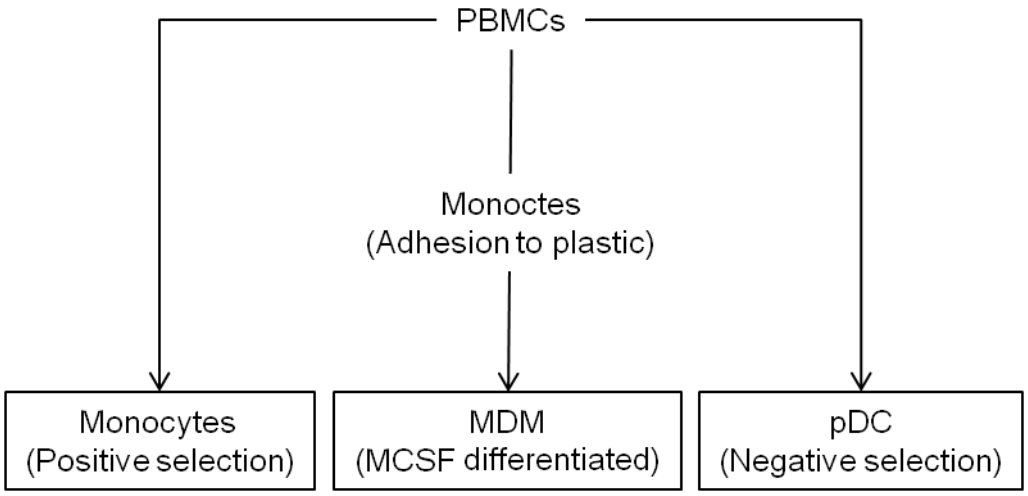
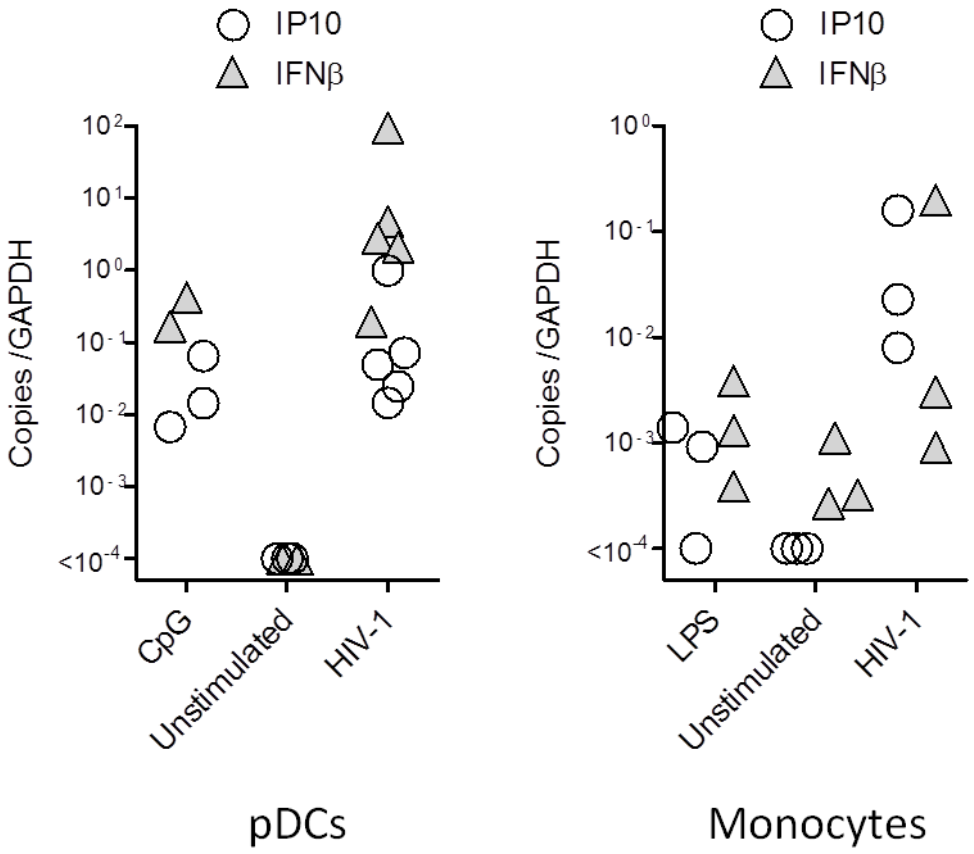


Diagram showing how the three cell types were isolated from PBMC. Adhesion to plastic was used to isolate monocytes, which were then differentiated into MDM in culture by the addition of M-CSF. The Miltenyi magnetic bead system was used to isolate monocytes by CD14 positive selecton. The Miltenyi magnetic bead system was used to negatively select for pDCs using the Plasmacytoid Dendritic Cell Isolation Kit.

Figure 28 - pDC and monocyte IFN responses to HIV-1



pDCs were isolated from PBMCs (or PBMCs which had been depleted of monocytes by adhesion to plastic for one hour) using the Miltenyi Plasmacytoid Dendritic Cell Isolation Kit, which purified by negative selection. Monocytes were isolated from PBMCs by the Miltenyi CD14 positive selection kit. Isolated cells were stimulated with CpG or LPS as a positive control or HIV-1 BaL. Four hours after stimulation RT-qPCR performed to look at the relative expression levels of IFNβ and IP-10. Both cell types were able to produce IFNβ and IP-10 mRNA in response to HIV-1.

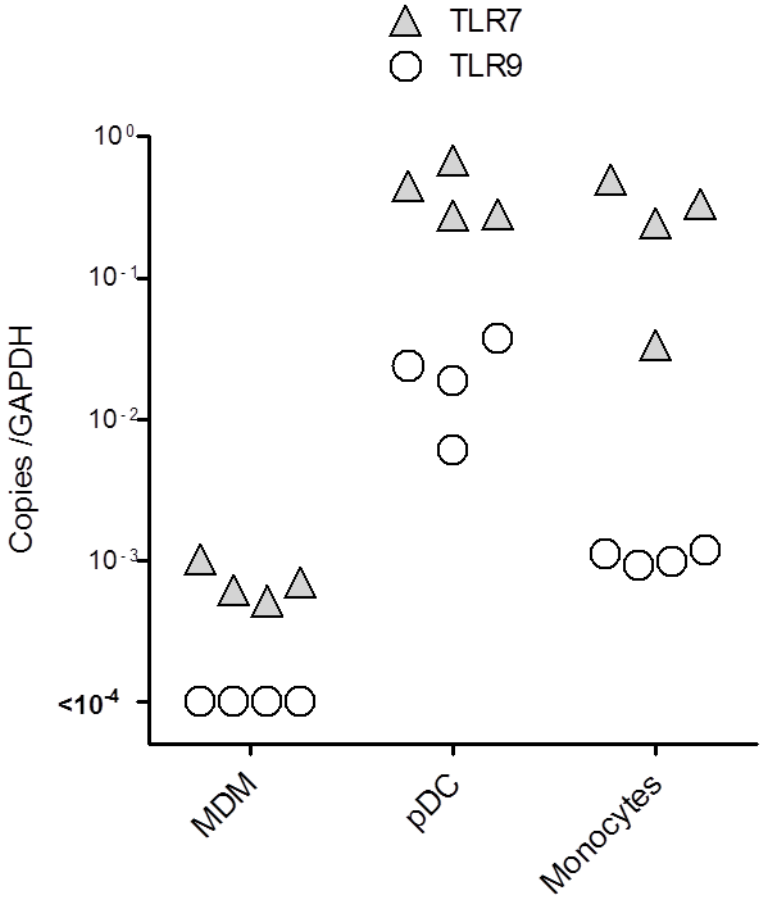
Each symbol represents an individual experiment/donor.

4.3.4.2 Differential expression of TLR7 and TLR9 in cells that do and do not respond to HIV-1

One of the major differences between MDM which do not response to HIV-1 and pDCs which do is the expression levels of some TLRs. pDCs are known for their high constitutive expression of TLR7 and 9, which are PRRs for ssRNA and unmethylated CpG DNA respectively. Either of these TLRs could potentially interact with HIV-1. The ssRNA viral genome can directly be recognised by TLR7, or the DNA intermediates formed after reverse transcription could be recognised by TLR9. The HIV-1 genome is relatively sparse in CpG motifs, and those that are found are present at the 5' and 3' LTRs (Krieg 1996) where they play and important in regulation of viral gene transcription (Gutekunst, Kashanchi et al. 1993).

I compared TLR7 and 9 expression in MDM, monocytes and pDCs using RT-qPCR. pDCs as expected had high levels of expression of both TLR receptors with over 100 fold more expression of both TLR7 and TLR9 compared to MDM (Figure 29). Monocytes, the other cell type I have shown able to mount innate immune responses to HIV-1, had similar levels of TLR7 expression as pDCs, and though they also expressed more TLR9 compared to MDM, the expression levels were much lower than those seen in pDCs (Figure 29).

Figure 29 - Differential expression of TLR7 and TLR9 in cells that do and do not respond to HIV-1



RT-qPCR was performed on RNA extracted from a selection of cell types looking at relative expression levels of the potential HIV-1 recrptors TLR 7 and TLR9. Monocytes and pDCs which can mount innate immune IFN responses to HIV-1 have higher expression of both TLR7 and TLR9 compared to MDM

4.3.5 IFN restriction of HIV-1 in MDM

4.3.5.1 Stimulation of MDM with IFN restricts HIV-1

Interferons were originally named because of their ability to interfere with viral replication (Isaacs and Lindenmann 1957; Isaacs, Lindenmann et al. 1957). The importance of the IFN response against viruses can be seen by the number of viruses which have evolved to evade or to suppress the IFN response in order to successfully infect their hosts.

Primary HIV-1 infection has been shown to invoke a systemic type I IFN response and this was clearly documented in a recent study (Stacey, Norris et al. 2009). Their data shows the IFN response beginning a number of days after measurable levels of HIV-1 is detected in serum samples. The data from my previous experiments show that macrophages are unlikely to be the cells producing this IFN (Section 4.2.2), but given that other sources of type I IFN responses to HIV-1 exist, I sought to test the effect of IFN stimulation on HIV-1 infection of MDM.

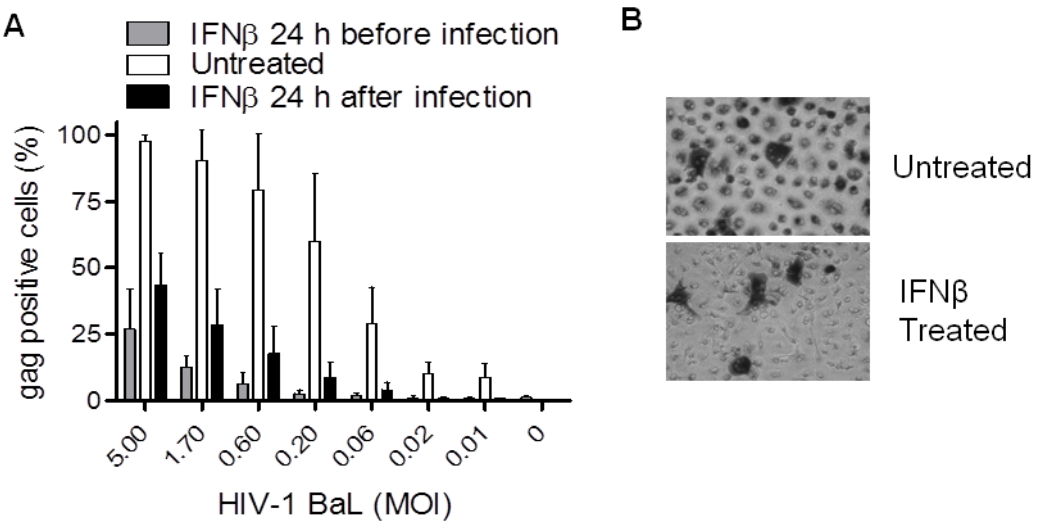
To test the effects of IFN on HIV-1 infection in our MDM model I treated MDM with either the type I IFN, IFNβ or the type II IFN, IFNγ. Antiviral assays for both of these proteins have ED_{50} at concentrations <1ng/ml (according to the manufacturers). I stimulated our MDM with 500U/ml (2ng/ml) of IFNβ or for IFNγ I used a dose of 20U/ml (1ng/ml) in order to test the effects of IFN restriction on HIV-1.

I tested the effects of IFNβ in our model of a spreading infection. IFNβ was added to MDM either 24 hours before or 24 hours after inoculation with HIV-1. In both cases a significant reduction in the number of HIV-1 infected cells were seen seven days after exposure to HIV-1 as determined by intracellular gag staining (Figure 30A). Pre-treatment with IFN leads to a slightly higher reduction in the number of infected cells, compared to addition of IFN 24 hours after HIV-1 infection.

The data from Stacey et al (Stacey, Norris et al. 2009) showed that initially as viral titres start to rise so do levels of type I IFNs. However IFNα levels peak and then decline to basal levels after a couple of weeks. IFNγ however, is induced later, and levels of which continue to increase beyond the timecourse of the data presented. Therefore I also tested the effects IFNγ stimulation had on HIV-1 infection of MDM. I found that the type II IFN, IFNγ, also restricted HIV-1 in MDM (Figure 31A).

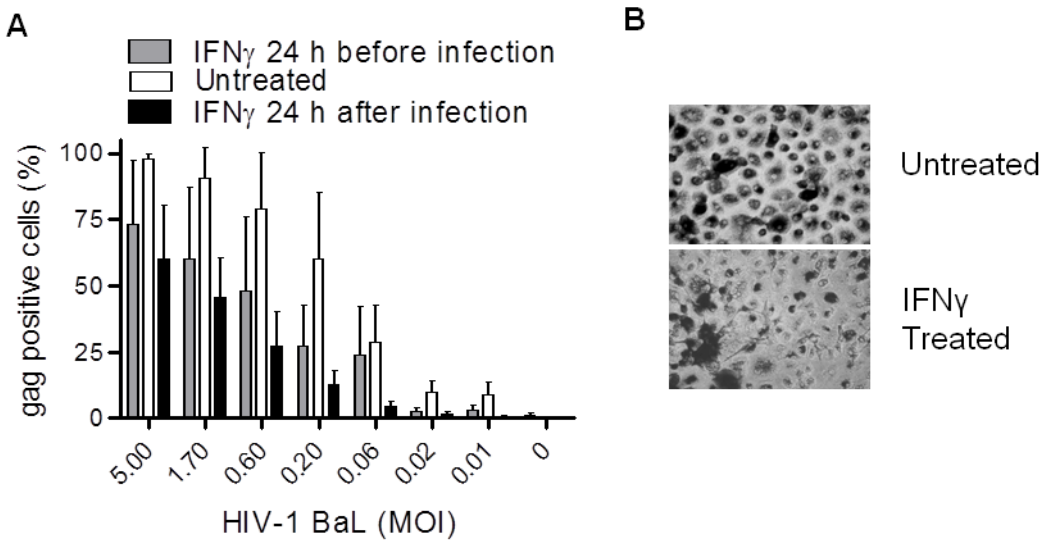
IFNs are known to induce apoptosis in stimulated cells. To confirm that this was true HIV-1 restriction and not just the proapoptotic effects of IFN killing cells indiscriminately the integrity of the cells was observed. Lack of gross cytopathic effects in IFN treated cells was verified by light microscopy after intracellular gag staining (Figure 30B and Figure 31B). Further confirmation was obtained by measuring the RNA integrity scores of MDM stimulated with the two IFNs, with no significant differences attributed to IFN stimulation.

Figure 30 - IFNβ treatment of MDM restricts HIV-1 infection



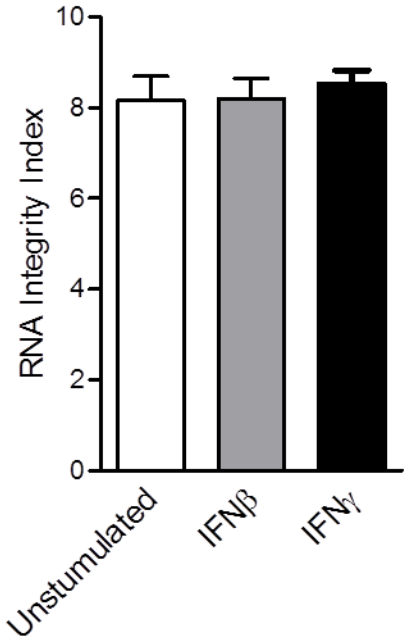
MDM were treated with 500U/ml of IFNβ for a period of 24h hours either 24 hours before or 24 hours after infection with HIV-1. **(A)** Seven days after infection, cells were stained for intracellular gag and the percentage of infected cells in the culture were measured over a range of MOI. **(B)** The reduction of HIV-1 gag positive cells in IFNβ stimulated cultures was not due to the proapoptotic effects of IFN as seen in the light microscopy image

Figure 31 - IFNγ treatment of MDM restricts HIV-1 infection



MDM were treated with 20U/ml of IFNγ for a period of 24 hours, either 24 hours before or 24 hours after infection with HIV-1. **(A)** After seven days of infection, cells were stained for intracellular gag and the percentage of infected cells in the culture were measured over a range of MOI. **(B)** The reduction of HIV-1 gag positive cells was not due to proapoptotic effects of IFN as seen in the light microscopy image

Figure 32 - MDM RNA integrity scores are not affected by IFN stimulation



MDM were stimulated with either 500U/ml of IFNβ or 20U/ml of IFNγ before RNA was extracted and run on a RNa bioanalyser. RNA integrity scores are used as a surrogate for cell viability. RNA integrity scores were not significantly affected by IFN stimulation.

