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The Influence of Antiviral Therapy and HIV/HCV Proteins on Matrix Metalloproteinase and Cytokine Production – an *in vitro* and *in vivo* study

A thesis submitted to the

University of Dublin,

Trinity College

In fulfilment of the requirement

for the degree of

Doctor of Philosophy

by

Alan Kennedy

BSc

2012

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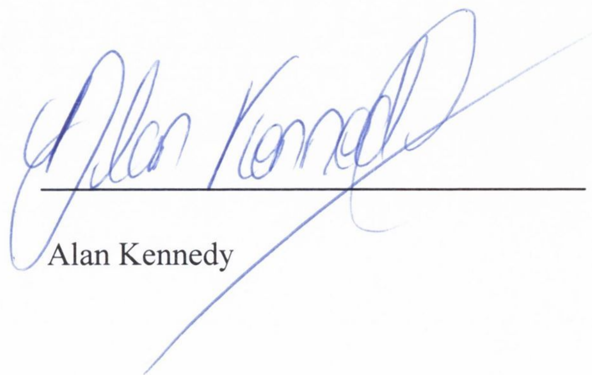

Alan Kennedy

Summary Methods and Results

The effect of antiviral therapy on MMP/TIMP expression in THP-1 and LX-2 cells and in HIV/HCV co-infected patients: MMP-9 activity was measured by gelatin zymography, MMP-9 mRNA expression by real-time RT PCR, and MMP-9, MMP-2, and TIMP-1 protein expression by ELISA. Cells were treated for 48 hr unless otherwise stated. In THP-1 cells, interferon-α2a (IFN-α2a) dose-dependently decreased MMP-9 activity relative to PMA (50 ng/ml) controls ($P<0.05$). IFN-α2a (250 IU/ml) alone, or in combination with ribavirin (RBV; 10 μM), decreased MMP-9 activity compared to PMA (44±4.2 and 60±1.4 versus 100±3.1 AU; $P<0.05$) while RBV increased MMP-9 activity by ~50 % ($P<0.05$). At the mRNA level, RBV alone and in combination with IFN-α2a increased MMP-9 expression by ~2.5 fold compared to PMA controls (771±116, 772±42 versus 488±45, $P<0.05$), while IFN-α2a had no effect. Investigating this disparity between extracellular MMP-9 activity and intracellular MMP-9 mRNA it was found that RBV caused a robust increase in intracellular MMP-9 protein levels, while IFN-α2a had no effect. However, in combination with RBV, IFN-α2a reduced the RBV-mediated increases from 5.5±0.4 to 3.3±0.4 ng/ml ($P<0.05$). Co-treatment of THP-1 cells with the proteasome inhibitor MG132 (200 nM) increased ($P<0.05$) MMP-9 activity compared to PMA alone (178±23 versus 100±6.1 AU). However, it did not alter the effect of IFN-α2a on RBV-mediated increased MMP-9 activity in THP-1 cells. However, assessment of the temporal effects of IFN-α2a on MMP-9 mRNA expression revealed an ~65 % reduction ($P<0.05$) at 24 hr compared to PMA controls, while no effect at 48 and 72 hr were recorded. RBV, IFN-α2a, and the combination of both drugs, did not affect cell viability, or the process of differentiation from monocytes to macrophages, at the concentrations used in this study.

In LX-2 cells, IFN-α2a alone, or in combination with RBV, did not alter MMP-9 activity. However, RBV increased MMP-9 activity compared to PMA (134±4.1 versus 100±0.9

AU; $P<0.05$). RBV and IFN-α2a alone, or in combination, did not alter MMP-9 mRNA expression compared to PMA controls.

Saquinavir (SQV; 5 μM) and lopinavir (LPV; 10 μM) decreased ($P<0.05$) MMP-9 activity in THP-1 and LX-2 cells, respectively. Abacavir, zidovudine, efavirenz, nevirapine, and atazanavir had no effect in either cell line. Both, SQV and LPV had no effect on cell viability at the concentrations studied.

MMP-9, activity was 4 fold ($P<0.05$) higher in HIV/HCV co-infected and HIV mono-infected patients than in healthy controls (86±16 and 82±15 versus 21±4.7 AU). In HCV mono-infected patients MMP-9 activity was similar to that of healthy controls (32.9±5.1 versus 21±4.7 AU). MMP-9 protein expression was also higher in HIV/HCV co-infected patients (367.6±73.6 ng/ml) and HIV mono-infected patients (332.2±54.1) compared to healthy controls (129±23.9 ng/ml; $P<0.05$). MMP-2 activity was similar in all groups studied, as was TIMP-2 expression. In HIV/HCV co-infected patients, RBV/PEG-IFN-α2b decreased ($P<0.05$) plasma MMP-9 activity by ~70 % (Baseline versus Day 14: 98.8±17.3 vs 28.1±11.6 AU; $P<0.05$). MMP-9 protein expression was also reduced (Baseline versus Day 14: 458.7±95.7 vs 120.4±46.8 ng/ml; $P<0.05$). MMP-2 activity and TIMP-2 expression was not altered following treatment with RBV/PEG-IFN-α2b in the HIV/HCV co-infected group.

The effect of HIV-1 Tat clades B and C and HCV NS3 on MMP-9, TIMP-1, TNF-α and IL-1β abundance in THP-1 cells: MMP/TIMP and cytokine expression was measured by ELISA and MMP-9 activity by gelatin zymography. Cells were exposed for 48 hr to Tat clades and NS3 at concentrations of 37.5, 75, 150, 300, and 400 ng/ml, and 0.5, 1, 5, and 10 μg/ml, respectively. HIV-1 Tat clade B increased MMP-9 expression at 300 and 400 ng/ml concentrations compared to PMA (20 ng/ml) controls (1.3±0 and 1.5±0

versus 1±0 fold induction; $P<0.05$) but had no effect on MMP-9 activity at any concentration studied. TIMP-1 expression was increased by Tat B at 300 and 400 ng/ml, with a >6 fold increase at the latter concentration compared to PMA (4.4±0.52 and 7.1±1.7 versus 0.99±0 fold induction; $P<0.05$). Tat B increased the expression of TNF-α dose-dependently against PMA (1.9±0.1, 1.9±0, 2.9±0.1, 3.3±0, and 4±0.2 versus 1±0.1 fold induction; $P<0.05$).

Over the concentration range studied, Tat clade C did not alter MMP-9 expression or activity. Tat clade C caused a subtle dose-dependent increase in TIMP-1 expression, reaching significance at concentrations of 150, 300, 400 ng/ml compared to PMA (1.9±0.2, 2+0.1, and 2.4±0.2 versus 1±0.1 fold induction; $P<0.05$). TNF-α production was not altered by Tat clade C against PMA controls.

HCV NS3 protein increased MMP-9 expression at 5 and 10 μg/ml compared to PMA (1.9±0.1 and 2.6±0.3 versus 1±0.2 fold induction; $P<0.05$) and increased MMP-9 activity at 10 μg/ml (1.6±0 versus 1±0 fold induction; $P<0.05$). NS3 also increased TIMP-1 expression at concentrations of 1, 5, and 10 μg/ml, compared to PMA controls, reaching significance at the latter two concentrations (4.7±0.9, and 4.4±1.3 versus 0.7±0.1 fold induction; $P<0.05$). NS3 induced TNF-α expression >3 fold at the highest concentration studied (10 μg/ml) compared to PMA (4.3±0.6 versus 1±0.6 fold induction; $P<0.05$). Finally, NS3 increased IL-1β expression in a dose-dependent manner at concentrations of 1, 5, and 10 μg/ml against PMA controls (1.8±0, 3.2±0, and 4.2±0.1 versus 1±0 fold induction; $P<0.05$).

The effect of HIV-1 Tat clades A, B, C, and D on IFN-γ and TNF-α production by $CD3^+$ T cells and Vγ9Vδ2 T cells: The percentage of cells staining positive for IFN-γ and TNF-α was determined by flow cytometry while cytokine secretion was quantified by

ELISA. Cells were exposed to Tat clades for 48 hr. The percentage of $CD3^+$ T cells that stained positive for IFN-γ were similar to background under non-stimulated conditions and HMB-PP/IL-2 (10 nM/50 U/ml)-stimulated conditions. PMA/I (10 ng/ml/1 μg/ml) stimulation increased ($P<0.05$) the number of cells staining positive for IFN-γ (38±6.8 versus 0.4±0.1 and 1.3±0.5 %). The percentage of TNF-α positive cells was also minimal in unstimulated and HMB-PP/IL-2-stimulated cells but increased in cells exposed to PMA/I (41±9 versus 0.53±0.2 and 1.4±0.6 %; $P<0.05$). When $CD3^+$ T cells were exposed to Tat clades A, B, C, and D (200 ng/ml) under either basal, or stimulated conditions, there was no effect on the percentage of cells producing IFN-γ or TNF-α.

The mean percentage of Vγ9Vδ2 T cells staining positive for IFN-γ under basal conditions was 2.0±0.4 % and was not altered by exposure to any Tat clades. Stimulation with HMB-PP/IL-2 induced an ~24 fold increase in IFN-γ staining (48±14 versus 2.0±0.4 %; $P<0.05$). PMA/I stimulation caused an ~34 fold increase in IFN-γ staining (67±14 versus 2.0±0.4 %; $P<0.05$). As in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter IFN-γ positive cells at the concentration studied. Mean percentage of Vγ9Vδ2 T cells staining positive for TNF-α under basal conditions was 2.9±0.6 % and was not altered by exposure to any of the Tat clades investigated. HMB-PP/IL-2 stimulation caused an ~14 fold increase in TNF-α staining (41±12 versus 2.9±0.6 %; $P<0.05$). PMA/I stimulation caused an ~24 fold increase in TNF-α staining (71±12 versus 2.9±0.6 %; $P<0.05$). As in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter the percentage of TNF-α positive cells at the concentration studied.

In unstimulated $CD3^+$ T cells, Tat clades A, B, C, and D did not alter IFN-γ production compared to untreated (medium) controls (49±1.6, 44±6.4, 46±3.5, and 35±2.9 versus 43±3.8 pg/ml respectively). Following stimulation by HMB-PP/IL-2, IFN-γ levels did not increase significantly. Tat clades A, C, and D did not alter IFN-γ secretion, but Tat clade B

elicited an ~fold increase in IFN-γ levels relative to HMB-PP/IL-2 controls (105±4.2 versus 57±5.9 pg/ml; $P<0.05$). PMA/I stimulation induced IFN-γ secretion (734±17 versus 43±3.8 pg/ml; $P<0.05$), however, this was not altered by any of the Tat clades investigated. TNF-α was undetectable under non-stimulated conditions. Exposure of cells to Tat clades at the concentrations studied did not induce TNF-α to detectable levels. Stimulation with HMB-PP/IL-2 induced ($P<0.05$) TNF-α secretion (252±33 pg/ml). However, none of the Tat clades studied altered this response. PMA/I also increased TNF-α production (752±13 pg/ml; $P<0.05$), and the response was not altered by the presence of Tat clades investigated.

In Vγ9Vδ2 T cell lines Tat clades A, B, C, and D significantly ($P<0.05$) increased IFN-γ secretion compared to untreated cells, with clade B generating the largest increase (26±1.5, 48±1.4, 24±1.2, and 22±2.7 versus 13±0.8 pg/ml). Re-stimulation with HMB-PP/IL-2 resulted in an ~4 fold increase of IFN-γ levels (51±8.7 versus 13±0.8 pg/ml; $P<0.05$), and under these conditions, Tat clade B, but not A, C, or D, further increased secretion (117±5.7 versus 51±8.7 pg/ml; $P<0.05$). PMA/I re-stimulation also resulted in increases of IFN-γ secretion by these cells (80±20 versus 13±0.8 pg/ml; $P<0.05$), and again Tat clade B, but not A, C, or D, induced an ~4 fold IFN-γ production compared to PMA/I controls (326±5.2 versus 80±20 pg/ml; $P<0.05$).

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Publications and Presentations

Publications

Kennedy A, Hennessy, M, Bergin, C, Mulcahy, F, Hopkins, S, Spiers JP. **Ribavirin and interferon alter MMP-9 abundance *in vitro* and in HIV/HCV co-infected patients.** *Antiviral Therapy 2011; **16**:1237-1247 (doi: 10.3851/IMP1867)*

Kennedy A, Hennessy M, Spiers, JP. **Differential MMP and cytokine regulation by HIV-1 Tat clades and HCV NS3 in THP-1 cells.** *In preparation*

Kennedy A, Doherty DG, Hennessy M, Spiers, JP. **HIV-1 Tat displays clade-specific induction of interferon-γ in human T cells and Vγ9Vδ2 T cells.** *In preparation*

Abstracts

Oral Presentations:

Infectious Diseases Society Ireland Annual Scientific Meeting, Dublin, Rep of Ireland, 7-8th June 2011 **HIV-1 Tat clade B increases interferon-γ production by γδ T cells.**

Poster Presentations:

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Awards

Roche Young Investigators Participation Grant, 1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Barcelona, Spain, 19-22 May, 2011

Abbreviations

ABC	Abacavir
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Allophycocyanin
ART	Antiretroviral therapy
ARV	Antiretroviral
AST	Aspartate aminotransferase
ATV	Atazanavir
AU	Arbitrary Unit
BSA	Bovine serum albumin
CCl_4	Carbon tetrachloride
CCR5	C-C chemokine receptor 5
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
C_{final}	Final concentration
C_{max}	Maximum concentration

CXCR4	C-X-C chemokine receptor 4
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
E.coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EFV	Efavirenz
ELISA	Enzyme-linked immuno-sorbant assay
ERK 1/2	Extracellular signal-regulated kinase 1/2
EtBr	Ethidium bromide
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HAART	Highly active antiretroviral therapy
HAD	Human immunodeficiency virus-associated dementia
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMB-PP	(*E*)-4-hydroxy-3-methyl-but-2 enyl pyrophosphate
HRP	Horseradish peroxidise
HSC	Hepatic stellate cell
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-λ	Interferon lambda
IgG	Immunoglobulin G
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
IL	Interleukin
IPP	Isopentenyl pyrophosphate
IU	International unit
IVDU	Intravenous drug use
kDa	Kilodalton
LPS	Lipopolysaccharide
LPV	Lopinavir
LTR	Long terminal repeat
M	mol/L
mAb	Monoclonal antibody

MAPK	Mitogen-activated protein kinase
MFI	Mean fluoresence intensity
MHC	Major histocompatibilty complex
MIPα/β	Macrophage inhibitory protein alpha/beta
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transcriptase inhibitor
NVP	Nevirapine
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PEG	Pegylated
PI	Protease inhibitor
PKA/C/R	Protein kinase A/C/R
PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated on activation and normal T cell expressed and secreted

RBV	Ribavirin
RFLP	Restriction fragment length polymorphism
RIBA	Recombinant immunoblot assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SQV	Saquinavir
TAE	Tris-acetate-EDTA buffer
TCR	T cell receptor
TE	Tris-EDTA buffer
Th	T helper
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor alpha
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v/v	volume/volume
w/w	weight/weight
ZDV	Zidovudine

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Chapter 1.

Introduction

1.1 Human Immunodeficiency Virus-1 (HIV-1)

1.1.1 Origin, classification and structure

Acquired immunodeficiency syndrome (AIDS) was first reported in the United States of America in 1981 and, following a subsequent period of intensive research, human immunodeficiency virus (HIV) was identified as the causative agent. Originally defined as lymphadenopathy-associated virus (LAV) and human T-lymphotropic virus type III (HTLV-III) [1, 2], the virus was most likely brought from Central Africa to Haiti, then from Haiti to the USA, before eventually spreading worldwide [3]. There are two major subtypes of the virus, HIV-1 and HIV-2, believed to have emanated from strains of simian immunodeficiency virus (SIV) in chimpanzees and sooty mangabey monkeys, respectively [4]. The predominant agent of global HIV infection is HIV-1, with HIV-2 being restricted mainly to specific regions of Western and Central Africa and perceived as less virulent [5]. The virus is classified as a member of the Lentivirus genus of the *Retroviridae* family and, typical of such viruses, disease progresses over a chronic course, with a long period of clinical latency and persistent viral replication. The retrovirus genome features two identical copies of single-stranded RNA molecules and is characterised by the presence of the structural genes Gag, Pol, and Env. The Gag gene encodes the structural proteins of the core (p24, p7, p6) and matrix (p17) and the Env gene encodes the envelope glycoproteins gp120 and gp41, responsible for the recognition of cell surface receptors. The Pol gene encodes for enzymes that are critical for successful viral replication, namely reverse transcriptase (responsible for the conversion of viral RNA into DNA), integrase (incorporates viral DNA into host chromosomal DNA), and protease (cleaves large Gag and Pol protein precursors into their components). A complement of three additional regulatory proteins, Tat, Rev, and Nef, and three accessory proteins, Vif, Vpr, and Vpu, not

found in other retroviruses, also have a fundamental role in the modulation of viral replication.

1.1.2 Replication cycle

HIV entry into target cells is initiated by gp120 binding to the monomeric glycoprotein CD4 receptor and one chemokine co-receptor, either CXCR4 or CCR5 [6] (Figure 1.1). The stability afforded by this double bind facilitates the N-terminal fusion peptide gp41 in successful penetration of the cell membrane. CD4 is expressed on the cell surface of the majority of circulating T lymphocytes, on T cell precursors within the bone marrow and thymus, on monocytes/macrophages, eosinophils, dendritic cells and microglial cells of the central nervous system. However, CXCR4 and CCR5 are differentially expressed on HIV cellular targets, giving rise to the phenomenon of viral tropism [7]. CXCR4 is present on many cells including T lymphocytes, while CCR5 is more abundant on monocytes/macrophages, dendritic cells and activated T lymphocytes. Preferential binding to either receptor therefore, distinguishes HIV-1 strains that are T lymphocyte-tropic (T-tropic) or macrophage-tropic (M-tropic), which is manifest in differential disease pathogenicity and progression [8].

Subsequent to the fusing of viral and cell membranes, the viral core is released into the cytoplasm of the cell where the process of uncoating liberates viral RNA. Through its ribonuclease H active site, the reverse transcriptase enzyme initiates the conversion of the viral RNA genome into full length, double-stranded, pro-viral DNA [9], which is in turn inserted into the host chromosome by the action of the enzyme integrase [10]. Upon cell activation, transcription of pro-viral DNA into mRNA is stimulated by the binding of Tat to the transactivation response element (TAR) site of the long terminal repeat (LTR), facilitating the formation of longer RNA transcripts and increasing the production of viral

mRNA several hundred fold [11]. Rev is responsible for the subsequent transportation of viral mRNA from the nucleus to the cytoplasm and the expression of the structural proteins Gag, Pol and Env. The Nef regulatory protein is considered a major virulence factor as it perpetuates high viral loads through Fas L-mediated apoptosis of uninfected bystander $CD4^+$ T cells [12]. It also facilitates infected cells in evading the host cellular immune response by downregulating cell surface CD4 and class I major histocompatibility (MHC) molecules [13]. Similarly, accessory proteins Vif, Vpr, and Vpu counteract innate antiretroviral factors such as the APOBEC3G enzyme [14], induce T cell depletion [15], and antagonise the antiviral function of tetherins, proteins which retard the cell surface release of virions [16], respectively.

Successfully released virions from the infected cell, emerge by budding as immature viral particles which, when the Gag polyprotein is cleaved by HIV-1 protease, undergo marked morphological change resulting in the formation of mature, infectious virus particles. The final virion is composed of an internal cone-shaped capsid built from $p24_{gag}$ capsid protein surrounded by the viral lipid envelope and scaffolded by matrix protein $p17_{gag}$ spiked with glycosylated gp160 (gp120 and gp41) [17].

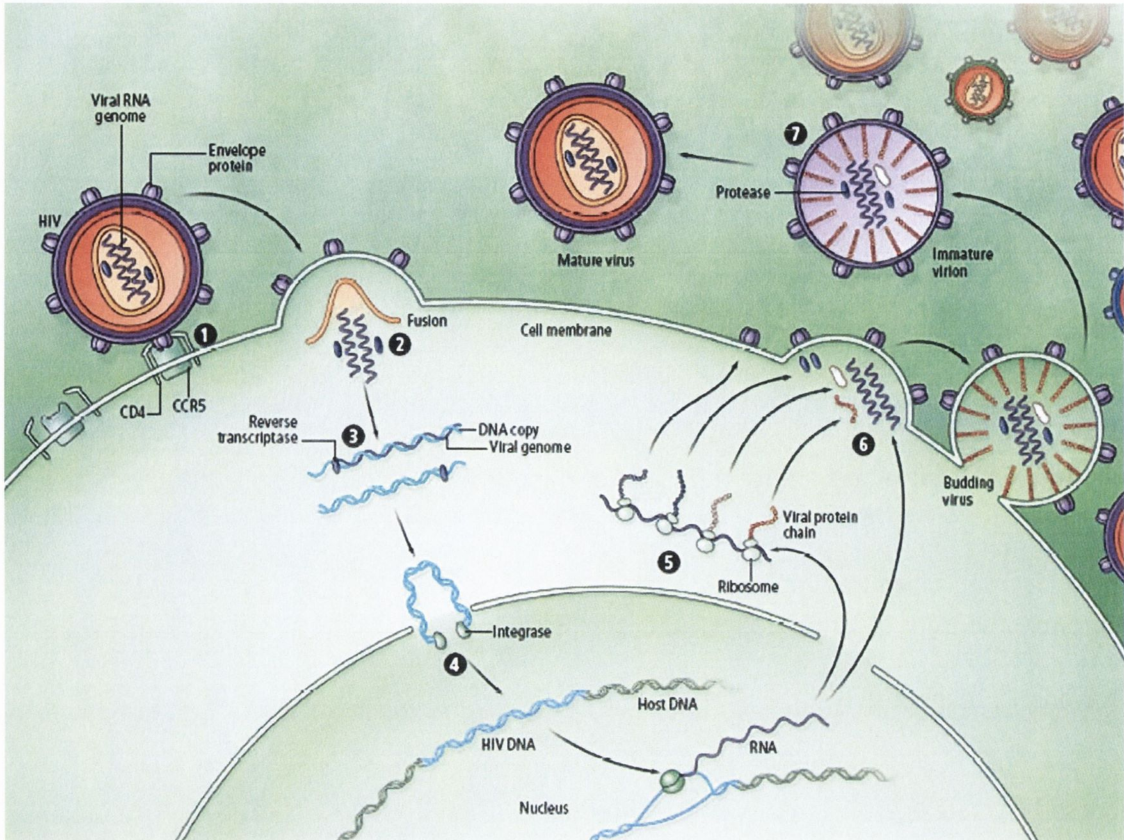


Figure 1.1 Schematic representation of the HIV-1 replication cycle. Binding of envelope proteins to CD4 and CCR5 cell surface receptors facilitates viral attachment (**1**). Fusion of the virus with the cell releases its contents into the cytoplasm (**2**). Reverse transcriptase (RT) initiates the reverse transcription of viral RNA into double-stranded DNA (**3**). Integrase then transfers the copy DNA into the cell nucleus and facilitates its integration into the host cell genome (**4**). Cellular machinery transcribes pro-viral DNA into mRNA that migrates to the cytoplasm, where ribosomes produce the encoded proteins (**5**). Viral RNA and replicative enzymes then move toward the cell membrane, where they form a budding virus particle (**6**). Modification of viral protein chains by the protease enzyme enables virions to mature into a form capable of infecting a new cell (**7**). Diagram adapted from Watkins, 2008 [18].

1.1.3 Distribution

In 2009, UNAIDS estimated that 33.3 million people worldwide were HIV-1 infected and that 2.6 million new cases of infection occurred that year (UNAIDS Report on the Global AIDS Epidemic, 2010). The same organisation state that HIV-1 is currently the fourth biggest killer in the world, with an annual death toll of about 2 million, the vast majority of which occur in sub-Saharan Africa where an estimated 22.5 million individuals have contracted the virus. The worst affected countries include South Africa, Botswana, Mozambique, Zimbabwe, United Republic of Tanzania and Ethiopia, where in some cases, the prevalence of infected adults can be greater than 10%. The estimated number of people currently living with HIV in Asia and the Pacific region is >4 million, in Latin America and the Caribbean, 1.4 million, in Eastern Europe and Central Asia, 1.4 million, in Western and Central Europe, 820,000, and in North America, 1.5 million.

1.1.4 Diversity

HIV-1 is characterised by an extensive genetic diversity, producing variants of distinct molecular subtypes and recombinant forms that are unevenly distributed throughout the world. Such variability is a distinct advantage for the virus in evading the host immune response, and to counter the effects of therapeutic and prophylactic measures [19]. Contributory factors to HIV variability are; an extremely high degree of error in the proof-reading capacity of the reverse transcriptase enzyme, introducing, on average, one substitution per genome per replication round [20], the rapidity of viral replication, estimated at 10^{10} virions / day in the infected individual [21], and the potential for an infective virus to mutate within the host, leading to the expression of a series of related molecular clones [22].

Three classes of HIV-1 have emerged across the globe: M (major), O (outlier) and N (non-M/non-O). Group M, which accounts for >90% of documented HIV-1 infections worldwide, can be further subdivided into 9 phylogenetic subtypes, or clades, of A-D, F-H, and J and K (Figure 1.2), in addition to several circulating recombinant forms [23]. HIV-1 clade B predominates in Western industrialised nations, and in Latin America and the Caribbean, but represents a mere 11% of global infections. In contrast, clade C comprises some 48% of worldwide infections, and is the predominant subtype in Southern and Eastern Africa and India [24]. Within group M, average inter-subtype genetic variability is 15% for the Gag gene and 25% for the Env gene [25]. Furthermore, there are emerging subtypes within subtypes, appearing to be phylogenetically more closely related to each other than to other subtypes, with, for example, clades A and F currently classified as A1 and A2, and F1 and F2, respectively [26, 27]. HIV-1 inter-clade recombinant forms have also been identified. 'Circulating recombinant forms' (CRFs) arise from individuals infected with two or more viral subtypes and are designated as such when an identical recombinant virus is characterised in at least three epidemiologically unrelated people [28]. Populations featuring multiple subtypes and CRFs increases the probability that individuals will become 'superinfected', resulting in the generation of several recombinants, referred to as 'unique recombinant forms' (URFs), which are then classified as CRFs when spread to others [29].

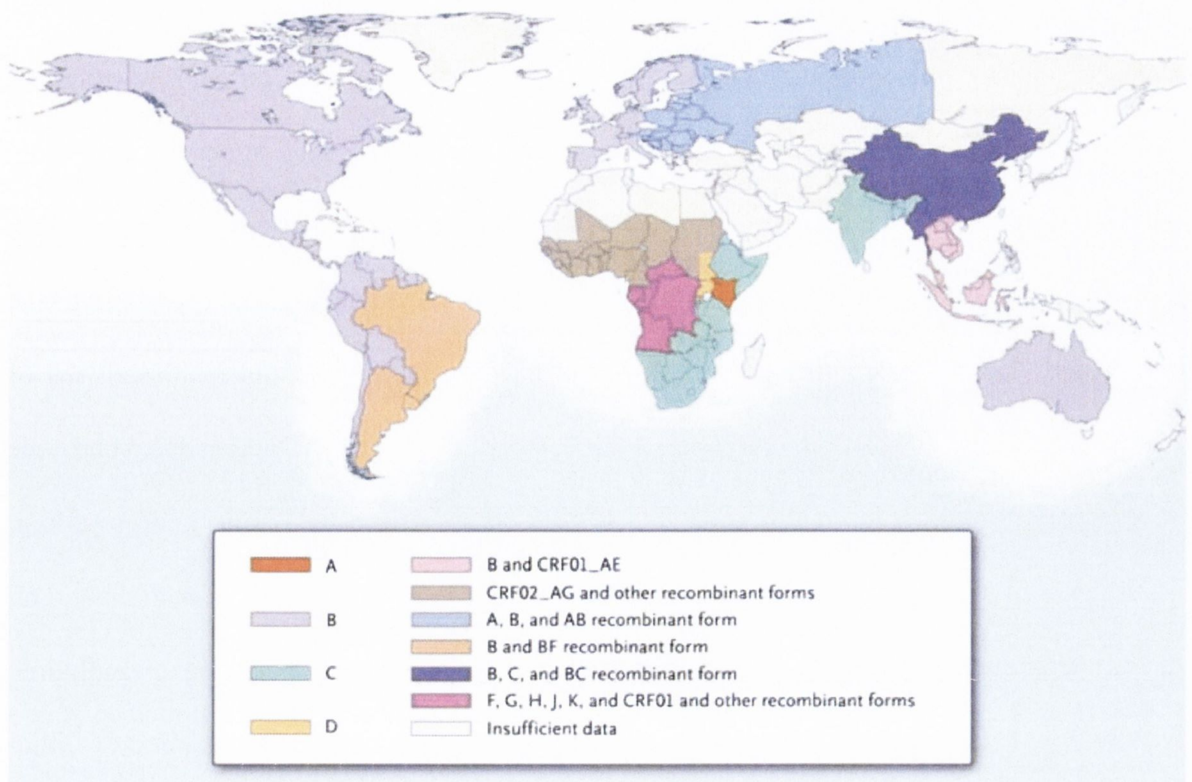


Figure 1.2 The global distribution of HIV-1 subtypes and circulating recombinant forms (CRFs). Diagram taken from Taylor *et al.,* 2008 [30].

1.1.5 Disease progression

Viral transmission is dependent on direct contact with infected blood or secretions, commonly through sexual intercourse or intravenous drug use (IVDU) [31]. Within 10-12 days of infection, viral RNA is detectable in the blood, and increases rapidly to peak levels during the acute phase of infection [32]. Concurrently, $CD4^+$ T cell numbers decline dramatically and most patients present with flu-like symptoms of fever, skin rash, oral ulcers, lymphadenopathy, pharyngitis, malaise, myalgia and weight loss [33]. Over a subsequent 7-14 day period, most patients become asymptomatic, HIV viremia declines,

and $CD4^+$ T cell numbers recover (although not to pre-infection levels), reflective of innate and adaptive antiviral immune responses [34]. Nonetheless, the disease continues to progress, viral replication continues, and immune cells are subjected to chronic activation, particularly in lymphoid compartments where tissues are destroyed as a result. Host failure to exert viral containment and reconstitution of memory T cells in mucosal lymphoid tissue and nodes, heralds a decline in $CD4^+$ T cells to < 200 cells/μl and an increased risk of opportunistic infections by bacteria, other viruses, fungi and parasites, and the development of tumours. Progression to AIDS is characterised by lymph node swelling, severe weight loss, fever, and respiratory, gastrointestinal, and neurological pathology, and the development of potentially fatal diseases such as Kaposi's sarcoma and non-Hodgkin's lymphoma [35]. The chronology and severity of disease progression from the time of initial infection is highly dependent on the infecting virus isolate and the ability of the host to mount an effective antiviral cellular and humoral response. Indeed, a proportion of infected individuals, dubbed 'elite controllers', maintain undetectable HIV viremia for a period of many years and show no signs of disease progression [36]. HIV-1 resistance in such patients may be directly attributable to cellular correlates such as $CD8^+$ T cell-mediated response [37], or genetic correlates such as homozygosity for the Δ32 alleic variant of the CCR5 protein [38, 39] or human leukocyte antigen (HLA) polymorphisms, notably HLAB*27 and B*57 alleles [40, 41].

The clinical manifestations of HIV-1-infected patients are primarily a consequence of the capacity of the virus and its components to disrupt immune system functions. The HIV-1 Tat protein, previously referred to for its role in viral replication (section 1.1.2), is a known pleiotropic factor that contributes to disordered immunity.