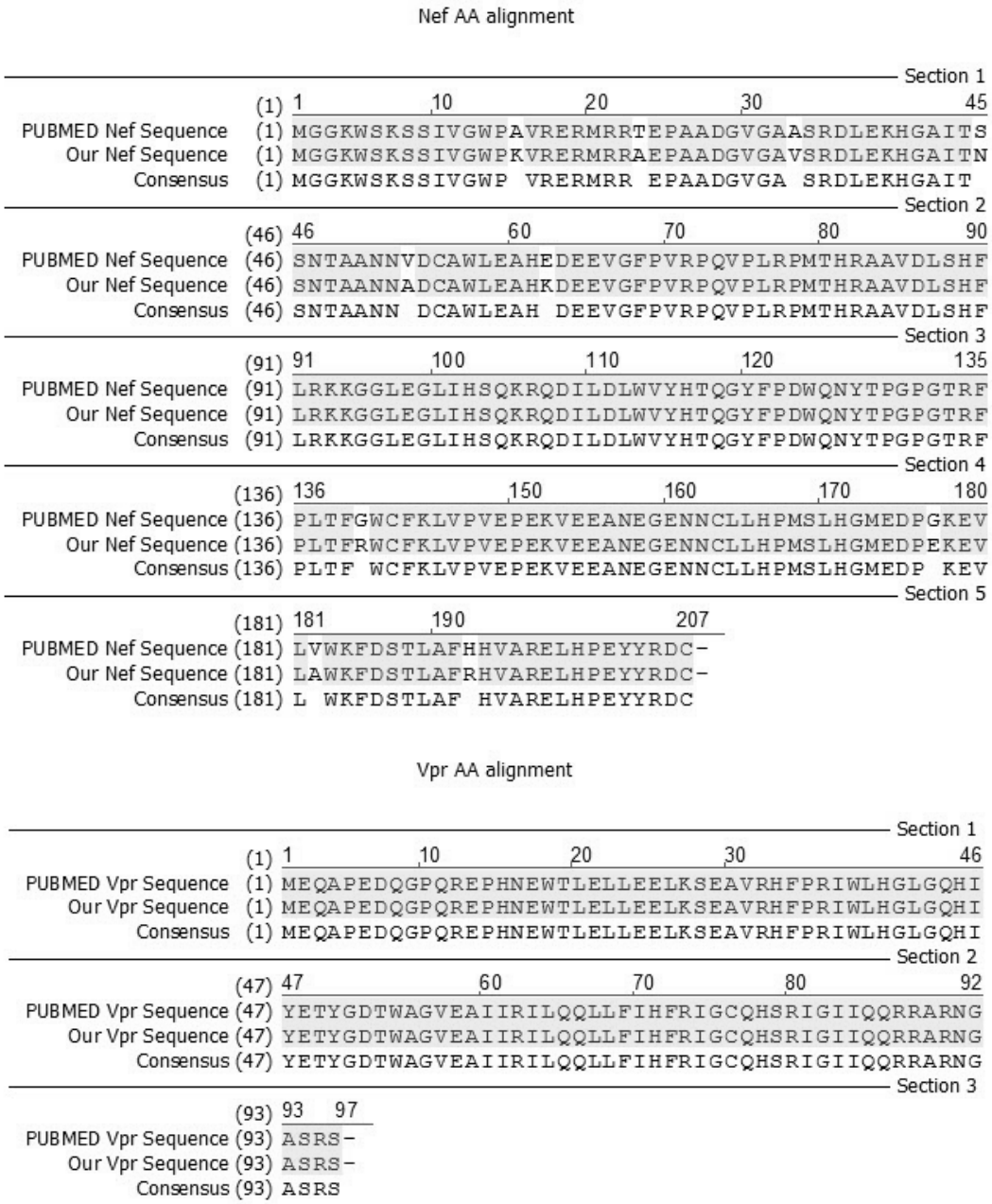
4.3.5.2 HIV-1 BaL contains full length accessory proteins and restriction of HIV-1 is not strain specific.

Because HIV-1 BaL is a laboratory strain, which is propagated by passaging through human cells, I verified if this strain contained functional accessory genes which are known to be involved in evasion of restriction (Kirchhoff 2010). RNA was harvested from MDM which had been infected with HIV-1 BaL, and RT-PCR was performed to confirm the presence of selected HIV-1 genes, Nef, Vpr and Vpu mRNA sequences.

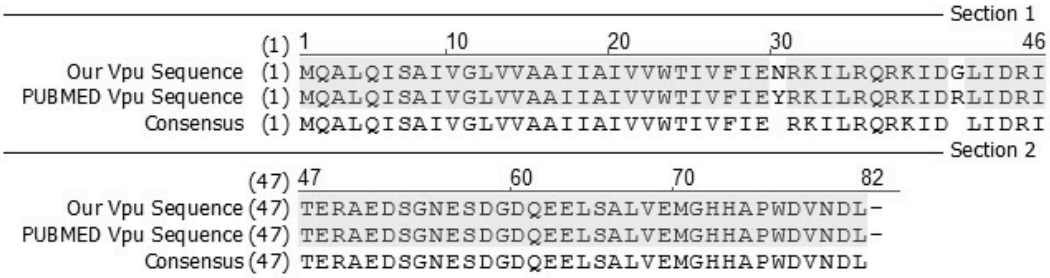
PCR products were then subcloned into pGEMT and an individual clone sent off for sequencing. As expected, there was a high degree of nucleotide mutation compared to the sequences from PUBMED clone (AB221005), however when analysed at the amino acid level there was still a high level of consensus between the accessory proteins in the HIV-1 viruses used in this study and the previously published sequences (Figure 33). Importantly none of the sequences contained a premature stop codon. Indeed the amino acid sequence for Vpu, was identical to the published data. This analysis shows that the HIV-1 BaL I used in these experiments, likely contain full length accessory proteins, however the functionality of the mutated proteins were not assessed.

To further validate these findings I show that the IFN restriction seen is not due to the specific laboratory strain of virus used and tested a variety of HIV-1 strains including common laboratory strains of HIV-1 generated from plasmid clones, which contain accessory proteins with known functionality, as well as some clinical isolates. All strains of HIV-1 tested, including a primary isolate (MM25-2) derived from a clinical blood sample from a patient with primary HIV-1infection (Aasa-Chapman, Aubin et al. 2006), were sensitive to restriction by IFNβ in MDM (Figure 34).

Figure 33 - HIV-1 BaL Accessory Protein Amino Acid Sequences

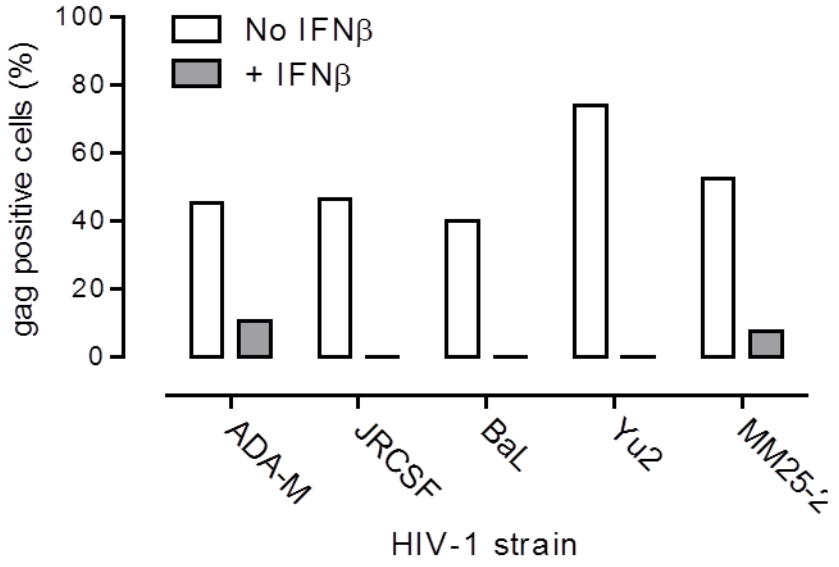


Vpu AA alignment



RT-PCR was performed on RNA extracted from MDM which had been infected with HIV-1 BaL. The cDNA for the accessory proteins Nef, Vpu and Vpr was sequenced and compared to a PUBMED HIV-1 BaL clone (AB221005). Amino acid sequence of the accessory gene shows full length protein with some substitutions compared to the published sequence, with the exception of Vpr for which had 100% consensus.

Figure 34 - IFN restricts a range of HIV-1 strains.

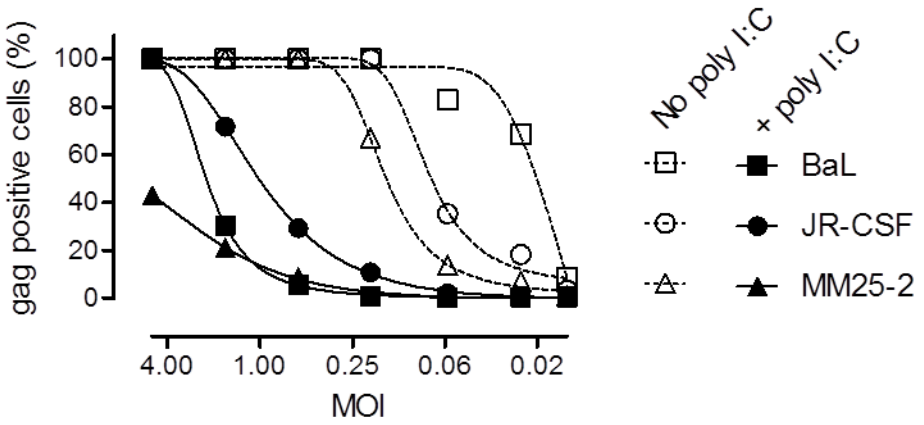


MDM were stimulated with 500U/ml of IFNβ for a period of 24 hours before infection with a variety of strains of HIV-1, including a primary clinical isolate, MM25-2. Seven days after infection cells were stained for intracellular gag and the percentage of infected cells were measured.

4.3.5.3 Stimulation of a endogenous IFN response restricts HIV-1 infection

I have shown that addition of exogenous IFN is able to restrict HIV-1 in this MDM model. It is possible that the doses of IFN used could be in excess of the amount the MDM are capable of producing themselves, so I examined the ability of an endogenous IFN response to restrict HIV-1. I did this by stimulating the MDM with poly I:C, at the same time as infection with HIV-1. My previous data showed stimulation by poly I:C induced moderate IFN responses (compared to stimulation with Influenza A) (Figure 19). Again a strong restriction of HIV-1 could be seen when an endogenous IFN response was induced by addition of poly I:C at the time of infection (Figure 35).

Figure 35 - An endogenous IFN response is able to restrict HIV-1 in MDM



MDM were infected with HIV-1 at the same time as receiving stimulation by poly I:C to induce an endogenous IFN response. After seven days of infection cells were stained for intracellular gag and the percentage of infected cells in the culture were measured over a range of MOI. Cells which received stimulation with poly I:C at the same time as infection with HIV-1 (Black symbols) showed lower levels of HIV-1 infected cells compared to MDM which did not receive poly I:C (White symbols).

4.3.5.4 Long term HIV-1 restriction by IFN and effects of IFN on established infection

So far I have shown that stimulation of MDM with IFN before or shortly after exposure to virus can effectively restrict HIV-1 infection. However as MDM do not mount IFN responses themselves, it is possible that in vivo, macrophages can be infected by HIV-1 well in advance of any IFN stimulation.

I therefore investigated the effects IFN stimulation has on an established HIV-1 infection and if IFN has any effect on ability of HIV-1 to replicate in these cells.

MDM were infected with HIV-1 and the infection allowed to spread for one week. MDM were then stimulated with IFNβ for 24hours and supernatant samples were harvested over a time course to follow the effects of IFN stimulation on HIV-1 replication. I found that IFNβ stimulation significantly reduced the p24 quantified in the supernatant of the cells, but this inhibition was only transient and the amount of p24 increased to baseline levels over 1 week (Figure 36).

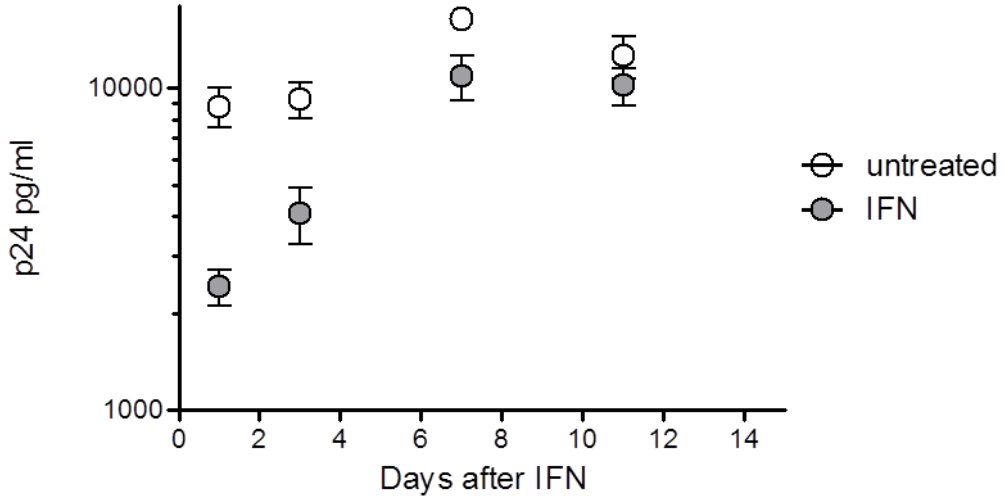
Having shown that IFN restricts the permissivity of MDM to HIV-1, I also investigated the duration of this effect. As macrophages are long lived cells previous exposure to IFN due a systemic IFN response to other pathogens could lead to HIV-1 encountering a cell which has previously been stimulated with IFN. Therefore it is of interest to see the long term effects IFN stimulation on MDM and whether the antiviral effect produced by IFN persists.

Day six or older MDM were exposed to 500U/ml of IFNβ for a period of 24 hours, before infection with HIV-1 at a later time. Figure 37 shows the experimental setup in more detail.

MDM become resistant to HIV-1 infection with time in culture which is why it was important to ensure that the age of the MDM were the same for all IFN stimulation time points. Despite the relative resistance of these “older” MDM to HIV-1 infection, HIV-1

restriction by IFN is still clearly evident. MDM treated with IFN 24 hours prior to HIV-1 infection show a significant decrease in the number of HIV-1 positive cells 1 week after infection. MDM which had been stimulated with IFNβ 1 and 3 weeks before exposure to HIV-1 still retained the ability to restrict HIV-1 though to a lesser extent the longer the time period between IFN stimulation and HIV-1 infection (Figure 38).

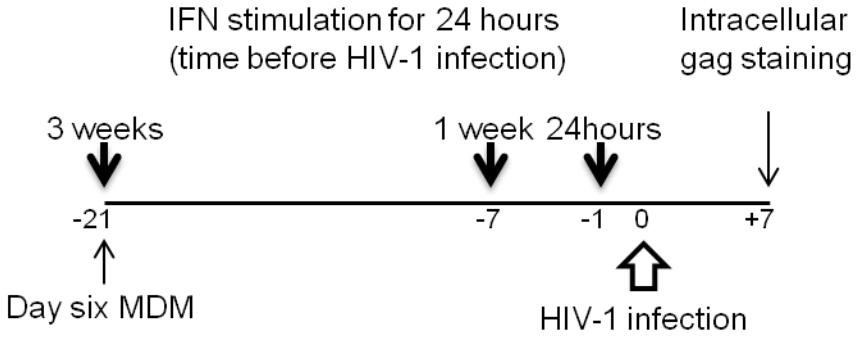
Figure 36 - Long term effects of IFN stimulation on established HIV-1 infection in MDM



MDM infected with HIV-1 BaL for seven days were stimulated with 500U/ml of IFNβ for a period of 24 hours. Supernatant was collected at various time points after IFN stimulation and p24 was detected by ELISA. IFN treated cells showed an initial decrease in p24 levels which recovered back to untreated levels around 11 days after IFN treatment

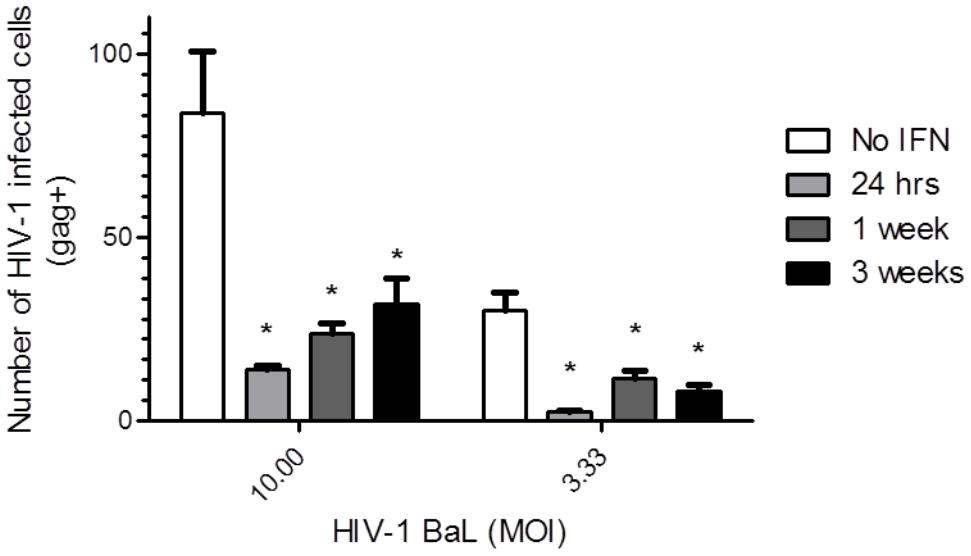
Datapoints represent mean±SD from three individual experiments/donors

Figure 37 - Experimental setup to investigate the long term effects IFN stimulation has on the ability of HIV-1 to infect and replicate.



MDM in culture are stimulated with IFNβ for a period of 24hours before infection with HIV-1 at a later time. All MDM are normalised for age in culture by varying the time of IFN stimulation. Intracellular gag staining is performed to detect HIV-1 infected cells seven days after infection with HIV-1.

Figure 38 - Long term restriction of HIV-1 in MDM exposed to IFN.



MDM were exposed to 500U/ml of IFNβ for a period of 24 hours, at varying timepoints after IFN exposure the MDM were infected with HIV-1 BaL. After seven days of infection, cells were stained for intracellular gag and the number of infected cells in the culture was measured over a range of MOI. Statistically significant (* $p<0.01$ ANOVA) reduction in the number of HIV-1 cells were seen in MDM treated with IFN irrespective of the time between stimulation and infection with HIV-1.

Bars represent mean values ±SD from three individual experiments/donors.

4.4 Discussion

I developed the nuclear translocation assay to measure innate immune activation in MDM by measuring nuclear translocation of transcription factors. The commonly used methods to measure NFκB activation, EMSA and Western blotting, utilise nuclear extracts. The main problems with using these assays are that it is difficult to quantitate and importantly for us requires a large amount of cells to obtain the nuclear extracts. Due to the nature of the MDM model used, where cells are derived from blood of healthy volunteers, there were not sufficient cell numbers to use this technique for our experiments.

I have shown that this confocal microscopy based assay for analysing transcription factor nuclear translocation compares favourably with an established NFκB reporter cell line, the differences between the assays could be attributed to the face that the reporter based assay measures reporter gene function which is expressed in response to NFκB nuclear translocation, and this could explain the imperfect regression analysis ($r^2 = 0.83$) between the two methods.

This confocal based assay for measuring NFκB activation and other nuclear translocating transcription factors allows for many experimental conditions to be performed on the limited number of cells obtained from blood of healthy volunteers, as well as being able to give quantitative measurements of activation. The analysis was later automated by using the software Metamorph, which also allowed for the analysis of NFκB status of individual cells, and will be especially useful for applications where MDM cultures are heterogeneously transduced. Until recently, by using virus like particles (VLPs) containing Vpx, it was very difficult to transduce more than 10-15% of a MDM population using HIV-1 based lentivectors, even so only about 70-80% of cells are transduced using Vpx VLPs. Coexpression of a reporter gene such as GFP which would allow for the software to segregate the cells based on GFP expression allowing for more reliable data from heterogeneously transduced populations. This would also

enable the distinction between the direct effect of transduction and bystander effects on cells which have not been transduced. This can be extended to cultures which are heterogeneous in HIV-1 infection status. This assay was used to study whether or not HIV-1 activates innate immune responses in MDM.

HIV-1 is a pathogen which is composed of multiple components which should trigger innate immune responses in cells containing the appropriate PRR. As macrophages are in essence a sentinel cell of the immune system, and acts as a link by detecting incoming pathogens and activating the rest of the immune system, I thought it should mount immune responses to incoming HIV-1. Though previous studies have shown that macrophages do mount immune responses to various components of HIV-1, from the envelope protein gp120 to the viral ssRNA, as well as to whole HIV-1 virions, the type and magnitude of responses vary with different responses produced in different studies.

The most commonly used surrogate for HIV-1 is the envelope protein gp120. HIV-1 gp120 has been shown to elicit a variety of innate immune responses. In monocytes and macrophages, gp120 and/or full length HIV-1 has been reported to induce innate immune responses but with different cytokines being produced in a variety of studies (Gessani, Puddu et al. 1994; Borghi, Fantuzzi et al. 1995; Canque, Rosenzwajg et al. 1996; Gessani, Borghi et al. 1997; Fantuzzi, Canini et al. 2001; Lee, Tomkowicz et al. 2005; Cheung, Ravyn et al. 2008; Fantuzzi, Spadaro et al. 2008). These differences could be due to how their macrophages are produced or possible contamination with other cell types. Also differences in the recombinant gp120 proteins could account for many of these findings. HIV-1 gp120 like many HIV-1 proteins can vary between viruses. Though the core structure is conserved, the variable loops which confer co-receptor specificity can be very different, as well as this, different strains of HIV-1 may also have different glycosylation of the gp120 protein (Poignard, Saphire et al. 2001). Because of this gp120 from different sources (Kong, Wilson et al. 1996) and even the

same gp120 protein produced by different methods have been shown to induce different responses (Clouse, Cosentino et al. 1991).

Another commonly used surrogate for HIV-1 is synthetic ssRNA oligonucleotides based on the HIV-1 sequence. Innate immune responses to RNA from influenza virus have been shown in plasmacytoid dendritic cells (pDC) to be dependent on TLR7 and 8 and the MyD88 dependant pathway (Diebold, Kaisho et al. 2004) and pDCs have also been reported to secrete IFNα in response to HIV-1 ssRNA (Hornung, Guenthner-Biller et al. 2005). NK cells can be activated, in coculture with pDC or CD14+ monocytes, by ssRNA40 (Alter, Suscovich et al. 2007), a uridine-rich ssRNA derived from the HIV-1 long terminal repeat. In PBMCs, ssRNA sequences derived from HIV-1 have also been shown to activate NFκB and stimulate secretion of TNFα (Sioud 2005). Another study showed that the human macrophage like cell line THP-1 can also respond to ssRNA derived from HIV-1 by producing TNFα, but only after IFNγ priming (Gantier, Tong et al. 2008). Less has been published about human macrophage responses to ssRNA. One study reports expression of IFNβ, IP-10 and IL-1β in MDM after stimulation with a specific HIV-1 RNA sequence that generates secondary structures (HIV-1 Tar). However the levels of gene expression were significantly lower than that seen when MDM were exposed to poly I:C, especially for IFNβ where poly I:C induced over 2000 times more expression compared to HIV-1 RNA (Berg, Melchjorsen et al. 2012).

From our previous experiments I have shown that HIV-1 is able to infect entire cultures of MDM and viral replication in these cells occurs at a high rate (Chapter 3). If these cells mounted immune responses I could expect lower levels of viral replication with time as the antiviral effect of IFN takes effect on the infected cells, or in fact some cells could resist infection altogether. Contradicting many of the published data, when I stimulated MDM with HIV-1 of different strains and sources I could find no evidence of any immune response to incoming HIV-1, even when exposed to very high MOI's. There was no activation of the major transcription factors involved in immune

responses, NFκB and IRF3, or induction of IFNβ or ISG transcription. Furthermore no innate immune activation profile could be seen on whole genome transcriptional microarrays.

There is evidence that M-CSF differentiated macrophages may be less inflammatory than MDM differentiated with GM-CSF (Verreck, de Boer et al. 2004; Fleetwood, Lawrence et al. 2007; Lacey, Achuthan et al. 2012). I therefore tested the hypothesis that it may be a function of how the MDM were differentiated causing them to be unresponsive to stimulation by HIV-1. Alternatively differentiated MDM also failed to mount innate immune responses to HIV-1 while retaining similar responsiveness to influenza A virus.

As much of the published data utilises parts of HIV-1 rather than full length replicative virus I wondered whether there may be either active suppression of the immune response by viral factors, or simply lack of accessibility of viral PAMPs such as ssRNA which may be reverse transcribed before it can be detected by PRRs. However neither Vif deficient viral particles in which viral reverse transcription is deficient (Guo, Cen et al. 2006; Iwatani, Chan et al. 2007; Bishop, Verma et al. 2008) or minimal HIV-1 based vectors lacking the accessory proteins activated an immune response. These data could have been improved by the addition of HIV-1 RT mutants which do not reverse transcribe. Though I had used a Vif deficient mutant which should be susceptible to APOBEC3G inhibition of reverse transcription, its binding to the HIV-1 genome may interfere with PRR interacting with ssRNA.

The HIV-1 accessory genes have many independent functions, including interfering with immune activation. Vpu and Vpr have both been shown to inhibit NFκB activation. Vpu suppresses NFκB activation by preventing the degradation of inhibitor IκBα (Bour, Perrin et al. 2001) whereas Vpr induces expression of IκB (Ayyavoo, Mahboubi et al. 1997). Nef too has been shown to inhibit NFκB responses, by inhibiting

phosphorylation of IκB (Lee, Park et al. 2005; Qiao, He et al. 2006). Therefore I tested minimal HIV-1 based vectors lacking the accessory proteins for their ability to activate immune responses, but none were detected. I also tested the hypothesis that the macrophages needed to be primed first, in order to respond to incoming virus. I saw that IFNβ stimulation of MDM did upregulate expression of some PRRs. Despite upregulation of a number of receptors involved in RNA sensing including the RLRs, TLR3 and TLR8, I could not detect any innate immune response to the virus after priming the MDM with IFNβ.

Interestingly when I infected MDM with HIV-1 without prior purification of the viral preparation by ultracentrifugation through sucrose, I did see immune responses in our MDM. This could explain also explain some of the literature showing MDM immune responses to HIV-1. This however doesn't account for how purified recombinant gp120 has been shown to induce immune responses. This could be due to different glycoslyation of gp120 by the producer cells compared to human cells. Alternatively the lack of response by whole viruses could be due to conformational differences of soluble monomeric gp120 compared to the trimeric form found on viral particles, or because of differences of how gp120 is presented to innate immune cells when it is part of the viral envelope.

At the time these experiments were performed, the PRR for HIV-1 was thought to be a RNA sensor. The lack of innate immune response by MDM suggested that they may lack the PRR for HIV-1. Though IFNβ priming of MDM led to increased expression of some PRRs involved with sensing RNA, the expression of others like TLR 7 remained low and the lack of TLR7 expression could be the reason why HIV-1 can infect these cells without triggering an immune response. The fact that pDCs can mount immune responses to HIV-1 and their constitutively high expression of TLR7 only reinforced this hypothesis. The finding that monocytes can mount immune responses against HIV-1 was surprising considering the lack of response from MDM. The high levels of TLR7

expression in monocytes further added to the evidence that TLR7 could be the PRR for HIV-1.

Following from these findings I sought to knockdown TLR7 expression using siRNA LVV. This approach was unsuccessful so I attempted to exogenously express TLR7 instead, in order to test if this enabled cells to respond to HIV-1. However I could not obtain functional expression of TLR7. A publication released after these experiments reported that CD14 is an important co-receptor for the endosomal TLRs and that mouse macrophages lacking CD14 secrete less type I IFN in response to viral stimulation (Baumann, Aspalter et al. 2010). The reduction in CD14 levels I see as monocytes differentiate into macrophages (Figure 3) could be linked to the decreased TLR7 levels in MDM compared to monocytes. Interestingly a commercial TLR reporter cell line THP-1–Xblue-CD14 (Invivogen) which all of the TLRs also fails to respond to TLR7 or TLR9 stimulation, however when CD14 is overexpressed in these cells they can then mount a modest response to the TLR7 ligand immiquimod (http://www.invivogen.com/PDF/THP1_XBlue_CD14_TDS.pdf). This suggests that perhaps the TLR7 and TLR9 pathways are either not fully functional in myeloid cells or that activation of TLR7 and TLR9 may require additional stimuli or adaptor proteins for signalling which is not required in TLR8 signalling.

Suppression of host cellular IFN responses is important for the successful infection and replication for many viruses. The importance of the IFN response against viruses can be seen by the number of viruses which have evolved to evade or to suppress the IFN response in order to successfully infect their hosts.

Flaviviruses utilise a number of different mechanisms in order to evade the host IFN response. This family of viruses has been shown to inhibit type I IFN signalling via disruption of STAT1 phosphorylation by the viral non structural proteins (Munoz-Jordan, Sanchez-Burgos et al. 2003; Lin, Liao et al. 2004; Munoz-Jordan, Laurent-

Rolle et al. 2005). Additionally West Nile Virus downregulates expression of STAT1, and inhibits phosphorylation of STAT2 as well (Liu, Wang et al. 2005). Dengue virus has also been shown to inhibit phosphorylation of STAT2 as well as targeting the protein for degradation (Mazzon, Jones et al. 2009).

Adenoviruses have also been shown to inhibit the formation of the ISGF3 transcription factor complex (Kalvakolanu, Bandyopadhyay et al. 1991). Poxviruses have been shown to encode for soluble receptor homologues for IFN thus preventing binding of IFN to its cognate cellular receptors (Upton, Mossman et al. 1992; Colamonici, Domanski et al. 1995; Symons, Alcami et al. 1995).

As well as inhibiting cellular responses to IFN stimulation, some viruses directly inhibit activation of the IFN response in the infected cell. Hepatitis C virus inhibits both type I and type II IFN signalling (Heim, Moradpour et al. 1999; Kim and Yoo 2010), and additionally has been shown to inhibit innate immune signalling through the PRR RIG-I and therefore preventing phosphorylation and activation of the transcription factor IRF3 (Breiman, Grandvaux et al. 2005). The NS1 proteins from influenza virus A and B also inhibit activation of IRF3, despite the low amino acid sequence similarity between them (Talon, Horvath et al. 2000; Donelan, Dauber et al. 2004; Haye, Burmakina et al. 2009).

The filoviruses Ebola and Marburg encode proteins which interfere with both IRF7 and IRF3. The virally encoded VP35 is a substrate for both IKK-ε and TBK-1 which are involved in phosphorylation and activation of the IRFs, and thus inhibits IFN production through these transcription factors by competition for their activating kinases (Basler and Amarasinghe 2009).

It was with some surprise that I found that HIV-1 does not trigger an IFN response at all in MDM despite all the potential PAMPS found in an RNA virus as well as the high levels of viral replication within these cells.

There have been a few clinical trials using IFN to treat HIV-1. Most of which have focussed on plasma levels of virus and CD4 cell counts, with many of the early studies finding that IFN treatment had no effect on HIV-1 (Sperber, Gocke et al. 1993; Katabira, Sewankambo et al. 1998) or a slight but transient increase in CD4 counts (Kaiser, Jaeger et al. 1992). The doses of IFN used in these studies however were low, with as little as 200IU of IFN administered daily. One study which used higher doses of IFN reported that IFN treatment in some patients cleared HIV-1 from PBMCs and that treatment also prevented onset of AIDS. However it was also noted that all patients treated with IFN developed side effects including flu like symptoms, granulocytopenia and elevated liver enzyme levels, with 35% withdrawing from the study (Lane, Davey et al. 1990).

The general consensus is that type I IFN does reduce viral loads and is efficient at restricting HIV-1 infection (Haas, Lavelle et al. 2000; Hatzakis, Gargalianos et al. 2001; Aguilar Marucco, Veronese et al. 2007; Asmuth, Murphy et al. 2010; Tavel, Huang et al. 2010).

However increased IFN levels can be seen in patients progressing to AIDS (Haas, Lavelle et al. 2000; Stylianou, Aukrust et al. 2000), with HIV-1 induced immune activation implicated. There have also been two clinical studies looking at the effects of vaccination against IFNα as a treatment for HIV-1 (Gringeri, Santagostino et al. 1996; Gringeri, Musicco et al. 1999). These studies found that patients who developed antibodies against IFNα after vaccination were less likely to have disease progression compared to non vaccinated or vaccinated patients which did not develop anti IFNα antibodies. These results from these clinical trials are in opposition to the others which show IFN treatment is beneficial and could be explained by the methods used. After initial vaccination against IFNα, patients were given monthly boosters. The adjuvant that was used in this study has been shown to activate inflammatory immune responses (Goto, Kato et al. 1997). Though vaccination produced antibodies against

IFNα I know that many type I IFNs exist and that the antibodies produced may in fact only be against a small fraction of the total IFN produced. Therefore each successive booster given to the patients may essentially been a dose of IFN through induction of the immune response.

In this study I looked at the effect exogenous IFN has on the ability of HIV-1 to infect and replicate within these cells. I found that despite containing full length accessory proteins (Figure 33), which have been demonstrated to be important in counteracting many of the restriction factors induced by IFN, exogenous stimulation of MDM with IFN still significantly restricts HIV-1 in MDM (Figure 34).

Stimulation of MDM with IFNβ before exposure to HIV-1 resulted in greater restriction compared to when MDM were stimulated after exposure to HIV-1. This was as expected as some of the antiviral effects of IFN act on the early part of the viral life cycle before integration and viral replication which likely would have occurred within 24 hours. IFNγ stimulation may possibly be more effective at restricting HIV-1 when done 24 hours after infection rather than before. This is surprising as others have demonstrated that the antiviral activity of IFNγ is delayed compared to the type I IFNs (Sim and Cerruti 1987) which would suggest that IFN stimulation before viral infection should be more efficient at restricting, though this was performed in a mouse model. Another publication reported that both type I and type II IFNs restrict virus, with IFN stimulation before infection restricting more potently (Mikloska and Cunningham 2001). However this study also reported that the antiviral effects were functional only within a short time frame of 6 hours before to 12 hours after viral exposure. The differences between these studies and the results I have obtained may be due to different effects of IFN on different cell types as well as the specific virus restricted. The higher levels of HIV-1 restriction I see when IFNγ stimulation of MDM is performed 24 hours after HIV-1 exposure may be due to IFNγ induction of autophagy (as discussed in section 1.2.3). Autophagic activity may peak at an early time point (Periyasamy-Thandavan,

Jiang et al. 2008), with little activity occurring upon infection with HIV-1. So in MDM stimulated with IFNγ 24hours before HIV-1 exposure, autophagy induction may peak too early , whereas when stimulated 24 hours after HIV-1 exposure, though the induction of other antiviral effects may be delayed and be less able to restrict the initial infecting virus, induction of autophagy may overcome this and help clear intracellular HIV-1.

I showed that despite the fact that the HIV-1 BaL used in our experiments likely contains full length accessory proteins, which have been reported to counteract IFN inducible restriction factors, IFN still potently restricts this virus. However not all of the HIV-1 proteins were sequenced and also due to the nature of the method used for cloning and sequencing the accessory proteins, this may not be representative of the HIV-1 population. I therefore confirmed these findings by expanding on the strains of HIV-1 tested, including strains obtained from plasmid clones with known functionality of the accessory proteins as well as a primary clinical strain (Figure 34).

The data from Stacey et al 2009, suggest that the IFN response to HIV-1 may not occur until a number of days post infection. I therefore investigated the effect IFN stimulation has on MDM with established HIV-1 infection, and found that although strong initial viral restriction occurs, viral replication rebounds and reaches normal levels within about two weeks.

There is also the possibility that macrophages may have been stimulated with IFN sometime before encountering HIV-1. Though there have been previous studies which have looked at the long term effects of type I IFN stimulation on HIV-1 infection and replication in MDM, they were based on repeated stimulations with IFN (Gendelman, Baca et al. 1990) which may not be the case as seen from the kinetic studies by Stacey et al, which shows a transient increase in IFNα which peaks around 6 days post infection and drops down back to near baseline levels after another week (Stacey,

Norris et al. 2009). I found that MDM exposed to IFN for a 24 hours period, even 3 weeks previous to HIV-1 infection still retained the ability to restrict HIV-1.