1.1.6 Tat and its contribution to disease pathogenesis

HIV-1 *trans*-activator protein, Tat, is an 86-101 amino acid polypetide (9-11 kDa) essential for initiating transcription and RNA chain elongation of the HIV-1 LTR. Two exons encode the full length 101 amino acid Tat, the first of which encodes residues 1-72, and the second, residues 73-101 [42]. Tat can be arbitrarily considered as containing several domains (Figure 1.14), with first exon encoding the N-terminal acidic domain 1 (aa 1-20), the highly conserved cysteine-rich domain 2 (aa 21-40), the core region domain 3 (aa 41-48), and the arginine and lysine-rich basic domain 4 (aa 49-72). The second exon encodes the C-terminal domain 5, which starts at amino acid position 73. The domains of the first exon are considered sufficient for full transactivating function, while the second exon is not required for transactivation [43]. In the absence of virally-encoded Tat, very low level gene expression is directed by the pro-viral LTR, but in the presence of Tat, transcription is increased several hundred-fold [44]. Tat binds to a short, *cis*-acting RNA target composed of a stem, a bulge and a loop, that is known as the *trans*-activation response region (TAR). Located at the 5' end of HIV LTR, TAR association enables Tat to recruit the complex of cyclin-dependent kinase 9 (CDK9) forming the positive transcription elongation factor B complex. CDK9, in turn phosphorylates the carboxy terminus domain of RNA polymerase II, enhancing elongation of the viral promoter [45].

Tat represents a highly significant factor in the pathogenesis of HIV disease. The protein is actively released from unruptured, HIV-infected cells and is detectable in *ex vivo* culture supernatants and in the serum of HIV-infected patients [46, 47]. Tat has the ability to enter cells, most likely through cell membrane heparan sulphate proteoglycans, and to translocate to the nucleus, maintaining an active form as it does so [48]. As a consequence, HIV LTR transcriptional activity within infected, but otherwise quiescent cells can be stimulated by Tat, thereby increasing the amount of infectious virions in circulation [49].

Furthermore, Tat can *trans*-activate cellular genes such tumour necrosis factor-α (TNF-α), interleukin-2 (IL-2), and interleukin-6 (IL-6) [50-52], in uninfected cells, potentially priming them for subsequent infection with HIV-1. Indeed, Tat activates T lymphocytes *in vitro*, rendering them highly permissive for productive HIV-1 infection [53]. Tat also activates uninfected B lymphocytes, indicating that it may contribute to B cell hyperactivation during early stage HIV-1 infection and activation-induced B cell death mediated by Fas during late stage HIV-1 infection [54]. Additionally, Tat impairs the cytotoxic activity of natural killer cells [55], induces chemokine HIV-1 coreceptors in PBMCs [56], and stimulates the chemotaxis of numerous cell types, including the major targets of HIV infection [57]. Finally, Tat is also a suspected cofactor in AIDS-associated pathologies such as Kaposi's sarcoma [58, 59] and HIV-associated dementia (HAD) [60, 61].

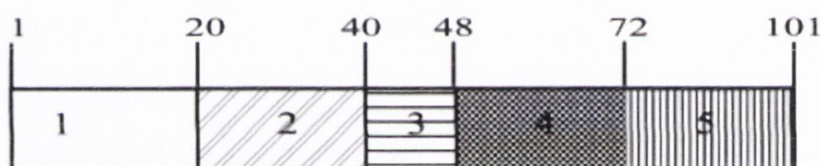


Figure 1.3 Diagram depicting the physical domains of the 101 amino acid isoform of HIV-1 Tat protein. Diagram adapted from Jeang *et al*, 1999 [62].

1.1.7 Co-infection with hepatitis C virus (HCV)

HIV and HCV co-infection is common among intravenous drug users (IVDUs) and haemophiliacs who received contaminated blood or blood products prior to routine serologic screening of donated blood for HCV. One-fourth to one-third of patients infected with HIV in Europe and the United States are co-infected with HCV, and up to 10% of all

HCV-infected patients are co-infected with HIV. This suggests a global co-infection prevalence of 4-5 million individuals [63, 64].

Co-infection with HCV, in addition to increasing the rate of HCV replication [65] and augmenting the dysregulation of the immune response [66, 67], accelerates the progression of liver fibrosis [68]. Furthermore, decompensated liver disease and hepatocellular carcinoma occur with greater frequency in co-infected patients than in HCV mono-infected patients [69]. The mechanistic basis for accelerated hepatic damage may be a consequence of generalised immune suppression resulting from diminished $CD4^{+}$ T cell numbers, or, as a number of studies have suggested, a result of intrahepatic interactions between HIV and/or viral proteins and resident cells. For example, Tuyama and colleagues have demonstrated that HIV can infect hepatic stellate cells (HSCs), promoting the expression and secretion of collagen I and monocyte chemoattractant protein-1 (MCP-1), and also enabling the transfer of infectious virus to lymphocytes in co-culture [70]. Type I collagen production by HSCs is a hallmark of fibrogenesis and MCP-1, a potent chemoattractant for monocytes and lymphocytes, is up-regulated during chronic hepatitis and correlates with inflammatory infiltration of the portal tract [71]. Notably, other researchers have shown that HIV envelope protein gp120 mediates the chemotaxis of HSCs by upregulating MCP-1, and additionally, induces the expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) [72]. Furthermore, gp120 induces HCV replication and enhances HCV-regulated, profibrotic, transforming growth factor-β1 (TGF-β1) in hepatoctyes [73].

Finally, therapeutic side-effects also warrant consideration as, in a percentage of co-infected patients in receipt of highly active antiretroviral therapy (HAART), particularly protease inhibitor-based regimens, hepatic damage is exacerbated by hepatotoxicity and necro-inflammatory lesions [74-76]. Nevertheless, the degree of pre-existing liver fibrosis [77],

and infection with HCV genotype 3 [78], are also important risk factors for hepatotoxicity, regardless of HAART composition.

1.2 Hepatitis C Virus (HCV)

1.2.1 HCV disease

Following the identification of hepatitis A and B viruses in the 1970s, a blood borne, non-A, non-B agent, responsible for the majority of transfusion associated cases of hepatitis, was recognised. The culprit was not identified until 1989 as the development of recombinant DNA technology allowed the cloning of a virus genome which was subsequently named hepatitis C virus [79]. In addition to contaminated blood transfusions, the main routes of transmission are intravenous drug use and reused medical supplies. Sexual transmission of HCV is possible, and recent evidence indicates that risk of transmission is commensurate to increasing numbers of sexual partners, particularly if those partners are co-infected with HIV [80-82].

HCV currently infects some 170 million people worldwide and is believed to kill an estimated 350,000 people each year [83]. Its global distribution is disparate [84], but the highest rates of transmission occur in Egypt, where at least 14 % of the population are infected, and in nations where HIV is widespread, there is the suspicion that large numbers of HCV infected individuals remain undiagnosed [85] (Figure 1.3). HCV infection causes acute hepatitis, which is self-resolving in 20-50 % of cases but does not confer permanent immunity. In the majority of cases (50-80 %), a chronic disease is manifested, resulting in cirrhosis (in ~10-20 % of cases after 10-20 years), and hepatocellular carcinoma (1-4 % incidence rate per year in patients with HCV-related cirrhosis) [84].

A feature of HCV, similar to HIV, is a propensity for genomic mutation leading to the generation of a number of viral subtypes. Phylogenetic analysis of full-length or partial sequences of HCV strains from different geographical regions has identified six main genotypes, numbered 1 to 6, and a large number of subgroups within each genotype, identified by lower case letters. Furthermore, HCV displays heterogeneity within an individual patient as a series of quasispecies, which are variants of the predominant infecting strain [86]. Such a large number of variants, a consequence of the high error rate of the viral RNA-dependent RNA polymerase (RdRP), has implications for the severity and aggressiveness of liver infection as well as response to therapy.

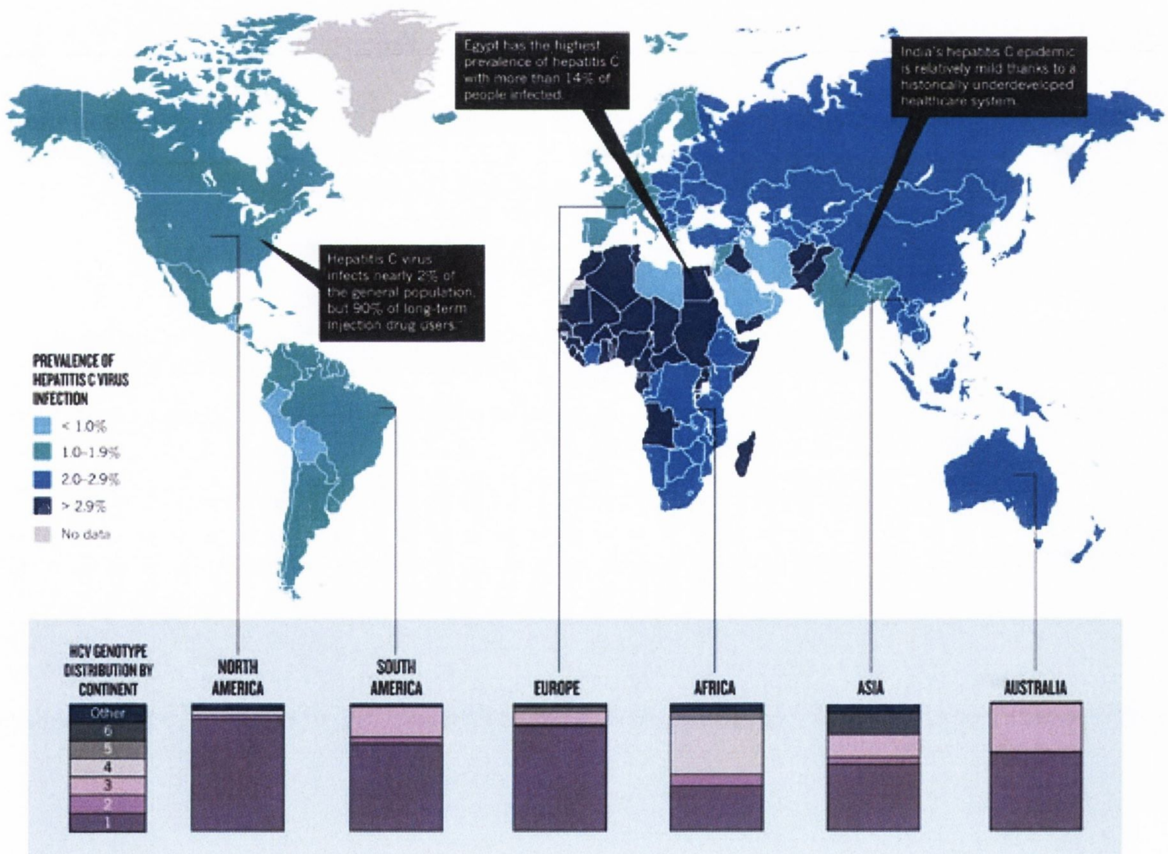


Figure 1.4 Global prevalence of HCV infection and the distribution of the six major genotypes by continent. Diagram adated from Gravitz, 2011 [85].

1.2.2 Molecular biology of HCV

Hepatitis C virus (HCV) is a small (~50 nm diameter), enveloped, positive strand RNA virus belonging to the Hepacivirus genus in the *Flaviviridae* family. The HCV genome is a single-stranded RNA molecule containing a single open reading frame (ORF) encoding a polyprotein of approximately 3,000 amino acids. The ORF is flanked by 5' and 3' untranslated regions (UTRs) bearing highly conserved RNA structures essential for replication and the initiation of translation. The 5' UTR contains an internal ribosome entry site (IRES) that binds the 40S ribosomal subunit and directs polyprotein translation. The polyprotein precursor is then subjected to processing by host and viral proteases both during and after translation on the endoplasmic reticulum (ER) to yield ten mature viral proteins [87]. Three of these proteins are structural, consisting of a core protein (C), which forms the viral nucleocapsid, and two envelope glycoproteins (E1 and E2). Six are non-structural proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Structural and non-structural proteins are separated by the small membrane peptide p7 (Figure 1.4).

1.2.2.1 HCV structural proteins

The HCV core protein (C) is a RNA-binding protein which forms the structural component of the virus particle [88]. It is cleaved from the viral polypeptide by a host signal peptidase cleavage at the C-terminus, producing the immature form of the protein, which when processed further by a host signal peptide peptidase, yields the mature form [89]. Most of the C protein is found in the cytoplasm, bound to ER membranes or located on the surface of lipid droplets (intracellular organelles involved in lipid storage and vesicular trafficking), while a small amount is also found in the nucleus. In addition to its role in nucleocapsid formation, HCV core can also function as a modulator of cell signalling, apoptosis, proliferation, and lipid metabolism [90].

HCV E1 and E2 are type-I transmembrane glycoproteins that form heterodimers on the ER where they are glycosylated, and, following cleavage by the host signal peptidase, their signal-like sequence is reoriented towards the cytosol, leading to a single transmembrane passage [91]. E1 and E2 are thought to induce fusion between the viral envelope of the host cell membrane [92], participate in the assembly of infectious particles [93], and, because of their exposure on the cell surface, act as a ligand for cellular receptors such as CD81, thus facilitating viral entry [94, 95].

1.2.2.2 HCV non-structural proteins

NS2 is a non-gylcosylated integral membrane protein containing a dimeric cysteine protease with two composite active sites. Considered non-essential for the formation of the replication complex [96], NS2 mediates the proteolytic cleavage of the NS2-NS3 junction, a zinc-dependent autocatalytic cleavage that detaches NS2 from the downstream portion of the precursor polyprotein [97].

NS3 contains a serine protease domain and an RNA helicase domain. Protease activity of NS3 is enhanced by NS4A as a co-factor, as it allows the induction of a conformational change that induces a repositioning of the catalytic triad [98]. Furthermore, NS3 has no transmembrane domain, but when co-expressed with NS4A, is found in association with ER or ER-like membranes [99]. The NS3-4A complex is responsible for downstream NS3 polyprotein cleavage, activity essential for the generation of components of viral RNA replication [100], and has also been shown to abrogate host cell antiviral immune responses [101]. The NS3 helicase domain comprises the C-terminal amino acids of NS3 and possesses multiple functions, including RNA-stimulated NTPase activity, RNA binding, and the unwinding of RNA regions at the termini of positive and/or negative strands [102].

NS4B is a hydrophobic non-structural protein containing four transmembrane proteins that are palmitoylated in its C terminal region. Palmitoylation assists the process of oligomerisation which is regarded as essential for HCV replication [103]. The protein is also believed to induce intracellular membrane alterations. Membranous structures augmented by NS4B most likely support RNA replication [104].

NS5A is a phosphoprotein containing a unique amphipathic alpha-helix at its N-terminus and is detected in association with ER or ER-derived membranes. NS5A is an integral part of HCV genome replication [105] and is being investigated as a modulator of the interferon immune response, a potential mechanism for viral evasion of host defences [106].

NS5B is a membrane-associated protein containing a C-terminal anchoring transmembrane domain. NS5B is an RNA-dependent RNA polymerase (RdRP), essential for viral replication, and is thus a key protein for specifically targeted antiviral therapy [107].

Finally, the p7 polypetide, located at the junction of the structural and non-structural proteins, is a small, intrinsic membrane protein composed of two transmembrane domains with both its N- and C- termini oriented toward the lumen of the ER [108]. It belongs to a family of viral proteins called viroporins that form ion channels and serves an essential role in the production of infectious viral particles during the HCV life cycle [109].

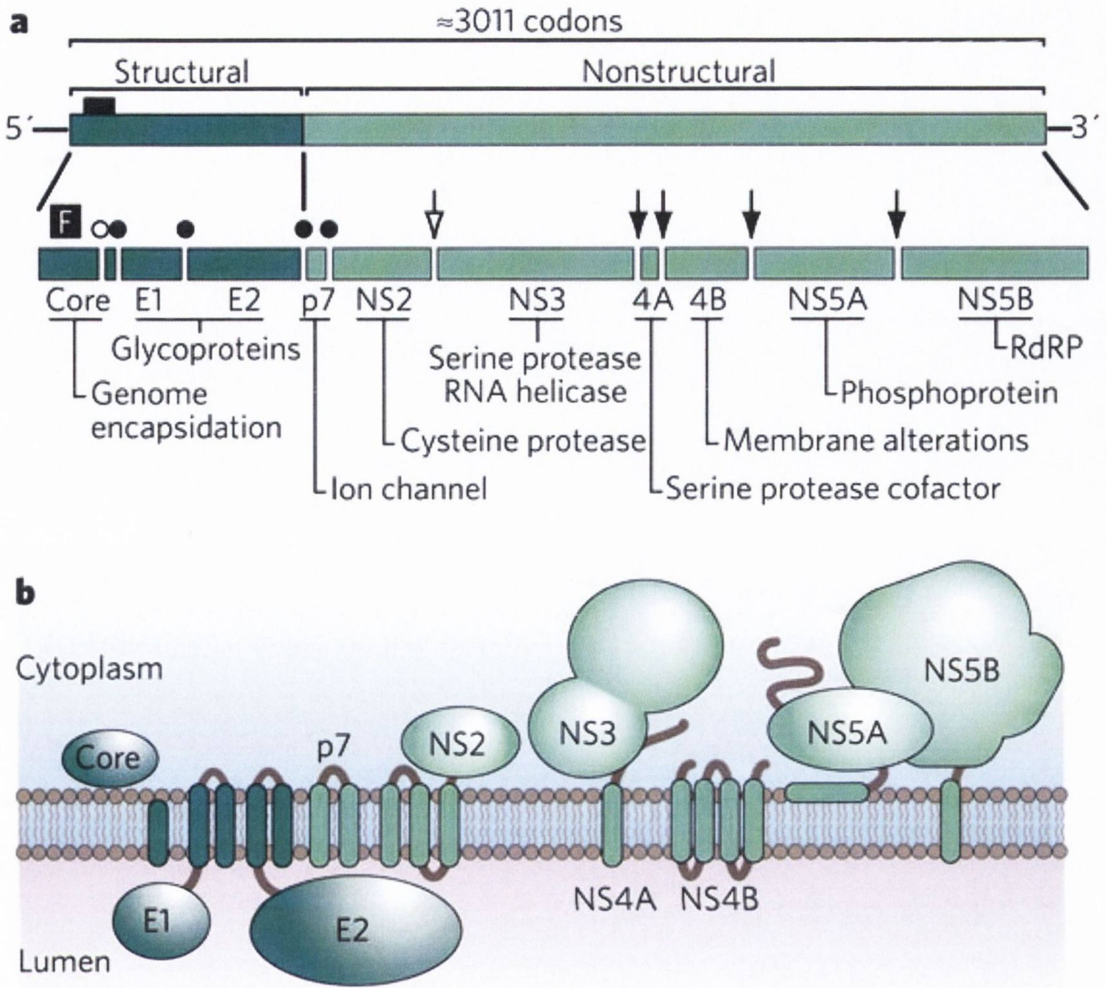


Figure 1.5 (**a**) Schematic representation of the structure of HCV genome showing the long open reading frame (ORF) encoding structural and non-structural genes, and 5' and 3' untranslated regions (UTRs). The polyprotein processing scheme is shown below. Closed circles indicate signal peptidase cleavage sites; the open circle indicates signal peptide peptidase cleavage site. (**b**) The topology of HCV proteins with respect to a cellular membrane. Diagram taken from Lindenbach and Rice, 2005 [110].

1.2.3 Virus entry, replication, and maturation

Infection by HCV occurs only in humans and chimpanzees and is mediated through interactions between its viral glycoproteins and a series of cell surface molecules, primarily in hepatocytes but also in B cells and dendritic cells amongst others [111] (Figure 1.5). Host proteins, CD81, scavenger receptor class B type 1 (SR-B1), and claudin-1 (CLDN1) are thought essential co-receptors for viral entry. Glycosaminoglycans (GAGs) and possibly, low density lipoprotein (LDL) receptors, have also been suggested to facilitate virus uptake. Notably though, *in vitro* cell models exist that express all of these entry factors yet remain resistant to HCV infection, suggesting that one or more essential molecule/s has yet to be identified [112]. The E2 envelope protein interacts with the large extracellular loop of CD81 and activates Rho GTPases, stimulating an actin-dependent re-localisation to intercellular contact regions, enabling virus contact with the CLDN1 co-receptor [113]. A clathrin-dependent endocytosis ensues [114], and fusion of the virus to the cell membrane is potentially assisted by the presence of heparan sulphate GAGs [115] and/or LDL [116]. While treatment of target cells with glycosidases has been shown to reduce HCV infectivity [117], and anti-LDLr antibodies demonstrate modest inhibition of HCV entry [118], a definitive case for the role of each remains to be established.

HCV enters the cell by clathrin-mediated endocytosis [114], leading to the release of a single-stranded, positive-sense HCV RNA genome which is directly translated. HCV translation initiation occurs through the formation of a binary complex between the internal ribosomal entry site (IRES) and the 40S ribosomal subunit. This formation is followed by the assembly of a 48S-like complex at the AUG initiation codon after the association of eukaryotic initiation factor 3 (eIF3) and the ternary complex of eIF2-Met-tRNA-GTP [119]. The rate limiting step is the GTPase-dependent association of the 60S subunit to form an 80S complex [120]. Translation of the HCV open reading frame (ORF) produces a

polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases to form the mature structural and non-structural proteins. The exact composition of a membrane-associated RNA replication complex requires much further elucidation. Studies have suggested interactions between HCV RNA replication and cellular lipid metabolism for example [121], and a diverse range of host factors including cyclophilin B (a cytosolic peptidyl-prolyl *cis-trans* isomerase), and FKBP8 (a member of the FK506-binding protein family) and Hsp90 (an abundant cellular heat shock protein), are thought to influence NS5A and NS5B activity [122, 123]. As systems allowing the production of sufficient amounts of virus particles have only recently been developed, little is known about late-stage HCV lifecycle. Particle formation may be initiated by core protein interactions with the RNA genome, leading to selective packaging and a repression of translation from the IRES, suggesting a potential mechanism for a translation / replication switch to assembly [124]. Additionally, E1 and E2 retention in ER compartments might facilitate nucleocapsid envelope acquisition by budding through ER membranes and eventual export via secretory pathways [125].

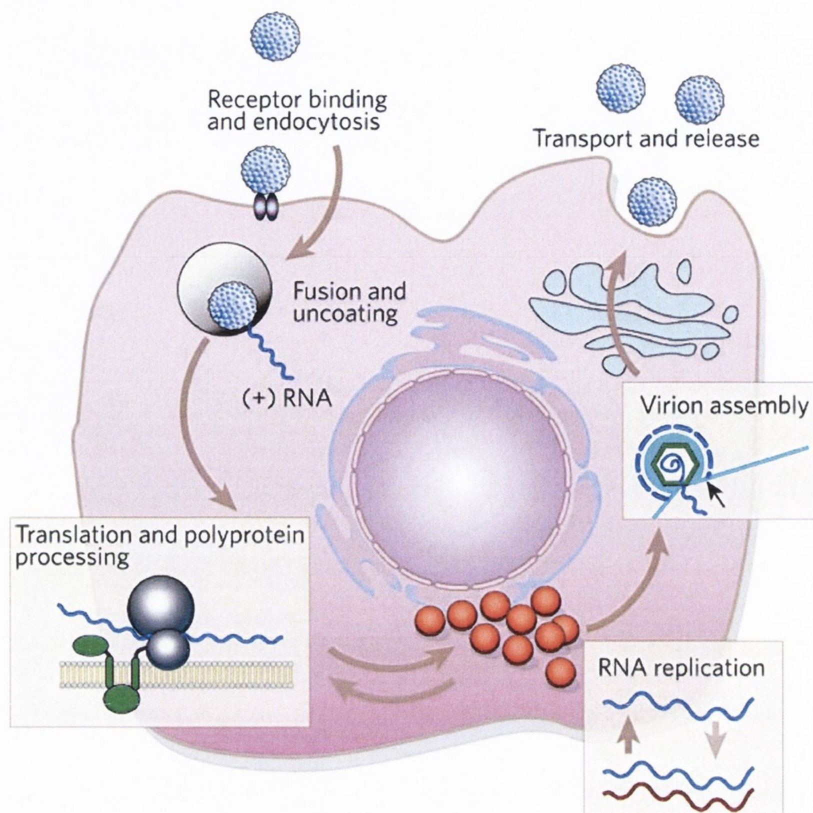


Figure 1.6 Schematic representation of HCV life cycle. After entry into the cell and uncoating, the HCV genome functions in three main roles: translation, replication and packaging into nascent virions. Diagram taken from Lindenbach and Rice, 2005 [110].

1.2.4 Immune response to HCV infection

Onset of HCV infection is typically asymptomatic and is usually not diagnosed until serum alanine aminotransferase (ALT) levels increase in response to the development of acute hepatitis, usually some 10-14 weeks later. At such time, HCV antibodies are detectable and HCV-specific T cell populations appear in the liver [126], a consequence of the adaptive immune response.

Within days of the initial infection however, innate immune responses are activated, the most significant of which is the induction of intrahepatic type I interferon (IFN) gene expression and associated IFN-stimulated genes (ISGs) [127]. Recognition of viral RNA by hepatocytes is accomplished by toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I), which signal through caspase activation and recruitment domain inducing IFN-beta (CARDIF), toll-interleukin-1R domain-containing adaptor inducing IFN-beta (TRIF), and IFN-beta promoter stimulator 1 (IPS-1). The subsequent phosphorylation of IFN regulatory factors 1 and 3 (IRF1 and 3) and nuclear factor-kappa B (NF-κB), and their nuclear translocation, induces transcription of both IFN-α and IFN-β. These molecules are in turn secreted and bind to receptors that signal through JAK-STAT pathways, which stimulate the transcription of inflammatory cytokines, the pattern recognition pathway proteins, and effector proteins, all of which induce an antiviral state within the cells.

However, HCV is concurrently subverting this innate response by counter-evasive actions at each critical step. For example, NS3/4A protease has been shown to cleave TRIF, thus reducing its abundance and inhibiting downstream IRF3 and NF-κB signalling in cell culture [128]. Core protein is also evidenced to bind to STAT-1, decreasing its phosphorylation and thus reducing ISG-3 binding to DNA, and ultimately disrupting IFN-stimulated gene transcription [129]. NS5A induction of interleukin-8 (IL-8) expression has also been shown to correlate to an inhibition of IFN antiviral activity [130]. E2 has also been demonstrated to possess a sequence that is identical to the phosphorylation site of protein kinase R (PKR), resulting in inhibition of its kinase activity [131]. PKR is an ISG with significant anti-HCV properties as it inhibits the protein synthesis of viral RNA.

Natural killer (NK) cells are an important component of the innate immune response to viral infection as they exert potent cytotoxic effects and are rapid producers of antiviral cytokines, namely type II IFN-γ. Several ISGs induced by HCV infection are known to

have roles in augmenting the effects of NK cell function. Notwithstanding, data derived from chronically infected HCV patients indicates that both NK number and function are significantly decreased compared to healthy controls [132, 133] and *in vitro*, HCV E2 has been shown to inhibit NK cell cytokine production, cytotoxic granule release, and proliferation [134, 135].

Innate immune responses regulate subsequent adaptive responses through cytokine activation of dendritic cells (DCs) and other antigen presenting cells. Studies have reported that DCs generated from HCV-infected patients indicate impaired functionality, reduced IFN-γ production, and a diminished ability to stimulate the proliferation of $CD4^+$ T cells [136]. $CD4^+$ T helper cells are critical to both the generation and maintenance of adaptive immune responses, mainly through their cytokine production which primes $CD8^+$ cytotoxic T cells for virus-infected cells and augments antibody production by B cells. Both chimpanzee and natural human infection studies have shown that clearance of HCV correlates with a sustained HCV-specific $CD4^+$ T cell response. In chimpanzee models of acute infection, the initiation of $CD4^+$ T cell responses is temporarily associated with a substantial decrease in viremia, and the accumulation of HCV-specific $CD4^+$ T cells in liver tissue appears to be essential for clearance of HCV [137, 138]. Furthermore, patients that generate a polyclonal HCV-specific $CD4^+$ T cell response are more likely to clear HCV, while those who do not are likely to develop persistent infection [139]. Similarly, the extent of $CD8^+$ T cell responses are positively correlated with clearance of the virus in primates and humans [140, 141].

Although neutralising antibodies to HCV have been identified [142], the precise role of the adaptive humoral immune response in HCV infection remains to be defined. Unlike acute hepatitis B, in which the development of antibody to hepatitis B surface antigen marks the onset of recovery, no distinct pattern of antibody production exists in HCV patients who

recover from infection or become chronically infected. The highly mutational nature of HCV also suggests that antibodies with potentially neutralising capacity may well be ineffective due to successful evasion by quasispecies [143, 144] (Figure 1.6).

Finally, host genetic determinants are undoubtedly a factor in the immune response to HCV. Differences with respect to prevalence and clearance of the virus have been documented amongst ethnic groups, with a more frequent occurrence of chronic infection and poorer response to therapy attributable to African Americans for example [145, 146]. Genetic associations between genes related to immune system function, such as HLA class II major histocompatibility complex (MHC) and effective viral clearance have been extensively studied, with meta-analysis indicating that DQB1*0301 and DRB1*1101 alleles confer the greatest advantage [147]. More recent evidence indicates that certain single nucleotide polymorphisms (SNPs) in close proximity to the type III IFN-λ IL28B gene, have strong predictive value in anticipating both spontaneous and treatment-induced clearance of HCV [148-150].

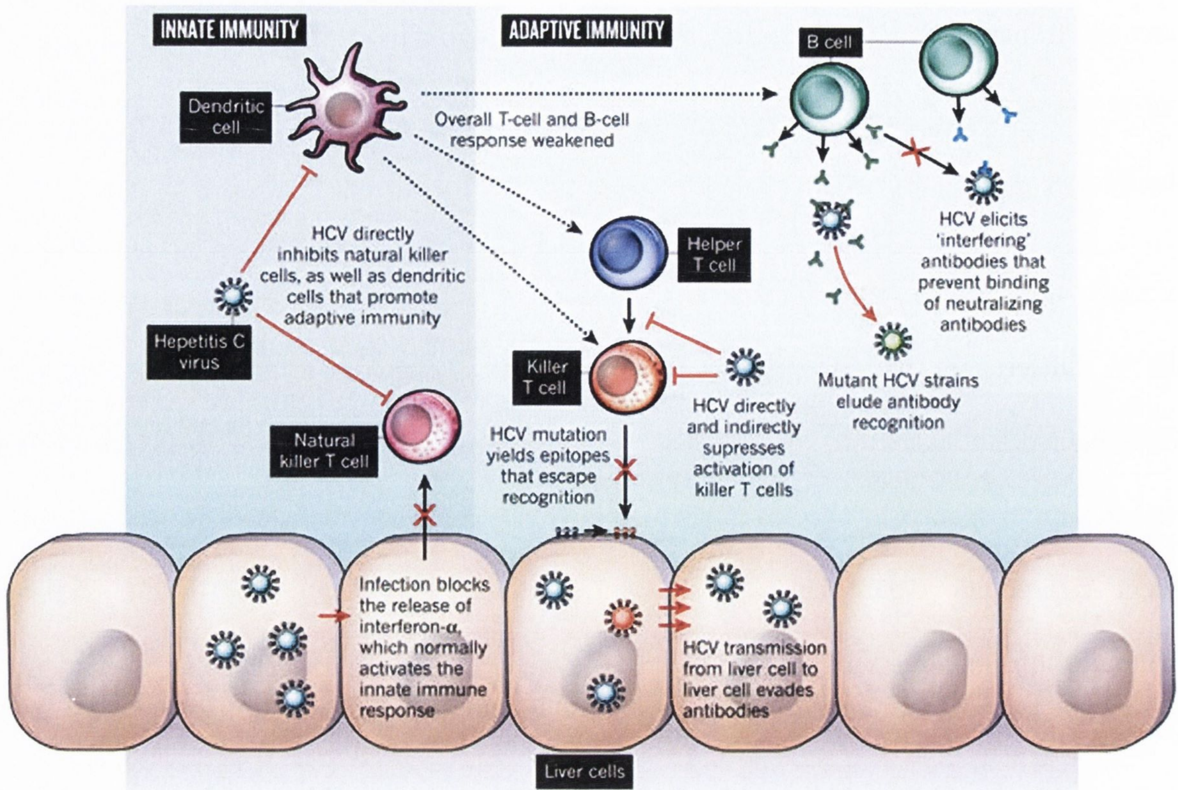


Figure 1.7 HCV conspires to evade (red) the host immune defences (black) by many possible routes. Diagram adapted from Eisenstein, 2011 [151].