Recently a number of new PRRs have been discovered, and there is mounting evidence that HIV-1 may be detected by cytosolic DNA sensors. Trex1 is a 3' DNA exonuclease and degrades DNA found in the cytoplasm. HIV-1 reverse transcripts were found to accumulate in Trex1 negative mouse cells as well as human cells where Trex1 function was inhibited by RNAi (Yan, Regalado-Magdos et al. 2010). Furthermore cells lacking Trex1 function produced type I IFN in response to HIV-1, which was found to be mediated by the adaptor proteins TBK1 and STING, leading to activation of the transcription factor IRF3 (Yan, Regalado-Magdos et al. 2010). It has also been reported that accumulation of RT products in T cells leads to activation of pro-apoptotic and pro-inflammatory immune responses (Doitsh, Cavrois et al. 2010). Interestingly when Trex was knocked down with siRNA, it was found to enable MDM to mount IFN responses to HIV-1 but only one week after infection (Yan, Regalado-Magdos et al. 2010). The lack of an earlier response is likely due to the low dose of virus used (200ng of p24/ml) , the authors of the study state that even after 1 week of infection only 20% of the cells were infected with HIV-1 BaL at a dose of 200ng/ml p24 (Yan, Regalado-Magdos et al. 2010). Though the infectious titre of virus can vary for a set amount of p24, the MOI of virus used in the previous study is likely to be very low (One study reported that 50ng of p24 per 10^6 cells corresponded a MOI of approximately 0.003 (Neri, Giolo et al. 2011)), explaining the low infection rates 1 week after virus inoculation.

Other evidence that may support the theory for DNA sensing of HIV-1 was described with the discovery of the myeloid cell restriction factor SAMHD1. SAMHD1 is a dNTP triphosphatase which is active in myeloid cells reducing the levels of dNTPs, inhibiting reverse transcription by the virus. DCs are normally refractory to HIV-1 infection and replication. Addition of HIV-2/SIV Vpx to these cells disrupted SAMHD1 function by

targeting the protein for proteasomal degradation, allowing HIV-1 to infect these cells. However these DCs are then able to mount an IFN response to HIV-1 which is also mediated through activation of IRF3 (Manel, Hogstad et al. 2010). Thought this does not rule out a DNA sensor for HIV-1, the actual PRR could be activated by any part of the virus lifecycle after reverse transcription and production of RT products.

This is interesting with the fact that SAMHD1 is also present in macrophages. The lack of a HIV-1 countermeasure against SAMHD1 suggests that macrophages may not be an important target for the virus, however I (Figures 2 and 3) and others have shown productive infection of MDM by full length replication competent HIV-1. This ability of HIV-1 to infect and replicate in MDM may be due to the long lifespan of these cells. Though reverse transcription may be slowed due to the lower concentration of dNTPs it is probably not completely inhibited. It is likely that removal of the SAMHD1 restriction factor could improve efficiency of infection in macrophages, however if like in DCs suppression of SAMDH1 activity leads to activation of the IFN response, a slower delayed infection of MDM may be a compromise for long term survival of the virus.

Following from my findings, that HIV-1 does not induce innate immune responses on MDM, recent work in our (Noursadeghi) lab in collaboration with the Towers lab has found that HIV-1 infection of MDM is dependent on recruitment of the cyclophilins, Nup358 and CypA, and cleavage and polyadenylation specificity factor subunit 6 (CPSF6) in order to evade the immune response (Rasaiyaah, Tan et al. 2013). Wildtype HIV-1 capsid interacts with the host cell cyclophilins and CPSF6 which stabilise the capsid and transports it to the nucleus, therefore bypassing cytoplasmic DNA sensors. Capsid mutants N74D and P90A which are impaired in their ability to interact with these cofactors cannot replicate in MDM as they stimulate the activation of NFκB and IRF3 via the PRR cGAS.

4.5 Conclusions

In this section I have looked at the ability of macrophages as sentinel cells of the innate immune system to detect and initiate immune responses to incoming HIV-1. I had already shown that HIV-1 can infect and replicate within MDM, and surprisingly this all occurs without triggering any detectable innate immune responses as assessed by measuring; the activation of the common innate immune transcription factors, NFκB and IRF3, the mRNA levels of IFNβ and the ISG IP-10 as well as selected proinflammatory cytokines. The lack of immune response was not due to specificities in MDM differentiation or different strains of HIV-1. More surprisingly the lack of detectable response is not due to active suppression by HIV-1. Unlike in other viruses which actively evade immune responses by suppression of the IFN response, HIV-1 appears to evade immune recognition by MDM altogether.

IFN stimulation of MDM does potently restrict HIV-1 despite the presence of the HIV-1 accessory proteins that have been shown to negate the effects of IFN inducible antiretroviral transcription factors. Once stimulated, MDM retain an antiviral state to HIV-1 for over 3 weeks. This finding suggests that once stimulated these MDM may be permanently refractory to HIV-1. The importance of HIV-1 evasion of innate immune responses in MDM is highlighted when IFN is added to MDM with an established infection. Although addition of IFN to MDM with established infection leads to a decrease in viral replication, this is only transient and viral replication recovers within days.

Recent discoveries suggest that HIV-1 may be detected by cytosolic DNA PRRs such as cGAS, and though this PRR is present in MDM the cellular factors CypA, CPSF6, Trex1 and SAMHD1 may limit the amount of cytoplasmic viral DNA preventing immune activation. SIV and HIV-2 encode for Vpx which counteracts the function of SAMHD1 allowing infection by HIV-1, but in DCs this also leads to activation of the IFN response. Rather than implying that HIV-1 does not naturally infect macrophages as it lacks a

countermeasure for this restriction factor, instead it may be a compromise to allow it to infect without activating an innate immune response. Although this may mean infection and replication is delayed, the long lived nature of macrophages would mean there is no long term disadvantages. Further evidence for macrophages as a natural host for HIV-1 comes from recent work. HIV-1 infection in MDM is dependent on capsid interactions with the host cellular factors CPSF6 and CypA, which aid the transport of the capsid to the nucleus bypassing cytoplasmic DNA sensors such as cGAS. Interestingly CypA in DCs has been reported to have a role in innate immune detection of HIV-1, suggesting adaptation of HIV-1 to utilise specific cellular factors in order to infect macrophages without activating immune responses.

Altogether these data suggest that macrophages may be the natural host cell for HIV-1 and are an important part of its lifecycle. Macrophages are permissive cells which do not mount restrictive IFN responses to the virus. They help disseminate the virus and the long lived nature of this cell type, along with the specialised compartment in which HIV-1 resides, protected from neutralising antibodies, means they are an effective viral reservoir.

Chapter 5. Effects of HIV-1 infection on MDM function

5.1 Introduction

HIV-1 infection can eventually lead to AIDS. Whilst reduced numbers of lymphocytes have correlated strongly to the loss of immune function in HIV-1 individuals, the role of macrophages in this immunodeficiency has largely been overlooked. Studies have reported that HIV-1 infected monocytic cells and macrophages can induce apoptosis in bystander T cells (Herbein, Van Lint et al. 1998). This effect has been attributed to upregulation of FasL (Badley, McElhinny et al. 1996; Badley, Dockrell et al. 1997), TRAIL (Badley, Dockrell et al. 1997) and TNF on the HIV-1 infected cell (Zhang, Li et al. 2001; Wang, Nadeau et al. 2010). This HIV-1 induced bystander cell death may be attributed to the accessory protein Tat, as monocytes treated with HIV-1 Tat has also been reported to be able to cause apoptosis in uninfected CD4+ T cells (Yang, Tikhonov et al. 2003).

As discussed earlier in section 1.1.2 macrophages are an important part of the immune system and are involved in both innate and adaptive immune responses (Miyata and van Eeden 2011). HIV-1 infection of these cells may impair their ability to carry out normal functions. A number of studies have reported inhibition of the transcription factors important in innate immune responses by HIV-1 accessory proteins. Vpu (Akari, Bour et al. 2001; Bour, Perrin et al. 2001) and Vpr (Kogan, Deshmane et al. 2013) have been reported to inhibit NFκB signalling. Recently Vpu was also reported to inhibit signalling through IRF3 by targeting it for degradation (Doehle, Chang et al. 2012), with Vpu deficient strains of HIV-1 activating innate immune responses (Doehle, Chang et al. 2012).

In addition to innate immune stimuli, certain cytokines are also key modulators of macrophage function. This is exemplified by IFNγ. Many macrophage immune functions are activated or augmented after stimulation with IFNγ. Stimulation of

macrophages with IFNγ can lead to activation of antimicrobial activity to intracellular pathogens such as leishmania, chlamydia and mycobacterium (Murray, Rubin et al. 1983; Rothermel, Rubin et al. 1983; Lehn, Weiser et al. 1989; Fabri, Stenger et al. 2011). IFNγ has also been shown to increase binding and phagocytosis as well as killing of Salmonella (Gordon, Jack et al. 2005), though there have also been reports of IFNγ reducing receptor mediated phagocytosis (Jungi, Brcic et al. 1991). This antimicrobial mechanism may be due to IFNγ induction of hydrogen peroxide (Nathan, Murray et al. 1983; Gluck and Weinberg 1987) and/or autophagy, a recycling mechanism where dysfunctional or no longer needed cellular organelles are degraded, but is also capable of killing of intracellular pathogens by the same mechanism (Al-Zeer, Al-Younes et al. 2013).

In addition to the potential reduction of this cytokine due to the loss of CD4+ T cells, HIV-1 infection may also interfere with macrophage responses to this cytokine. HIV-1 has been reported to inhibit autophagy (Zhou and Spector 2008; Li, Au et al. 2011), via induction of suppressor of cytokine signaling-2 (SOCS-2) by HIV-1 Tat (Cheng, Li et al. 2009) which suppresses IFNγ signalling through STAT1 (Li, Au et al. 2011).

5.2 Objectives

- Assess the effects of HIV-1 infection on MDM innate immune responses to other pathogens, using LPS and Pam3CSK4 as minimal TLR stimuli.
- Assess the effects HIV-1 has on MDM responses to stimulation by IFNγ

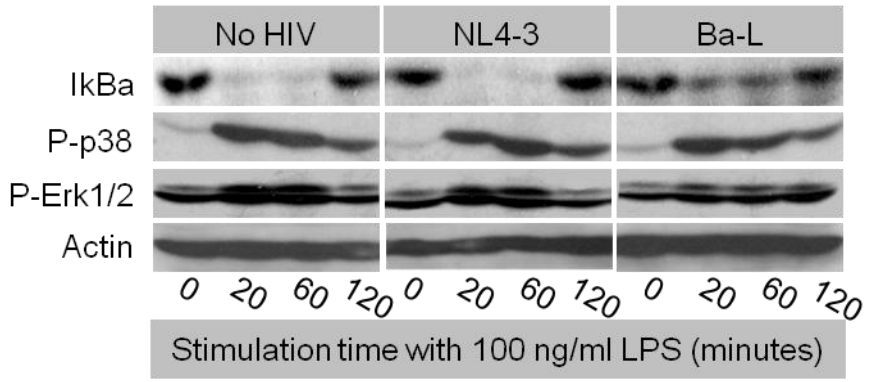
5.3 Results

5.3.1 HIV-1 infected MDM show impaired IκBα degradation after stimulation with LPS

Macrophages are sentinel cells of the immune system able to detect and respond to pathogens using PRRs. The innate immune cellular activation pathways employ a complex network of receptors, adaptor molecules and kinases which appear to converge onto selected intracellular signalling events which can be detected by Western blotting.

Western blotting was therefore used to examine degradation of IκBα and phosphorylation of p38, Erk1/2 and JNK in MDM after stimulation by LPS, representing a typical innate immune stimulus from bacteria (Figure 39). In uninfected MDM, stimulation with LPS induces degradation of IκBα. The IκBα signal was diminished at 20 and 60 minutes after stimulation, and regenerated by 120 minutes. Rapid p38 and increased Erk1/2 phosphorylation was also evident, but JNK phosphorylation was not detected. This pattern of signalling events was replicated in control MDM cultures inoculated with HIV-1 NL4-3, a strain that is unable to establish host cell infection. In HIV-1 BaL infected MDM, p38 and Erk1/2 phosphorylation patterns were comparable to controls, but degradation of IκBα was attenuated with the same time course profile.

Figure 39 - HIV-1 attenuates IκB degradation in MDM stimulated with LPS



MDM were inoculated with HIV-1 at a MOI three and cultured for seven days for infection to fully establish before stimulation with 100ng/ml LPS for 0, 20, 60 or 120 minutes. Western blotting was used to probe for IκBα, phosphorylated p38, phosphorlated ERK1/ 2 and beta actin as a loading control. After stimulation with LPS, all of the IκB is rapidly phosphorylated and degraded and new IκBα is synthesised after two hours. In MDM infected with HIV-1 BaL IκB degradation at the 20 and 60 min time points is attenuated and a IκB band is still present after stimulation. P38 and ERK phosphorylation in response to LPS stimulation is not affected by HIV-1 BaL infection. Representative images from multiple experiments are shown.

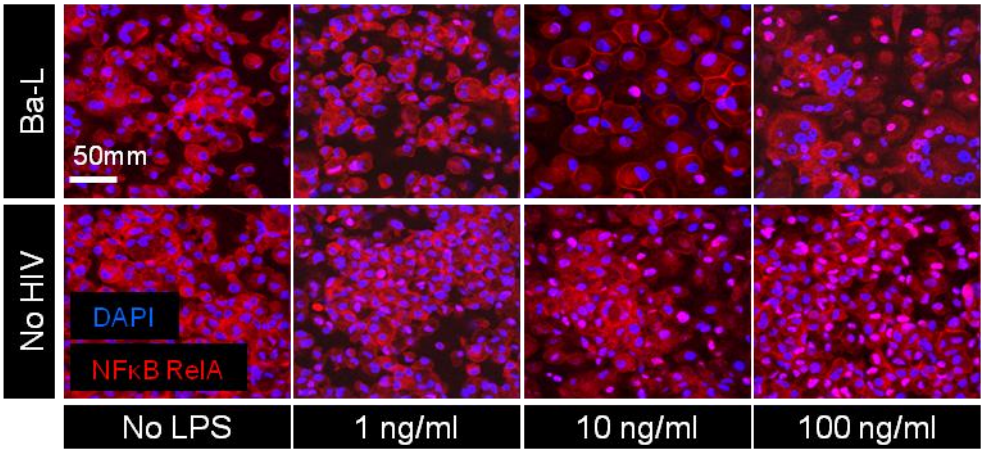
5.3.1.2 Impaired IκBα degradation leads to attenuation of NFκB activation

IκBα binds to the NFκB p50-RelA heterodimer and inhibits the transcription factor activity of RelA, primarily by sequestering it in the cytoplasm. In the classical NFκB activation pathway, IκBα is phosphorylated then ubiquitinated before being degraded, allowing for nuclear translocation of the NFκB complex. Therefore in addition to measuring the degradation of IκBα by Western blotting, NFκB RelA nuclear translocation was also directly measured using a confocal immunofluorescence assay allowing for the assessment of the functional consequences attenuated IκBα degradation has in HIV-1 infected MDM. Additionally the measurement of NFκB nuclear translocation by this assay would allow for more accurate quantitation compared to densitometry on Western blots.

RelA exhibited mostly cytoplasmic staining in control and HIV-1 BaL infected MDM before stimulation (Figure 40). In response to stimulation with increasing concentrations of LPS, greater nuclear staining was clearly evident. Nuclear: cytoplasmic RelA staining and the proportion of cells showing nuclear RelA staining, was then used to compare the dose response to LPS in HIV-1 infected and uninfected MDM cultures (Figure 41). Nuclear translocation of RelA was significantly attenuated in HIV-1 infected MDM across the LPS dose range. This effect was evident in 9/10 separate experiments using cells from different donors (Figure 42A). Testing multiple donors in this way provided a powerful illustration of the natural variance of this response and the effect of HIV-1 infection. The proportion of cells showing RelA nuclear translocation in control (uninfected) MDM cultures stimulated with 10 ng/ml LPS ranged between 17-97%, and was reduced by a mean value of 28.9% (95% confidence interval of 9.8-46.8% inhibition) in HIV-1 infected MDM. The specificity of these observations was also tested in MDM infected with the dual tropic HIV-1 strain 89.6 and with a synthetic TLR1/2 stimulus (Pam3CSK4). Similar attenuation of NFκB RelA nuclear translocation was seen (Figure 42B), suggesting that HIV-1 dependent

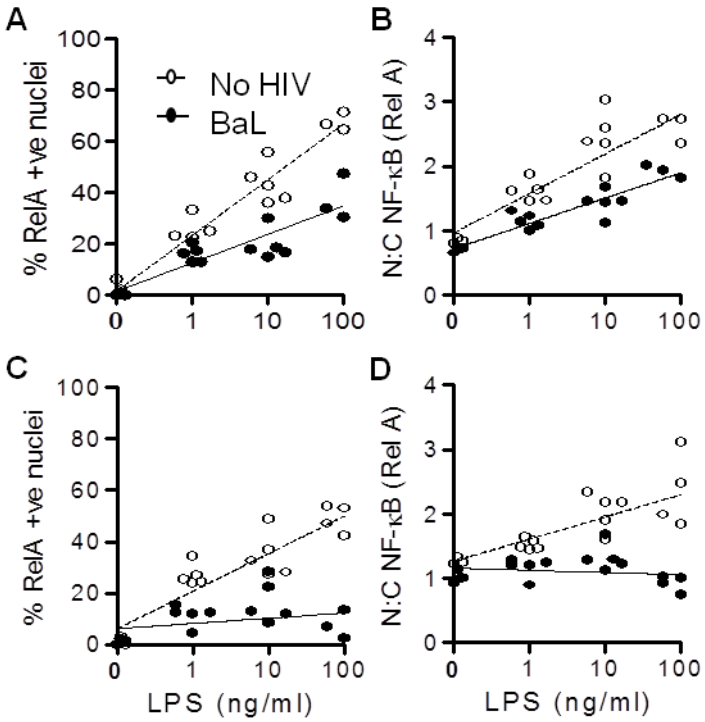
attenuation of NFκB activation in response to innate immune stimuli may show broad strain and stimulus specificity.

Figure 40 - HIV-1 infection of MDM attenuates NFκB nuclear translocation in response to LPS stimulation



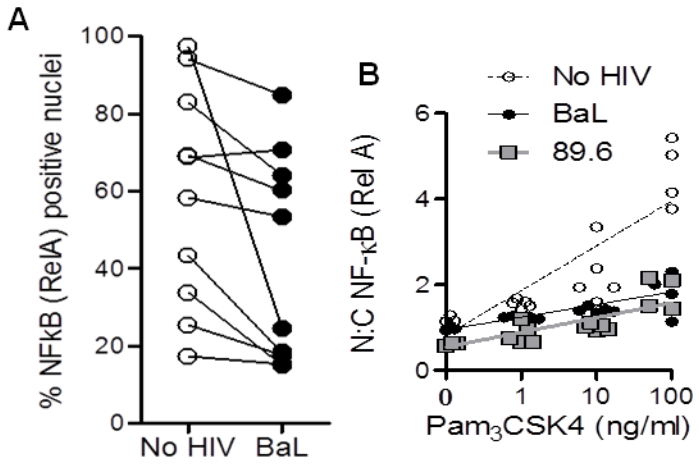
MDM, cultured on glass coverslips, were inoculated with HIV-1 at a MOI three and cultured for seven days for infection to fully establish before stimulation with LPS at various doses for 60 minutes. Cells were fixed in PFA permeablised and stained for NFκB RelA (red) and the nuclei were counterstained with DAPI (blue). Increased nuclear RelA staining is evident with increasing LPS concentrations, with HIV-1 infected MDM showing reduced nuclear translocation compared to uninfected cells. Representative images of multiple experiments shown.

Figure 41 - HIV-1 infected MDM show attenuated RelA nuclear translocation across all doses of LPS tested.



Quantitation of nuclear RelA staining is presented as % RelA positive nuclei **(A and C)** and nuclear:cytoplasmic (N:C) ratios **(B and D)** in MDM stimulated with a dose range of LPS. Data points represent image analysis from five separate high power fields with best-fit lines across the stimulus dose range. Data from **(A and B)** and **(C and D)** show two paired examples of response to LPS stimulation. In each experiment HIV-1 infected MDM showed attenuated RelA nuclear translocation dose responses to innate immune stimulation ($p<0.01$, ANOVA).

Figure 42 - HIV-1 Attenuation of NFκB nuclear translocation in MDM is not HIV-1 strain specific and can be seen with other TLR stimuli

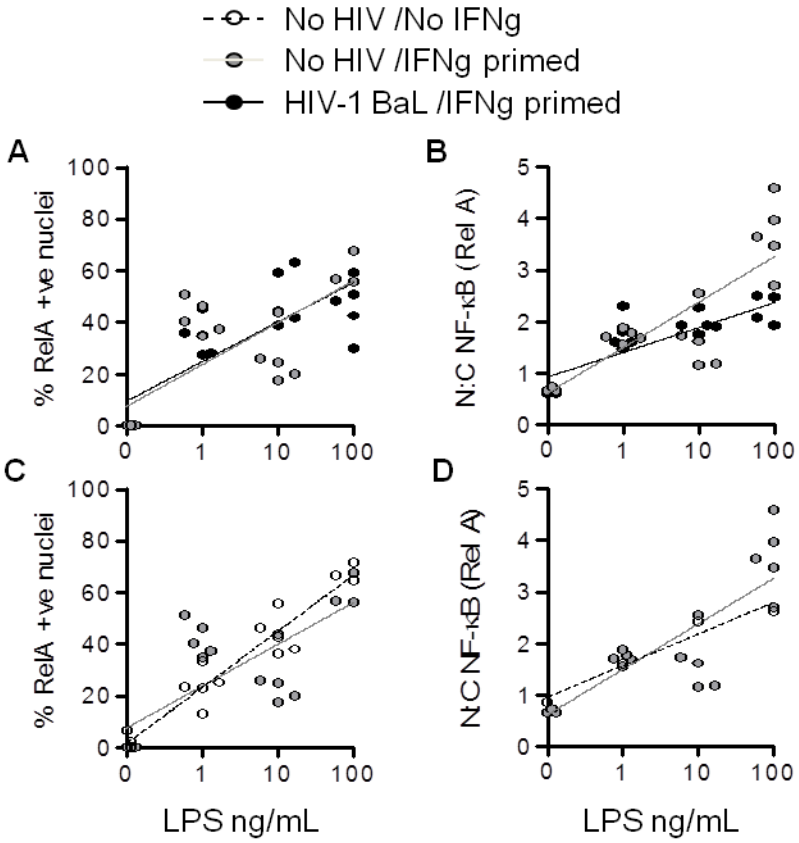


(A) Shows summary data from 10 separate experiments of RelA nuclear translocation in MDM stimulated with 10 ng/ml LPS. Lines indicate paired HIV-1 infected and control MDM from the same donor. Analysis of paired responses show less RelA nuclear translocation in HIV-1 infected MDM in 9/10 experiments ($p<0.01$, Wilcoxon signed rank test). **(B)** Shows an example of the response to Pam3CSK4. In each of these experiments, HIV-1 BaL- or HIV-1 89.6-infected MDM showed attenuated RelA nuclear translocation dose responses to innate immune stimulation

5.3.1.3 IFNγ priming of HIV-1 infected MDM restores normal NFκB activation

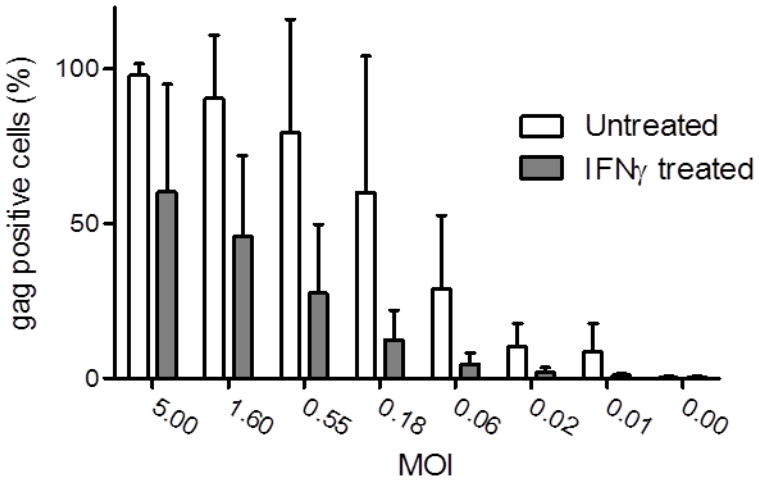
Innate immune responses by macrophages are known to be augmented by IFNγ from T cells in the classical TH1 type paradigm for adaptive immune responses (Boehm, Klamp et al. 1997; Schroder, Hertzog et al. 2004). Therefore the effect of IFNγ priming on activation of the NFκB pathway in HIV-1 BaL infected MDM was investigated. In HIV-1 infected MDM, the attenuation of the nuclear translocation is abolished when MDM were primed with 10ng/ml IFNγ 24 hours before stimulation (Figure 43A-B). In view of this it was possible that some of the variability in HIV-1 mediated inhibition of NFκB activation may be due to the presence of IFNγ, as a result of minor T cell contamination of MDM cultures. Therefore all cell culture supernatants from the experiments performed in this study were analysed for the presence of IFNγ, but none was detected by ELISA at a sensitivity of 10pg/ml. The mechanism by which IFNγ corrects attenuated NFκB activation responses in HIV-1 infected cells may be due to priming of the innate immune activation pathway or by an effect of IFNγ on HIV-1. Comparison of LPS induced nuclear translocation of NFκB RelA in uninfected MDM with and without IFNγ pre-stimulation showed no significant differences (Figure 43C-D), suggesting that there is no priming effect. However, in keeping with previous reports (Meylan, Guatelli et al. 1993; Creery, Weiss et al. 2004), I found that addition of IFNγ 24 hours after infection of MDM infection by HIV-1 BaL potently inhibited the number of HIV-1 infected cells (Figure 44), supporting the hypothesis that the IFNγ priming of NFκB activation in HIV-1 infected MDM may be mediated through an inhibitory effect on HIV-1.

Figure 43 - Attenuated NFκB activation by HIV-1 is rescued by priming infected MDM with IFNγ



Quantitative image analysis of confocal nuclear and RelA staining, presented as percentage of RelA-positive nuclei (N:C) ratios **(A and C)** and nuclear:cytoplasmic **(B and D)** in MDM stimulated with a dose range of LPS. MDM pretreated with 10 ng/ml IFNγ 24 hours before stimulation with a dose range of LPS show equivalent RelA nuclear translocation responses in HIV-1-infected and control-uninfected MDM **(A and B)**. Similar IFNγ pretreatment does not enhance RelA nuclear translocation in uninfected MDM **(C and D)**. Data points are derived from image analysis from five separate high power fields, with best-fit lines across the stimulus dose range, and are representative of three separate experiments.

Figure 44 - IFNγ priming of HIV-1 infected MDM reduces the number of HIV-1 positive cells after 7 days.



The inhibitory effect of IFNγ on HIV-1 replication in MDM is shown by significant ($p < 0.01$, ANOVA) reduction of p24-positive cells in MDM cultures stimulated with 10 ng/ml IFNγ 24 hours after inoculation. Bars represent mean +SD of three separate experiments

5.3.1.4 HIV-1 infection attenuates the upregulation of gene expression in the MDM response to LPS stimulation

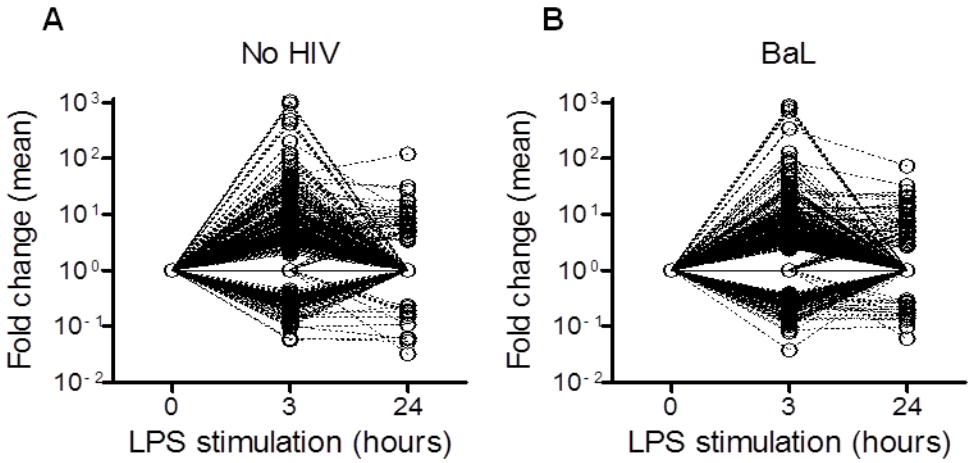
NFκB is a central transcription factor for many cellular innate immune functions. To assess the effects attenuation of the NFκB activation has on downstream responses, whole genome transcriptional microarrays were used to compare the transcriptome of uninfected and HIV-1 infected MDM stimulated with LPS (Figure 45). The transcriptome was analysed 3 and 24 hours after LPS stimulation to look at the primary response and the steady state transcriptome respectively. Gene expression changes were clearly greater at 3 hours (approximately 400 genes up-regulated and 100 genes down-regulated) when compared with 24 hours (approximately 40 genes up-regulated and 15 genes down-regulated) (Figure 46). Analysis of significant changes in gene expression in response to LPS stimulation showed similar overall expression profiles between uninfected and HIV-1 infected MDM when compared to unstimulated cells.

The transcriptional responses to LPS were assessed quantitatively by aligning all significantly affected genes in an expression matrix showing mean fold change in 3 or 24 hour LPS stimulated cells compared with the corresponding HIV-1-infected or uninfected unstimulated MDM (Figure 46). Functional annotation analysis of these genes was performed by identifying statistically overrepresented gene ontology clusters using the online DAVID bioinformatics database (Dennis, Sherman et al. 2003; Hosack, Dennis et al. 2003) and indicating the alignment of individual genes within the expression matrix. This analysis shows LPS stimulation induced upregulation of a wide range of genes which cluster within functionally related immune response ontology groups, which demonstrates highly significant enrichment in comparison with the whole human genome (Figure 47). In contrast the genes which are downregulated by LPS stimulation do not cluster into immune response ontology groups and are not significantly enriched. Genome wide transcriptional responses to LPS at 3 and 24 hours appear to be similar for uninfected and HIV-1 infected MDM by this analysis.

To assess the effects of HIV-1 infection on MDM responses to LPS, the expression levels of LPS responsive genes (up and downregulated genes at 3 and 24 hours) were analysed, comparing HIV-1 infected to uninfected MDM. Statistically significant differences in gene expression in 85 of the 581 LPS responsive genes were found by paired testing of HIV-1 infected and uninfected MDM from individual donors. The major effect was attenuation of a proportion of up-regulated LPS responsive genes in HIV-1 BaL infected MDM compared with uninfected cells and the extent of attenuation was a 1.2 - 3 fold reduction in expression levels. Alignment of these genes with the gene ontology groups identified by the functional annotation clustering analysis of LPS responsive genes showed that LPS responses attenuated by HIV-1 included a range of pro-inflammatory cytokines and immune-related genes (Figure 48). However these differences were only evident when pairing individual donor sample uninfected and HIV-1 infected MDM. When they were analysed as two groups no significant differences were found, suggesting that differences in gene expression attributable to HIV-1 infection were smaller than the variability between different donors.

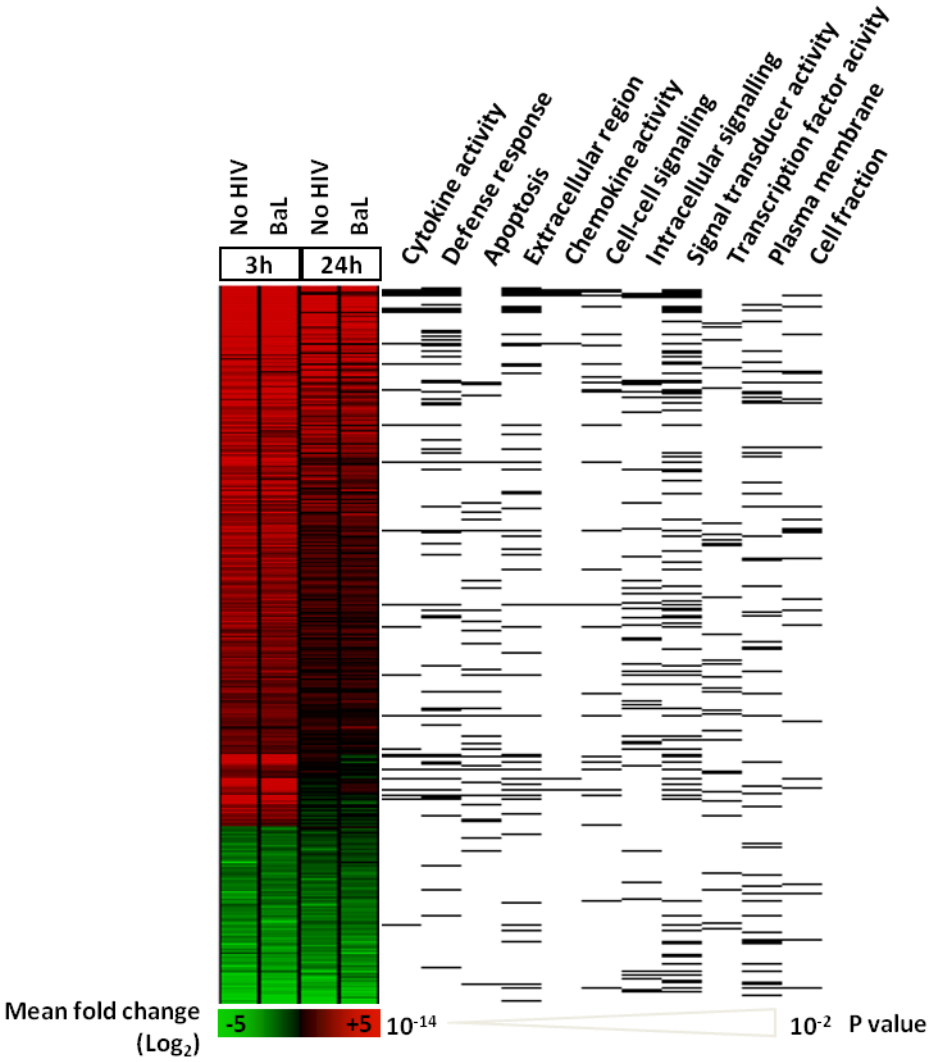
This is supported when performing MDS analysis, looking for dissimilarities between the samples (Figure 49). This 2D representation of gene expression differences between the samples clearly shows segregation of samples based on LPS stimulation on dimension 1. With 3hr stimulated samples clustering together on the right and 24hr stimulated samples clustering together on the left independent of HIV-1 infection status. Though the effect of HIV-1 infection is more pronounced in dimension 2, the differences between different donors are greater than the differences seen between uninfected and HIV-1 infected cells.