1.2.5 NS3 as an immunomodulator during HCV infection

The HCV genome, as related in section 1.2.2, encodes a polyprotein featuring a structural region (C-p7 proteins) and a non-structural region (NS2-NS5B proteins). The NS3 protein is a 70 kDa bifunctional enzyme containing a serine protease and an RNA helicase, essential for HCV replication. The N-terminal third of the protein contains the protease activity responsible for processing of the non-structural region of the polyprotein [152, 153]. The C-terminal two thirds of NS3 is an RNA helicase of DExH/D, a subgroup of DNA and RNA helicases within helicase superfamily 2 [154]. Members of this family feature a core

helicase structure consisting of paired RecA-like folds (domains 1 and 2). With these two domains, NS3 helicase has a third domain that forms a single-stranded DNA/RNA binding groove [155, 156] (Figure 1.13). Helicase activity is essential for replication of the viral RNA genome [157] and is believed to complex with NS5B polymerase during viral replication [158]. NS3 also participates in the intracellular assembly and packaging of infectious virus particles [159].

Further to its replicative functions, NS3 possesses considerable immunomodulatory properties. Probably the most researched extravirological effect is its ability to suppress antiviral signalling, the mechanisms of which were touched on briefly before (section 1.2.4). In greater detail, activation of TLR3 and RIG-I receptors in response to HCV viral entry and uncoating, and cytosolic attachment, respectively, results in activation of IRF3. Subsequent IRF3 nuclear transactivation of the IFN-β promoter induces IFN-β expression and leads to ISG activation, the products of which are effectors of the innate immune response. However, the signalling pathways from TLR3 and RIG-I to activated IRF3 are dependent on the specialist proteins TRIF and CARDIF [160, 161]. Evidence derived from *in vitro* studies suggests that HCV-induced blockage of IRF3 activation is mediated by NS3. Li and colleagues [128] for example have demonstrated that specific proteolysis of TRIF by the NS3/4A complex reduces its abundance and inhibits upstream IRF-3 signalling. NS3/4A has also been shown to cleave the RIG-I adaptor protein IPS-1 from the mitochondrial membrane, thereby diminishing interaction with RIG-I and preventing downstream activation of IRF-3 [162]. Supportive of these findings, disruption of NS3/4A function by mutation or pharmacological inhibition restores RIG-I signalling and IRF-3 phosphorylation [101, 162]. Further evidence indicates that NS3 impairs the function of dendritic cells by inducing the expression of FasL-mediated apoptosis, interfering with allostimulatory capacity, inhibiting nuclear translocation of NF-κB, and reducing TLR

signalling, all possible mechanisms for the impaired T cell response typical of chronically infected individuals [163]. The data outlined above is consistent with an NS3-mediated viral evasion of host cellular antiviral defences.

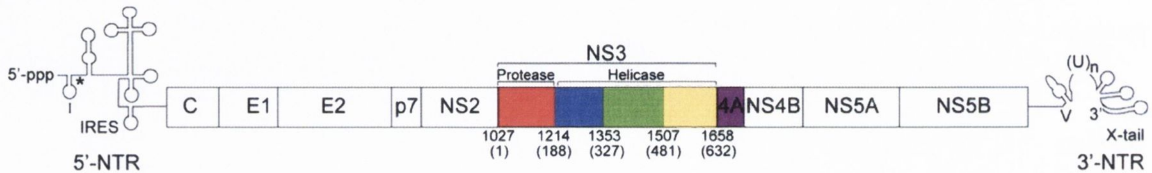


Figure 1.8 Diagram featuring the location and enzymatic composition of the NS3-coding sequence in the HCV genome. Full-length NS3 protein is located from amino acids 1027 to 1658 of genotype 1b polyprotein consensus sequence (NCBI accession number AJ238799) [164]. Adapted from Raney *et al,* 2010 [165].

1.2.6 Development of HCV-induced liver fibrosis

During the course of chronic HCV infection necrotic damage of liver tissue represents the end result of the targeted host immune response toward HCV-infected hepatocytes. The early events of liver fibrosis are characterised by an increase of cytokine and growth factor secretion, such as interleukin-1β, tumour necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) [166-168], by infiltrating $CD4^+$ and $CD8^+$ T cells, monocytes/macrophages, and resident inflammatory cells [169]. These soluble factors activate hepatic stellate cells (HSCs), which then adopt a myofibroblast phenotype that is characterised by the expression of the intermediate

filament α-smooth muscle actin (α-SMA) and profibrotic genes including collagen I and select matrix metalloproteinsases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [170]. Recognising tissue damage, HSCs proliferate and migrate to the site of injury, initiating the secretion and deposition of extracellular matrix (ECM) proteins rich in fibrillar collagen type I and III, the main components of fibrotic tissue in the cirrhotic liver [171, 172] (Figure 1.7). *In vitro* studies indicate that secreted TIMPs exert marked biological effects, significantly inhibiting the proteolytic activity of concurrently expressed MMPs and thereby protecting newly formed matrix from degradation [173]. However, regression of fibrosis, as witnessed in patients who successfully clear HCV infection, corresponds to an alteration in the MMP/TIMP balance that favours matrix degradation, and extensive apoptosis of activated HSCs [174, 175]. Progression of fibrosis is responsible for the development of cirrhosis [176].

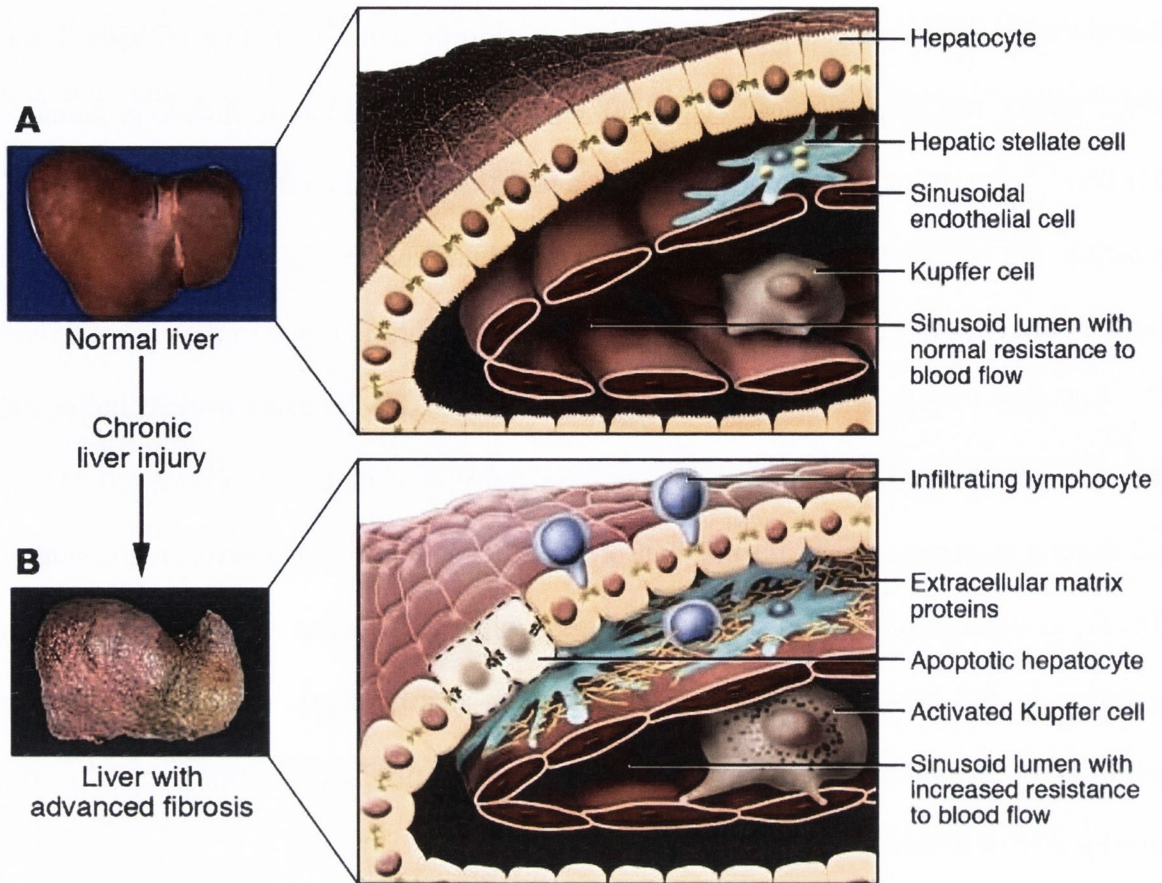


Figure 1.9 Alterations to normal hepatic architecture (**A**) characteristic of advanced hepatic fibrosis (**B**). Following liver injury, stimuli from infiltrating lymphocytes causes resident HSCs to become activated and proliferate, secreting large amounts of extracellular matrix proteins. Hepatic macrophages, such as the activated Kupffer cell, play an important role in regulating tissue remodelling by their release of soluble factors. Deposition of fibrillar matrix results in damage to hepatic parenchyma including hepatocyte apoptosis, loss of microvilli and loss of endothelial fenestration. Diagram taken from Bataller and Brenner, 2005 [177].

1.3 Pharmacological management of HIV and HCV

1.3.1 HIV antiretroviral therapy

Whilst the first pharmacological agent shown to inhibit HIV replication was suramin [178, 179], the first compound licensed for clinical use was zidovudine in 1987. Since then, in excess of twenty five anti-HIV compounds have been approved for treatment, categorised according to their specific pharmacological target: nucleoside reverse transcriptase inhibitors (NRTIs); nucleotide reverse transcriptase inhibitors (NtRTIs); non-nucleoside reverse transcriptase inhibitors (NNRTIs); protease inhibitors (PIs); fusion inhibitors (FIs); co-receptor inhibitors (CRIs); and integrase inhibitors (INIs) (Table 1.1) (Figure 1.8).

Zidovudine (AZT), like other members of the nucleoside reverse transcriptase inhibitor (NRTI) class, is a nucleoside analogue which undergoes a three step intracellular phosphorylation to its 5'-triphosphate derivative in order to compete with cellular triphosphate substrates for viral DNA synthesis by the reverse transcriptase enzyme. Incorporation into the growing chain as an alternate substrate results in chain termination [180]. With a similar mode of action, nucleotide reverse transcriptase inhibitors (NtRTIs) such as tenofovir (TDF) are nucleotide analogues that require only a two step phosphorylation.

Reverse transcriptase is also the target for non-nucleoside reverse transcriptase inhibitors (NNRTIs) such as nevirapine (NVP), with their site of action being the allosteric site. Due to the proximity of this site to the catalytic site, NNRTIs interfere with the binding of RT to viral RNA and in doing so, disrupt its function [181].

Ten protease inhibitors (PIs) are currently licensed for clinical use. With the exception of tipranavir (which has a coumarin scaffold), PIs are based on the peptidomimetic principle in which their hydroxyethylene scaffold mimics the peptide linkage cleaved by HIV

protease, but is itself resistant to cleavage [182]. Acting as substrate, PIs thus prevent HIV protease from initiating proteolytic processing of precursor viral proteins into mature viral proteins.

Enfuvirtide (DP178) comprises the sole licensed fusion inhibitor (FI) currently available. Enfuvirtide, as a result of interactions with the heptad repeat (HR) regions of gp41, blocks fusion of the virus particle and the extracellular membrane [183]. In contrast to all other antiretrovirals, enfuvirtide must be administered parenterally, owing to its polymeric structure, and is thus limited to salvage therapy for treatment-experienced patients. The co-receptor inhibitors (CIs), maraviroc (MVC) and vicriviroc (VCV), the latter awaiting Food and Drug Administration (FDA) approval, are CCR5 chemokine receptor antagonists [184] and thus have limited clinical utility as they are effective against M-tropic viral strains only. Raltegravir (MK0158) is the sole integrase inhibitor (INI) licensed for clinical use, although elvitegravir (EVG) is currently undergoing clinical trials. Both compounds are designed to inhibit integrase insertion of pro-viral DNA into host genomic DNA [185]. Finally, the maturation inhibitor, bevirimat (BVM), acts by blocking the final cleavage event in Gag processing, the separation of the capsid protein from its C-terminal spacer peptide 1, resulting in the formation of non-infectious virions [186]. BVM is not yet in receipt of FDA approval for clinical use.

The optimal time at which to start antiretroviral therapy has, historically, been a contentious issue in the clinical manangement of HIV infection. Current European AIDS Clinical Society (EACS) guidelines recommend treatment of primary HIV infection if $CD4^+$ T cell count is <350/μl at month 3 or beyond [187]. This threshold was established, in part, because it was midway between the lower limit of normal (500 cells/μl) and the threshold typically used to define AIDS (200 cells/μl). A majority of cohort studies would

seem to suggest that 350 cells/μl could be close to the threshold at which the benefits of starting therapy outweigh the risk of delaying treatment [188, 189].

Combination treatment with three or more anti-HIV agents is known as highly active antiretroviral therapy (HAART), and since its adoption as standard of care in 1996, the clinical profile of the disease has changed to a chronic, manageable condition. Individualised regimens selected from the major NRTI/NNRTI and PI classes have facilitated HIV infected patients in achieving viral suppression to undetectable levels, restoration of $CD4^+$ T cell counts to near norms, and as a consequence, significantly reduced morbidity and mortality [190]. However, despite the successes of antiretroviral therapy, many limitations remain. As the virus cannot be eradicated, treatment is lifelong, thus requiring sustained patient compliance to a complex therapeutic regimen. Owing to the highly mutational nature of the virus, resistance has been attributed to the three major classes (NRTIs, NNRTIs, and PIs) [191, 192], and cross-resistance between individual class members has also been documented [193, 194]. Furthermore, a range of adverse effects have been reported in treated patients. Several reverse transcriptase inhibitors may induce mitochondrial toxicity, resulting in peripheral neuropathy and myopathy, and also rare, but potentially fatal, incidences of lactic acidosis [195]. Hypersensitivity reactions have also been attributed to these agents, with abacavir treatment in particular resulting in fever, skin rash, and a range of respiratory and gastrointestinal symptoms [196]. Protease inhibitor-based regimens are most closely associated with the development of metabolic complications such as dyslipidaemia, lipodystrophy, and insulin resistance and diabetes [197-200], increasing risk for the development of cardiovascular disease in patients.

Newer therapeutic classes such as entry, fusion, and maturation inhibitors may help to circumvent cross resistance with older compounds, but optimal clinical management of the virus will undoubtedly be an ongoing challenge.

Table 1.1 Current HIV-1 antiretroviral agents and their mechanism of action

Class	Compound (abbreviation)	Mechanism of action
NRTI	Zidovudine (AZT), abacavir (ABC), lamivudine (3TC), stavudine (d4T), didanosine (ddI), zalcitabine (ddC), emtricitabine ((-)FTC)	Nucleoside analogues whose triphosphate form acts as substrate for HIV-1 RT.
NtRTI	Tenofovir (TDF)	Nucleotide analogue with a similar mechanism of action to NRTIs.
NNRTI	Nevirapine (NVP), efavirenz (EFV), fetravirine (TMC125), rilpivirine (TMC278)	Bind to non-catalytic site of HIV-1 RT inducing conformation change.
PI	Saquinavir (SQV), lopinavir (LPV), ritonavir (RTV), atazanavir (ATV), nelvinavir (NFV), fosamprenavir (FPV), amprenavir (APV), indinavir (IDV), darunavir (DRV), tipranavir (TPV)	Mimic the peptide linkage cleaved by HIV-1 protease.
FI	Enfuvirtide (DP178)	Binds to HR region of gp41 and blocks fusion of virus particle to cellular membrane
CRI	Maraviroc (MVC), vicriviroc (VCV)	CCR5 chemokine antagonists.
INI	Raltegravir (MK0158), elvitegravir (EVG)	Inhibits integration of strand transfer of viral DNA into host cell DNA
MI	Bevirimat (BVM)	Prevents cleavage of precursor polyprotein by protease.

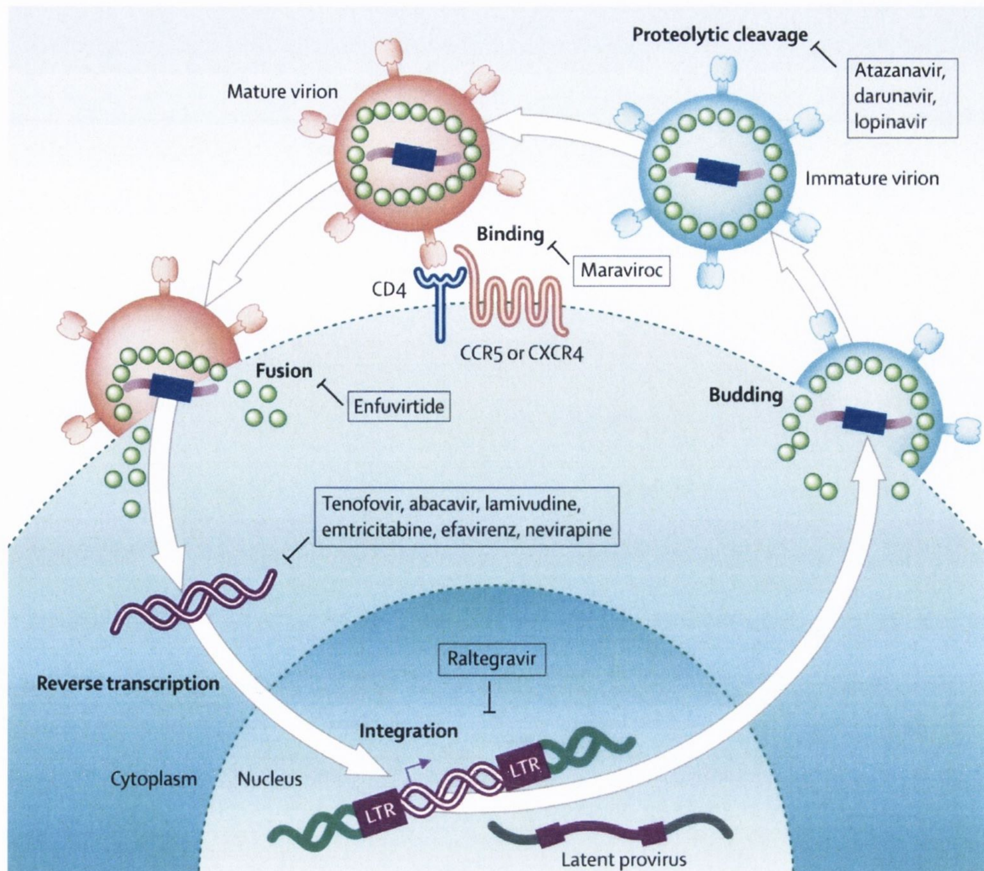


Figure 1.10 Diagram illustrating the HIV-1 life cycle and its major antiretroviral drug targets. Entry of extracellular virions to their target cell is achieved by attachment to the CD4 receptor, binding to CCR5 or CXCR4 coreceptors, and membrane fusion. Maraviroc is designed to block CCR5 binding and enfuvirtide blocks fusion. Transcription of viral RNA into pro-viral DNA is catalysed by the reverse transcriptase enzyme, a step inhibited by nucleoside/nucleotide analogues (NRTIs/NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs). HIV integrase facilitates the incorporation of pro-viral DNA into host chromosomes, a process inhibited by integrase inhibitors (INIs) such as raltegravir. Following transcription and translation of the HIV genome, immature virions bud from the cell surface requiring proteolytic cleavage by HIV protease to produce mature, infectious viruses. This step is inhibited by protease inhibitors (PIs). Diagram taken from Volberding and Deeks 2010 [201].

1.3.2 HCV therapy

Following the completion of large scale clinical trials [202, 203], the standard of care (SOC) for chronic hepatitis C during the last decade has consisted of pegylated interferon-alpha 2a (PEG-IFN-α2a), or pegylated interferon-alpha 2b (PEG-IFN-α2b), in combination with ribavirin (RBV).

RBV (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), is a guanoside nucleoside analogue, synthesised at ICN Pharmaceuticals in 1970, and originally marketed as an anti-HCV therapeutic by Schering-Plough as Rebetol in 2001. PEG-IFN-α2a, a 40 kDa branched molecule, and PEG-IFN-α2b, a 12 kDa linear molecule, were developed by Hoffmann-La Roche and Schering-Plough respectively, and granted approval by the FDA in 2001. The development of these two polyethylene glycol-conjugated interferons represented distinct pharmacokinetic/pharmacodynamic advantages over their non-conjugated predecessors. PEG-IFN-α has a reduced rate of degradation and clearance with an extended half-life, and a doubling of patient sustained virological response has been achieved following its introduction [204, 205]. Both PEG-IFN-α and RBV constitute non-specific pharmacological inhibitors of HCV. Exogenous recombinant IFN therapy is believed to mimic the effects of endogenous IFN in creating a non-virus-specific antiviral state in cells via increased expression of ISGs, while RBV potentially mediates a variety of antiviral effects, one being the inhibition of RdRP [206].

In advance of commencing anti-HCV therapy patients are typically tested for HCV RNA with a quantitative amplification assay, establishing baseline levels against which virologic response can be measured and providing an indication of the likelihood of that response, as high viral loads are negatively correlated to treatment response [207]. Subsequent determination of important clinical parameters such as early virological response (EVR), a

$\geq$2 $\log_{10}$ reduction in HCV RNA levels within 12 weeks of initiating therapy, and sustained virological response (SVR), undetectable HCV RNA $\geq$6 months after completion of therapy, can also be measured by such means. Determination of HCV genotype is also important as patients with treatment-refractory genotypes 1 and 4 may require treatment of longer duration and higher dosage than treatment-favourable genotypes 2 and 3. Baseline liver biopsy may also be performed in genotype 1 and 4 patients to assess histologic grade and stage in advance of treatment but, due to its highly invasive nature, is often foregone in genotype 2 and 3 patients in whom the benefits of treatment are thought to outweigh considerations of disease severity and potential for progression [63].

Treatment regimens consist of weight-based PEG-IFN-α2b (1.5 μg/kg) or fixed-dose PEG-IFN-α2a, administered weekly by subcutaneous injection, with daily oral RBV. Dosage of RBV is dependent on patient genotype and body mass. Genotype 1 patients are recommended for 1000 mg/day for those $<$75 kg, and 1200 mg/day for those $\geq$75 kg, for a total of 48 weeks. While genotype 2 and 3 patients can be treated with 800 mg/day, regardless of body mass, for only 24 weeks [208]. PEG-IFN-α/RBV combination therapy has led to the achievement of SVRs in 42-52 %, 65-85 %, and 76-82 % of individuals infected with HCV genotype 1, genotypes 4, 5 and 6, and genotypes 2 and 3, respectively [209]. In HCV/HIV co-infected patients, across all genotypes, SVR rates range between 27-55 % [210]. Combination treatment is not without significant toxicity and side-effects however. Reported adverse events in patients undergoing PEG-IFN-α/RBV therapy include neutropenia, thrombocytopenia, anaemia, psychiatric disorders, and thyroid dysfunction [211-213]. Adverse effects are relatively common and may lead to a discontinuation of treatment in an estimated 10-15 % of patients [214].

Recent advances in the understanding of HCV genomic organisation and life cycle, allied to the development of HCV replicons and infectious viral particles in tissue culture, have

facilitated the rational design of compounds that specifically target HCV replication. Telaprevir and boceprevir are two protease inhibitors that have been licensed for clinical use in 2011 and represent the prototype direct acting antivirals (DAAs) for HCV. Telaprevir, produced by Vertex Pharmaceuticals in the USA, is a peptidomimetic serine protease inhibitor that binds covalently to the NS3/4A protease, with slow binding and dissociation kinetics [215]. Early *in vitro* characterisation of the drug demonstrated a time and dose dependent inhibition of HCV RNA in replicon cells, culminating in a complete elimination after a two week incubation [216]. Boceprevir is also a covalent, linear protease inhibitor that binds reversibly to the NS3 protease active site and exhibits potent activity in the HCV replicon system (EC_{50} 0.3-0.4 μM), a potency that is enhanced in combination with IFN [217]. Phase III clinical trial data showed that the addition of telaprevir to PEG-IFN-α/RBV for 12 wks of a 48 wk treatment course for gentotype 1 patients increased SVR rates from 24 % to 88 % in relapsers, from 15 % to 59 % in partial responders, and from 5 % to 33 % in null responders [218]. Adding boceprevir to SOC for genotype 1 patients for 32-44 wks resulted in an increased SVR from 29 % to 75 % in relapsers and from 7 % to 52 % in partial responders (null-responders were not included) [219]. These are encouraging results for the future treatment of chronic hepatitis C, particularly for patients with treatment-refractory genotypes. A plethora of additional DAAs are moving toward approval for clinical use such as mericitabine, an NS5B inhibitor, BMS-790052, an NS5A inhibitor, and alisporivir, a cyclophilin inhibitor, that may eventually permit IFN-free regimens, but important issues surrounding cost, drug resistance, and side effects remain to be overcome.

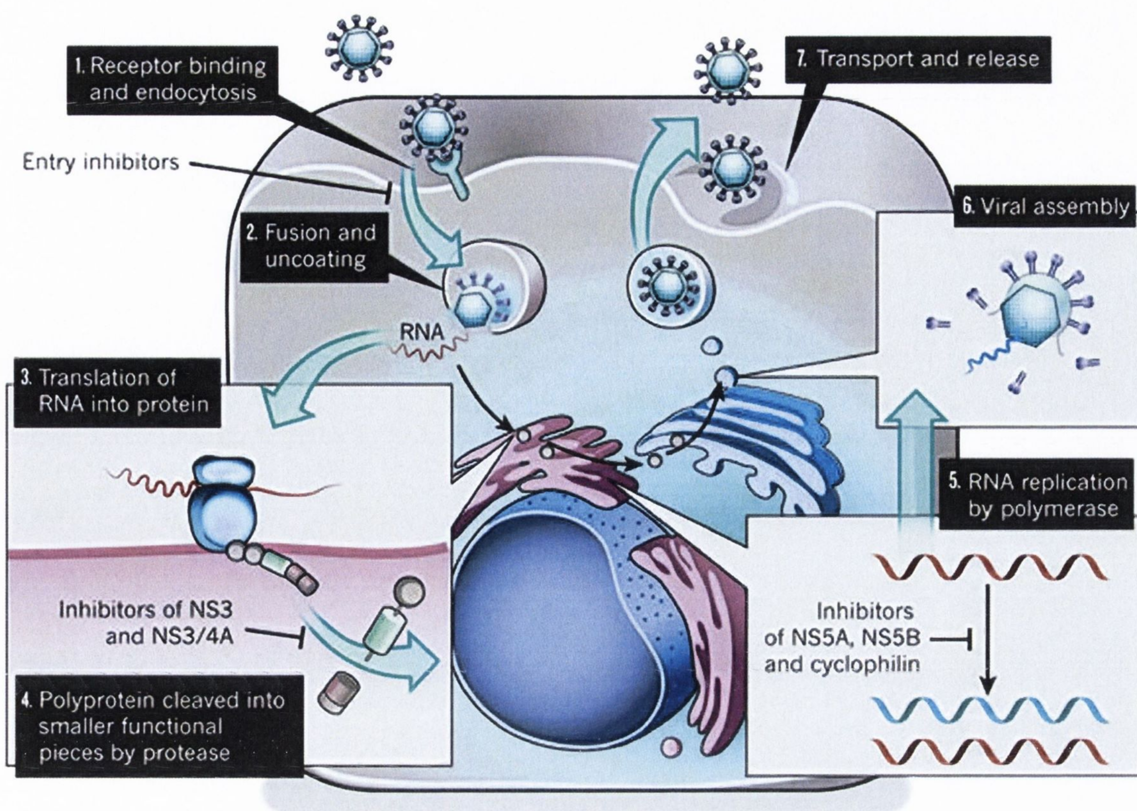


Figure 1.11 Diagram illustrating the HCV life cycle and the stages of which that have been targeted for development of direct acting antiviral (DAA) compounds. After entry into the cell, viral RNA is translated into a single polyprotein which is subsequently cleaved into a series of functional proteins by NS3/4A. Boceprevir and telaprevir block NS3/4A protease activity. Replication of viral RNA is dependent on the action of NS5A, NS5B, and cyclophilin B, a protein which interacts with NS5B to stimulate its RNA binding activity. Specific inhibitors of each of these proteins are currently undergoing clinical trials. In theory, all steps of the HCV life cycle are targets for pharmacological intervention, and further classes such as entry inhibitors are under development. Diagram taken from Schlutter 2011 [220].

1.4 Matrix metalloproteinases (MMPs) and their role in HIV and HCV infection

1.4.1 Background

Matrix metalloproteinases (MMPs), also known as matrixins, are a group of highly conserved proteolytic enzymes that have important roles in normal physiological processes such as embryogenesis, morphogenesis, bone elongation, menstruation, and wound healing, mainly by regulating the turnover of ECM components. MMPs can also mediate cell migration, and the activation or inactivation of numerous soluble factors. Dysregulation of MMP expression is implicated in the pathophysiology of arthritis, cancer and cardiovascular disease [221-223]. MMPs are also highly significant immunomodulators as they facilitate leukocyte recruitment, cytokine and chemokine processing and defensin activation [224-226], and, with their endogenous tissue inhibitors of metalloproteinases (TIMPs), are known to contribute to HIV/HCV disease pathogenesis by involvement in HIV-associated dementia and HCV-related liver fibrosis and cirrhosis [227-229].

1.4.2 Classification and structure

MMPs are a subfamily of zinc- and calcium-dependent enzymes belonging to the metzincin super-family and have traditionally been categorised into groups in accordance with substrate specificity, primary structure and cellular location as collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs). Alternatively, MMPs can be classified based on differences in domain structure (Figure 1.10). The majority of MMPs share a common domain structure comprising of an N-terminal predomian, which is a signal peptide for secretion, a prodomain to maintain latency, a catalytic domain

containing a zinc ion (Zn^{2+}) in the active site, and a C-terminal hemopexin (HPX)-like domain linked to the catalytic domain through a hinge region [230]. However, MMP-23, in contrast to the other MMPs, lacks the HPX domain which is substituted by a C-terminal cysteine array region and an immunoglobulin G-like domain, and, instead of the N-terminal peptide, an N-terminal type II transmembrane domain. The gelatinases, MMP-2 and -9, feature a catalytic domain containing an insert of three fibronectin type II repeats. MMP-9 also has a collagen-like sequence at one end of this domain. Finally, four of the six MT-MMPs are anchored to the cell surface by a carboxyl-terminal transmembrane domain, the other two by a glycosylphosphatidylinositol (GPI) anchor, following the HPX domain.

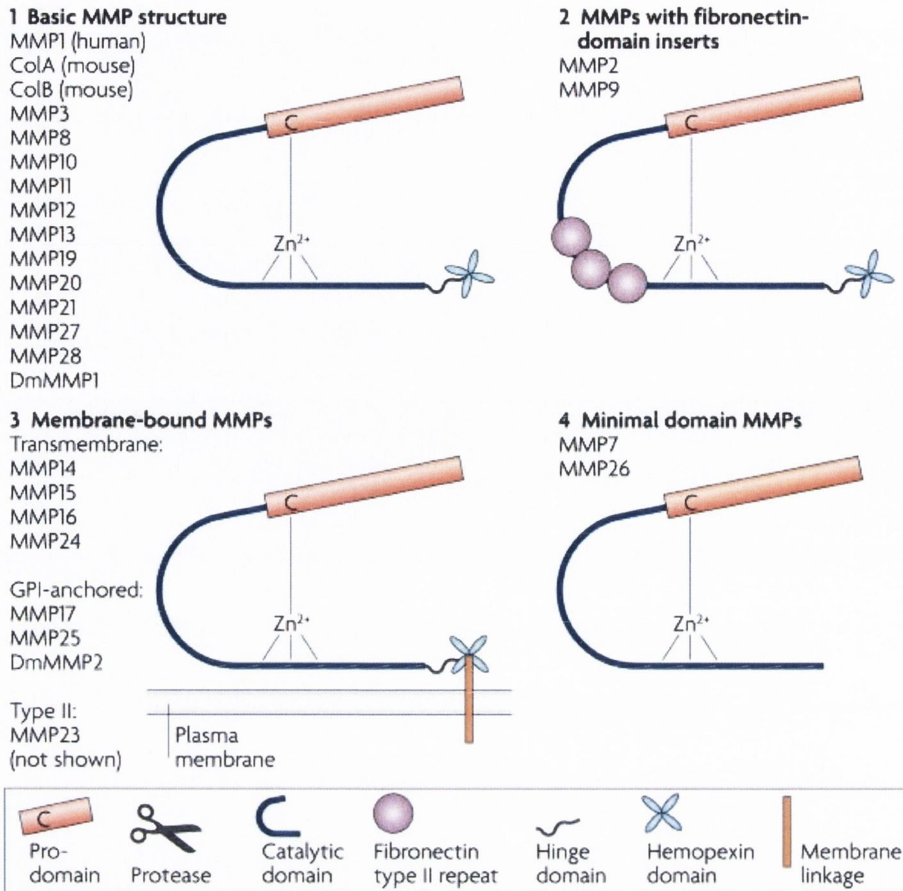


Figure 1.12 Classification of MMPs based on domain structure. The majority of MMPs feature a conserved domain structure of pro-domain, catalytic domain, hinge region and hemopexin (HPX) domain (**1**). Uniquely, MMP-2 and MMP-9 have three fibronectin type II repeats in their catalytic domains (**2**). Membrane type-MMPs (MT-MMPs) are linked to the plasma membrane by a transmembrane domain or a glycosylphosphatidylinositol (GPI) linkage, attached to the HPX domain (**3**). MMP-7 and -26, the matrilysins, lack hinge and HPX domains (**4**). Diagram adapted from Page-McCaw *et al.*, 2007 [231].

1.4.3 Regulation of MMP activity

Appropriately for enzymes that may be deleterious to the host, the expression and activity of MMPs is a tightly regulated process, occurring at several levels including gene transcription, post-transcriptional modification, localisation, pro-enzyme activation and enzyme inhibition.

Changes in expression of MMP genes are the result of targeting by signal transduction pathways of particular elements within promoter regions in response to stimuli such as inflammatory cytokines (IL-1β, IL-6, IL-11, TNF-α) or growth factors (TGF-β). Recruited by activation of signalling intermediates such as mitogen activated protein kinases (MAPK) and signal transducers and activators of transcription (STAT), MMP promoters feature the *cis*-acting elements, activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), and nuclear stimulating protein-1 (SP-1), that cooperate to enhance transactivation of most MMPs [232]. Impairing these signal pathways, by reduced synthesis or by inhibition of phosphorylation, blocks transactivation and leads to silenced gene expression [233].

Post-transcriptional modification of MMPs involves secretion as proMMPs, or zymogens, maintained in a catalytically inactive state by the interaction between the thiol group of a pro-domain cysteine residue and the zinc ion of the catalytic site. Disruption of this interaction by proteolysis of the pro-domain or by modification of the cysteine thiol group results in conversion to active proteinases, a process often referred to as the 'cysteine switch' [234]. Plasmin, which is produced by the action of urokinase on plasminogen, can activate a number of MMPs by cleavage of the pro-domain. While activation of most MMPs occurs extracellularly, MMP-11 and the MT-MMPs are activated intracellularly via cleavage of their pro-peptide by the pro-hormone convertase furin. This results in secretion of an active enzyme form [235].

Localisation also represents a significant regulatory pathway of MMP activity. Membrane-bound MMPs for example are anchored by means of transmembrane (TM) (MMP-14, -15, -16, -24), glycosylphosphatidylinositol (GPI) (MMP-17, -25), or N-terminal signal (SA) (MMP-23) linkages. Anchoring to the cell surface thus enables select MMPs to maintain high local enzyme concentrations and also results in more targeted enzyme catalysis toward specific pericellular substrates [225].

A family of four proteins known as TIMPs (TIMP-1, -2, -3, and -4) represent the predominant natural MMP inhibitors, forming non-convalent binding complexes in a 1:1 stoichiometry to the catalytic site [230]. Whilst most of their biological activity is attributable to sequences within the N-terminal domain, C-terminal domain interactions are possible with the catalytic sites of some MMPs and with the HPX domains of MMP-2 and -9 [236] (Figure 1.12). All four TIMPs are broadly inhibitory towards most active forms of MMPs, although TIMP-1 is a poor inhibitor of MMP-19 and a number of the MT-MMPs [237]. Paradoxically, MMP/TIMP interactions may result in activation. Indeed, TIMP-2 participates in the MMP-14 activation of proMMP-2, forming a trimeric complex in which the C termini of proMMP-2 and TIMP-2 interact while the N termini of MMP-14 and TIMP-2 interact [238]. The other major endogenous inhibitor of MMP activity is α2-macroglobulin (α2-M), a glycoprotein consisting of four identical subunits found in the blood and tissue fluids. α2-M is a broad-spectrum proteinase inhibitor, entrapping mainly circulatory MMPs [237] and effecting irreversible clearance via scavenger receptor-mediated endocytosis [239].

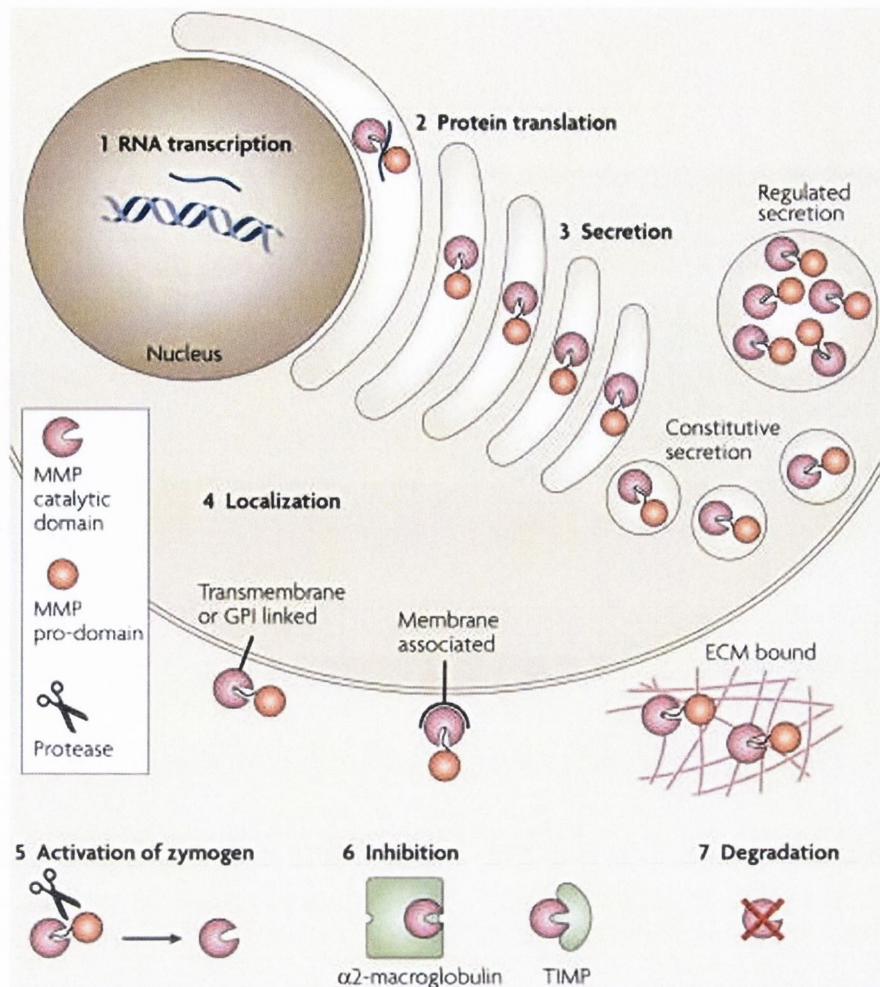


Figure 1.13 Schematic diagram of MMP regulation. MMP RNA transcription (**1**) and protein synthesis (**2**) can be controlled at the level of secretion as latent pro-MMPs, or zymogens, in which the cysteine residue in the pro-domain is bound to the catalytic zinc ion (**3**), and subcellular or extracellular localisation in which MT-MMPs are anchored to the cell surface (**4**). Extracellular control of MMP activity is mediated by zymogen activation via numerous proteolytic cascades (**5**), and the expression of endogenous inhibitors (**6**) and protease degradation (**7**). In addition, substrate availability and accessibility may determine the extent of MMP activity. Diagram taken from Page-McCaw *et al.*, 2007.

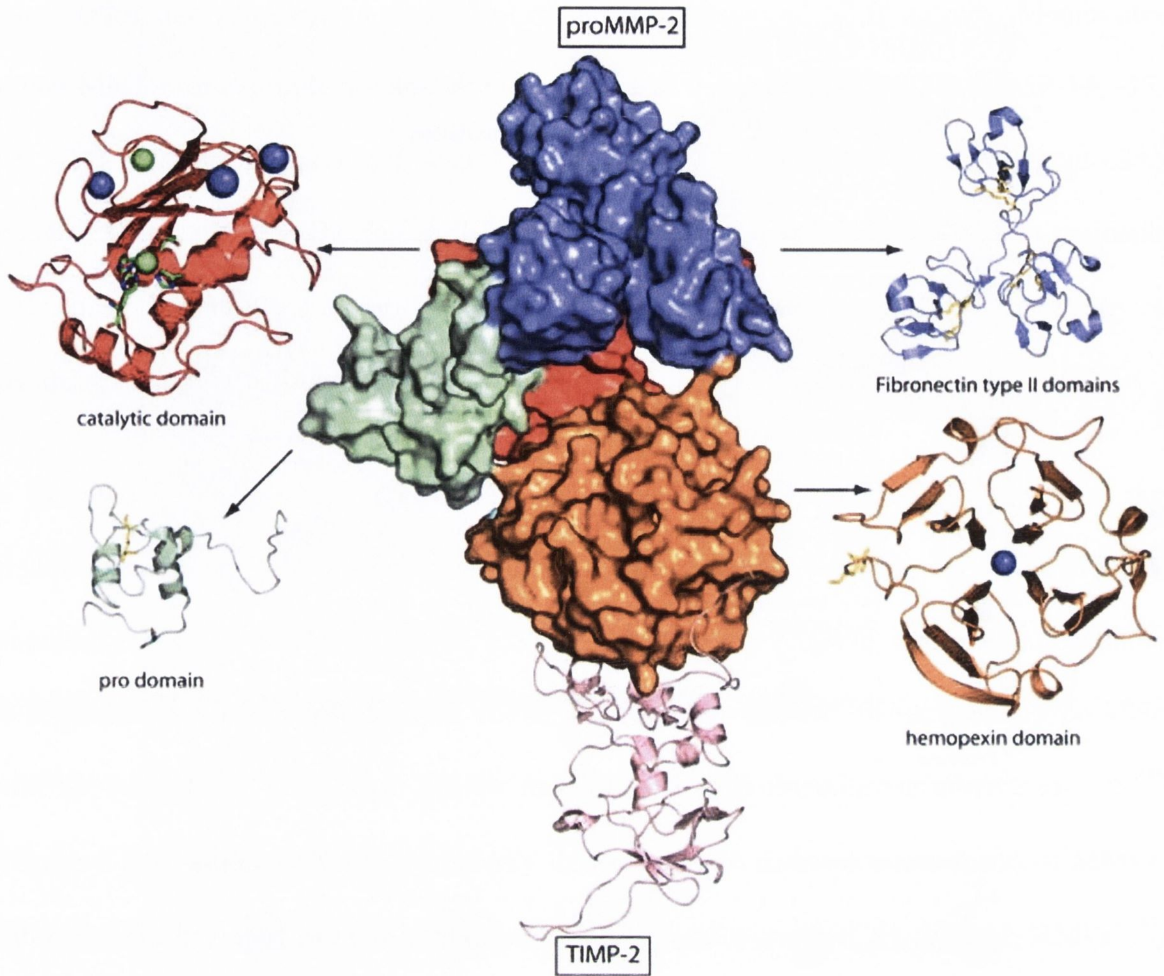


Figure 1.14 3D diagram of human proMMP-2 / TIMP-2 complex. Zinc and calcium ions are indicated by green and blue spheres, respectively, in the catalytic domain. Diagram taken from Murphy and Nagase, 2008 [240].

1.4.4 MMP dysregulation during HIV and HCV infection – implications for pathogenesis

Dysregulated MMP expression and activity is indicated in both HIV- and HCV-infected patients. While appropriate MMP production facilitates an effective immune response, abberant production may enhance disease progression and host tissue damage by altering cellular migration and inducing pathological ECM remodelling. Changes in circulatory and tissue levels of the gelatinases, MMP-2 and MMP-9, and their natural inhibitors TIMP-1 and TIMP-2, have been recorded in HIV-infected patients [241-243] and similarly in HCV-infected groups [244-248]. While the MMP status of co-infected patients is poorly characterised to date, increases in plasma and serum TIMP-1 levels have been described [249, 250].

1.4.4.1 MMP / TIMP mediated pathogenesis during HIV infection

One of the earliest rationales for MMP-mediated pathogenesis in HIV was described by Dhawan and colleagues [251], who showed that increased invasion of a basement membrane model by HIV-infected monocytes was associated with an upregulation of a 92kDa metalloproteinase (MMP-9). Later, the same authors showed that infected monocytes expressing high levels of this enzyme increased the permeability of endothelial cell layers and that incubation with TIMP-1 or TIMP-2 abrogated the effect [252]. These observations are consistent with MMPs facilitating the dissemination of the virus through degradation of cellular and ECM barriers. Alterations of brain microvasculature and the blood-brain barrier (BBB), common in HIV-1 infection, particularly in patients with HIV-associated dementia (HAD), facilitate the entry of activated and infected mononuclear cells into the central nervous system (CNS) [253]. Induction of MMP-9 has also been noted in the cerebrospinal fluid (CSF) of patients with HAD [228, 254], and both MMP-9 and MMP-2 are

associated with neural damage by dint of myelin degradation and neurotoxic protein induction, respectively [255, 256]. Deleterious MMP activity is also suspected in a number of other HIV-associated diseases. Kaposi's sarcoma (KS) is a virally-induced, AIDS-defining cancer which manifests as lesions on the skin, mouth, gastrointestinal, or respiratory tracts. Increases in MMP-2 have been found in the plasma of HIV-infected KS patients compared to non-infected KS patients and both MMP-2 [257] and MMP-9 [258] are overexpressed in cells from KS lesions. Invasion of endothelial cells signifies the initial phase of tumor-associated angiogenesis, and *in vitro* inhibition of MMP-2 by TIMP-2 has been shown to inhibit endothelial cell invasion induced by AIDS-KS cell supernatants [259]. Such data suggest that MMP activation is central to AIDS-KS progression and might represent an appropriate therapeutic target [260]. In addition, overexpressed MMP-9 and TIMP-1 mRNA has been reported in the renal tissue of patients with HIV-associated nephropathy [242], and induction of various MMPs, including MMP-9, have been recorded in the saliva and gingival tissue of HIV-infected patients [261, 262], though evidence for a pathological role for MMPs in both conditions remains to be established.

1.4.4.2 MMP/TIMP mediated effects on the pathology of HCV infection

MMPs play a fundamental role in modulating the course of HCV-related pathology as they contribute to both hepatic fibrogenesis and fibrolysis. Activated hepatic stellate cells (HSCs), the predominant cellular source of collagen production during liver fibrosis, express MMP-2, -14, and -9, and TIMP-1 and -2. MMP-2, and its endogenous activator MMP-14, are increased in liver tisue from HCV patients with chronic hepatits and/or liver fibrosis, compared to normal tissue [245]. Temporal analysis of experimental carbon tetrachloride (CCl_4)-induced liver fibrosis in rats suggests a relationship between progression of liver fibrosis and increased hepatic MMP-2 expression and activity [263]. MMP-2 is also an autocrine proliferation and migration factor for HSCs [264, 265], and as

MMP-14 is a membrane-bound molecule, localisation of active MMP-2 on the cell surface of HSCs might optimise its ability to disrupt normal cell-matrix interactions and enhance the progression of fibrosis. In rodents, MMP-9 is produced by a range of hepatic cell types [266], during CCl_4-induced liver injury [267], and in hepatic stellate cells stimulated with IL-1 *ex vivo* [268]. Futhermore, the expression and activity of MMP-9 are increased in HCV-infected patients [246, 269]. Interestingly, the clinical studies suggest a transient activation of MMP-9 that is more indicative of liver inflammation than fibrosis stage. Factoring evidence that MMP-9 activates latent TGF-β [270], a stimulant for HSC activation and collagen deposition, reinforces the idea that MMP-9 might participate primarily in early stage fibrogenic events.

Increased plasma and serum levels of TIMP-1 and -2 have been recorded in HCV patients compared to healthy controls [248, 271] , with positive correlations to fibrosis stage shown in one of these studies. To what extent circulatory TIMP levels are reflective of the tissue status is uncertain, but Yata and colleagues [272] have reported induction of hepatic TIMP-1 mRNA in HCV patients compared to controls that are commensurate with the degree of infection and cirrhosis. By utilising immunoelectron microscopy techniques, the authors also demonstrated localisation of TIMP-1 to resident stellate and fibroblast cell types. Studies using animal models of progressive fibrosis and explanted human liver tissue also support an associated upregulation of TIMP-1 and TIMP-2 [273, 274].

Liver fibrosis is a dynamic process, undergoing phases of progression and regression, and also resolution in the case of patients who successfully clear the hepatitis C virus, and when experimental liver injury is ceased. In a superbly conceived study, Iredale and colleagues [174] demonstrated that alterations to the MMP/TIMP balance that favour matrix degradation might provide a mechanistic basis for such phenomena. This study, using a rodent model, showed that CCl_4 treatment for a 4 week period generated significant liver

fibrosis, which progressed to cirrhosis after an additional 8 weeks exposure. Cessation of the insult at the 4 week timepoint however, resulted in complete resolution of fibrosis and restoration of normal liver histology. The authors documented that resolution coincided with marked apoptosis of myofibroblast-like HSCs, a reduction in both TIMP-1 and TIMP-2, and perhaps of greatest significance, an increase in collagenolytic activity that coincided with degration of fibrotic matrix. Decreased TIMP expression may be a direct consequence of HSC apotosis of course, but an additional finding of this study was that MMP-13 levels remained unchanged throughout the regression phase. This suggests that a cellular source of matrix degrading MMPs, other than HSCs, mediate the process of fibrosis resolution, quite possibly a circulatory infiltrate such as the macrophage. During fibrosis progression, as previously mentioned, macrophages can promote the activation of HSCs via TGF-β1 secretion, but in contrast, Duffield and colleagues [275] have shown that experimental deletion of macrophages at the onset of fibrosis resolution suppresses ECM degradation and the loss of activated HSCs. This opens the possibility that macrophages induce apoptosis of HSCs, possibly through expression of TNF-related apoptosis-inducing ligand (TRAIL) and other apoptotic stimuli [276] (Figure 1.13). A potential regulator of this macrophage duality could be TNF-α, or indeed its downstream signalling mediator, NF-κB [277]. Macrophages may also indirectly mediate fibrosis resolution by recruitment of neutrophils, an adundant source of collagen degrading MMP-8, to liver tissue [278]. The extent to which macrophages directly mediate matrix degradation, most likely through MMP-9, is an important future question that has implications for both HIV and HCV infection in terms of immunoregulation and immunopathogenesis.

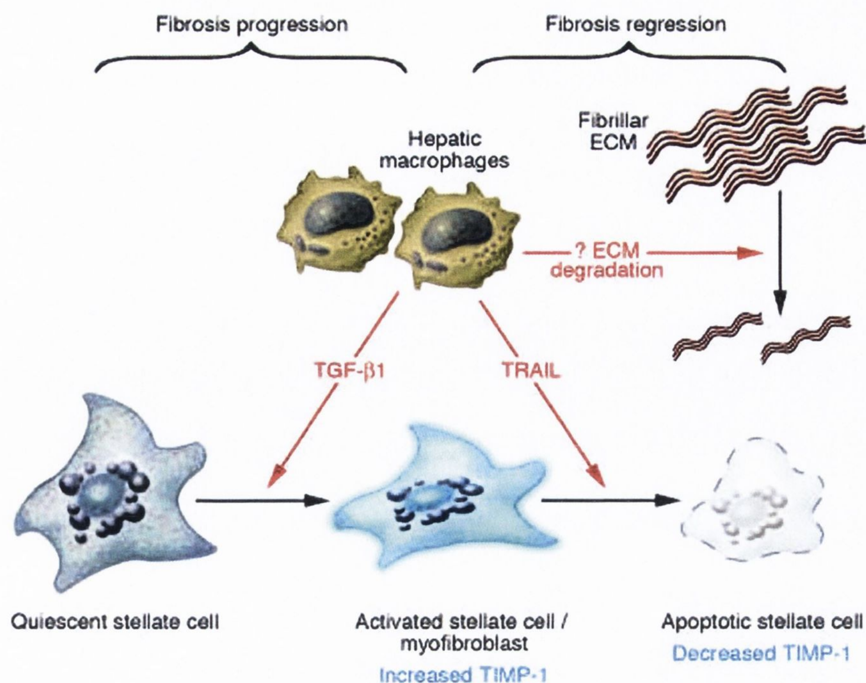


Figure 1.15 Schematic diagram depicting the potential role of macrophages in both the progression and regression of hepatic fibrosis. Duality of macrophage function is indicated by possible TGF-β1-mediated promotion of stellate cell activation during fibrosis progression and potentially TRAIL-mediated stellate cell apoptosis during fibrosis regression. Declining stellate cell numbers is associated with reduced expression of TIMP-1, thus facilitating MMP secretion by macrophages, stellate cell subsets, or alternative cell types. Diagram taken from Friedman, 2005 [277].

1.4.5 MMP/pro-inflammatory cytokine and chemokine interactions

In the context of viral infection, MMPs function not only as effectors of tissue remodelling but also mediate important aspects of the inflammatory response to infection by interactions with cytokines, chemokines and other immunomodulatory proteins. The

cellular components of the inflammatory response include monocytes, macrophages, B- and T-lymphocytes, and neutrophils, each with their own specific patterns of MMP production, secretion, and activation. Monoctyes and macrophages are sources of MMP-1, -2, -3, -9, -14, -17 and the elastase, MMP-12 [279]. T-cells, predominantly MMP-2 and -9 [280, 281], and neutrophils, MMP-8, and -9 [282, 283]. In addition, these MMP producing immune cells are also significant sources of cytokines, including the major pro-inflammatory cytokines, TNF-α and IL-1β, both of which contribute to the pathogenesis of HIV and HCV infection. TNF-α and IL-1β are upregulated in monocytes infected with HIV [284, 285] and are overexpressed in the serum and culture supernatants of cells from HIV patients [286, 287]. Either alone, or in synergy with other cytokines such as IL-6, evidence suggests that both TNF-α and IL-1β enhance HIV replication in infected cells, mainly through NF-κB-mediated transactivation of the viral LTR [288-290]. Increased TNF-α and IL-1β have also been detected in the serum of HCV patients, particularly amongst chronically infected patients with liver cirrhosis and hepatocellular carcinoma [291], and as previously mentioned (section 1.2.6), both are considered pro-fibrogenic as they contribute to the activation of collagen-producing HSCs during fibrosis progression. Upregulation of IL-1β may be particularly damaging as it has been shown in culture to increase the activation and proliferation of myofibroblasts while dose-dependently increasing collagen synthesis [292 293]. Given their common cellular sources, prominent MMP/cytokine interactions pertain, realising often divergent effects. TNF-α and IL-1β both modulate the expression and regulation of MMPs [294-296] and are known to induce MMP-9 in human monocytes and macrophages [297 294]. Exposure of monocytes to exogenous TNF-α and IL-1β, in the presence of macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (regulators of differentiation to macrophages) induces the production of MMP-1, MMP-9, and TIMP-1 *in vitro* [298]. Cell-cell contact between activated T cells and monocytes is also sufficient to upregulate these MMPs and to

simultaneously enhance TNF-α and IL-1β production [299, 300]. By cleavage of cell surface proteins, MMPs can in turn, function as regulators of TNF-α and IL-1β. Gearing et al [301] were one of the first investigators to provide evidence that MMP proteolytic cleavage of the membrane-bound TNF-α precursor protein was responsible for the release of mature TNF-α from leukocytes. Subsequent studies in monocytes implicated additional cytokines dependent on similar activation by MMPs, including M-CSF and TGF [302]. Conversely, the ability of IL-1β to increase MMP expression is negatively regulated by MMPs themselves, as MMP-1, -2, -3, and -9 have been shown to degrade IL-1β [303]. It is also noteworthy that inflammatory mediators may act as inhibitors of MMP production, as both type I and II IFNs, and TGF-β1, have been shown to reduce MMP-9 expression in monocytes [297].

Chemokines, proteins which facilitate the recruitment of lymphocytes, neutrophils and monocytes to sources of inflammation [304], are also targets for cleavage by MMPs. C-C motif ligand-7 (CCL7) and CXC-motif ligand-12 are substrates for MMP-2 [305], while MMP-9 cleaves and activates CXCL6 and CXCL8 (also known as IL-8) and inactivates CXCL-1 and CXCL-4 [306, 307]. Matrix degradation by MMPs also facilitates leukocyte extravastion, a critical event for successful immune surveillance during the inflammatory response. Monocyte migration for example, is dependent on MMP-induced cleavage of basement membrane components and cell surface molecules including CD16 [308], and L-selectin [309], and also the potential regulation of β_1 and β_2-integrin receptor function [310]. Taken together, the above data points to MMPs functioning as both effectors and regulators of the innate immune response, exerting both pro- and anti-inflammatory effects.

1.4.6 Tat and NS3 as mediators of MMP and cytokine dysregulation

Several lines of evidence indicate that both HIV Tat and HCV NS proteins may contribute to the inflammatory response to viral infection by upregulating MMP activity and the production of pro and anti-inflammatory cytokines. In monoctyes, a major target for HIV infection and a significant source of MMPs and cytokines during the course of the disease, exposure to extracellular Tat has revealed a plethora of pathogenic effects. For instance, monocytes subjected to Tat *in vitro* aggregate, adhere to, and disrupt endotheial cell monolayers in tandem with increasing MMP-9 synthesis and release [311]. These findings were highly significant as they implicated Tat as a potential mediator of the MMP-associated extravastion of monocytes into tissues and the subsequent tissue and organ damage witnessed during conditions such as HAD. Indeed, brain-derived Tat sequences from demented AIDS patients display increased MMP-2 and -7 when expressed in primary human macrophages and conditioned medium from these cultures is neurotoxic [312]. Significantly, it has also been shown in astrocytes, a source of inflammatory activity in the CNS, that MMP-9 expression induced by Tat is in turn dependent on Tat-induced TNF-α production [313]. In the context of KS development, Tat and basic fibroblast growth factor (bFGF), a major angiogenic factor, combine synergistically to increase MMP-2 secretion from enthothelial cells [257]. Enhanced bFGF production by endothelial cells is augmented by IFN-γ, TNF-α, and IL-1β [314], a further example of MMP/cytokine relatedness during AIDS pathogenesis.

Cytokine dysregulation during HIV infection is extensive and Tat-mediated cytokine dysregulation is implicated in host damage and the development of opportunistic infections [315]. IL-10, a significant cytokine during HIV infection which shifts the immune response from a Th1 dominance to that of a Th2 [316], has been shown by a number of studies to increase in monocytes as a result of Tat exposure [317-319]. Inductions of pro-inflammatory

IL-6 [320] and IL-8 [321] have also been recorded in Tat-transfected HeLa cells. TNF-α is a major mediator of inflammation that is mainly produced and secreted by macrophages. In HIV-infected monocyte-derived macrophages *in vitro* TNF-α is upregulated and constitutively secreted, and also augments the infectivity of macrophages in an autocrine fashion [322, 323]. PBMCs and CD4$^+$ T cells also produce TNF-α following HIV infection *in vitro* [324]. Addition of recombinant TNF-α to these cultures increases viral replication by up to 10,000-fold, an effect abrogated by neutralisation antibodies. Increased TNF-α is also detected in the sera and *ex vivo* culture supernates of HIV-infected patients [325, 326]. Tat has been demonstrated to upregulate TNF-α in human macrophages via protein kinase C (PKC) pathways [327]. PKR and calcium signalling have also been identified as other potential Tat-induced regulators of TNF-α production [328, 329]. Additionally, Tat has been shown to interact with CD40 to induce TNF-α in monocytes and microglia thereby increasing inflammatory processes within the CNS [330]. IL-1β is another important inflammatory cytokine mainly produced by cells of the macrophage lineage in response to infection and inflammation. *In vitro* infection of MDMs with HIV-1 upregulates IL-1β secretion [331] and monocytes isolated from HIV-infected individuals are seen to express the two biologically active forms of the cytokine, IL-1α and IL-1β [332]. The effects of Tat on IL-1β expression are limited; Buonaguro *et al* [333] found no direct effect in transfected T cell and monocytic cell lines, while Nath *et al* [334] have described increased production in monocytic but not astrocytic cell types. IFN-γ is a highly pleiotropic cytokine produced by activated T cells and NK cells in response to pathogen invasion. HIV-infected macrophages exposed to IFN-γ leads to a reduction in viral replication [335, 336], while exposure of macrophages and monocytes to IFN-γ in advance of infection results in subsequently increased viral replication [337]. IFN-γ secretion is significantly reduced in the supernates of HIV patient-derived PBMCs including both CD4$^+$ and CD8$^+$ T cell subsets [338]. Evidence suggests that Tat can synergise with IFN-γ to increase the production of

CXCL10, a chemokine implicated in the progression of HAD, in macrophages [339]. Combined with other data indicating that IFN-γ enhances the neurotoxicity of Tat and gp120 [340], suggests a pro-inflammatory role for IFN-γ in association with Tat.

The genetic diversity of HIV, the extent of which was discussed in section 1.1.4, not only contributes to successful circumvention of host immune responses but also has profound implications for the relative pathogenesis of individual viral subtypes. Recent evidence suggests differential cytokine expression in response to Tat clades of B and C subtype. Investigating effects in monocytes, Campbell *et al* [341] relate that Tat B induces TNF-α and IL-6 to a greater extent than Tat C, and in a follow-up study by Wong and colleagues [342], a similar comparative effect was reported for IL-10. The only other published study to assess Tat clade differences in respect to cytokine production is that of Gandi *et al* [343], where the authors relate alternate findings as Tat C was evidenced to increase IL-10 secretion above that of Tat B.

HCV proteins have also shown potential for extravirological MMP and cytokine augmentation. In a study by Nunez and colleagues [344], which documented increased intrahepatic cyclooxygenase 2 and MMP-2 and -9 in a group of chronically infected HCV patients, both core and NS5A were shown to upregulate MMP-9 gene expression in hepatocye-derived transfected cells. A paper by Mazzocca *et al* [345] also determined that E2 binding to cell surface receptor CD81 on primary human HSCs increased MMP-2 synthesis and activity in a time-dependent fashion until experiments were terminated at 24 hr. Similar effects were recorded by the same study in NIH3T3 mouse fibroblasts. Such results could well reflect the early stages of fibrogenic events in which increased gelatinase activity contributes to inflammation and deleterious cellular activation (see section 1.3.5).

In a recent study using DCs isolated from healthy donors, Krishnadas and co-workers [346] demonstrated selective IL-12 suppression by core, NS3, NS4, and NS5 proteins confirming earlier findings by others using macrophages [347]. In PBMCs of both healthy donors and HCV chronically-infected patients, Brady *et al* [348] have also shown that NS4 induces IL-10, the source of which was monocytes, as determined by cell separation techniques. Additionally, core and NS3 (but not E2) can also increase TNF-α and IL-10 secretion by monocytes from healthy and HCV-infected donors [349]. All of these studies relate either direct or indirect cellular impairments as a result of interactions with viral proteins, indicating that extracellular core and NS proteins in particular are significant immunomodulators.