Figure 45 - Global transcriptional changes in response to LPS stimulation in uninfected and HIV-1 infected MDM are similar



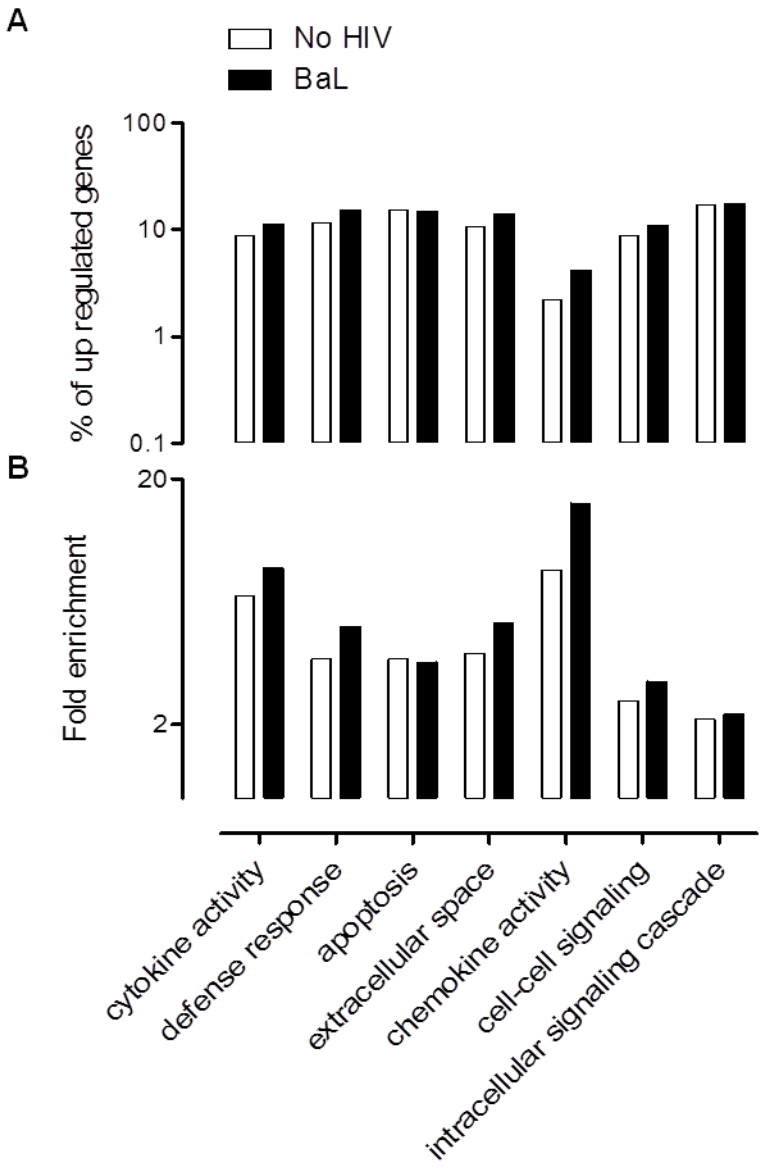
Analysis of mean fold changes (three separate experiments) in expression of all significantly (SAM, 1% FDR) up-regulated (>1) or down-regulated (<1) genes at 3 and 24 hours after LPS stimulation (10ng/ml) compared with unstimulated cells in HIV-1 BaL-infected **(B)** and uninfected **(A)** MDM cultures shows comparable overall profile.

Figure 46 - Effects of HIV-1 infection on MDM transcriptional responses to LPS stimulation



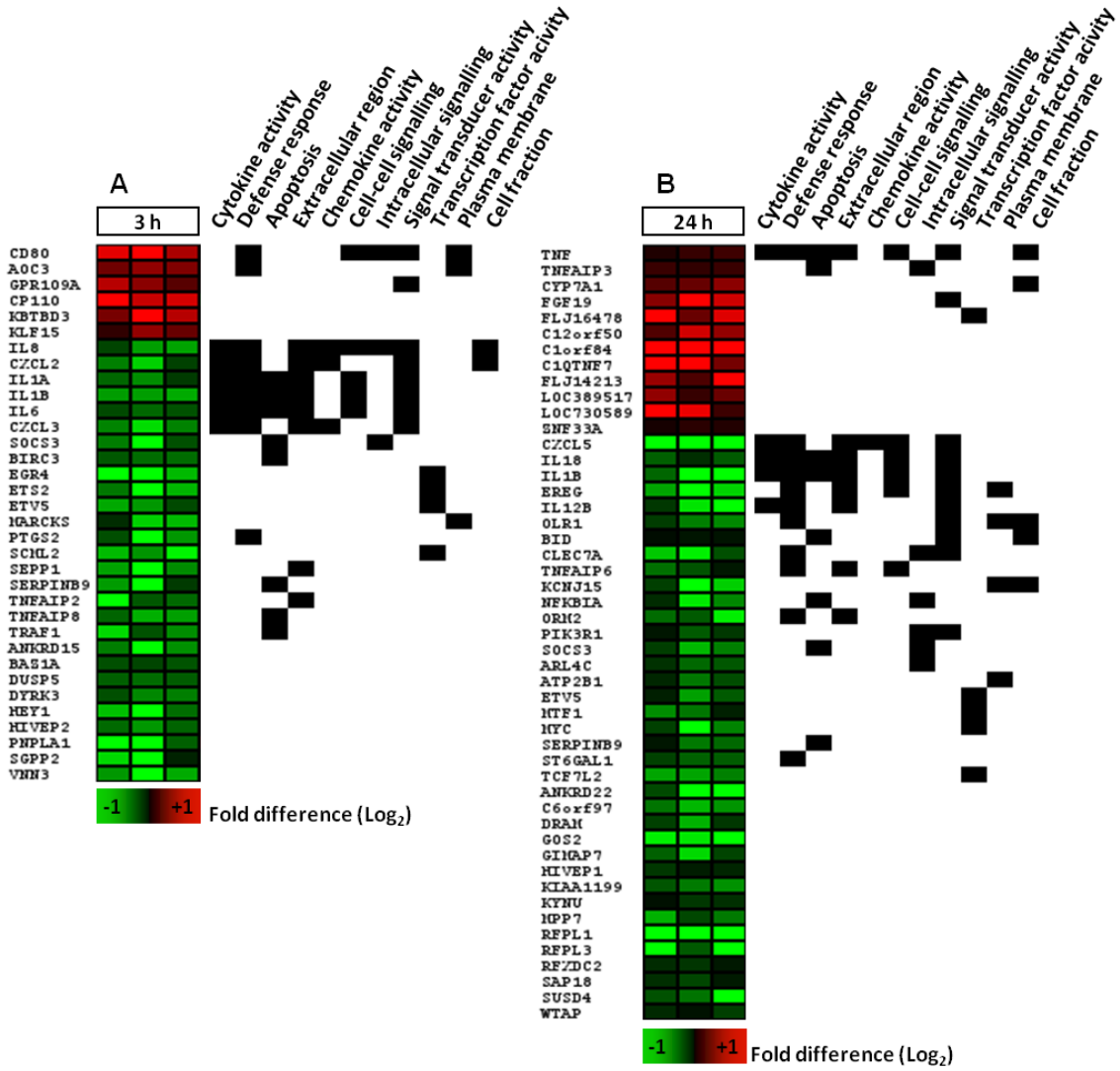
Significantly up-regulated and down-regulated genes in the transcriptional response to LPS (10ng/ml) at 3 and 24 hours from HIV-1-infected and uninfected MDM are aligned for comparison. Gene ontology term associations for each gene (row) are shown for significantly ($p < 0.05$) overrepresented functionally related gene clusters, using the online DAVID functional annotation clustering analysis tool. Gene ontology terms are arranged in order of statistical significance (p values 10^{-14}–10^{-2}). This expression matrix and analysis are restricted to genes with refseq accession numbers, for which contemporary functional annotation is available

Figure 47 - Gene clusteres upregulated by LPS stimulation and the effect of HIV-1



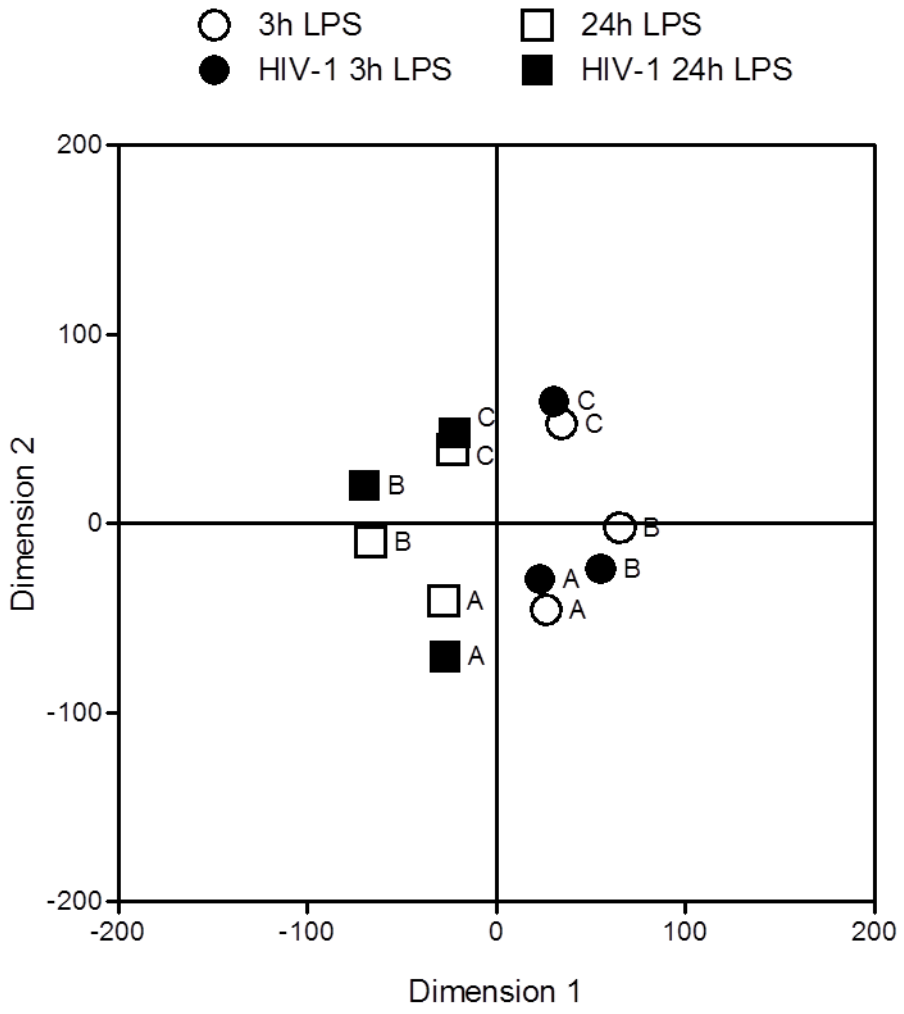
The proportion of genes up-regulated by LPS (derived from three separate experiments) associated with each significantly overrepresented gene cluster **(A)** and the fold enrichment of each gene cluster compared with the whole human genome **(B)** is comparable in HIV-1-infected and uninfected MDM.

Figure 48 - HIV-1 attenuation of LPS responses in MDM



Differences in the transcriptional response to LPS between HIV-infected and uninfected MDM are tested by analysis of LPS-responsive genes in paired HIV-infected and uninfected samples from three separate donors for statistically significant differences (SAM, 1% FDR) at 3-h (A) and 24-h (B) LPS stimulation. The fold difference in HIV-infected MDM compared with uninfected cells, in each of three separate experiments, is shown together with the gene symbol and gene ontology term associations.

Figure 49 - MDS analysis showing no significant differences between uninfected and HIV-1 infected MDM stimulated with LPS at different time points

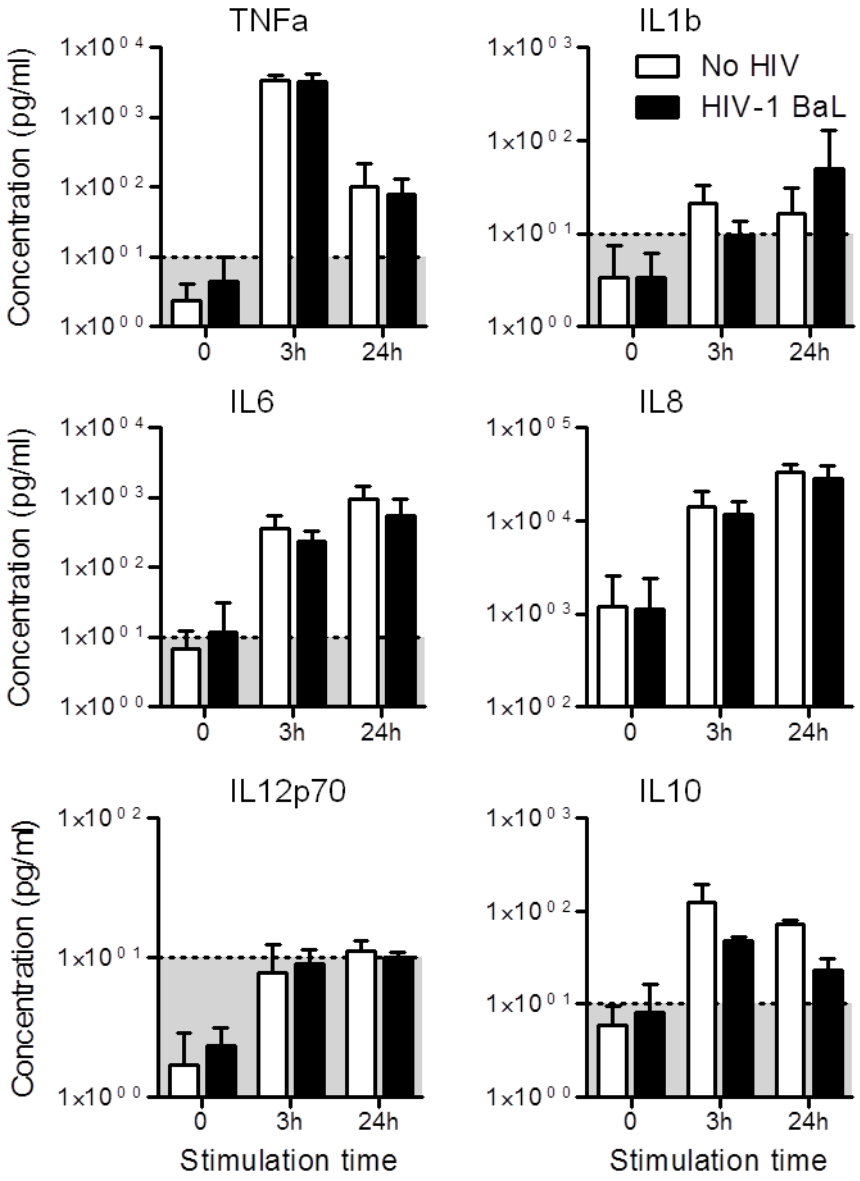


A 2D representation showing limited gene expression differences in MDM stimulated with LPS for either 3 (circles) or 24 (Squares) hours. Data is from 3 seperate donors represented by a different letter, HIV-1 infection is represented by a black filled symbol. LPS stimulation can be clearly seen to influence the position on dimension (Dim) 1, with 3h stimulated cells clustering together to the right and 24hr stimulated cells clustering together on the left. Whereas HIV-1 infection has a lesser effect with HIV-1 infected cells clustering together with the corresponding uninfected sample.

5.3.1.5 Despite attenuation of the NFκB response to LPS in HIV-1 infected MDM, cytokine release is unaffected

The release of inflammatory cytokines is one of the major responses seen in macrophages after innate immune stimulation. It was therefore of significant interest that expression levels of IL-1, IL-6, IL-8 and IL-12 were included amongst the LPS transcriptional responses which were attenuated by HIV-1 infection. In order to establish if these transcriptional differences translated to significant differences in protein levels, MDM culture supernatants were collected from the same experiment which the transcriptional profiling experiments were performed. Supernatants from unstimulated, 3 hour and 24 hour LPS stimulated cells with and without seven days HIV-1 infection were analysed for the presence of these cytokines (Figure 50). In addition TNFα and IL-10 levels were measured, as well established components of MDM cytokine responses to LPS that were not found to have attenuated gene expression levels in HIV-1 infected MDM. Significant concentrations of IL-8, but none of the other cytokines tested, were detectable in unstimulated MDM culture supernatants and were unchanged by HIV-1 infection. Concentrations of TNFα, IL-6, IL8 and IL-10 clearly increased following LPS stimulation, but were equivalent in HIV-1 infected samples. Similarly, no significant difference was evident in levels of IL-1β, which increased just above the detection threshold in response to LPS. IL-12p70 was not detected in any samples, an observation in keeping with previous analyses of this MDM model (Smith, Feldmann et al. 1998; Martinez, Gordon et al. 2006).

Figure 50 - Cytokine secretion in response to LPS in HIV-1 infected MDM



MDM were infected with HIV-1 for seven days before stimulation with LPS for 3 hours or 24hours. Supernatants were collected and cytokine secretion measured. Cytokine secretion in HIV-1 infected MDM was not significantly affected compared to control uninfected MDM despite a decrease seen in gene expression. Bars represent mean±SD from 3 seperate experiments

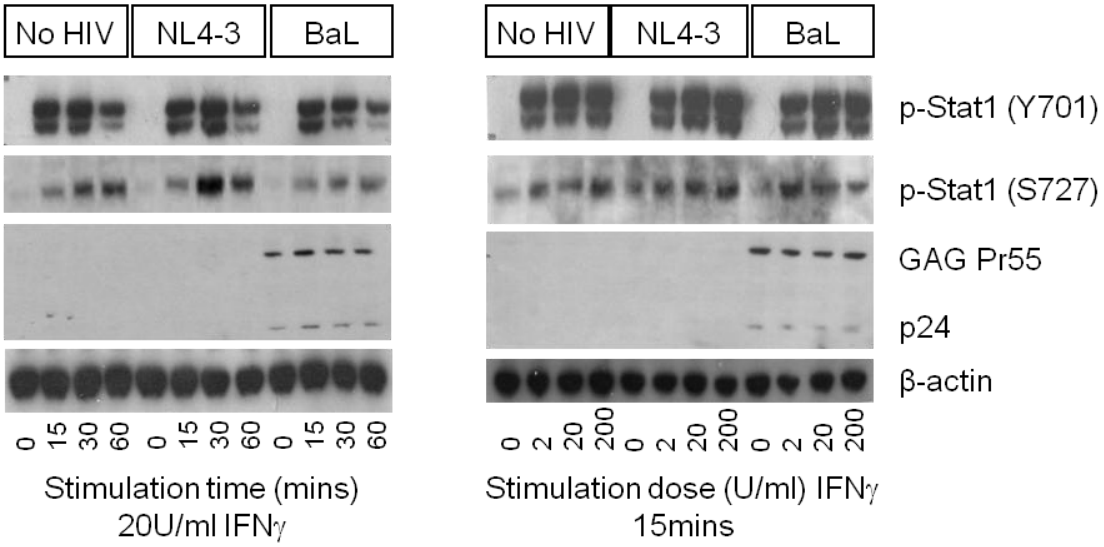
5.3.2 HIV-1 does not affect Stat1 phosphorylation or gene transcription in response to IFNγ stimulation

IFNγ has been shown to function via signalling through the JAK-STAT signalling pathway, and specifically through STAT1 (Durbin, Hackenmiller et al. 1996; Stark, Kerr et al. 1998). Interestingly in mice disruption of the STAT1 gene leads to compromised innate immune responses to viral disease (Durbin, Hackenmiller et al. 1996). As discussed in section 4.3 a number of viruses have evolved mechanisms to evade both type I and type II IFN responses in order to infect their hosts. Specifically hepatitis C virus has been reported to downregulate IFNγ signalling in T cells by reducing STAT1 protein levels (Kondo, Sung et al. 2007).