Most published studies to date feature HIV-1 Tat B isolates, the most common subtype in North America and Western Europe but only a minority of the global HIV population compared to clade C strains, which comprise over 55 % [23]. The clade-specific studies of Tat effects on cytokine production mentioned above are an important step in addressing this considerable shortfall in the literature and continuing research should help to characterise the significant differences in the pathogenesis of the major clades. Furthermore, the effect of Tat clades on MMP/TIMP expression is unknown at the present. Extracellular HCV NS3 protease activity is also deserved of much futher investigation. Its immunomodulatory potential, in part, may be better understood by an assessment of its effects on cytokine and MMP/TIMP production in relevant cell models.

1.4.7 MMP expression in response to antiviral therapy

Considering the implications for dysregulated MMP expression in the context of HIV and HCV infection, the effects of antiretroviral agents on MMPs have been relatively poorly

explored to date. Assessing the neuropathological implications of altered MMP activity during HIV infection, a study by Liuzzi *et al* [350] was one of the earliest to demonstrate direct MMP modulatory effects by anti-HIV agents. Subjecting neuronal cell cultures to nano molar concentrations of zidovudine, an NRTI, and indinavir, a PI, resulted in a dose-dependent reduction of MMP-9 activity and mRNA expression. These findings were further investigated by examination of MMP-9 levels in PBMCs isolated from HIV-infected patients relative to treatment status. Supernatants from cell cultures isolated from treatment-naïve patients displayed significantly higher MMP-9 activity and mRNA expression than from HAART-treated patients. Indeed, MMP-9 abundance in treated patients was comparable to healthy donors [351]. In examining the potentially deleterious role of reduced MMP-9 in PI-mediated alterations of adipocyte function, successive studies by Bourlier *et al* [352] and De Barros *et al* [353] also related reductions of gelatinolytic activity by a range of PIs including, indinavir, saquinavir, ritonavir, and nelfinavir, at concentrations $\leq$ 10 μM.

Interferon, as a therapeutic with general anti-inflammatory properties, is a potential MMP modulator. While affirmative data exists from *in vitro* and *in vivo* studies, effects are divergent and a lack of consensus pervades clinical studies. In demonstrating reduced MMP-9 levels in HIV-infected monocytes *in vitro* through IFN-γ treatment, Dhawan and co-authors [354] reported that IFN decreases MMP abundance. Cancer-related studies have also reported that IFN-α therapy suppressed MMP-9 expression [355, 356], with IRF1 activation suggested as the mechanistic basis for such an effect [357]. In HSCs, stimulated with IL-1β and TNF-α, Gianelli and colleagues [358] report that MMP-9 mRNA is decreased by the addition of IFN-α. Contrary to this, Diaz-Sanjuan and colleagues [359] showed that IFN-α increased MMP-13 mRNA expression in cultures of the same cell type. Interestingly, IFN-γ has been shown to synergise with IL-1β to increase MMP-9 activity in

tuberculosis-infected monocytes [360]. Patient based studies of IFN effects on MMPs centre in the main on HCV mono-infected groups and in more recent years have included assessments of combination therapy with RBV. IFN monotherapy increases the serum MMP-1/TIMP-1 ratio and improves fibrosis score in responders [361], and reduces serum TIMP-1 and alanine aminotransferase (ALT) levels in patients who achieved a SVR [362]. In a study by Flisiak *et al* [363], the combination of PEG-IFN-α2b with RBV was found to increase MMP-1 and reduce TIMP-1 in the plasma of HCV-infected patients with a SVR. In non-reponders, TIMP-1 remained significantly elevated compared to controls during treatment and follow-up. The same treatment in another group of HCV-infected patients resulted in decreased levels of MMP-9 but not MMP-2, TIMP-1 or -2, in SVRs, findings also confirmed at the liver tissue level [364]. Interestingly, in a group of HIV/HCV co-infected patients who were treatment naïve for anti-HCV therapy, but HAART experienced in the majority of cases, plasma TIMP-1 was significantly elevated compared to healthy controls, with no differences in MMP-9 levels [249].

Overall, the findings of these studies would seem to suggest pharmacological effects by anti-HIV and anti-HCV therapeutics beyond inhibition of viral replication and indicate that disease-associated pathologies in which MMPs are prominent may be influenced by the effects of such agents. Importantly though, in HIV/HCV co-infection where disease pathogenesis is exacerbated, data is lacking, and the cellular source of potential therapy-induced alterations to circulatory MMP/TIMP levels requires identification. Given the capacity of cells of the monocyte / macrophage and HSC lineage to mediate disease pathogenesis, *in vitro* models would represent an appropriate area of investigation.

1.5 Innate immunity and cytokine regulation of the immune response to infection

1.5.1 The components of innate immunity

Innate immune responses are rapidly activated against HIV and HCV infection and are a critical determinant of both the degree of infectivity and the subsequent course of disease progression. Indeed, the functions of later T and B cell-mediated adaptive immune responses to viral infection are thought closely related to the quality of the initial innate immune response. The innate immune system consists of cellular components such as monocytes, macrophages, dendritic cells (DCs), natural killer cells (NKs), and γδ T cells, and their respective antiviral and immunomodulatory factors.

Innate cells of myeloid lineage including monocytes, macrophages, and bone marrow-derived DCs play an important role in initial viral infection and contribute to its pathogenesis throughout the course of infection. By virtue of CD4 and CCR5 receptor expression, these cells represent a major target for HIV-1 infection and act as subsequent viral reservoirs, contributing to continuous residual virus replication [365]. Macrophages, unlike T cells, are resistant to the cytopathic effects of the virus [366], and their ability to migrate to organs and survive in tissues facilitates viral dissemination. Furthermore, acting as antigen presenting cells (APCs) or as a scource of chemotactic cytokines, their interaction with $CD4^+$ T cells may favour intercellular virus transmission. However, macrophages are also central to immune control of infection, either by directly destroying invading pathogens by the release of antiviral enzymes such as APOBEC3G [367], or by secreting cytokines such as type I IFN and TNF-α/β that are capable of viral inhibition or activation of alternate innate and adaptive defences [368, 369].

DCs are instrumental in pathogen-specific immune responses via expression of pattern recognition receptors such as Toll-like receptors (TLRs) and C-type lectin DC-SIGN (DC-specific intercellular adhesion molecule-grabbing non-integrin). TLRs are capable of detecting dsRNA and ssRNA during viral infection [370, 371], have been shown to recognise simian immunodeficiency virus (SIV) *in vivo* [372], and recent findings indicate that specific TLR blockade could result in impaired DC recognition of HCV [373]. An initial target for HIV infection, DCs also facilitate viral transmission to $CD4^+$ T cells and macrophages via binding of virions to DC-SIGNs [374, 375]. TLR signalling also induces the differentiation of immature DCs to mature DCs, leading to an overexpression of major histocompatibility complex (MHC) antigens, inflammatory chemokine receptors, and a range of inflammatory cytokines, including IL-2, IL-10, IL-12, IL-18, and TNF-α [376-379].

NK cells are specialised in their recognition of virus-infected cells [380] and are activated by IL-12 release from macrophages and DCs, resulting in cell lysis of the latter [381]. They express soluble anti-viral factors such as IFN-γ [382], perforin and granzymes [383], FasL and TRAIL [384], and also secrete a range of CC chemokines [385]. Indeed, NK cells isolated from HIV-infected patients have been shown to secrete MIP-1α and MIP-1β (macrophage inhibitory protein-1α and -1β), and RANTES (regulated upon activation, normal T cell expressed and secreted), all CC chemokines capable of macrophage-tropic HIV-1 suppression [386].

The immunomodulatory potential of γδ T cells is becoming increasingly apparent and it is thought that these cells may play a critical role in linking innate and adaptive immunity. An explanation of their role in HIV-1 infection follows.

1.5.2 γδ T cell response to HIV-1 infection

The majority of $CD3^+$ T cells in peripheral blood feature a T cell receptor (TCR) composed of α and β chains, while a smaller population bear a TCR comprising γ and δ chains [387]. The predominant γδ T cell within adults is the Vγ9Vδ2-encoded TCR subset, which comprises some 1-5% of peripheral blood T lymphocytes [388]. Vγ9Vδ2 T cells are innate lymphocytes that recognise nonpeptidic phosphoantigens [389, 390], alkylamines [391], and aminobisphosphonates [392], and do so without the requirement for uptake, processing, or major histocompatibility class (MHC) I or II expression [393]. Stimulation of Vγ9Vδ2 T cells *in vitro* with phosphoantigens induces their activation and expansion, and the production of Th1 cytokines IFN-γ and TNF-α [394, 395]. Under certain conditions, Vγ9Vδ2 T cells can also secrete IL-2, IL-4, IL-10 [396] and IL-17 and IL-22 [397]. Antigen-stimulated Vγ9Vδ2 T cells also produce β-chemokines such as MIP-1α and MIP-1β and RANTES, both natural ligands for the CCR5 HIV-1 co-receptor [398, 399]. Thus, Vγ9Vδ2 T cells, in response to antigen, can rapidly produce an array of cytokines and chemoattractants likely to facilitate the host immune response to infection.

Vγ9Vδ2 T cells display diverse responses to HIV *in vitro* and *in vivo*, including the mediation of cytotoxicity, modulation of viral expression, recognition of lymphomas, and repertoire alteration.

Vγ9Vδ2 T cell clones have been demonstrated to lyse (in a MHC-unrestricted manner) HIV-infected T cell lines more efficiently than uninfected control cells [400]. A study investigating the influence of mycobacterial co-infection on HIV disease progression in which HIV-infected promonocytic cells (U1) were exposed to Vγ9Vδ2 T cells stimulated with mycobacterial Ag, noted two distinct but connected effects; U1 cell death and viral expression [401]. Both effects were mediated by the Vγ9Vδ2 T cell release of IFN-γ and

TNF-α, although Fas ligand (FasL) also contributed to U1 apoptosis. The extent of U1 survival, and thus HIV expression, was dependent on mycobacterial Ag concentration, coupled to the differential secretory potentcy of Vγ9Vδ2 cells. When Ag concentration was low, cytokine production by Vγ9Vδ2 cells was reduced and viral expression perpetuated. Indeed, HIV patients co-infected with mycobacterium tuberculosis exhibit increased γδ T cell percentages that are associated with a greater disease severity than mono-infected patients [402]. Simian γδ T cells can suppress SIV replication in infected T cells and induce cytoxic effects that are calcium-dependent, indicating that cytotoxicity is mediated by the perforin/granzyme pathway rather than the Fas-FasL pathway [403].

B cell lymphomas such as Burkitt's lymphoma (BL) are a common AIDS-associated cancer [404], and in response to co-culture with a BL-derived cell line, Daudi, Vγ9Vδ2 T cells can proliferate in a HLA-unrestricted manner [405]. Furthermore, in a similar *in vitro* model, Vγ9Vδ2 specifically lyse Daudi cells and secrete low levels of IFN-γ and GM-CSF in reponse to stimulation with Daudi cells [406].

Increases in relative and absolute numbers of γδ T cells have been recorded in the peripheral blood of HIV-1-infected individuals, including those free of clinical infections caused by opportunistic pathogens [407-409]. Such increases are likely reflective of enhanced Vδ1 subsets, with a resultant inversion of the normal adult peripheral blood Vδ2 to Vδ1 ratio [410]. In the absence of a clonal expansion of Vδ1 T cells, increased numbers in the circulation are thought a consequence of increased trafficking from various tissues [410], potentially a result of heightened MMP and cytokine production. A restoration of normal γδ T cell repertoire has been shown in HIV patients as a result of HAART. Bordon *et al* [411] reported recovery of normal Vγ9 TCR repertoire to correlate positively with longer duration of therapy and attainment of viral suppression. Martini *et al* [412] also showed improved γδ T cell reactivity after only 3 months of HAART. However, Poles and

colleagues [413] describe an expansion of Vδ1, and a contraction of Vδ2 T cells, in both the mucosa and the peripheral blood of HIV-infected patients that persists without reversion despite treatment with HAART. It is notable though that nearly 43% of the treated subjects had detectable viremia despite chronic HAART and a further 25% had only recently initated therapy.

A previous study suggested that HIV-1 Tat could interfere with chemokine receptor function as its N-terminal cysteine-rich domain contains CXC and CC chemokine-like sequences, and thus contribute to the disordered distribution of γδ T cells observed in HIV-infected patients [414]. Despite these findings no further studies have been conducted on the effects of Tat on γδ T cell repertoire or function to date. Furthermore, in light of differential modulation by subtypes B and C of cytokine expression in monocytes shown recently, it remains to be established whether distinct Tat clades influence γδ T cells similarly.

1.5.3 The role of interferon-γ and tumour necrosis factor-α in regulating the immune response to HIV-1 infection

1.5.3.1 Interferon-γ

Interferons (IFN-α, -β, -γ, -λ) possess a wide variety of antiviral and antiproliferative effects and are capable of non-specific viral inhibition. The IFN family consists of type I IFN (α/β), type II IFN (γ), and the recently characterised type III IFN (λ). Originally defined as an antiviral agent [415], IFN-γ, distinct from type I and III IFN, is involved in the regulation of nearly all phases of the immune and inflammatory response to infection, and is thus better defined as an immunoregulatory cytokine. IFN-γ is secreted by activated NK cells [416], T helper cells of the Th1 subset [417], $CD8^+$ cytotoxic T (CTL) cells [418], and γδ T

cells (see section 1.5.2), and exerts its biological functions through the transcriptional regulation of interferon-stimulated genes (ISGs) upon binding to its cognate receptor (IFNGR). The signals initiated by IFN-γ receptor binding are mediated predominantly by the classic JAK/STAT signalling pathways [419].

IFN-γ stimulates antigen presentation by upregulating class I and II antigen presentation pathways. Cell surface class I MHC upregulation by IFN-γ is important for host response to intracellular pathogens, as it increases the potential for CTL recognition of foreign peptides and thus promotes the induction of cell-mediated immune surveillance [420]. Induction of class II MHC molecules by IFN-γ promotes antigen presentation properties in macrophages [421] and peptide-specific activation of $CD4^+$ T cells [422]. IFN-γ also induces *de novo* class II MHC expression on fibroblasts, keratinocytes, and endotheial and epithelial cells, cells that would not otherwise express MHC molecules, thus enabling these cell types to function as temporary APCs at sites of immune activity [423, 424].

Disease progression subsequent to infection with HIV-1 has been shown to correlate with a shift from a T helper type 1 (Th1) to a T helper type 2 (Th2) cytokine response [425-427]. Th1 cells are characterised by secretion of antiviral cytokines, IFN-γ and IL-2, while Th2 cells are characterised by secretion of predominantly proviral cytokines such as IL-4, IL-10, and IL-13. IFN-γ plays an important role in regulating the balance between Th1 and Th2 cells by increasing IL-12 synthesis [428]. IL-12 is the primary effector that drives developing $CD4^+$ cells to become Th1 cells [429, 430]. In a positive feedback loop, IL-12 directly induces IFN-γ gene transcription and secretion in antigen-stimulated naïve $CD4^+$ T cells [431, 432], and also NK cells [433].

IFN-γ can also have direct effects on humoral immunity by regulating the development, proliferation, and immunoglobulin (Ig) secretion and chain switching, of B cells. As

different Ig isotypes promote distinct effector functions in the host, IFN-γ can facilitate interaction between the humoral and cellular components of the immune response and increase antiviral defences by selective induction of particular Ig isotypes [434, 435].

1.5.3.2 Tumour necrosis factor-α

Tumour necrosis factor-α (TNF-α) was originally characterised for its ability to induce tumour cell apoptosis and cachexia but is now recognised as a central mediator of a broad range of biological activities, encompassing inflammatory and immune responses. TNF-α, and other members of the TNF cytokine family, interact with more than one receptor of a corresponding superfamily of cognate receptors that include TNFR1, TNFR2, lymphotoxin-β receptor, and the herpes virus entry mediator [436-438]. TNFR1 is widely expressed throughout human tissues and acts as the main receptor for TNF-α, while TNFR2 is exclusive to leukocytes and binds both TNF-α and TNF-β. Binding of TNF-α to these two receptors results in downstream activation of caspases (3, 6, 7, and 8) and the transcription factors, AP-1 and NF-κB [439].

As a result of its ability to induce NF-κB, a transcriptional activator of the HIV-1 LTR, pro-viral effects have been attributed to TNF-α in chronically infected T cell and monocytic cell lines [440-443]. However, pre-incubation of human macrophages with TNF-α inhibits HIV-1 entry [444, 445], suggesting that TNF-α may also confer host cellular resistance to infection. The CC chemokines RANTES and MIP-1α and -1β have been identified as effective inhibitors of HIV-1 entry into susceptible cells by competing with the virus for receptor binding [446, 447]. Lane and colleagues [448] showed that TNF-α treatment of freshly HIV-infected monocytes and alveolar macrophages increased RANTES production, thereby suppressing viral replication. This effect was abrogated by immunodepletion of RANTES alone, or in combination with MIP-1α and -1β. Additionally, it was reported that

exposure to TNF-α reduced CCR5 expression in both cell types. Given that NK cells are a significant source of TNF-α [449] and MIP-1α [450], these cells may also mediate a similar antiviral pathway during early stage infection. The above data would suggest that TNF-α exerts differential modulation of monocytes/macrophages latently infected, and newly infected, by HIV.

TNF-α may also interact with other cytokines to regulate the immune response to HIV infection. IL-10 is a cytokine produced by DCs and Th2 cells that inhibits the production of pro-inflammatory cytokines and chemokines [451] and displays predominantly pro-viral activity [452]. During *in vitro* HIV infection of macrophages, IL-10 has been shown to inhibit viral replication based on the prevention of synthesis and release of endogenous TNF-α and IL-6 [453]. However, lower concentrations of IL-10 resulted in enhanced HIV replication, an effect correlated to the cooperation of the released TNF-α and IL-6 [454]. TNF-α and IL-12 synergise to increase IFN-γ secretion by NK cells [455], and, interestingly, IL-10 production in response to infection with Listeria monocytogenes inhibits this response in immunodeficient mice [456].

1.6 Rationale and aims

MMPs and their natural inhibitors, TIMPs, and the cytokines TNF-α, IL-1β and IFN-γ, are an integral part of the inflammatory and immune response to HIV and HCV infection, exerting potentially significant pathological and immunoregulatory effects during the course of both infections. Some evidence suggests that MMP/TIMP and cytokine expression is altered by both exogenous therapeutic agents and endogenous viral proteins.

As such, select anti-HIV and anti-HCV compounds have been shown to alter the MMP/TIMP balance *in vitro* in a limited number of cell types and *in vivo* in HIV and HCV mono-infected patients. The HIV-1 regulatory protein Tat has been evidenced to induce MMP and cytokine expression, with recent data indicating a differential modulation of cytokine expression in respect to individual HIV subtypes, or clades. HCV non-structural (NS) proteins have also demonstrated extravirological functions including the potential to mediate changes to cytokine production in non-infected cells.

The effects of HIV/HCV antiviral therapy on important cellular sources of MMPs and TIMPs during infection such as monocytes/macrophages and hepatic stellate cells requires much further characterisation and the effects of combination ribavirin/pegylated interferon-α (RBV/PEG-IFN-α) therapy on the MMP/TIMP status of HIV/HCV co-infected patients have not been described to date by any investigators. The effects of HIV-1 Tat clades on MMP and TIMP production in immune cells has also not been studied and considering their often concomitant effects, a parallel assessment involving pro-inflammatory TNF-α and IL-1β cytokine secretion may serve to further delineate the respective pathogenesis of HIV-1 genetic subtypes, which to date is poorly understood. Furthermore, an additional investigation into the effects of HCV NS3 protein on the same parameters might contribute to an understanding of how viral proteins contribute to impaired immunity and accelerated disease progression in HIV/HCV co-infection. Additionally, the potential for Tat clades to affect differential cytokine expression patterns in T lymphocytes and their Vγ9Vδ2 subset has not been explored to date, despite the significant immunomodulatory properties of these cells.

Therefore, the overall aims of this thesis are to investigate the effects of antiviral therapy and HIV and HCV proteins on MMP/TIMP abundance and cytokine production in both *in vitro* and *in vivo* settings where appropriate. Specifically, THP-1 monocyte/macrophage

and LX-2 hepatic stellate cell models are employed to assess the effects of HIV PIs, NRTIs, and NNRTIs, and RBV and IFN-α on MMP-9 activity and mRNA expression. An accompanying clinical study also investigates the MMP/TIMP status of patients either mono-infected with HIV or HCV, or co-infected with both viruses. The effect of initiating RBV/PEG-IFN-α2b therapy on the MMP/TIMP status of the latter patient group is also examined.

In view of evidence that HIV-1 Tat protein trans-activates numerous non-viral genes differentially based on genetic subtype, and that HCV NS3 protein may also exert pleiotropic effects, THP-1 monocytes/macrophages are subjected to Tat clades B and C and NS3 protease. Employing a range of concentrations for each protein, effects on MMP-9/TIMP-1 abundance and TNF-α/IL-1β secretion are examined.

Factoring the importance of T lymphocyte cytokine production during viral infection and the emerging significance of the Vγ9Vδ2 T cell subset with respect to immunomodulation, the principal aims of the third study are to; 1.) quantify the percentage of these cells producing IFN-γ and TNF-α, and, 2.) determine the levels of IFN-γ and TNF-α secreted by these cells, when challenged with Tat clades, A, B, C, and D, under both stimulated and non-stimulated conditions.

Chapter 2.

Cells and materials

2.1 Cells and cell culture reagents

THP-1 Cells:

THP-1 cells are a pro-monocytic human leukaemic cell line cultured from the blood of a boy with acute monocytic leukaemia [457]. These cells grow in suspension, and can be differentiated into adherent cells of a macrophage phenotype by exposure to phorbol esters [458]. THP-1 cells were a gift from Prof. Joseph Keane, Trinity College Dublin.

LX-2 Cells:

LX-2 cells are an adherent human hepatic stellate cell line, which were selected through spontaneous immortalisation in low serum conditions [459]. Prof. Scott L. Friedman (Mount Sinai School of Medicine, New York) kindly donated these cells.

Peripheral Blood Mononuclear Cells (PBMCs):

PBMCs were isolated from the venous blood of healthy male donors by gradient density centrifugation using Lymphoprep™ immediately before experimentation.

Table 2.1 The main cell culture reagents used in the study.

Product	**Supplier**
RPMI 1640	Invitrogen (Paisley, UK)
Dulbecco's Modified Eagles Medium (DMEM)	Sigma-Aldrich (Arklow, Rep of Ireland)
Foetal bovine serum	Sigma-Aldrich
Penicillin	Sigma-Aldrich
Streptomycin	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
Trypsin	Sigma-Aldrich

2.2 Consumables

Table 2.2 Major consumable items used in the study.

Product	Manufacturer
12-well plate, Nunclon™Δ Surface, sterile	Nunc (Kamstrupvej, Denmark)
24-well plate, sterile	Sarstedt (Numbrecht, Germany)
48-well plate, Nunclon™Δ Surface, sterile	Nunc
96-well plate, Nunclon™Δ Surface, sterile	Nunc
White 96-well plates	Greiner Bio-One (Frickenhausen, Germany)
MicroAmp® Fast Optical 96-Well Reaction Plate	Applied Biosystems (Carlsbad, USA)
T75-cell culture flask, Nunclon™Δ Surface, filter cap, sterile	Nunc
T150-cell culture flask, Nunclon™Δ Surface, filter cap, sterile	Nunc
Cryogenic vials, sterile	Corning (Amsterdam, Netherlands)
Serological pipettes, sterile	Corning
Syringes and needles, sterile	Becton, Dickinson and Company (Oxford, UK)
Microtubes	Sarstedt

2.3 Pharmacological agents and recombinant proteins

Table 2.3 comprises the antiretroviral agents and recombinant proteins, with their suppliers, used in this study. Pharmacological agents and proteins not mentioned were obtained from Sigma-Aldrich (Arklow, Co. Wicklow, Rep of Ireland).

Table 2.3 Antiretroviral agents and recombinant proteins

Compound	Class	Supplier
Efavirenz	NNRTI	Boehringer Ingelheim (Ingelheim, Germany)
Nevirapine	NNRTI	Boehringer Ingelheim
Abacavir sulphate	NRTI	NIAD, NIH (Bethesda, MD, USA)
Zidovudine	NRTI	USP Reference Standards (Rockville, MD, USA)
Lopinavir	PI	Boehringer Ingelheim
Atazanavir sulphate	PI	GlaxoSmith-Kline (Uxbridge Middledex, UK)
Saquinavir mesylate	PI	USP Reference Standards (Rockville, MD, USA)
HCV NS3	n/a	Prospec (East Brunswick, NJ, USA)
HIV-1 Tat Clades	n/a	Prospec
Interferon alpha 2a	n/a	Prospec
proMMP-9	n/a	AnaSpec (Fremont, CA, USA)

2.4 Enzymes and primers for real-time RT-PCR

Table 2.4 Enzymes used and their suppliers.

Enzyme	Company
Deoxyribonuclease I, Amplification Grade	Invitrogen (Paisley, UK)
M-MLV Reverse Transcriptase	Sigma-Aldrich
QuantiTect SYBR Green PCR Kit,	Qiagen (Crawley, UK)

Table 2.5 QuantiTec Primers for real-time RT-PCR. All RT-PCR primers were purchased from Qiagen as part of a predesigned QuantiTec Primer Assay and selected according to species and target gene of interest.

Target	Species	Detected transcript	Amplicon length	Product code
MMP-9	Human	NM_004994	115 bp	QT00040040
GAPDH	Human	NM_002046	119 bp	QT01192646

2.5 Antibodies

Table 2.6 Details of the antibodies used for flow cytometric analysis, their source, conjugation, and suppliers.

Target	Source	Conjugate	Supplier
CD3	Mouse	FITC	Immunotools (Friesoythe, Germany)
CD14	Mouse	FITC	Immunotools
CD3	Mouse	Pacific Blue	BD Biosciences (Oxford, UK)
CD4	Mouse	PE	BD Biosciences
CD19	Mouse	APC	BD Biosciences
IFN-γ	Mouse	APC	BD Biosciences
TNF-α	Mouse	FITC	BD Biosciences
Vδ2	Mouse	PE	BD Biosciences
IgG1	Rat	-	Miltenyi Biotec (Bergisch Gladbach, Germany)

2.6 Equipment

Table 2.7 Details of the equipment used in the study and their suppliers

Equipment	Model/Supplier
7900HT Fast Real-time PCR System	Applied Biosystems
Analytical balance	Mettler, AE240
Autoclave	Dixons (Wickford, UK)
Automated pipettes	Gilson, Inc. (2 μl-5000 μl) (20–300 μl)
Pipetman Ultra 8-channel	Gilson Inc. (20–300 μl)
Cell Sorter	Beckman Coulter, MoFlo™ XDP
Centrifuge	Hettich Zentrifugen, EBA 12R/mikro 22R
Flow Cytometer	Beckman Coulter, CyAn™ ADP
Freezer (-80°C)	Thermofisher Scientific, Revco Value Plus
Gel documentation system	Syngene, GeneGenius
Gel electrophoreses system	Bio-Rad, Mini-Protean
Incubator (37 °C, 5% CO_2, 95 % rh)	Memmert, Inco2
Inverted microscope	VWR, VistaVision™
Laminar Flow Hood	Mason Technology, BioBan 48

Table 2.7 cont.

Equipment	Model/Supplier
Luminometer	Thermofisher Scientific, Fluoroskan AscentFL
Microplate reader	BioTek, EL 808
Microplate washer	Bio Tek, ELx405
Micro-Volume UV-Vis Spectrophotometer	Thermofisher Scientific, Nanodrop ND 8000
Neubauer haemocytometer	BRAND GMBH + CO KG, Blaubrand®
pH meter	Mettler-Toledo Inc., MP230
Thermocycler	MJ Research Inc, PTC-100

Chapter 3.

Effect of ribavirin and interferon on MMP-9 abundance in THP-1 and LX-2 cells and in HIV/HCV co-infected patients

3.1 Introduction

Since the advent of highly active antiretroviral therapy (HAART) in the mid-nineties, mortality associated with human immunodeficiency virus (HIV) has declined and the clinical profile of the disease has diminished in severity [460]. However, immunopathological complications remain a significant factor in the management of the disease, and co-infection with hepatitis C virus (HCV) has emerged as a major detriment to the survival of patients [461].

The pathophysiology of both viruses is well characterised and key cellular targets have been identified. For example, monocytes/macrophages are known reservoirs of the HIV virus and disruption of the vascular endothelium during infection permits the migration of activated cells to tissues, thus facilitating viral dissemination and organ damage [462]. With regard to HCV infection, activation of resident hepatic stellate cells (HSCs) characterises remodelling of liver tissue. Upon activation, HSCs adopt a myofibroblast-like phenotype and express profibrogenic cytokines and type I and III collagen [463]. The net result of which is the accumulation of extracellular matrix (ECM) and the formation of scar tissue [464]. Liver macrophages are also implicated in this process as they can activate hepatic stellate cells by secreting paracrine factors such as transforming growth factor-β1 (TGF-β1) [277].

Matrix metalloproteinases (MMPs), a large group of proteolytic enzymes regulated via gene expression, precursor activation and inhibition by their endogenous tissue inhibitors of metalloproteinases (TIMPs), contribute to cell migration, activation and inactivation of soluble factors, and the regulation of ECM constituents [230]. As activated monocytes/macrophages and HSCs both express a range of MMPs and TIMPs, MMPs may play a fundamental role in HIV/HCV-associated pathology. In experimental models of CCl_4-induced liver fibrosis MMP-2 and -9 are increased [244, 465], and have been shown to

activate HSCs via cytokine stimulation, which in turn stimulates collagen production during the early stages of fibrogenesis [466, 467]. During later stages, enhanced MMP-9-mediated degradation of type I collagen, coupled with a corresponding reduction in TIMP expression, may contribute to fibrolysis, a process facilitated by increased apoptosis of both HSCs [468] and macrophages [275]. This indicates that *in vivo*, liver fibrosis is probably a process of continual flux, involving phases of progression and regression [463]. Such data suggests a prominent role for cells of the monocyte/macrophage and hepatic stellate cell lineage in immunopathological events in which MMPs and their inhibitors are prominent.

Data on the effect of anti-HIV and anti-HCV pharmacological agents on MMP abundance in relevant cell models is limited [350, 359], and while it is well documented that MMPs are highly dysregulated in HIV and HCV mono-infected patients [228, 246, 249, 261, 351, 469, 470], little consensus exists and their status in co-infected groups is poorly investigated. Furthermore, it remains to be established if anti-HCV therapy alters MMP and TIMP expression in co-infected patients. Therefore, we conducted an *in vitro* study to examine the effects of RBV, IFN-α, and a selection of clinically prescribed HIV antiviral agents on MMP-9 activity and expression in cells of an inflammatory and fibrotic phenotype, namely THP-1 monocytes/macrophages and LX-2 hepatic stellate cells. Additionally, as MMP-9 and MMP-2 are contributory to disease progression in HIV/HCV patients, and factoring their endogenous regulation by TIMPs, we also investigated if MMP-9, MMP-2 and TIMP-2 activity and abundance differ in HIV/HCV co-infected patients compared to HIV and HCV mono-infected patients and healthy controls. We also assessed the effects of initiating HCV therapy (ribavirin and pegylated-interferon-α2b) on MMPs in HIV/HCV co-infected patients.

3.2 Methods

3.2.1 Cell culture

THP-1 cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640, L-glutamine, 25 mM HEPES) (Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Sigma-Aldrich, Arklow, Rep of Ireland) in a humidified atmosphere at 37 °C and 5 % CO_2. Flasks were divided 1:3 every 3-4 days. LX-2 cells were cultured under identical atmospheric conditions in Dulbecco's Modified Eagle's Medium (DMEM, 4500 mg/l glucose) (Sigma-Aldrich) supplemented with 10 % foetal bovine serum, 2 mM glutamine and 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were subcultured every 2-3 days using Trypsin solution (Sigma-Aldrich). Both cell lines were periodically tested for mycoplasma contamination using MycoAlert® Mycoplasma Detection kit (Lonza, Slough, UK). In order to determine cell number and viability, cells were stained with Trypan Blue (Sigma-Aldrich) (0.4 % w/v; 1:5) for 5 min and counted using a Neubauer haemocytometer. Cell number per ml of culture medium was calculated using the following equation:

$$Cells/ml = \frac{number\ of counted\ cells \times 10000 \times 6}{8\ squares \times 5}$$

3.2.2 Preparation of pharmacological agents

HIV antiretrovirals (see Table 2.2), phorbol 12-myristate 13-acetate (PMA), MG132, and doxorubicin hydrochloride (DOX) (all Sigma-Aldrich) drug stocks were solubilised in dimethyl sulphoxide (DMSO) (Sigma-Aldrich), while RBV (Sigma-Aldrich), and IFN-α2a

(see Table 2.3) were solubilised in sterile dH_2O (18 $M\Omega^{-cm}$). All working solutions were prepared in medium and cells exposed to drugs for 48 hr unless otherwise indicated. Final solvent concentrations were as follows; atazanavir (C_{final}= 0.05 % v/v DMSO), lopinavir (C_{final}=0.16 % v/v DMSO), saquinavir (C_{final}= 0.04 % v/v DMSO), abacavir (C_{final}= 0.05 % v/v DMSO), zidovudine (C_{final}= 0.04 % v/v DMSO), efavirenz (C_{final}= 0.1 % v/v DMSO), nevirapine (C_{final}= 0.07 % v/v DMSO), PMA (C_{final}= 0.05 % v/v DMSO), MG132 (C_{final}= 0.01 % v/v DMSO), and doxorubicin (C_{final}= 0.05 % v/v DMSO).

3.2.3 Patient selection and treatment

The patient study was approved by the local ethics committee and informed consent obtained.

HIV/HCV co-infected patients and age, gender, and risk factor matched HIV mono-infected, and HCV mono-infected patients (*n*=10/group), were recruited from patients attending the Department of Genitourinary Medicine and Infectious Diseases, St James's Hospital. Age and gender matched healthy controls were recruited from staff at the hospital. Exclusion criteria included smoking, a disease (cancer, inflammatory condition or obstructive airway disease) or therapies known to alter MMP activity (e.g., statins). The HIV/HCV co-infected patients, a subset of a larger study group [471], received RBV (1000 mg/day for patients <75 kg or 1200 mg/day for patients ≥75 kg orally) and PEG-IFN-α2b (1.5 μg/kg/week subcutaneously) and were followed for 14 days upon commencing treatment. Venous blood was reserved at baseline in all groups, and on days 3 and 14 in the HIV/HCV co-infected group. Blood samples were collected in EDTA and lithium heparin tubes, centrifuged (1650 rpm, 25 min, 4 °C), and the top plasma layer was aliquoted into 1.5 ml screw-cap tubes and stored at -80 °C until required.

3.2.4 Hospital laboratory analysis

HCV infection was confirmed using HCV antibodies (Abbott IMX V3.0; Abbott/Murex Laboratories Ltd., Maidenhead, UK) and recombinant immunoblot assay (RIBA) (Chiron V3.0; Chiron Corporation, Emeryville, USA). Patients were genotyped by restriction fragment length polymorphism (RFLP) as described by others [472]. HIV viral load was quantified using Cobas Amplicor V1.5 (Roche Diagnostics Ltd, Lewes, UK), which has a limit of detection of 50 HIV-1 RNA copies/ml. Biochemical and haematological parameters were measured using standard laboratory techniques.

3.2.5 Gelatin zymography of culture medium and plasma

THP-1 monocytes were seeded at a density of 5 x 10^5 cells/well in 24-well plates in serum-free RPMI and exposed to either ribavirin (RBV) (10 μM), interferon-α2a (IFN-α2a) (250 IU/ml), HIV protease inhibitors (PIs); atazanavir (ATV), lopinavir (LPV) and saquinavir (SQV), HIV nucleoside reverse transcriptase inhibitors (NRTIs); abacavir (ABC) and zidovudine (ZDV), or HIV non-nucleoside reverse transcriptase inhibitors (NNRTIs); efavirenz (EFV) and nevirapine (NVP) at concentrations of 1, 5 and/or 10 μM. One hour later, cells were activated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), to initiate differentiation to macrophages. Experiments were also conducted in the presence of the proteosomal inhibitor MG132 (200 nM), in which case cells were pre-treated with the compound for 1 hour in advance of RBV/IFN-α treatment as above.

LX-2 hepatic stellate cells were seeded at a density of 4 x 10^4 cells/well in 24-well plates in DMEM containing 10% FBS and allowed to attach overnight. Wells were then washed once with phosphate buffered saline (PBS; containing $MgCl_2$ and $CaCl_2$) (Sigma-

Aldrich), serum-free DMEM substituted, and cells exposed to antiviral agents and PMA as above. For both cell lines, all treatments were done in duplicate and terminated after 48 hr. Medium or PMA acted as controls where appropriate. The concentrations of the antiviral drugs used in the *in vitro* experiments reflect mean/median plasma C_{max} values derived from the following published clinical studies: ribavirin (2.7 μg/ml [473]), interferon (12.1 ng/ml [474]); PIs atazanavir (3.2 μg/ml [475]), lopinavir (9.7 μg/ml [476]), saquinavir (3 μg/ml [477]); NRTIs abacavir (3.2 μg/ml [478]), zidovudine (1 μg/ml [479]); NNRTIs efavirenz (3.3 μg/ml [480]), and nevirapine (1.9 μg/ml [481]). Conditioned medium was then collected, cleared by centrifugation (5000 g; 5 min), aliquoted, and stored at -80 °C. In advance of zymographic analysis both culture medium supernatants and plasma samples were mixed with a 2X non-reducing sample buffer (50 mM Tris-HCl, pH 6.8; 0.1 % w/v bromophenol blue, 10 % v/v glycerol and 2 % w/v SDS; final concentrations), vortexed, and centrifuged (1800 g, 5 min, 4° C) to remove any remaining particulate matter. Equal volumes of samples along with recombinant human MMP-2 and MMP-9 standards were subjected to electrophoresis on an 8 % SDS-polyacrylamide gel (0.75 cm thickness; 5 % stacking gel; Table 3.3) co-polymerised with 0.1 % (w/v) gelatin. Gels were run at 100 V for 1.5 hr in a buffer of composition: 25 mM Tris base, 192 mM Glycine, 0.1% (w/v) SDS. Gels were then washed in Triton X-100 (2.5 % v/v) for 30 min and incubated for 18 hr at 37 °C in an incubation buffer of composition 50 mM Tris HCl, pH 7.6, 10 mM $CaCl_2$, 50 M NaCl. Gels were rinsed in destain (1:3:6; glacial acetic acid: methanol: dH_2O) followed by staining (0.05 % w/v coomassie brilliant blue R250 in destain solution) for 1 hr followed by destaining (5 min). Gelatinolytic activity, clear band on blue background, was quantified using a Gel documentation system (Syngene, Cambridge, UK). Samples were normalised to control (PMA). To ensure that non-specific protease activity did not confound the results, identical gels were incubated in

either incubation buffer or one supplemented with 20 mM EDTA to inhibit MMP activity.

Table 3.1 Composition of the resolving and stacking gels used for gelatin zymography.

	Resolving gel (8 %)	Stacking gel (5 %)
dH_2O	2.6 ml	1.4 ml
Gelatin (5 mg/ml)	2 ml	-
30 % (w/v) Acrylamide/Bis-acrylamide	2.7 ml	0.33 ml
Tris HCl (1.5 M, pH 8.8)	2.5 ml	-
Tris HCl (1 M, pH 6.8)	-	0.25 ml
10 % (w/v) SDS	100 μl	20 μl
10 % (w/v) ammonium persulphate	100 μl	20 μl
TEMED	6 μl	2 μl

3.2.6 Real-time RT-PCR

3.2.6.1 RNA isolation and cDNA Synthesis

THP-1 and LX-2 cells were seeded and treated as described previously (section 3.2.5). Total cellular RNA was extracted using TRI Reagent™ (Sigma-Aldrich). In brief, cells were lysed in the wells by addition of TRI Reagent™ (200 μl/well) and the content of the wells transferred to sterile microtubes. Chloroform (40 μl) was added to each sample,

mixed for 15 s, and incubated at room temperature for 15 min. The aqueous and organic layers were separated by centrifugation (12,000 g, 15 min, 4 °C), and the colourless upper aqueous phase containing the RNA was carefully transferred to a sterilised tube. RNA was precipitated by addition of isopropanol (100 μl) and pelleted by centrifugation (12,000 g, 10 min, 4 °C). The pellet was washed with 75 % (v/v) ethanol (200 μl), vortexed and pelleted by centrifugation (12,000 g, 10 min, 4 °C). The resulting RNA pellet was air-dried for 5-10 min and dissolved in 0.1 % (v/v) diethylpyrocarbonate (DEPC)-treated water (13 μl).

The integrity of the RNA samples was assessed using agarose gel (1 % w/v) electrophoresis (Figure 3.1). As can be seen all samples showed 2 clear bands corresponding to 28S rRNA and 18S rRNA in a ratio of 2:1, respectively. There was no smearing noted between bands, which would represent RNA degradation. A third minor band corresponding to 5S rRNA was also noted. Further, concentration and possible contamination were assessed by UV spectrophotometry. Samples (1 μg) were then treated with 1 U of DNase I in 1X DNase I Reaction Buffer and incubated at room temperature (<15 min) to remove any DNA contamination. The reaction was stopped by addition of EDTA (25 mM; 1 μl) and heat inactivation at 65 °C for 10 min. DNase I treated RNA was stored at -20°C until required. cDNA was synthesised by reverse transcription of 800 ng of DNase I treated RNA using M-MLV Reverse Transcriptase in a two-step reaction. RNA samples were denatured and annealed with anchored oligo$(dT)_{23}$ primers (5 μM) in a mixture containing dNTPs (1 mM) at 70 °C for 10 min. The reaction was then cooled (4 °C) and the remaining components added (M-MLV Reverse Transcriptase Buffer, 200 U M-MLV Reverse Transcriptase, 20 U RNase Inhibitor). This was then incubated for 10 min at room temperature, followed by 50 min at 37 °C. The reaction was terminated by heat inactivation at 92 °C for 10 min.

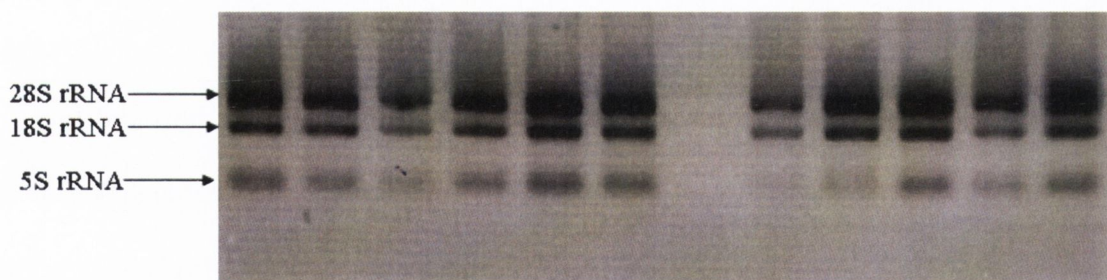


Figure 3.1 Assessment of the integrity of RNA samples isolated using TRI Reagent™.A typical midi gel (1 % w/v agarose) showing 28S rRNA, 18S rRNA and 5S rRNA bands. Image is presented as a negative for clarity.

3.2.6.2 Real-time RT-PCR

Real-time RT-PCR was performed using a QuantiTect SYBR Green Assay and a QuantiTec Primer Assay (Qiagen, Crawley, UK) with primers for human MMP-9 (sense 5'-CCCGGAGTGAGTTGAACCA-3'; antisense 5'-GGATTTACATGGCACTGCCA-3' [NM_004994]) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense 5'-GAAGGTGAAGGTCGGAGTC-3'; antisense 5'-GAAGATGGTGATGGGATTTC-3' [NM_002046]). cDNA (1.5 μl) was added to a reaction mix containing 7.5 μl of QuantiTect SYBR Green, 1.5 μl of the Primer set and 4.5 μl of RNase-free water (PCR-grade) in a fast optical 96-well plate. This was covered with an optical adhesive film, briefly shaken, and analysed in a 7900HT Fast Real-time PCR System (Applied Biosystems) (see Table 3.2 for cycling parameters).

Correction for background fluorescence was achieved through setting ROX dye as an internal fluorescence reference. Furthermore, each real-time PCR run contained a melting point analysis ranging from 60 °C to 95 °C over 30 min and a continuous fluorescence

measurement to identify formation of primer-dimers. Data collection and analysis was acquired using SDS software v2.2.1, in which the threshold level was set to 0.4. Threshold cycle (Ct) was defined as the point where the fluorescence crosses the threshold line and was set in the exponential phase of the amplification. Amplification efficiencies of the individual primer sets was obtained by plotting the mean threshold cycle (Ct) values against the logarithm of DNA mass, in order to determine slope and linearity. Relative mRNA expression was analyzed according to the following equation [482]:

$$ratio = \frac{(E_{target})^{\Delta Ct_{target}\,(control-sample)}}{(E_{ref})^{\Delta Ct_{ref}\,(control-sample)}}$$

In order to make valid comparisons between samples, amplification efficiency for each primer set was determined using a serial dilution from 40 ng of control cDNA (1, 1:3, 1:10, 1:30, 1:100). Recorded amplification efficiencies for MMP-9 and GAPDH gene transcripts were 87.5 % and 89.8 %, respectively ($r^2 \geq 0.98$).

Real-time PCR efficiency (E) was calculated using the slope of the amplification efficiency of the individual primer sets, according to $E = 10^{[-1/slope]}$. E_{target} describes real-time PCR efficiency of the target gene transcript and E_{ref} is the real-time PCR efficiency of a house-keeping gene transcript [482]. ΔCt_{target} is the difference of mean Ct value of control minus treated sample of the target gene transcript. Furthermore, the ΔCt_{ref} value describes the difference of the mean Ct value of the control minus the treated sample of the house-keeping gene transcript.

Samples were analysed in duplicate. Each assay included a no-template control and a no reverse transcriptase-control.

Table 3.2 Real-time theromcycler conditions

Step	Time	Temperature	Comment
Initial activation step	15 min	95 °C	
Denaturation	15 sec	94 °C	
Annealing	30 sec	55 °C	
Extension	30 sec	72 °C	Fluorescence data collection
		40 cycles	

3.2.7 Determination of MMP-9 and TIMP-2 expression by ELISA

THP-1 cells were seeded in 12-well plates at a density of 1 x 10^6 cells/well and treated with RBV/IFN-α2a as described previously (section 3.2.5). After 48 hr medium was removed, wells washed twice with cold PBS, and attached cells lysed by the addition of lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA, 1 % v/v Triton X-100, 0.1 % w/v SDS) supplemented with a protease inhibitor cocktail (4 µl/ml) on a plate shaker (100 rpm, 5 min, 4 °C). Lysates underwent two subsequent freeze/thaw cycles. Following centrifugation (800 g, 5 min, 4 °C), supernatants were removed and stored at -80 °C. For analysis, THP-1 cell lysates were assayed undiluted, while plasma samples were diluted 1:50 in assay diluent. MMP-9 and TIMP-2 abundance was quantified by using the Human MMP-9 Quantikine ELISA Kit and Human TIMP-2 Quantikine ELISA Kit (R&D

Systems, Abingdon, UK) respectively, in accordance with manufactures protocol. The lower limit of detection for MMP-9 and TIMP-2 were <0.156 ng/ml and 0.011 ng/ml, respectively. The intra-assay coefficients of variation and the inter-assay coefficients of variation for MMP-9 were 2.0 % and 7.9 %, respectively. For TIMP-2 these values were 4.4 % and 6.8 %, respectively. Optical density was read at 450 nm with a reference wavelength of 540 nm. MMP-9 and TIMP-2 levels were determined by interpolation from their respective standard curves. Samples and standards were analysed in duplicate and expressed as ng/ml.

3.2.8 Assessment of differentiation markers by flow cytometry

THP-1 cells were seeded in 12-well plates at a density of 1 x 10^6 cells/well and treated with RBV/IFN-α2a as described previously (section 3.2.5). After a 48 hr incubation, culture medium from all wells was discarded and 1.5ml of EDTA (10 mM) added. Plates were placed on a shaker (100 rpm, 15 min, 21 ºC), and subsequently cells from each well were detached by gentle agitation using a cell scraper and transferred to microtubes tubes. After centrifugation (800 g, 5 min, 21 °C), cells were resuspended in 100 µl of PBA buffer (1 % v/v BSA; 0.02 % w/v NaN_3; dissolved in PBS), and 50 µl of each sample was added to FACS tubes containing 0.8 µg of FITC-conjugated mouse anti-Human CD14 mAb (Immunotools, Friesoythe, Germany). Tubes were incubated in the dark for 15 min. Cells were then washed with 2 ml of PBA/tube and resuspended in 500 µl of 2 % w/v paraformaldehyde (PFA). The mean fluorescent intensity of each sample was determined by electronic gating on the cell population using a CyAn ADP (Beckman Coulter, High Wycombe, UK) flow cytometer running Summit software (Dako, Fort Collins, USA), with unstained cells acting as control.

3.2.9 Cell viability assay

THP-1 cells were seeded at a density of 4 x 10^4 cells/well in 96-well plates using serum-free RPMI. LX-2 cells were seeded at a density of 8 x 10^3 cells/well in 96-well plates in complete DMEM and were allowed to attach for 24 hr, after which time cells were washed with PBS and the medium changed to serum-free DMEM. Cells were treated with ribavirin (RBV 10 μM), interferon-α2a (IFN-α2a) (250 IU/ml), saquinavir (SQV, 5 μM), lopinavir (LPV, 10 μM), doxorubicin hydrochloride (DOX, 10 and 50 μM) and medium in the presence of phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) for 48 hrs. At the end of the incubation time 20 μl of CellTiter-Blue® (Promega, Madison, USA) reagent was added to each well and incubated for 3 hours at 37 °C. After a brief shake (10 s), the absorbance was measured at a wavelength of 540 nm with a reference wavelength of 650 nm using a mircoplate reader (BioTek, EL 808).

3.2.10 Data and statistical analysis

MMP activity was expressed as arbitrary units (AU) based on densitometric measures of gelatin lysis. Data was normalised to PMA controls. MMP-9 mRNA expression was calculated as described previously (section 3.2.6.2) and expressed as fold changes. Data was again normalised to PMA controls. MMP and TIMP abundance was determined by interpolation of unknown data from their respective standard curves using KCjunior™ software (Bio-Tek, Vermount, USA). Data from cell viability experiments were normalised to medium controls and expressed as a percentage. Mean fluorescent intensities (MFIs) were obtained from gated flow cytometry histograms using Summit© software (Dako, Fort Collins, USA).

Data were analysed by one-way ANOVA with *post hoc* analysis (Bonferroni or Dunnetts; GraphPad Prism, version 5.01) or paired Student's *t*-test where appropriate. Data are expressed as mean ± SEM. All samples were assayed in duplicate or triplicate and experiments were repeated at least three independent times (n). A value of $P<0.05$ was taken to indicate statistical significance.

3.3 Results

3.3.1 Effects of RBV/IFN-α2a on MMP-9 abundance in THP-1 cells

MMP-9 activity and mRNA expression were non-detectable in inactivated THP-1 cells of the monocyte phenotype. However, following exposure to PMA for 48 hr, MMP-9 activity and expression increased as cells underwent differentiation to macrophages.

In PMA activated cells IFN-α2a dose-dependently decreased MMP-9 activity (Figure 3.2 A). In all subsequent experiments, IFN-α2a was used at a concentration of 250 IU/ml. IFN-α2a (250 IU/ml) alone, and in combination with RBV (10 μM), decreased MMP-9 activity (44±4.2 and 60±1.4 versus 100±3.1 AU; $P<0.05$), while RBV increased activity by approximately 50 % ($P<0.05$; Figure 3.2 B).

At the mRNA level, RBV alone and in combination with IFN-α2a increased MMP-9 expression compared to PMA controls (RBV, RBV/IFN-α versus PMA: 771±116, 772±42 versus 488±45; $P<0.05$; Figure 3.2 C), while IFN-α2a had no effect. The disparity between extracellular MMP-9 activity and intracellular MMP-9 mRNA was investigated further by looking at effects on intracellular MMP-9 abundance, the effect of proteosomal inhibition and temporal mRNA expression patterns. RBV caused a robust increase in intracellular MMP-9 protein levels, while IFN-α2a had no effect. However, in combination with RBV,

IFN-α2a reduced the RBV-mediated increases from 5.5±0.4 to 3.3±0.4 ng/ml ($P<0.05$; Figure 3.2 D). To ascertain if this was due to increased proteosomal degradation, the experiments were repeated in the presence of MG132, a proteosomal inhibitor. Co-treatment of THP-1 cells with the proteasome inhibitor MG132 (200 nM) increased ($P<0.05$) MMP-9 activity compared to PMA alone (178±23 versus 100±6.1 AU; Figure 3.3 A). However, it did not alter the effect of IFN-α2a on RBV-mediated increased MMP-9 activity in THP-1 cells. When the temporal effects of IFN-α2a on MMP-9 mRNA expression were assessed, it was found that it reduced ($P<0.05$) MMP-9 mRNA by approximately 65% at 24 hrs, while having no effect at the later time points studied compared to PMA treated controls (Figure 3.3 B).

3.3.2 Effects of RBV/IFN-α2a on THP-1 cell viability and surface markers of differentiation

To ensure our results were not confounded by cytotoxic effects of the drugs used, we assessed their effect on cell viability using a CellTiter-Blue cell viability assay. RBV, IFN-α2a, and the combination of both drugs, did not affect cell viability at the concentrations used in this study. Doxorubicin (10 μM) was employed as a positive control, and reduced MMP-9 activity by ~85 % (14±3 versus 100±0.5 AU; $P<0.05$; Figure 3.4 A). Additionally, to ensure that our results were not simply due to effects on the differentiation process of monocytes to macrophages, we analysed their effects on CD14, a monocyte cell surface marker that is down-regulated during differentiation. All PMA treated samples showed significant reduction ($P<0.05$) in CD14 mean fluorescent intensities (MFI) compared to untreated monocytes and CD14 expression was not altered by RBV or IFN-α2a, alone, or in combination (Figure 3.4 B).

3.3.3 Effects of RBV/IFN-α2a on MMP-9 activity and expression in LX-2 cells

In LX-2 cells, MMP-9 activity and mRNA expression were undetectable prior to activation with PMA. PMA upregulated MMP-9 activity and mRNA expression at 48 hr. In this cell line, IFN-α2a alone, or in combination with RBV, did not alter MMP-9 activity. However, RBV increased MMP-9 activity compared to PMA alone (134±4.1 versus 100±0.9 AU; $P<0.05$; Figure 3.5 A). RBV and IFN-α2a alone, or in combination, did not alter MMP-9 mRNA expression compared to PMA controls at 48 hr (Figure 3.5 B).

3.3.4 Effects of HIV ART on MMP-9 activity in THP-1 cells

Incubation of THP-1 cells with the HIV antiretroviral drugs abacavir, zidovudine, efavirenz, nevirapine, lopinavir, and atazanavir did not alter MMP-9 activity, with the exception of saquinavir (SQV). At a concentration of 5 μM, SQV decreased MMP-9 activity (44±2.3 versus 100±1.6 AU; $P<0.05$; Figure 3.6 A). To discount the influence of cytotoxicity in this finding, we conducted parallel cell viability assays, demonstrating SQV had no cytotoxic effects at this concentration. Doxorubicin (10 μM), employed as a positive control, reduced MMP-9 activity by ~85 % (14±3 versus 100±0.5 AU; $P<0.05$; Figure 3.6 B).

3.3.5 Effects of HIV ART on MMP-9 activity in LX-2 cells

Exposure of LX-2 cells to the same HIV antiretroviral drugs did not alter MMP-9 activity, with the exception of lopinavir (LPV). LPV (10 μM) decreased MMP-9 activity (53±6.8 versus 100±4.6 AU; $P<0.05$; Figure 3.7 A). To rule out the possibility of cytotoxicity, we

also conducted parallel cell viability assays, showing that LPV had no cytotoxic effects at the concentration studied. Doxorubicin (50 μM) was employed as a positive control, and reduced MMP-9 activity by ~40 % (60±2 versus 100±1.9 AU; P<0.05; Figure 3.7 B).

3.3.6 Patient characteristics and hospital laboratory analysis

The majority of HIV/HCV co-infected patients included in this study were male, prior injecting drug users, of HCV genotype 3. Full baseline characteristics for the HIV/HCV co-infected cohort are outlined in Table 3.3.

3.3.7 Effects of disease and RBV/PEG-IFN-α2b therapy on the MMP/TIMP status of patients

A representative *in vitro* zymogram (Figure 3.8 A) illustrates plasma MMP-9 and MMP-2 activity in the patient study groups and shows the effect of RBV/PEG-IFN-α2b treatment at follow-up on days 3 and 14. A zymogram of the same samples incubated in the presence of EDTA (20 mM) is presented at (B).

MMP-9, activity was 4 fold (P<0.05) higher in HIV/HCV co-infected and HIV mono-infected patients than in healthy controls (86±16 and 82±15 versus 21±4.7 AU). In HCV mono-infected patients MMP-9 activity was similar to that of healthy controls (32.9±5.1 versus 21±4.7 AU) and was approximately 60 % lower (P<0.05) than in the HIV/HCV or HIV infected groups (Figure 3.9 A). MMP-9 protein expression was also higher in HIV/HCV co-infected patients (367.6±73.6 ng/ml) and HIV mono-infected patients (332.2±54.1) compared to healthy controls (129±23.9 ng/ml; P<0.05, Figure. 3.9 B).

MMP-2 activity was similar in all groups studied, as was TIMP-2 expression (Table 3.4). In HIV/HCV co-infected patients, RBV/PEG-IFN-α2b caused a 70 % decrease (*P*<0.05) in plasma MMP-9 activity by day 3, and was maintained for the study duration (Baseline versus Day 14: 98.8±17.3 vs 28.1±11.6 AU; *P*<0.05; Figure 3.9 C). MMP-9 protein expression was similarly reduced (Baseline versus Day 14: 458.7±95.7 vs 120.4±46.8 ng/ml; *P*<0.05; Figure 3.9 D). MMP-2 activity was not altered following treatment with RBV/PEG-IFN-α2b in the HIV/HCV co-infected group, and similarly TIMP-2 expression was unchanged (Table 3.4).

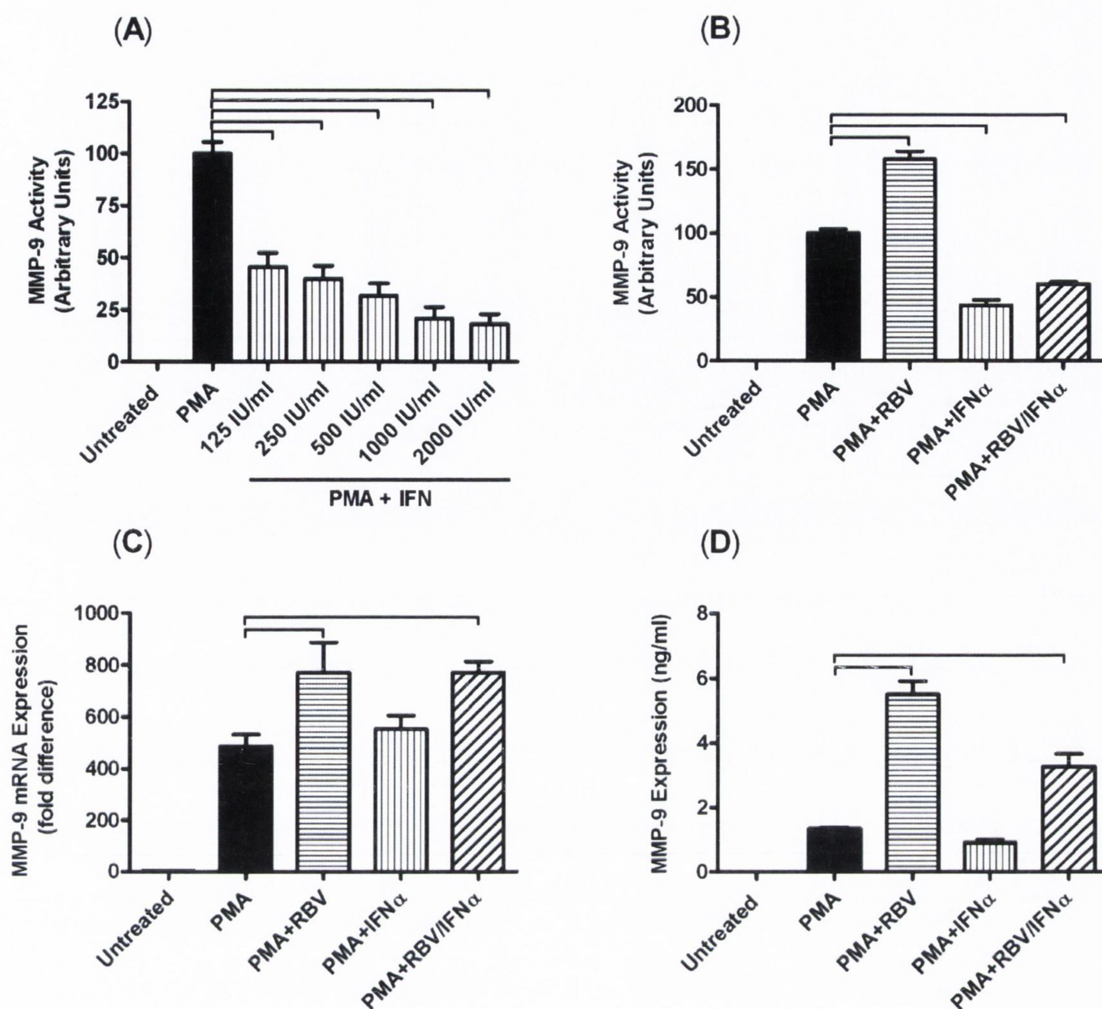


Figure 3.2 Effect of IFN-α2a concentration (A) and RBV/IFN-α2a (250 IU/ml/10 μM) (B) on MMP-9 activity in THP-1 cells. The effect of RBV/IFN-α2a (250 IU/ml/10 μM) treatment on MMP-9 mRNA expression (C) and intracellular protein expression (D) are also shown. MMP-9 activity was measured in culture medium supernatants, and mRNA and protein levels in whole cell lysates (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at $P < 0.05$).

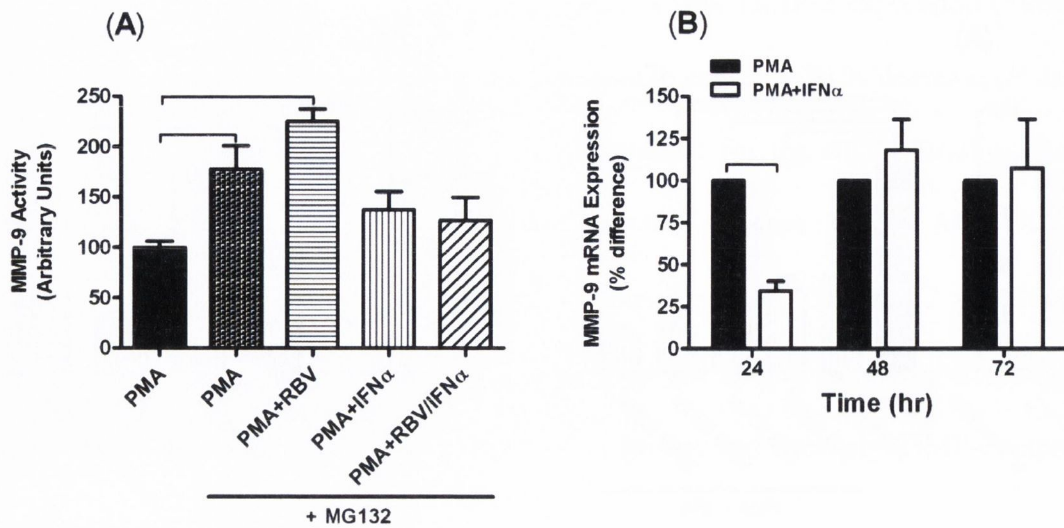


Figure 3.3 Effect of RBV/IFN-α2a (250 IU/ml/10 μM) treatment on MMP-9 activity when combined with the proteasomal inhibitor, MG132 (200 nM) (A). The temporal effects of IFN-α2a (250 IU/ml) on MMP-9 mRNA expression are also shown (B). For all experiments, cells were seeded in serum-free medium and incubated with treatments for 48 hr before analysis (with the mRNA time course including additional analyses at 24 and 72 hr). MMP-9 activity was measured in culture medium supernatants and mRNA in whole cell lysates (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test or paired t-test; horizontal capped bars indicate statistically significant differences from PMA positive controls at P <0.05).