In light of this, the activation of STAT1 was assessed in HIV-1 infected MDM in response to IFNγ stimulation. Activation of STAT1 occurs through phosphorylation at two residues (S727 and Y701) and Western blotting was used to detect for the presence of the phosphorylated forms of STAT1 in MDM which had been stimulated with IFNγ. No significant differences in STAT1 phosphorylation were seen for the two different phosphorylated residues, between uninfected or HIV-1 infected MDM, either in a dose or a time course response to IFNγ stimulation (Figure 51). STAT1 signalling in response to IFNγ stimulation appears to be unaltered in HIV-1 infected MDM. To rule out the possibility that IFNγ signalling was modulated via alternative STAT proteins, or by another pathway altogether, whole genome expression microarray profiling was performed comparing uninfected and HIV-1 infected MDM responses to IFNγ. Analysis of the microarray data showed no significant differences between uninfected and HIV-1 infected MDM after stimulation with IFNγ as shown by the MDS analysis (Figure 52). This analysis is a 2D representation gene expression differences between individual samples. The response to IFNγ stimulation is clearly seen in dimension 1, with stimulated cells clustering together on the left with unstimulated cells clustering together on the right. There appears to be no significant differences between

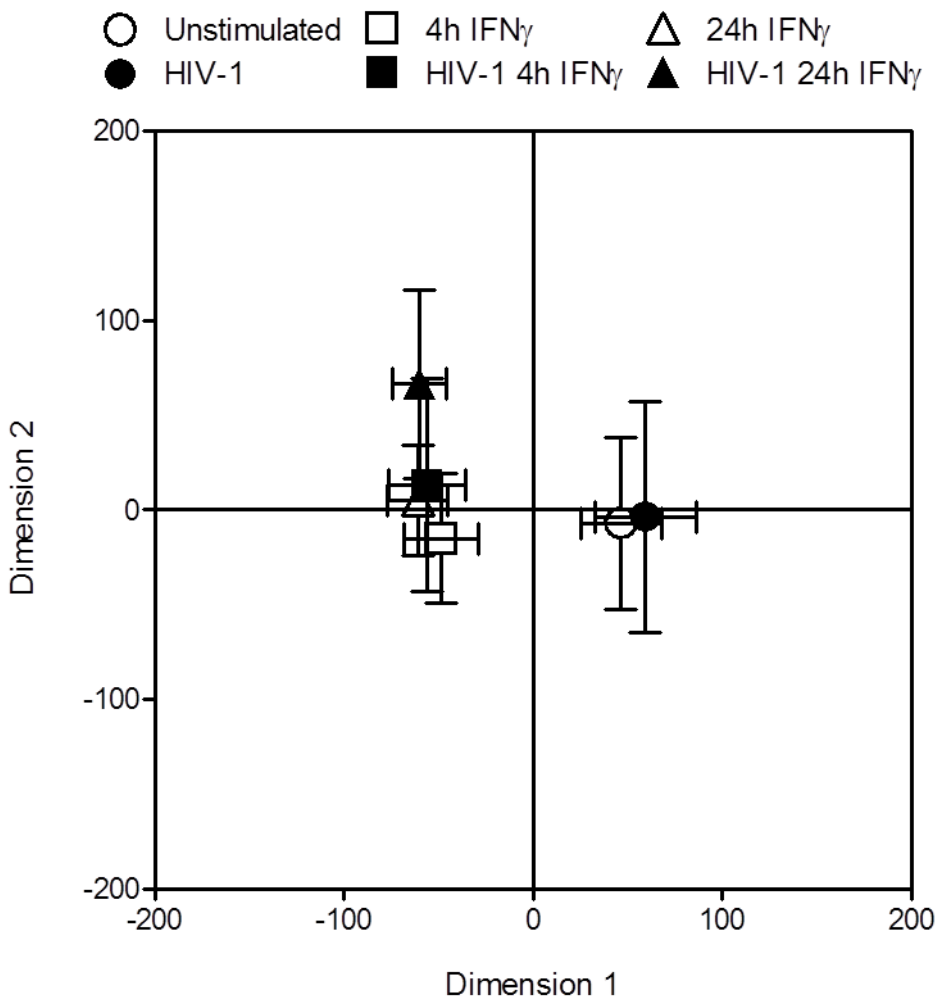
uninfected and HIV-1 infected MDM when looking at dimension 1 at all time points. In dimension 2 there may be some differences in gene expression attributable to HIV-1 infection when MDM are stimulated with IFNγ for 24 hours, though when paired donor analysis is performed the effect is evident only in two out of four donors (Figure 53).

Figure 51 - HIV-1 infection of MDM does not alter STAT1 signalling in response to IFNγ stimulation



MDM inoculated with HIV-1 were left for seven days for infection to establish before stimulation with IFNγ. The left panel was stimulated with 20U/ml of IFNγ and samples harvested at 15, 30 and 60 minutes after stimulation. The right panel was stimulated for 15 minutes with a dose response of 0, 2, 20 or 200U/ml of IFNγ. Western blot probing for phosphorylated Stat1 at two separate phosphorylation sites (Y701 and S727) showed no significant differences in Stat1 phosphorylation for MDM infected with HIV-1 in response to IFN γ stimulation. HIV-1 p24 was used to confirm infection of the MDM by HIV-1 (This antibody also picks up the unprocessed 55KDa GAG precursor). Beta actin was used as a loading control. Representative images of three independent experiments shown.

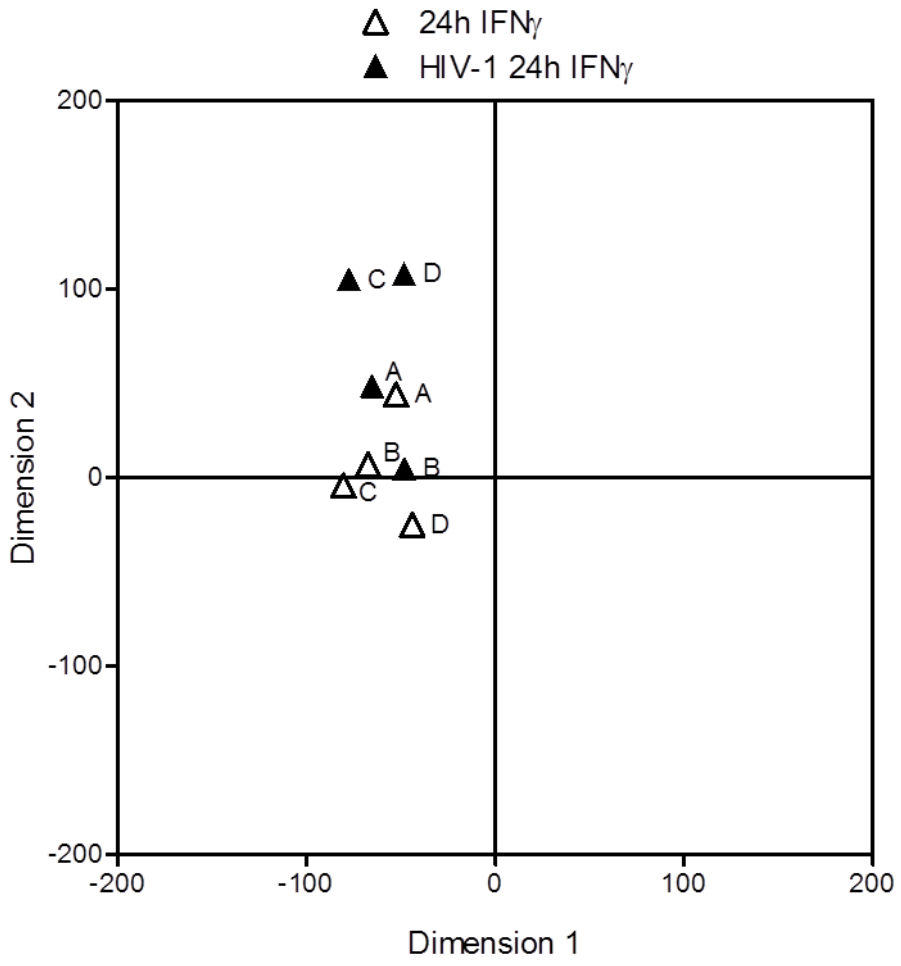
Figure 52 - MDS analysis of gene expression differences in uninfected and HIV-1 infected MDM after stimulation with IFNγ



A 2D representation showing gene expression differences in uninfected and HIV-1 infected MDM stimulated with IFNγ for either 4 or 24 hours. Gene expression differences between unstimulated and IFNγ stimulated MDM are evident in dimension 1, while HIV-1 infection status appears to have no significant effect on IFNγ responses with uninfected and HIV-1 infected cells clustering together.

Data points and error bars represent mean±SD for at least 4 individual donors.

Figure 53 - MDS analysis of gene expression differences on uninfected and HIV-1 infected MDM stimulated with IFNγ for 24 hours



A 2D representation showing gene expression differences in uninfected and HIV-1 infected MDM stimulated with IFNγ for 24 hours. HIV-1 infection appears to have little effect on the major response to IFNγ represented by dimension 1. There may be some differences between uninfected and HIV-1 infected MDM in dimension 2, but this is only seen in two out of four donors.

Each data point represents a individual donor paired samples from the same donor are denoted by the letters A-D.

5.4 Discussion

The effects of HIV-1 infection on macrophage immune functions along with CD4+ cell depletion could be an important step in understanding how HIV-1 leads to AIDS.

HIV-1 infected individuals are often more susceptible to opportunistic infections. Using this MDM model I are able to productively infect 100% of the MDM in the culture and could use these infected cells to identify any modifications made by HIV-1 infection on the normal innate immune responses of macrophages.

It is possible to look at the innate immune response to whole pathogens, however they often trigger innate immune activation through more than one PRR. To be able to more precisely study the effects of HIV-1 infection on the innate immune response, I decided to first use minimal innate immune stimuli such as LPS as a surrogate for bacteria. MDM which had been infected for seven days (when HIV-1 replication is at a peak as seen in Figure 4A) were stimulated with LPS and protein samples were collected at different time points to be analysed by Western Blotting. The MAPK signalling pathways were analysed, but there was found to be no effect on phosphorylation of p38 and ERK by HIV-1 infection in response to LPS stimulation. I also looked at NFκB activation via IκBα degradation and found that HIV-1 infection of MDM attenuated IκB degradation, after stimulation with a dose of LPS which induced maximal NFκB activation.

This attenuated IκBα degradation lead to a decrease in NFκB activation as seen by nuclear translocation. The attenuation of NFκB signalling seen was not specific to the stimuli used or the strain of HIV-1 as I could replicate the results using the 8.96 strain of HIV-1 and the TLR1/2 stimuli Pam3CSK4. This is consistent with the data of others which report HIV-1 accessory proteins inhibiting NFκB activation (Niederman, Garcia et al. 1992; Akari, Bour et al. 2001; Kogan, Deshmane et al. 2013). However, others have reported HIV-1 activation of NFκB (Fiume, Vecchio et al. 2012). HIV-1 gene

expression has been reported to be activated by NFκB (Kretzschmar, Meisterernst et al. 1992) which would suggest that it may beneficial for the virus to activate this transcription factor. Alternatively this HIV-1 gene expression induced by NFκB activation may be a method for the virus to quickly replicate in cells which mount innate immune responses in response to infection.

Interestingly the attenuation of NFκB signalling in this model could be reversed by stimulating the infected MDM with IFNγ 24 hours prior to stimulation with LPS. This could be due to a priming effect (Ma, Chow et al. 1996; Bosisio, Polentarutti et al. 2002) making the MDM more sensitive to LPS overcoming the attenuation by HIV-1. Priming of MDM was not evident from the experiments performed here, with NFκB activation similar in uninfected cells independent of IFNγ stimulation. Alternatively the antiviral effects of IFNγ may be reducing the HIV-1 product(s) responsible for the attenuation.

Inhibition of NFκB signalling by HIV-1 infection led to attenuation of the MDM response to LPS stimulation. The extent of the attenuation was modest affecting only 85 out of the 581 LPS responsive genes and with 1.2 – 3 fold reduction in genes expressed in response to stimulation. This is highlighted by the fact that inter donor variability is greater than the effect exerted by HIV-1 infection as these differences could only be seen using paired sample analysis. Of interest the expression of a number of cytokines was attenuated with HIV-1 infection including, IL-1β, IL-6, and IL-8. However these differences seen at the transcriptional level did not translate into altered protein expression. No significant differences in cytokine release were seen both at early (3 hour) and late (24 hour) time points after stimulation with LPS, between uninfected and HIV-1 infected cells. This could be attributed to the finding that the NFκB signal is attenuated and not completely blocked. Combined with the intact MAPK signalling pathways which were unaffected by HIV-1 infection, could compensate for the loss of some NFκB signalling due to redundancy in the innate immune signalling pathways

(Mestre, Mackrell et al. 2001). Although the inhibition of NFκB signalling and the reduction in gene expression to LPS stimulation were not associated with a corresponding decrease in protein expression in this study, using a lower dose of LPS may have a greater effect. Also, though LPS stimulation through TLR4 is a well characterised pathway of innate immune activation, whole pathogens are much more complicated, and HIV-1 attenuation of multiple signals may prove to have more effect than the attenuation of the signal from a single minimal TLR stimuli. Additionally the advances in proteomics in a similar method to microarray analysis could be used to analyse any differences in the proteome caused by HIV-1 infection.

These results contrast to those seen in studies looking at innate immune responses in alveolar macrophages or ex vivo MDM generated from patients with HIV-1. The cells in these studies however are likely to be infected by HIV-1 only at low frequencies (Spear, Ou et al. 1990; Nakata, Weiden et al. 1995). Therefore the phenotype of these cells is unlikely due to a direct effect of HIV-1 infection and may be due to the in vivo effects of HIV-1 on other components of the immune system. For example T cell depletion is likely to have an effect on macrophage functions, where the loss of these IFNγ producing cells likely to influence the activation and polarisation of macrophages.

Overall the results from these experiments support the hypothesis that HIV-1 infection of MDM may have a selective inhibitory effect on innate immune signalling pathways. It would be interesting to extend these experiments to see whether attenuation of NFκB signalling pathway occurs for signalling through other TLRs as well as other PRRs. Recent data suggests that HIV-1 may also attenuate innate immune signalling through IRF3 (Doehle, Chang et al. 2012), and these experiments could be extended to assess this although another publication suggests that this may not be true (Hotter, Kirchhoff et al. 2013).

Individuals with HIV-1 seem to be more susceptible to opportunistic infections that would normally be controlled by macrophages (Biggs, Hewish et al. 1995). Studies have reported the inhibition of phagocytosis in HIV-1 infected macrophages (Kedzierska, Ellery et al. 2002). I therefore tested the hypothesis that HIV-1infection may interfere with IFNγ signalling in macrophages due to its importance in activating macrophage functions such as phagocytosis. The major protein involved in IFNγ signal transduction is STAT1. Western blot analysis revealed that HIV-1 infection has no significant effect on STAT1 phosphorylation. This is in contrast to other reports where STAT1 phosphorylation was inhibited by HIV-1 Tat protein (Cheng, Li et al. 2009; Li, Au et al. 2011). However these studies used recombinant Tat which may be differentially glycoslyated compared to that produced in the infected cells. Additionally these studies used Tat at a minimum concentration of 0.1nM, which may be in excess of that seen in vivo.

Viral interference with IFN signalling has been reported to occur at different points in the signalling pathway. To investigate if HIV-1 could be affecting interfering further downstream, whole genome transcriptional microarrays were utilised to get an overview of the effects HIV-1 had on IFNγ responses in MDM. These showed that HIV-1 infected MDM behave in a similar fashion to uninfected MDM with both clustering together in a MDS analysis. Though there may be a HIV-1 effect at the 24 hour timepoint, this was only present in two out of four donors (Figure 53) and more data points would be required to confirm whether or not HIV-1 has an effect on gene expression responses to IFNγ stimulation.

The lack of modulation to MDM responses to IFNγ at the four hour time point suggests that HIV-1, unlike other viruses, has not evolved a mechanism to interfere with IFNγ signalling. This could be due to a number of factors. Firstly HIV-1 is able to infect MDM without initiating an immune response which could recruit and activate other immune cells to produce IFNγ. Though HIV-1 may directly activate these other cells,

macrophages may have already been infected before IFNγ is produced, and as I have shown that IFN restriction is transient, the requirement for an IFN evasion strategy in these cells may not be essential due to their long life span. Secondly selection pressure for evading IFNγ responses may also decrease as the host becomes immunocompromised and CD4+ T cell numbers decline.

Though there was no detectable changes to the MDM responses to IFNγ stimulation, HIV-1 may still have an effect on autophagy. HIV-1 could directly interact with components of the autophagy system which would not detected by screening at the gene expression level. This study could be expanded to investigate whether HIV-1 infection interacts with components essential to autophagy. It has been reported HIV-1 is dependent on modulation of the autophagy pathway to replicate in MDM. Induction of the early stages of autophagy has been reported to enhance viral replication combined with inhibition of late stage autophagy events which would lead to lysosomal degradation of HIV-1 (Kyei, Dinkins et al. 2009; Killian 2012) Additionally vitamin D3 has been shown to induce autophagy in MDM (Fabri and Modlin 2009; Yuk, Shin et al. 2009). It may be interesting to see if HIV-1 infection modulates responses to this chemical, though this may be unlikely as induction of autophagy by vitamin D3 has also been shown to inhibit HIV-1 replication. (Campbell and Spector 2011; Campbell and Spector 2012).

5.5 Conclusions

Due to the progression into AIDS by HIV-1 infection and the fact that HIV-1 can infect and replicate within macrophages without cytopathic effect maintaining a long lived viral reservoir, it was speculated that HIV-1 could modulate the innate immune responses of the host cell.