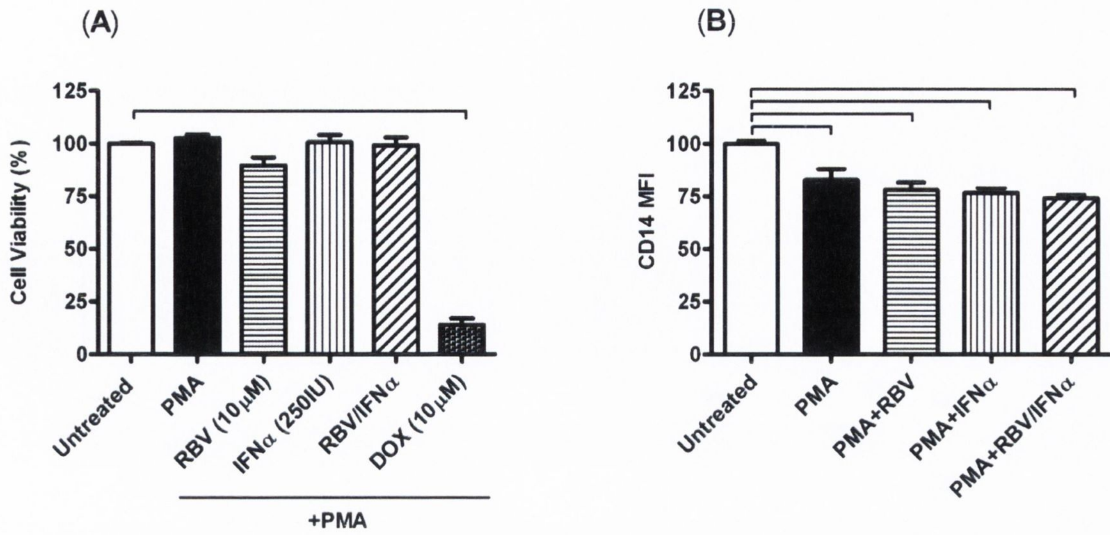


Figure 3.4 Effect of RBV/IFN-α2a (10 μM/250 IU/ml) treatment on cell viability (A) and the expression of the cell surface marker CD14 (B) in THP-1 cells. For all experiments, cells were seeded in serum-free medium and incubated with treatments for 48 hr before analysis. The effects of treatment with respect to cytotoxcity were determined by CellTiter-Blue Cell Viability Assays using doxorubicin (DOX; 10 μM) as a positive control. CD14 expression was determined by flow cytometric analysis (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from untreated (medium) controls at P <0.05).

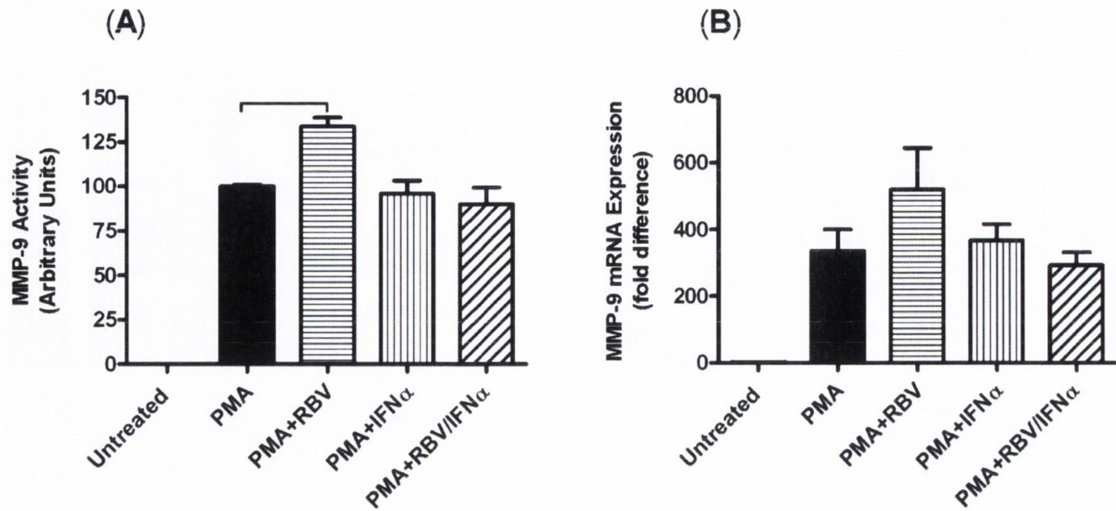


Figure 3.5 Effect of RBV/IFN-α2a (10 μM/250 IU/ml) treatment on MMP-9 activity (A) and MMP-9 mRNA expression (B) in LX-2 cells. For all experiments, cells were seeded in serum-free medium and incubated with treatments for 48 hr before analysis. MMP-9 activity was measured in culture medium supernatants and mRNA in whole cell lysates (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at P <0.05).

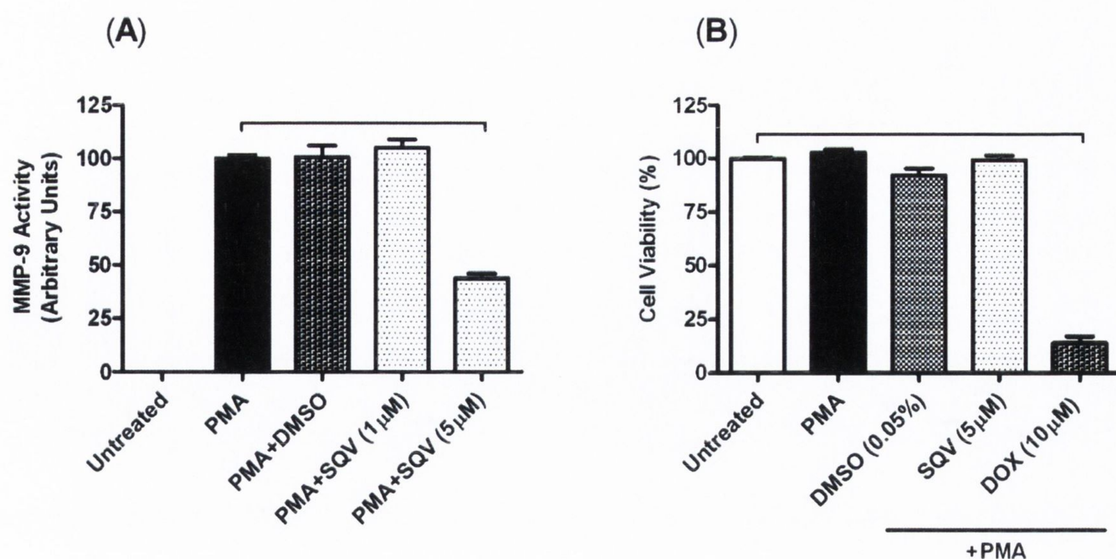


Figure 3.6 Effect of SQV (1 μM and 5 μM) treatment on MMP-9 activity in THP-1 cells (A). Corresponding cytotoxicity assays for SQV (5 μM) are presented at (B) using doxorubicin (DOX; 10 μM) as a positive control. For all experiments, cells were incubated with treatments for 48 hr in serum-free medium before analysis (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls or untreated (medium) controls at $P < 0.05$).

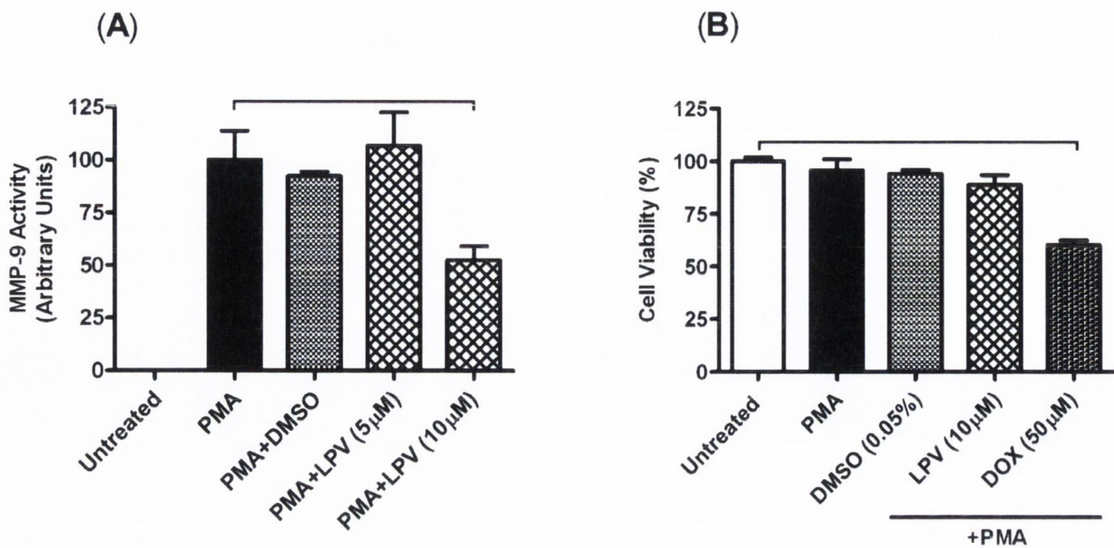
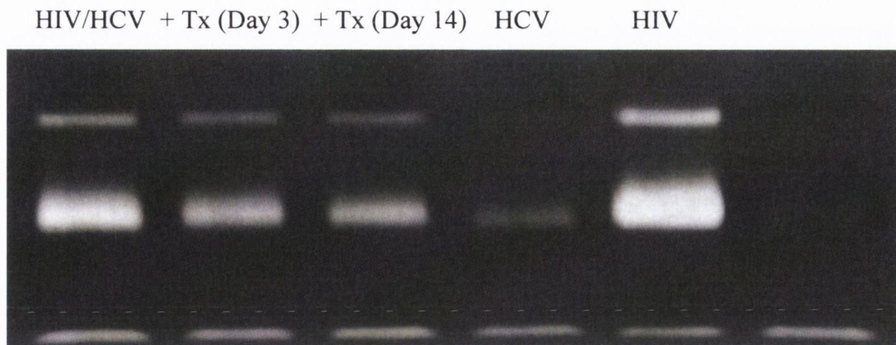


Figure 3.7 Effect of LPV (5 μM and 10 μM) treatment on MMP-9 activity in LX-2 cells (A). Corresponding cytotoxicity assays for LPV (10 μM) are presented at (B) using doxorubicin (DOX; 50 μM) as a positive control. For all experiments, cells were incubated with treatments for 48 hr in serum-free medium before analysis (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls or untreated (medium) controls at P <0.05).

(A)



(B)

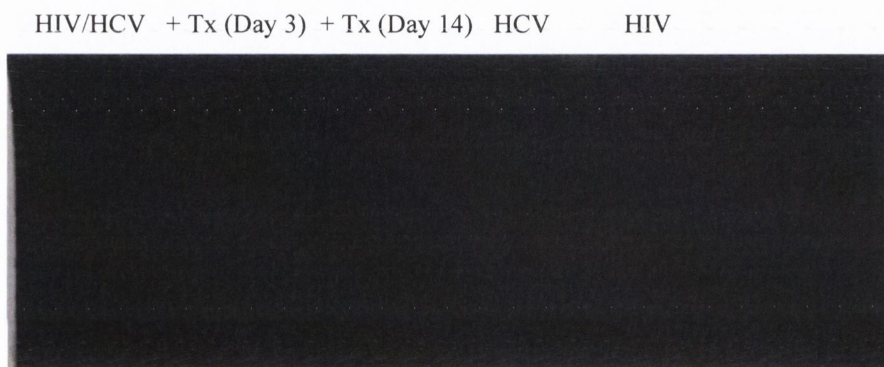


Figure 3.8 A representative zymogram (A) showing gelatinolytic activity at 92, 82 and 72 kDa, corresponding to pro-MMP-9, active-MMP-9, and active-MMP-2, respectively, in the plasma of HIV/HCV co-infected, HCV mono-infected, and HIV mono-infected patients. For HIV/HCV co-infected patients, the effect of RBV/PEG-IFN-α2b is also shown subsequent to 3 and 14 days of treatment. A zymogram of the same samples incubated in the presence of EDTA (20 mM) is presented at (B).

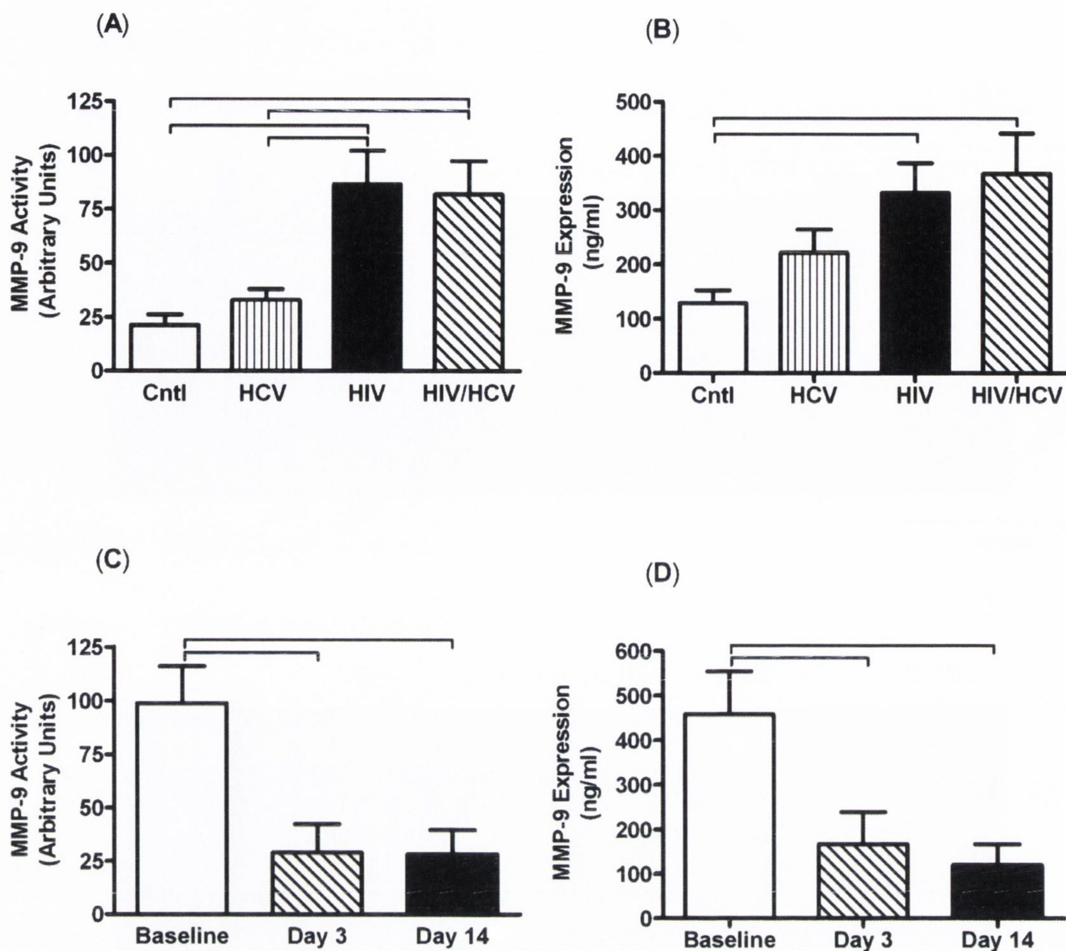


Figure 3.9 Quantitation of active plasma MMP-9 activity (A) and MMP-9 protein expression (B) in HIV/HCV co-infected, HIV mono-infected, and HCV mono-infected patient groups. Effect of RBV/PEG-IFN-α2b on plasma MMP-9 activity (C) and MMP-9 protein expression (D) when measured after 3 and 14 days of treatment in HIV/HCV co-infected patients (n=10/group; mean ± SEM; one-way ANOVA with *Bonferroni* post hoc test; horizontal capped bars indicate statistically significant differences between patient groups or baseline levels at $P < 0.05$).

Table 3.3 Main characteristics of HIV/HCV co-infected patients (n = 10)

Age (years)	38 (33-42)
Sex (*n*, male)	8
Weight (kg)	61 (55-89)
HIV-1 acquisition risk factor (*n*)	
Injecting drug user	6
Haemophiliac	3
Sexual	1
Baseline HIV viral load (copies/ml)	50 (50-621)
Baseline CD4 count (cells/μl)	652 (325-787)
Antiretroviral treatment (*n*)	
2 NRTIs + 1 PI	3
2 NRTIs + 1 NNRTI	4
3 NNRTIs	1
Untreated	2
HCV genotype (*n*)	
1	3
2	1
3	6
4	0
AST/ALT ratio	0.91 (0.82-1.4)
Baseline HCV viral load (IU/ml)	8.5×10^6 (2.8×10^6-1.3×10^7)

All continuous variables are reported as median and interquartile range as a measure of central tendency. Categorical or dichotomous variables are reported as the number of patients to which they correspond.

HIV, human immunodeficiency virus; HCV, hepatitis C virus: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

Table 3.4 MMP-2/TIMP-2 abundance in plasma of patients HIV/HCV co-infected, HIV mono-infected, and HCV mono-infected, and the effect of initiating RBV/PEG-IFN-α2b combination treatment on these parameters in the co-infected group at day 14.

Patients (*n*=10/group)	MMP-2 activity (AU) (Baseline / Day 14)	TIMP-2 expression (ng/ml) (Baseline / Day 14)
HIV/HCV	71.6±10.3 / 75.1±17.9†	78.2±6.1 / 85.3±11.8†
HIV	74.3±12.4	62.9±6.3
HCV	79.3±13.7	74.3±4.2
Healthy controls	88.9±9.5	75±3.3

All data are expressed as mean ± SEM. †indicates values measured at day 14 subsequent to start of therapy. RBV, ribavirin; PEG-IFN-α2b, pegylated-interferon alpha 2b; MMP-2, matrix metalloproteinase-2; TIMP-2, tissue inhibitor of metalloproteinase-2; HIV, human immunodeficiency virus; HCV, hepatitis C virus.

3.4 Discussion

In this study we demonstrated that RBV/IFN-α alters MMP-9 activity and mRNA in THP-1 monocyte/macrophage cells, but not in LX-2 hepatic stellate cells and that this effect is mediated by IFN-α. We also show that the HIV protease inhibitors, saquinavir and lopinavir, inhibit MMP-9 activity in THP-1 and LX-2 cells, respectively. In a follow-up *in vivo* study, we show that MMP-9 levels in plasma from a HIV/HCV co-infected, and a HIV mono-infected patient group, were approximately 3 fold higher than a HCV mono-infected group and healthy controls. MMP-2 and TIMP-2 levels were similar in all groups. Furthermore, in the HIV/HCV co-infected group, RBV/PEG-IFN-α2b therapy reduced plasma MMP-9 activity and protein expression.

Activated macrophages and hepatic stellate cells (HSCs) are central to the pathophysiology of HIV and HCV infection, with MMP-9 over-expression being implicated in tissue remodelling *in vivo*. For instance in patients with HIV-associated dementia (HAD), infiltration of activated macrophages is increased [483] and up-regulated MMP-9 activity contributes to degradation of ECM components of the blood-brain barrier [228]. During chronic HCV infection, the activation of HSCs, augmented by macrophages, is a key event in hepatic fibrosis that is mediated by an interplay between numerous pro-inflammatory cytokines and MMP-9 activity [229]. Although few studies describe the potential relationship between MMPs and antiretroviral agents *in vitro*, it has been reported that anti-HIV drugs zidovudine and indinavir, inhibit MMP-9 expression and activity in neuronal cells [350], while saquinavir and nelvinavir exert similar effects in preadipocytes [352]. However, information on the effect of anti-HCV agents on MMP-2, or MMP-9, abundance is sparse. Nevertheless, we found that RBV/IFN-α inhibits MMP-9 activity in THP-1 monocytes/macrophages, while having no effect in LX-2 HSC cells. This is attributable to IFN-α as it inhibited the inductive effect of RBV on MMP-9 activity and protein

expression in THP-1 cells. We also show that IFN-α dose-dependently inhibits MMP-9 activity. However, this was not reflected at the transcriptional level as IFN-α did not alter the RBV-mediated increase in MMP-9 mRNA expression. This disparity may reflect an early (24 hr) inhibitory effect of IFN-α on MMP-9 mRNA expression which is reflected in a decrease in intracellular/extracellular MMP-9 protein expression at 48 hr compared to RBV alone; no transcriptional effects were noted at 48 and 72 hr. This hypothesis is strengthened by the observation that the inhibitory effects of IFN-α on extracellular MMP-9 is unaffected by proteasomal inhibition.

We also screened a selection of anti-HIV agents from the three major classes prescribed to patients [484] and discovered that exposure of activated THP-1 and LX-2 cells to nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors did not alter MMP-9 activity. However, the protease inhibitors saquinavir and lopinavir, inhibited MMP-9 activity in THP-1 and LX-2 cells, respectively. Although the mechanism underlying this was not investigated in the present study, De Barros et al [353] found that in human preadipoctyes, saquinavir, nelfinavir and the proteasome inhibitor lactacystin inhibit the activity of the 20S proteasome resulting in accumulation of I-*κ*B*β*, which prevents NF-κB from initiating MMP-9 transcription. Therefore, HIV-protease inhibitors may inhibit MMP-9 expression through modification of NF-*κ*B signalling. Our data, and that of de Barros et al, also indicate that these effects appear to be drug-, rather than class-, specific and may also be cell-type specific.

Our clinical study found increased MMP-9 abundance in plasma from HIV and HIV/HCV co-infected, compared to HCV mono-infected, patients. Indeed, MMP-9 levels in the latter group were comparable to healthy controls. This would indicate that HIV infection is driving the increase in MMP-9 expression. Our patient data, indicating increased plasma MMP-9 and unaltered MMP-2 and TIMP-2 abundance compared to healthy controls,

contrasts with an earlier study assessing the MMP/TIMP status of co-infected patients [249]. In that study, Mastroianni and co-authors showed that in HIV patients, who were either HCV-positive, or HCV-negative, plasma TIMP-1 was increased and MMP-9 was reduced compared to healthy controls. Furthermore, TIMP-1 levels were significantly higher in co-infected patients versus HIV mono-infected patients. Similar to our cohort, the co-infected patients were treatment naïve for anti-HCV therapy but treatment experienced for anti-HIV therapy at the time of sampling. Unfortunately, though, the study lacks a matched HCV mono-infected group for comparison of TIMP-1 plasma levels.

In a HCV treatment naïve group, the initiation of RBV/PEG-IFN-α2b combination therapy in our co-infected cohort significantly reduced their enhanced plasma MMP-9 activity and protein expression, a finding which is unique in the literature. The combination of RBV and PEG-IFN-α represents the consensus treatment for chronic HCV patients [485], and in addition to its main function of viral suppression, may also possess antifibrotic properties [486]. In HSCs for example, IFN-α inhibits collagen synthesis *in vitro* and reduces α-smooth muscle actin (a marker of cell differentiation to a myofibroblast phenotype) in patients with chronic HCV subsequent to treatment [362]. More recently, RBV has been shown to decrease HCV sera-stimulated HSC proliferation, with or without IFN-α [464]. As MMPs regulate ECM turnover and influence HSC activation through a range of soluble factors, the modulation of MMP activity may be significant to these outcomes. There has also been much investigation over the last decade as to the influence of HIV antiretroviral therapy on liver fibrosis in co-infected patients, and, notwithstanding the substantive risk of hepatotoxicity, data would suggest that the use of PI-based HAART is associated with a reduction in both the severity of fibrosis and its rate of progression [487, 488]. Longitudinal studies documenting fibrosis stage allied to MMP status in treated patients may in the future provide evidence as to whether PI/MMP interactions are significant in this context.

While we have not presented histological data for our HIV/HCV co-infected patients, median AST/ALT ratios of 0.91 at baseline are indicative of impaired liver function. Correlations to the extent, or indeed absence, of liver fibrosis with circulatory MMP-9 expression and treatment status in HCV infected groups would have been of interest, but of somewhat limited value in view of the fact that liver fibrosis proceeds over a time-course well in excess of RBV/PEG-IFN-α treatment duration.

In conclusion, the present study shows that monocyte/macrophage cells are a target for MMP-9 modulation by antiretroviral therapies, as evidenced *in vitro* in THP-1 cells. The combination of RBV/PEG-IFN-α2b reduces plasma MMP-9 *in vivo*, as over-expression of circulatory MMP-9 in a HIV/HCV co-infected group was attenuated by the initiation of this therapy. This provides evidence that these agents possess pharmacological activity, beyond viral inhibition, that may influence pathological processes involving abnormal MMP expression.

Chapter 4.

Effects of HIV-1 Tat clades B and C and HCV NS3 on inflammatory markers in THP-1 cells

4.1 Introduction

HIV-1 infection is characterised by systemic chronic immune activation that is believed to be the primary driver of disease progression [489, 490]. HIV/HCV co-infected patients display increased immune activation compared to HIV mono-infected patients [491], and co-infection is known to alter the natural history of chronic hepatits C, disrupting innate immune responses [67, 492], augmenting viral replication [65], and accelerating the progression of liver fibrosis [493]. As previously discussed (section 1.5.1), the innate immune response is critical for defence against viral infection and is also a determinant of disease progression and outcome. Dysregulated innate immunity could, therefore, be a contributory factor to the immunopathogenesis of HIV/HCV infection. Some evidence suggests that HIV-1 Tat and HCV NS3 proteins contribute to innate immune dysfunction by mediating aberrant MMP and cytokine expression, particularly in cells of the monocyte/macrophage lineage.

The HIV-1 transactivator protein Tat, is an 86-101 residue (9-11 kDa) regulatory protein essential for viral replication [494]. Tat transactivates HIV-1 gene expression by interacting with sequences in the HIV-1 long terminal repeat (LTR) to promote transcription initiation of the integrated proviral genome and to stimulate the elongation of newly initiated viral transcripts [11]. In addition to its primary function and localisation in the nucleus, Tat is actively released from unruptured, HIV-infected cells and is detectable in *ex vivo* culture supernatants and in the serum of HIV-infected individuals [47, 495]. While extracellular Tat can enter and transactivate the HIV-1 LTR in neighbouring cells [46, 496], it can also transactivate a host of non-viral genes in uninfected cells, including those of MMPs and cytokines. For example, Tat has been shown to increase the expression and activity of MMP-9 in monocytes and neuronal cells [313, 497], MMP-2 in endothelial cells [257], and induce the expression of pro-inflammatory cytokines IL-1 [498], IL-6 [320], IL-8 [321], IL-10 [317], and TNF-α [321, 327], in monocytes and macrophages. HIV-1 comprises a range of viral subtypes

known as clades, and this diversity has important implications for the pathogenicity of the virus [499]. Indeed, recent studies conducted using Tat of clade B and C (the most prevalent subtypes in North America and Western Europe, and Sub-Saharan African regions, respectively) suggest contrasting regulation of cytokine production. Campbell and colleagues [341] reported an impaired ability by Tat clade C to induce TNF-α in comparison to Tat clade B. A subsequent study also confirmed clade B Tat to be a potent inducer of TNF-α, and also IL-6, but not IL-10, which was preferentially increased by clade C Tat [343]. Conversely, investigations conducted by Wong and colleagues [342] indicated reduced ability to augment IL-10 production by Tat C in comparison to Tat B. This differential modulation of monocyte/macrophage cytokine expression indicates one possible mechanism for differences in the pathogenicity of HIV-1 viral strains.

The HCV non-structural protein NS3, is a 70 kDa serine protease belonging to the trypsin/chymotrypsin protease superfamily [500]. It occupies amino acids 1027 to 1658 of the HCV polyprotein, followed in sequence by the NS4 (A, B) and NS5 (A, B) proteins. The NS3-NS5B region is essential for genome replication [501]. The N-terminal amino acids of NS3 contain protease activity [502, 503], while the C-terminal portion encodes helicase activity that assists RNA folding [504], polymerase processivity [505], and/or genome encapsidation [506], necessary steps for successful viral replication. In addition to these functions, a number of reports attribute extravirologic properties to HCV NS proteins. NS3 has been demonstrated to increase TNF-α and IL-10 in monocytes and to inhibit dendritic cell (DC) differentiation from PBMCs of HCV-infected patients [349]. Others have shown that impaired DC functionality subsequent to NS3 (and NS4 and NS5) exposure correlates with marked reductions in IL-12 [346]. NS4 also mediates IL-12 inhibition in monocytes isolated from HCV-infected patients [507], and NS5A upregulates IL-8 expression in HeLa cells [130]. One

study to date has shown NS-mediated MMP induction, with NS5B transfected hepatocytes displaying increased MMP-9 gene expression [344].

The data outlined above indicates that HIV-1 Tat and HCV NS3 proteins dysregulate MMP and cytokine expression, effects which may have significant immunomodulatory potential during the course of infection. For example, induction of IL-10, a T helper 2 (Th2) cytokine, is implicated in suppression of cellular immune responses during HIV infection [508, 509] and persistence of viral replication in chronically infected HCV patients [510]. Increased IL-6 expression has also been shown to augment HIV-1 replication in latently infected macrophages [511] and impair macrophage functionality [512], while decreased IL-12 production has been identified as a potential factor in impaired innate and Th1 cell-mediated responses observed in AIDS patients [513]. Furthermore, upregulated MMP expression, particularly MMP-2 and MMP-9, contributes to viral dissemination and is implicated in the pathogenesis of HIV/HCV infection through a myriad of pathways (see section 1.1.1).

TNF-α and IL-1β, which are up-regulated during HIV/HCV infection [287, 291] and contribute significantly to disease progression [288, 290, 292], maintain a broadly reciprocal relationship with MMP expression and activity. Both cytokines have been demonstrated to induce MMP-9 by complexing with ECM components [294, 296] and in turn, MMP-9 has been shown to regulate their expression by cleavage of membrane-bound precursor proteins and/or protein degradation [514, 515]. An up-regulation therefore, of any element of this inflammatory triad could have significant implications for disordered immune responses and thus accelerated disease progression. In the previous chapter we related increased MMP-9 levels in HIV mono-infected and HIV/HCV co-infected patients and showed that activated monocytes/macrophages expressing high levels of MMP-9 are a target for pharmacological inhibition of its activity. The extravirological effects of HIV-1 Tat and HCV NS proteins in

cells of this lineage as described above, suggests a possible pathway for increased MMP expression in HIV/HCV co-infection that may well be associated with up-regulated TNF-α and IL-1β production. However, no studies to date have assessed the effects of HIV-1 Tat subtypes and HCV NS3 on the expression of MMPs and their endogenous inhibitors, TIMPs, in which concurrent regulation of immunomodulatory cytokines such as TNF-α and IL-1β could be of major significance.

The aims of this *in vitro* study are therefore, to investigate the effects of HIV-1 Tat clades B and C and HCV NS3 on MMP-9, TIMP-1, TNF-α, and IL-1β production by activated monocytes/macrophages.