These experiments showed that HIV-1 did attenuate innate immune signalling, with HIV-1 infected MDM showing attenuated IκBα degradation, leading to decreased NFκB nuclear translocation and activation in response to minimal TLR stimuli. However despite this NFκB attenuation it appears that either the lower levels of activation is by itself enough to maintain normal immune responses or that redundancy in the immune signalling pathways, such as via p38 or ERK which was not affected by HIV-1 means that downstream cytokine secretion in response to stimulation was unaltered.

Furthermore, stimulation of MDM with IFNγ an important cytokine for macrophage functions was also unaffected by HIV-1 infection, I could find no evidence for modulation of Stat1 signalling the major transcription factor involved in IFNγ signalling. This was confirmed by whole genome transcriptional microarrays which found no differences between uninfected and HIV-1 infected cells when stimulated with IFNγ.

Overall I could find no compelling evidence that HIV-1 infection of MDM adversely affects their ability to respond to secondary infection by other pathogens, nor does HIV-1 infection alter the activation status of MDM when stimulated with IFNγ. However macrophage responses to whole pathogens is more complex than that of minimal TLR ligands and HIV-1 inhibition of multiple signalling pathways may be more potent.

6. Final discussion and future work.

6.1 Macrophage immune responses and HIV-1

This study utilizes M-CSF differentiated MDM to investigate effects of HIV-1 infection on the immune responses of macrophages. M-CSF differentiated MDM have been reported to be more permissive to infection to HIV-1 compared to GM-CSF differentiated MDM. These M-CSF differentiated MDM were indeed able to be infected with macrophage tropic HIV-1 strains with active viral replication detectable as early as three hours after infection (Chapter 3). Unlike HIV-1 infection of T cells, infection of MDM is not cytopathic, and HIV-1 infected MDM were still viable several (>6) weeks after infection when viral replication was still occurring. Interestingly despite high levels of viral replication in these cells, the steady state transcriptome was found to be relatively unaffected. Only when paired analysis of uninfected and HIV-1 infected cells from the same donor were significant differences found and even then these genes were only changed by a factor of less than two. Functional annotation clustering analysis of the significantly modified genes showed no concerted modulation of genes involved in immune responses.

The lack of apparent immune response to HIV-1 was surprising, considering the breadth of literature available reporting innate immune responses to various HIV-1 components as well a whole virions on a variety of cell types including macrophages. Therefore the ability of M-CSF MDM to mount an immune response to incoming HIV-1 was assessed (Chapter 4). In order to quantify innate immune responses in these primary cells, a confocal based microscopy assay was developed which could detect and measure the relative amounts of transcription factors present in the cytoplasm and nucleus of the cells. In this study the major transcription factors involved in innate immune responses, NFκB and IRF3 were measured in MDM exposed to HIV-1. At the time of these experiments HIV-1 was thought to be detected by ssRNA sensors such

as TLR7 and TLR8. Analysis of NFκB and IRF3 activation to a panel of TLR agonists revealed that MDM are able to respond to the TLR7/8 ligands gardiquimod and CL075 suggesting that they should also be able to detect HIV-1 ssRNA. However a variety of strains of HIV-1 did not induce immune responses in these cells. Furthermore microarray analysis of the transcriptome could find no evidence of induction of either inflammatory or IFN regulated genes. This lack of immune response was not specific to MDM differentiated with M-CSF which have been reported to be less inflammatory, as both GM-CSF differentiated MDM and MDM grown in serum alone also did mount immune responses to HIV-1. This lack of immune response did not appear to be due to viral suppression as a HIV-1 based vector lacking the HIV-1 accessory genes, which have been reported to inhibit immune responses, did not induce immune responses. Additionally IFNβ priming which upregulated expression of a number of PRRs including those involved in sensing RNA did not sensitise MDM to HIV-1, though it did enhance the production of IFN in response to stimulation with poly I:C. HIV-1 virus which had not undergone purification by ultracentrifugation through sucrose did induce immune responses which may partially explain some of the literature where HIV-1 has been reported to induce immune responses in MDM. Though one early publication suggested that as monocyte differentiate into macrophages they lose the ability (Francis, Fan et al. 1996) the vast majority of the literature reports that HIV-1 infection of macrophages does stimulate immune responses. More recently however other groups have replicated our data. One group reported that HIV-1 infection does not induce expression of inflammatory cytokine expression although they did find upregulation of programmed cell death 1 ligand 1 (PDL1) and PDL2 (Rodriguez-Garcia, Porichis et al. 2011). Another publication only found very low levels of ISG56 induction (around 4 fold increase compared to unstimulated cells) after HIV-1 infection which was around 100 fold less than that seen when stimulated with sendai virus, however this was induced using an amount of HIV-1 correlating to 5,000 × TCID50 compared to a MOI of 0.5 for sendai virus (Diget, Zuwala et al. 2013).

The lack of immune response to HIV-1 by MDM led to the hypothesis that MDM may lack the required PRR to detect HIV-1. The lack of TLR7 expression on MDM compared with pDCs and monocytes, which are both able to mount immune responses against HIV-1, suggest that this may be the PRR required for detecting HIV-1. Attempts to knockdown TLR7 by siRNA as well as exogenous expression of TLR7 were unsuccessful. Articles published after this work reported TLR7 dependent and independent immune responses in pDCs, and also that in TLR7 negative cells viral access to the cytoplasm was required for activation of IRF3 (Lepelley, Louis et al. 2011). In monocytes and mDCs HIV-1 derived TLR7/8 ligands were reported to induce distinctly different cytokine profiles compared to whole HIV-1 virions suggesting that pathways other than TLR7/8 are able to mediate immune responses to HIV-1 (Simmons, Scully et al. 2013). Further studies point towards cytosolic DNA sensors (Yan, Regalado-Magdos et al. 2010; Hrecka, Hao et al. 2011; Laguette, Sobhian et al. 2011; Lahouassa, Daddacha et al. 2012) which detect the HIV-1 RT products. Recently the Noursadeghi lab in collaboration with the Towers lab has discovered that HIV-1 evasion of the innate immune response is dependent on capsid recruitment of the host cellular proteins, cyclophillins and CPSF6 which are involved in viral uncoating and nuclear entry (Rasaiyaah, Tan et al. 2013). Capsid mutants which are impaired in their ability to bind these host proteins activate NFκB and IRF3 leading to the expression of IFNβ. This immune response is dependent on reverse transcription suggesting HIV-1 RT products are the PAMP responsible for induction of the immune responses in MDM.

The functional consequences of HIV-1 being able to evade an innate immune IFN response in macrophages were examined. Both type I and type II IFNs were able to potently restrict HIV-1 BaL in MDM despite the presence of the HIV-1 accessory proteins which have been documented to counteract various IFN inducible restriction factors. This restriction was not specific to the laboratory strain of HIV-1 used as

restriction was demonstrated against a variety of strains of HIV-1 including a primary clinical isolate. To see if physiological levels of IFN could also restrict HIV-1, poly I:C, as a mild stimulator of IFNβ, was added at the same time as HIV-1, stimulating an endogenous innate immune IFN response. This also was able to restrict HIV-1, suggesting that if MDM could detect incoming HIV-1 then the immune response would be able to restrict the virus. Subsequent to this work, other groups have also shown the ability of TLR ligands to inhibit HIV-1 infection in MDM (Campbell and Spector 2012; Victoria, Temerozo et al. 2013). The restriction of HIV-1 may be modulated by the newly discovered restriction factor Mx2, which is thought to recognize HIV-1 via CypA (Liu, Pan et al. 2013), for which no HIV-1 countermeasure has yet been described. However it is probably more likely to be an as of yet unidentified restriction factor which is expressed specifically in myeloid cells or macrophages. One group showed that TLR stimulation of MDM restricts HIV-1 but the same is not true for TLR stimulated PBLs (Wang, Chao et al. 2011) though at least some of the cells in the PBL mixture, such as pDCs, can already mount TLR responses to HIV-1. Additionally supernatant from TLR stimulated MDM can induce this antiviral state in other MDM but not PBLs.

The consequences of the lack of immune response upon encountering the virus can be seen when type I IFN is added to MDM with established HIV-1 infection. Though viral replication is clearly inhibited by IFN stimulation of MDM, this was found to be transient with viral replication rebounding to normal levels within a week. Interestingly IFNβ stimulation of MDM as long as three weeks before HIV-1 infection was still able to restrict HIV-1. As macrophages are long lived cells, prior exposure to IFN may be possible due to immune responses to other pathogens.

HIV-1 infected individuals have been reported to be more susceptible to opportunistic infections with pathogens normally controlled by the immune system. Though this may be due to the loss of CD4+ T cells, macrophages have an important part to play in the

immune system both as a direct effector cell as well as recruiting and activating the adaptive immune response. Therefore the ability of HIV-1 infected cells to respond normally to stimuli was examined (Chapter 5). HIV-1 infection of MDM did not appear to effect responses to IFNγ. Though when I tested the response to LPS as a surrogate for a bacterial pathogen, HIV-1 infection was found to impair the degradation of IκB and subsequently inhibit NFκB activation and nuclear translocation. This inhibition of NFκB was also evident when MDM were infected with other strains of HIV-1 and stimulated with an alternative TLR stimulus. This inhibition of NFκB signaling led to a modest decrease the expression of a subset of genes expressed upon LPS stimulation. Although modest decreases in the levels of mRNA were found for a number of cytokines, no differences were seen at the level of protein expression. This lack of attenuation of cytokine secretion may have been due to redundant innate immune signaling pathways compensating for the attenuated NFκB response and/or due to the dose of stimuli used. Additionally use of whole pathogens may be more informative as HIV-1 may attenuate signaling via multiple pathways.

Taken altogether these data support the theory that macrophages are an important target for HIV-1. They are permissive cells at the site of infection. They also contribute to the spread of HIV-1 by disseminating virus to other cells by cell to cell contact. Macrophages may also be involved in the immunodeficiency seen in AIDS. Along with the potential impairment of macrophage functions and loss of CD4+ Tcells by direct infection, HIV-1 infected macrophages cause bystander cell apoptosis in uninfected T cells and have also been reported to impair B cell functions by the transfer of HIV-1 Nef protein to the uninfected B cells.

Macrophages are also an important reservoir for HIV-1. They are long lived cells and viral replication occurs without causing cell death unlike in T Cells. Macrophages also do not mount innate immune responses to the incoming virus which can potently restrict HIV-1. HIV-1 in macrophages localises in a specialised plasma membrane

invagination which is protected from neutralizing antibodies (Koppensteiner, Banning et al. 2012) and after antiretroviral therapy HIV-1 is often detectable in macrophages when it has been cleared from other cell types. Also even though IFN does reduce viral replication in HIV-1 infected MDM, I have found that this restriction is transient in an established infection model. Finding a way to prevent HIV-1 infection of macrophages could therefore be important in treating HIV-1.

6.2 Future Work

The work in this thesis as well as recent publications has opened up interesting avenues which can be pursued.

The mechanism by which HIV-1 evades innate immune responses in MDM has been recently been discovered by the Noursadeghi lab in collaboration with the Towers lab following from the work presented in this thesis. HIV-1 capsid specifically interacts with the host cell factors, cyclophillins and CPSF6 which are involved in capsid uncoating and nuclear transport, bypassing the cytoplasmic DNA sensors which can detect HIV-1 RT products (Rasaiyaah, Tan et al. 2013).

One question raised by these findings is if this evasion mechanism is active in other cell types such as pDCs. It is evident that TLR7 is a receptor for HIV-1 as can be seen by pDC responses to the virus as well as HIV-1 derived ssRNA sequences. Would the immune response in pDCs be suppressed if this evasion mechanism was active in pDCs, or does viral replication and the production of viral ssRNA activate TLR7 despite evading this PRR in the early part of the viral lifecycle? Therefore it is of interest to investigate the mechanism for the loss of TLR7 expression on monocytes as they differentiate into MDM. As macrophages are sentinel cells of the immune system why does it lose expression of TLR7? Is the reduction in TLR7 expression linked to the decrease in cell surface CD14 expression as the cells differentiate? CD14 is known to be a coreceptor for TLR2, TLR4 and more recently TLR7 and TLR8 so a reduction in CD14 expression could impact the activity of these TLRs. It would be interesting to investigate whether the decreased expression of CD14 affects the sensitivity and signalling activity of these TLRs by further reducing CD14 expression using siRNA. Conversely would overexpression of CD14 by lentiviral transduction enhance TLR signalling in response to stimulation, specifically to TLR7 ligands?

Are other mechanisms involved in HIV-1 evasion of macrophage immune responses? It would be interesting to knockdown SAMHD1 to see if like in DCs it would enable MDM to mount immune responses to HIV-1 RT products. Or are the RT DNA products confined to the nucleus in these cells?

The role of macrophages in HIV-1 infection is still debated; there are arguments for and against macrophages as one of the initial targets for infection during transmission. The recent advances in chimeric mice which have a functional human immune system (Dudek, No et al. 2012) may provide the perfect opportunity to examine the role of macrophages in HIV-1 infection in an *in vivo* setting. Chimeric mice with humanised immune systems have been reported to support HIV-1 infection and replication (An, Poon et al. 2007; Dudek, No et al. 2012). Recently in the BLT mouse model (NOD/SCID mice reconstituted with a human immune system as well containing transplanted human bone marrow, liver and thymus tissue), immune responses to HIV-1 infection has been described in as well as HIV-1 adaptations to immune selection mirroring the response seen in acute infection in humans (Dudek, No et al. 2012). The female reproductive tract of BLT mice are also reconstituted with human immune cells and supports productive HIV-1 infection (Denton, Estes et al. 2008). If macrophages can be selectively depleted in these chimeric mice then the role of macrophages during transmission and progression of HIV-1 disease could be assessed. The importance of evading macrophage immune responses in the context of viral transmission could also be investigated by infecting humanised mice with HIV-1 capsid mutants known to induce immune responses in macrophages.

The lack of IFN response by MDM combined with the findings in this study and that of others which report inhibition of HIV-1 infection in MDM by TLR stimulation suggests that TLR ligands could be used as a potential treatment to control HIV-1 infection along with antiretrovirals. The mechanism by which IFN restricts HIV-1 in macrophages is also worth investigating. Despite expressing accessory proteins which are able to

counteract some of the IFN inducible restriction factors, IFN still potently restricts HIV-1 in MDM. Is this restriction due to the activities of Mx2, or are there other yet unidentified restriction factors involved? Also what is the biological significance of IFN restriction of HIV-1 in macrophages *in vivo*? Will exposure of macrophages to IFN before exposure to HIV-1 protect against infection. This could be investigated using humanised mouse models. These mouse models could also be used to reassess the viability of using IFN or TLR ligands as a treatment for HIV-1 infection in combination with currently used antiretrovirals.

Further work needs to be performed looking at HIV-1 effects on IFNγ stimulation. Does HIV-1 affect gene expression after IFNγ stimulation after 24 hours? If the data presented in figure 53 is representative, what is the mechanism by which HIV-1 interferes with gene expression in response to IFNγ stimulation and why does it only affect MDM from some donors and not others? Even if HIV-1 does not affect gene transcription, it may still modulate macrophage effector mechanisms; HIV-1 infection has been reported to inhibit macrophage responses to intracellular pathogens as well as interfering with autophagy.

Finally although there was no significant effect of HIV-1 infection on MDM cytokine secretion to stimulation by the TLR ligand LPS, despite attenuation of NFκB activation, immune responses to whole pathogens are more complex with activation of multiple PRRs. Recent work by our lab has shown HIV-1 infection does modulate innate immune responses to *Mycobacterium tuberculosis* (Mtb). HIV-1 attenuated both p38 and ERK phosphorylation in response to Mtb stimulation, leading to augmentation of the proinflammatory response to Mtb by attenuating expression of IL-10 (Submitted). These studies could be extended to other live organisms such as influenza and other viruses, as well as investigating if other macrophage functions are affected by HIV-1 infection. Does HIV-1 infection affect macrophage intracellular killing, antigen

presentation or apoptosis after exposure to live organisms both before and after stimulation with IFNγ which is an important regulator of macrophage functions?

7. Publications

Data generated during this thesis have contributed to the following publications

Chain, B., H. Bowen, J. Hammond, W. Posch, J. Rasaiyaah, J. Tsang and M. Noursadeghi (2010). "Error, reproducibility and sensitivity: a pipeline for data processing of Agilent oligonucleotide expression arrays." BMC Bioinformatics **11**: 344.

Chain, B. M., M. Noursadeghi, M. Gardener, J. Tsang and E. Wright (2008). "HIV blocking antibodies following immunisation with chimaeric peptides coding a short N-terminal sequence of the CCR5 receptor." Vaccine **26**(45): 5752-5759.

Noursadeghi, M., J. Tsang, T. Haustein, R. F. Miller, B. M. Chain and D. R. Katz (2008). "Quantitative imaging assay for NF-kappaB nuclear translocation in primary human macrophages." J Immunol Methods **329**(1-2): 194-200.

Noursadeghi, M., J. Tsang, R. F. Miller and D. R. Katz (2008). "Comment on "Transcription factor FOXO3a mediates apoptosis in HIV-1-infected macrophages"." J Immunol **180**(12): 7783; author reply 7783-7784.

Noursadeghi, M., J. Tsang, R. F. Miller, S. Straschewski, P. Kellam, B. M. Chain and D. R. Katz (2009). "Genome-wide innate immune responses in HIV-1-infected macrophages are preserved despite attenuation of the NF-kappa B activation pathway." J Immunol **182**(1): 319-328.

Tomlinson, G. S., L. C. Bell, N. F. Walker, J. Tsang, J. S. Brown, R. Breen, . . . M. Noursadeghi (2013). "HIV-1 Infection of Macrophages Dysregulates Innate Immune Responses to Mycobacterium tuberculosis by Inhibition of Interleukin-10." J Infect Dis.

Tomlinson, G. S., T. J. Cashmore, P. T. Elkington, J. Yates, R. J. Lehloenya, J. Tsang, . . . M. Noursadeghi (2011). "Transcriptional profiling of innate and adaptive human immune responses to mycobacteria in the tuberculin skin test." Eur J Immunol **41**(11): 3253-3260.

Tsang, J., B. M. Chain, R. F. Miller, B. L. Webb, W. Barclay, G. J. Towers, . . . M. Noursadeghi (2009). "HIV-1 infection of macrophages is dependent on evasion of innate immune cellular activation." AIDS **23**(17): 2255-2263.

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