4.2 Methods

4.2.1 Cell culture

THP-1 cells were cultured in RPMI 1640 medium (L-glutamine, 25 mM HEPES) (Invitrogen, Paisley, UK), supplemented with 10 % foetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Sigma-Aldrich, Arklow, Rep of Ireland) in a humidified atmosphere at 37 °C and 5 % CO_2. Flasks were divided 1:3 every 3-4 days. To determine the amount of viable cells, an aliquot were stained with Trypan Blue (Sigma-Aldrich) (0.4 % w/v; 1:5) for 5 min and counted using a Neubauer haemocytometer. Cell number per ml of culture medium was calculated using the equation specified previously in section 3.2.1. For experiments cells were seeded in serum-free RPMI. Phorbol 12-myristate 13-acetate (PMA) was prepared in DMSO (C_{final}= 0.02 % v/v), HCV NS3 was prepared in buffer (20 mM Tris-HCl, pH 8, 8 M urea, 10 mM B-ME), while all other

agents were prepared in sterile dH_2O (18 $M\Omega^{-cm}$). All working solutions were prepared in medium.

4.2.2 Determination of MMP-9, TIMP-1, TNF-α, and IL-1β expression by ELISA

Cells were seeded in 24-wellplates at a density of 3.75 x 10^5 cells/well and exposed to either recombinant Tat clade B (37.5, 75, 150, 300, and 400 ng/ml), Tat clade C (as for Tat B), HCV NS3 (0.1, 0.5, 1, 5, and 10 μg/ml), or medium. One hour later, cells were activated with PMA (20 ng/ml) to initiate differentiation to macrophages. All treatments were done in duplicate and terminated after 48 hr.

Cell debris was removed by centrifugation (5000 g, 5 min, 4 °C) and samples were aliquoted and stored at -80 °C until required. Samples were diluted 1:150 for MMP-9, and 1:100 for TIMP-1, analysis in reagent diluent (PBS, pH 7.2; 1 % w/v bovine serum albumin) prior to measurement of protein levels by ELISA (Human MMP-9 DuoSet, Human TIMP-1 DuoSet, Human TNF-α DuoSet, and Human IL-1β DuoSet; R&D Systems) according to manufactures protocol. Optical density was read at 450 nm with a reference wavelength of 540 nm. Samples and standards were assayed in duplicate and protein levels were determined by interpolation from their respective standard curves.

4.2.3 Determination of MMP-9 activity by gelatin zymography

Cells were seeded in 24-wellplates (3.75 x 10^5 cells/well) and treated as above. After 48 hr, the conditioned medium was collected and cell debris removed by centrifugation (5000 g, 5 min, 4 °C). Briefly, samples were diluted 1:10 with non-reducing sample buffer and

subjected to gelatin zymography utilising 8 % SDS-polyacrylamide gels containing 1 mg/ml gelatin. Gels were washed in Triton X-100 (2.5 % v/v) for 30 min and incubated for a further 18 hr at 37 °C in incubation buffer of composition 50 mM Tris HCl, pH 7.6, 10 mM $CaCl_2$, 50 M NaCl. Gels were rinsed in destain (1:3:6; glacial acetic acid: methanol: dH_2O) followed by staining (0.05 % w/v coomassie brilliant blue R250 in destain solution) for 1 hr followed by destaining (5 min). Gelatinolytic activity was quantified using a gel documentation system (Syngene, Cambridge, UK).

4.2.4 Data and statistical analysis

MMP-9, TIMP-1, TNF-α, and IL-1β abundance were determined by interpolation from respective standard curves using KCjunior™ software (Bio-Tek, Vermount, USA). Data was normalised to PMA controls and expressed as relative fold increases. MMP-9 activity data was also normalised to PMA controls and expressed as arbitrary units (AU) based on densitometric measures of gelatin lysis.

Data were analysed by one-way ANOVA with *post hoc* analysis (*Dunnetts*; GraphPad Prism, version 5.01). Data are expressed as mean ± SEM. All samples were assayed in duplicate and experiments were repeated at least three independent times (*n*). A value of $P<0.05$ was taken to indicate statistical significance.

4.3 Results

4.3.1 Effects of Tat clade B on MMP-9, TIMP-1, and TNF-α abundance

MMP-9 expression and activity, and TIMP-1 and TNF-α expression, were non-detectable in inactivated THP-1 cells of the monocyte phenotype. However, following exposure to PMA for 48 hr, MMP, TIMP and cytokine expression increased as cells underwent differentiation to macrophages. In PMA treated cells, HIV-1 Tat clade B increased MMP-9 expression at 300 and 400 ng/ml concentrations (1.3±0 and 1.5±0 versus 1±0 fold induction; $P<0.05$; Figure 4.1 A) but had no effect on MMP-9 activity at any concentration studied (Figure 4.1 B).

TIMP-1 expression was significantly increased by Tat B at 300 and 400 ng/ml, with a >6 fold increase at the latter concentration compared to PMA controls (4.4±0.52 and 7.1±1.7 versus 0.99±0 fold induction; $P<0.05$; Figure 4.1 C).

Tat B increased the expression of TNF-α dose-dependently against PMA (1.9±0.1, 1.9±0, 2.9±0.1, 3.3±0, and 4±0.2 versus 1±0.1 fold induction; $P<0.05$; Figure 4.1 D).

4.3.2 Effects of Tat clade C on MMP-9, TIMP-1, and TNF-α abundance

Over the concentration range studied (37.5-400 ng/ml) Tat clade C did not alter MMP-9 abundance or activity in conditioned medium from THP-1 cells activated by PMA (Figure 4.2 A and B, respectively).

Tat clade C caused a subtle dose-dependent increase in TIMP-1 expression, reaching significance at concentrations of 150, 300, 400 ng/ml compared to PMA (1.9±0.2, 2+0.1, and 2.4±0.2 versus 1±0.1 fold induction; $P<0.05$; Figure 4.2 C).

TNF-α levels were unchanged relative to PMA controls following exposure of cells to Tat clade C over the range of concentrations studied (Figure 4.2 D).

4.3.3 Effects of HCV NS3 on MMP-9 and TIMP-1 abundance

HCV NS3 protein increased MMP-9 expression in THP-1 cells at concentrations of 5 and 10 μg/ml compared to PMA (1.9±0.1 and 2.6±0.3 versus 1±0.2 fold induction; $P<0.05$; Figure 4.3 A). The increase in MMP-9 protein expression was also accompanied by enhanced MMP-9 activity at a concentration of 10 μg/ml (1.6±0 versus 1±0 fold induction; $P<0.05$; Figure 4.3 B).

Interestingly, NS3 also increased TIMP-1 expression at concentrations of 1, 5, and 10 μg/ml, compared to PMA controls, reaching significance at the latter two concentrations (4.7±0.9, and 4.4±1.3 versus 0.7±0.1 fold induction; $P<0.05$; Figure 4.3 C).

4.3.4 Effects of HCV NS3 on TNF-α and IL-1β expression

NS3 induced TNF-α expression in THP-1 cells >3 fold at the highest concentration studied (10 μg/ml) compared to PMA (4.3±0.6 versus 1±0.6 fold induction; $P<0.05$; Figure 4.4 A). In contrast, NS3 increased IL-1β expression in a dose-dependent manner at concentrations of 1, 5, and 10 μg/ml against PMA controls (1.8±0, 3.2±0, and 4.2±0.1 versus 1±0 fold induction; $P<0.05$; Figure 4.4 B).

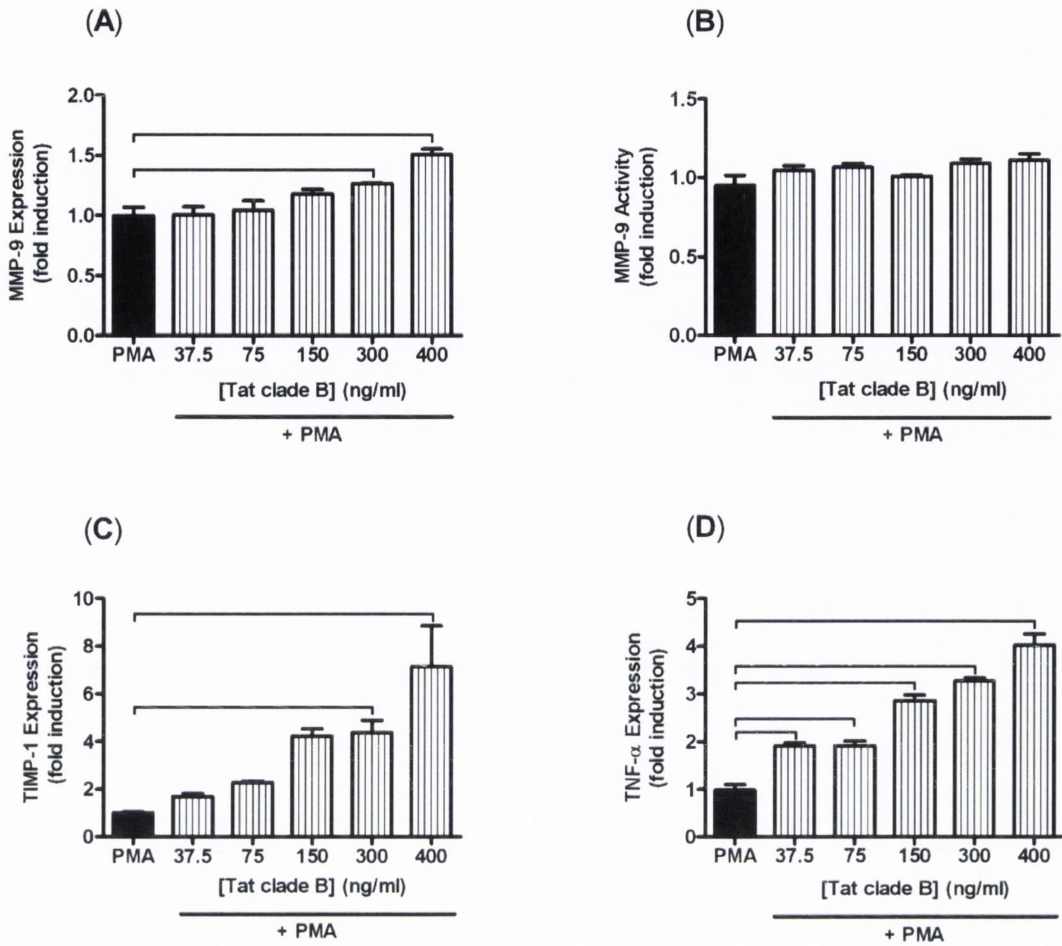


Figure 4.1 The concentration-dependent effects of HIV-1 Tat clade B on MMP-9 expression (A), MMP-9 activity (B), TIMP-1 expression (C), and TNF-α expression (D) in THP-1 cells activated by PMA (20 ng/ml). All experiments were performed in serum-free medium and supernatants collected after 48 hr exposure to Tat (*n*=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at $P < 0.05$).

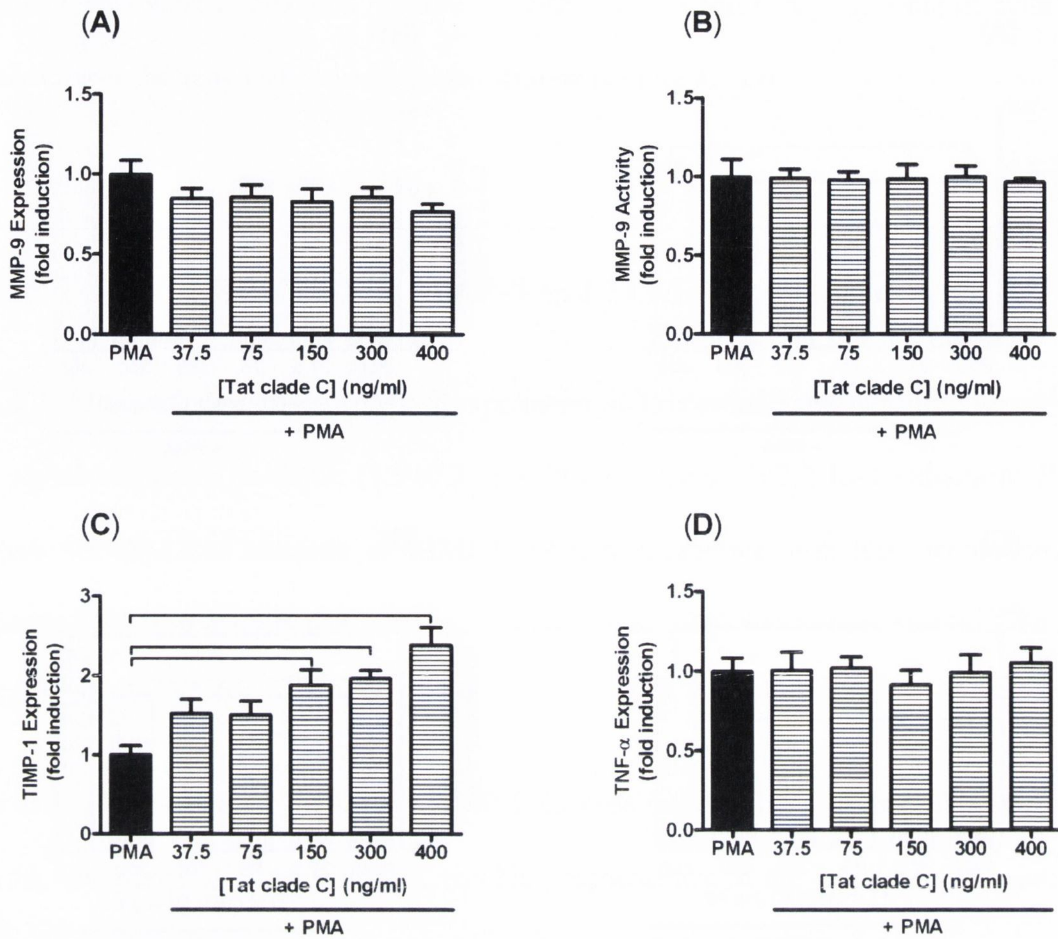


Figure 4.2 The concentration-dependent effects HIV-1 Tat clade C on MMP-9 expression (A), MMP-9 activity (B), TIMP-1 expression (C), and TNF-α expression (D) in THP-1 cells activated by PMA (20 ng/ml). All experiments were performed in serum-free medium and supernatants collected after 48 hr exposure to Tat (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at P <0.05).

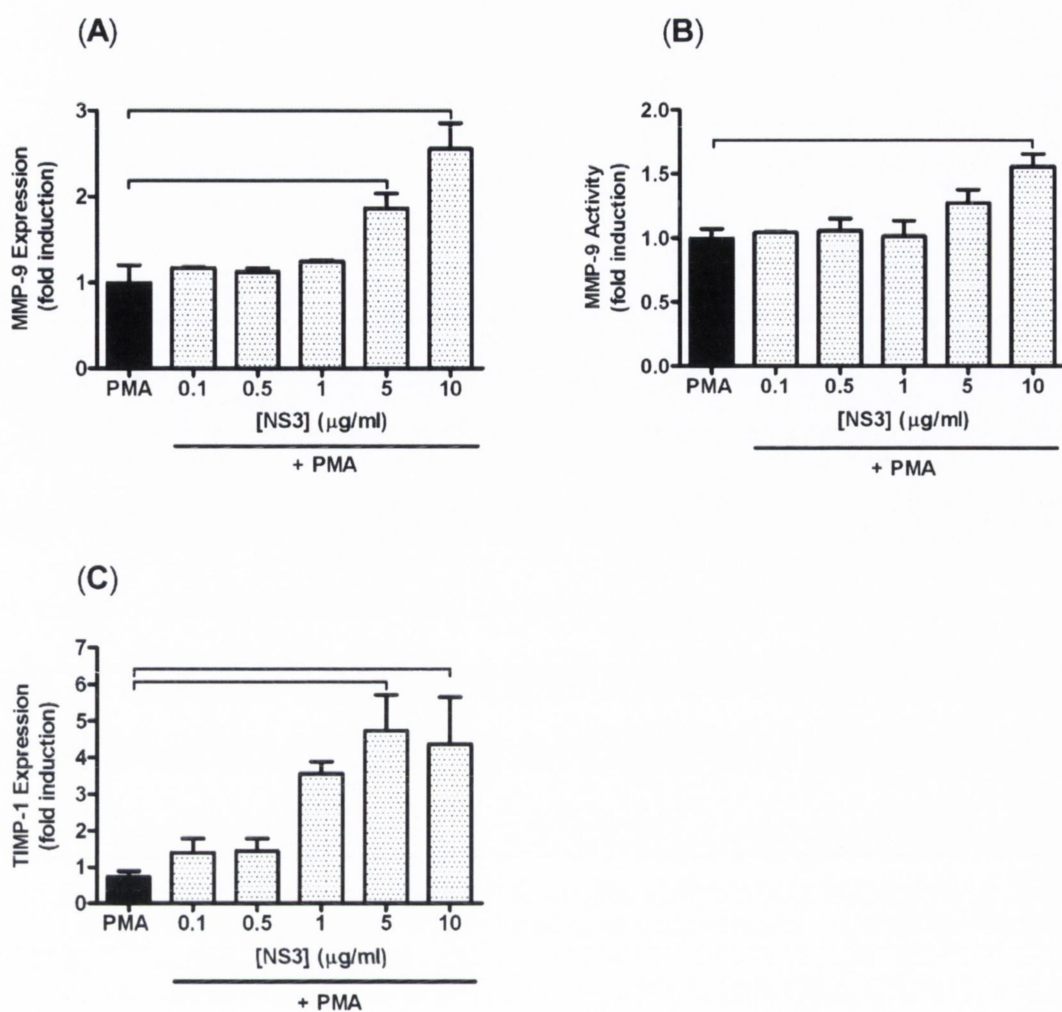


Figure 4.3 The concentration-dependent effects of HCV NS3 on MMP-9 expression (A), MMP-9 activity (B), and TIMP-1 expression (C) in THP-1 cells activated by PMA (20 ng/ml). All experiments were performed in serum-free medium and supernatants collected after 48 hr exposure to NS3 (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at $P < 0.05$).

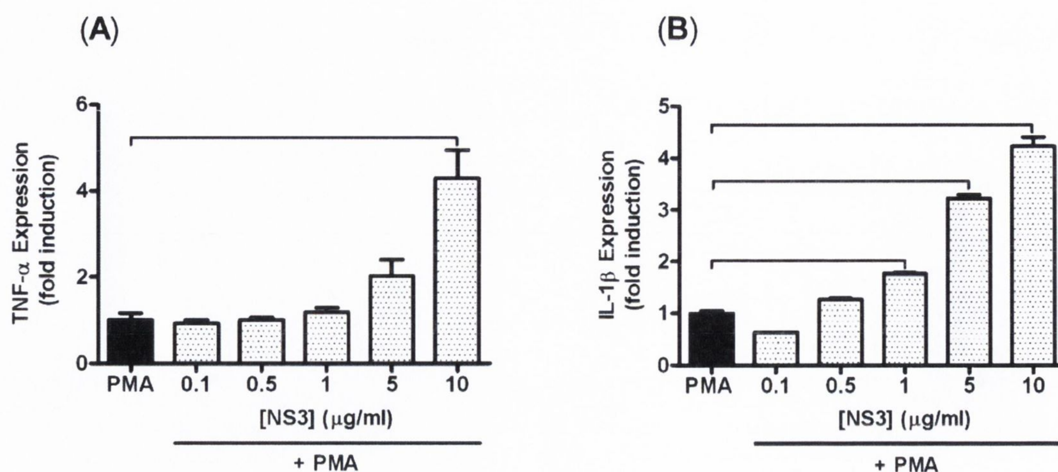


Figure 4.4 The concentration-dependent effects of HCV NS3 on TNF-α expression (A) and IL-1β expression in THP-1 cells activated by PMA (20 ng/ml). All experiments were performed in serum-free medium and supernatants collected after 48 hr exposure to Tat (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from PMA positive controls at P <0.05).

4.4 Discussion

In this study we have demonstrated that HIV-1 Tat clades B and C differentially modulate MMP-9, TIMP-1, and TNF-α production in THP-1 monocytes/macrophages. We have also shown that HCV NS3 upregulates MMP-9, TIMP-1, TNF-α, and IL-1β in these cells.

Dysregulated MMP/TIMP and pro-inflammatory cytokine expression has been ascribed to HIV/HCV infection and contributes to the pathogenesis of both viruses [229, 279, 462, 516, 517]. Imbalance of these soluble factors may result from direct virus/cell interactions or could be a consequence of non-specific immune activation. HIV-1 Tat and HCV NS proteins have indispensible roles in productive viral replication [125, 494] but also possess pleiotropic properties that influence the expression of several non-viral genes [349, 518, 519]. Recent evidence indicates that Tat exerts its extravirological effects differentially according to viral subtype, as exposure of monocytes to Tat clades B and C has been shown to augment the expression of cytokines such as TNF-α, IL-6, and IL-10 to varying degrees [341-343]. In the current study we demonstrate that Tat clade B increases TNF-α production by PMA-activated THP-1 cells in a concentration-dependent manner, while Tat clade C has no effect. This finding supports evidence presented by Gandhi and colleagues [343] who showed significant upregulation of TNF-α and IL-6, both pro-inflammatory cytokines, in monocytes treated with Tat B. They also demonstrate that expression of anti-inflammatory IL-4 and IL-10 was higher in Tat C treated cells, suggesting distinct Tat modulation of inflammatory pathways based on genetic subtype. Indeed, we also show that Tat B augments MMP/TIMP expression to a greater degree than that of Tat C in THP-1s. We report increased MMP-9 expression in Tat B treated cells, a finding that was not associated with increased MMP-9 activity, possibly reflecting enhanced levels of the pro-enzyme, and a marked induction of TIMP-1. Tat C failed to alter MMP-9 abundance but did increase

TIMP-1 by a modest 1.5 fold at the highest concentration studied. To our knowledge, these are the first data to document the effects of Tat clades on MMP/TIMP expression.

HIV-1 clade B remains the predominant subtype in Western countries, with a progressive introduction of non-B clades from countries with higher incidences of epidemic disease, while clade C represents the most prevalent form globally [24]. Subtype diversity has important clinical implications for differences in transmission and replication of the virus, but also for differential disease pathogeneity [520, 521]. For example, the incidence of neurological disease in HIV-1B-infected individuals within the US and Europe is estimated at 15-30 %, while only 1-2 % of clade C-infected individuals suffer from such complications [522, 523]. Tat is known to play a significant neuropathogenic role during HIV infection. Release from infected macrophages and glial cells facilitates its interaction with neurons and it has been demonstrated that Tat induces cell death by apoptosis of neuron cultures [524-526]. TNF-α has been shown to potentiate apoptosis in primary human and rat neurons exposed to Tat [527, 528], and to synergise with Tat to induce the neurotoxic chemokine CXCL10 in human astocytes [529]. Furthermore, Tat sequences derived from the brains of demented AIDS patients induce MMP expression when expressed in human macrophages, and the conditioned media from these cultures is highly neurotoxic [530]. Indeed, inductions of MMP-9 have been reported in the CSF of patients with HIV-associated dementia [228, 254], and it is likely that MMP proteolysis facilitates the extravasation of infected mononuclear cells across the blood-brain barrier [253]. Our data, indicating that clade B Tat stimulates the production of MMP-9 and TNF-α, is therefore supportive of HIV-1B augmenting neuropathgenesis *in vivo*. However, the increases observed in MMP-9 and TNF-α expression were accompanied by a prominent induction of TIMP-1 expression in Tat B treated cells, suggesting an inhibition of proteolysis. Indeed, the results of zymographic analysis, a sensitive measure of gelatinolytic activity by the

active enzyme, revealed no effect. Concurrent MMP-9 and TIMP-1 upregulation is common in a number of neuroinflammatory settings [531, 532], and may well reflect compensatory mechanisms to mitigate on-going tissue destruction [533]. Of course, the physiological functions of TIMPs are not restricted to MMP inhibition, and a direct neuroprotective effect has been attributed to TIMP-1 by its reduction of glutamic acid-induced excitotoxic injury [534]. Tat clade C induced TIMP-1 expression in a dose-dependent manner to a maximal 1.5 fold increase at the upper concentration studied (400 ng/ml) and did not affect changes to MMP-9 and TNF-α abundance. A deleterious role for clade C Tat in immunopathogenesis involving aberrant ECM degradation and/or activated pro-inflammatory pathways would therefore, seem unlikely.

The current study also demonstrates that HCV NS3 alters MMP/TIMP expression in THP-1 cells, significantly increasing MMP-9 abundance and TIMP-1 expression. TNF-α production was also increased by NS3, and a dose-response induction of pro-inflammatory IL-1β was shown. These data almost certainly provide the first evidence that NS3 modulates MMP/TIMP expression and could, in tandem with the data already discussed, provide a rationale for accelerated disease progression in HIV/HCV co-infection. Indeed, production of TNF-α and IL-1β correlate positively with disease severity during HCV infection [535, 536], contributing to the most significant pathology of HCV infection, that of liver fibrosis, by synergistic cellular and MMP activation [266, 537, 538], and as discussed indepth in section 1.1.1.2, the relative expression of MMP-9 and TIMP-1 has a profound effect on fibrogenic and fibrolytic activity in the liver. In addition, others have documented impairments to innate immune responses as a result of NS3-mediated changes to cytokine expression, effects that may well mirror the cellular abnormalities observed in HCV-infected individuals. In this respect the findings presented by Dolganiuc e*t al* [349] show that TNF-α production is increased in monocytes exposed to NS3 over 48 hr periods (directly

reflective of our own data), and this effect results in inhibited differentiation and allostimulatory capacity of immature dendritic cells (DCs). Supporting these results, others have demonstrated that NS3 mediates a downregulation of IL-12, a significant immunomodulatory cytokine during infection, inducing similar impairments to DC functionality [346].

The concentration range selected for Tat clades and NS3 in this study is in keeping with values adopted by other comparable studies [539, 540]. Although we have not investigated the mechanistic basis for our findings, previous reports indicate common transcriptional pathways for both viral proteins. HIV-1 Tat induces MMP-9 in moncytes through protein tyrosine phosphatase (PTPase) activation of nuclear transcription factor NF-κB [497], and induces TNF-α in macrophages by initiating the protein kinase C (PKC) pathway and downstream mitogen-activated kinases (MAPK) p38 and extracellular regulated (ERK) 1/2, and NF-κB [327]. NS3 increases TNF-α production in monocytes via toll-like receptor (TLR) 2 and subsequent induction of IL-1 receptor associated kinase (IRAK) activity, phosphorylation of p38, ERK, and c-jun N-terminal (JNK) kinases, and ultimate activation of NF-κB [540].

In conclusion, the results of this study have shown that exposure of THP-1 monocytes/macrophages to HIV-1 Tat clades B and C alters the MMP-9/TIMP-1 balance in favour of TIMP-1. In Tat B treated cells this effect is more prominent and is accompanied by increased production of pro-inflammatory TNF-α. HCV Exposure of these cells to NS3 also induces MMP-9 and TIMP-1 abundance, again favouring TIMP-1 dominance, and markedly increases both TNF-α and IL-1β cytokines. These results raise the possibility that HIV and HCV viral proteins might combine to enhance inflammatory pathways and chronic activation of innate immune responses leading to the accelerated disease progression characteristic of HIV/HCV co-infected patients.

Chapter 5.

Effects of HIV-1 Tat clades A, B, C, and D, on cytokine production in human $CD3^+$ T cells and Vγ9Vδ2 T cell subsets

5.1 Introduction

HIV-1 has evolved from its initial discovery and characterisation to what is now a genetically diverse range of viral subtypes (clades A, B, C, D, E, F, G, K and O). According to recently published data, the most globally prevelant of these HIV-1 genetic forms are clades A, B, C, and D, with clade C accounting for almost 50 % of all HIV-1 infections worldwide [24]. The highly mutational nature of the virus, allied to a propensity for genomic recombination and the influence of wide-ranging host, environmental, and/or therapeutic selection pressures are factors contributing to genetic diversification [23]. Such diversity has implications for differential disease transmission and replication rates, responses to antiretroviral therapy (including the development of resistance) and vaccine development, but also for the pathogenicity of the virus.

The HIV-1 transcription factor Tat, is a significant mediator of viral pathogenesis and AIDS-related diseases [518]. Tat can be released into the extracellular environment and has the ability to cross the membrane of neighbouring cells, via interactions with specific cell surface receptors, and to translocate to the nucleus, maintaining an active form as it does so [48]. As a consequence, HIV-1 LTR transcriptional activity within infected, but otherwise quiescent cells, can be stimulated by Tat, thereby increasing the amount of infectious virions in circulation [49]. Tat also demonstrates additional pathogenic properties as it activates uninfected primary T and B lymphocytes [53, 54], induces chemokine HIV-1 coreceptors in PBMCs [56], stimulates the chemotaxis of numerous cell types [57], and is also a suspected neurotoxin, implicated in HIV-associated dementia (HAD) [60].

Some studies also demonstrate that Tat is a modulator of cytokine production [318, 319, 327, 334], with recent evidence indicating clade-specific effects as subtypes B and C were shown to differentially augment TNF-α, IL-6, and IL-10 secretion by monocytes [342, 342, 343].

Dysregulation of the cytokine profile is symptomatic of HIV-1 infection with decreases in T-helper type 1 (Th1; IL-2, IFN-γ) and increases in T-helper type 2 (Th2; IL-10, IL-13), and pro-inflammatory (IL-1, TNF-α) cytokines typically observed during the course of the disease [541]. Such abnormalities in cytokine secretion impairs cell-mediated immunity, an important consequence as long-term control of infection is dependent on an appropriate innate immune cytokine response [542]. Studies concerning the influence of Tat on cytokine dysregulation are largely confined to the monocyte/macrophage, and whilst cells of this lineage are a major target for infection by HIV-1 and are an important source of cytokine production, a critical event in early HIV-1 infection is the disruption of T lymphocyte homeostasis, with alterations to their cytokine profile particularly significant for disease progression [426, 427, 543, 544].

γδ T cells are a subset of human T lymphoctyes that, similar to αβ T cells, recognise antigen with their TCRs, perform comparable cellular functions, retain an immunological memory, and act as potent antigen-presenting cells [545]. The adult human γδ T cell repertoire comprises Vδ1 and Vδ2 subsets with a predominant population bearing Vγ9Vδ2-encoded TCRs which form approximately 1 to 5 % of peripheral blood T cells. Vγ9Vδ2 T cells respond to phosphoantigens such as (*E*)-4-hydroxy-3-methyl-but-2 enyl pyrophosphate (HMB-PP) and isopentenyl pyrophosphate (IPP), rapidly producing the Th1 antiviral cytokine IFN-γ and the pro-inflammatory cytokine TNF-α [394]. Activated Vγ9Vδ2 T cells demonstrate potent anti-HIV activity *in vitro*, inhibiting viral replication by both cytolytic and non-cytolytic effects [398, 546, 547] and can influence dendritic cell maturation [548] along with T cell and B cell responses [545, 549, 550], thus exhibiting indirect antiviral properties. However, successive reports indicate a depletion and loss of functionality of Vγ9Vδ2 cells in HIV-infected patients [410, 551-553], the consequence of which may be increased susceptibility to opportunistic infections [554].

Despite the substantial anti-viral potential of Vγ9Vδ2 T cells, effects that may well be cytokine dependent, and their paradoxical anergy in the face of HIV infection *in vivo*, no studies to date have assessed the impact of Tat on their production of IFN-γ and TNF-α. As these two cytokines mediate variable effects on the pathogenicity of HIV infection, IFN-γ being broadly anti-viral and TNF-α broadly pro-viral, modulation of either by Tat could profoundly affect T cell immunoregulatory potential as a whole. Furthermore, as Tat clade may differentially influence cytokine expression, this may impact on disease progression and outcome. Therefore, the aims of the present study were to; 1.) assess the effects of Tat clades A, B, C, and D on the percentage of $CD3^+$ T cells and their $V\gamma9V\delta2^+$ subsets producing IFN-γ and TNF-α, and, 2.) determine if this is reflected in changes to their secretion pattern in both cell types.

5.2 Methods

5.2.1 Blood samples and isolation of PBMCs

For all experiments, venous blood samples were obtained from healthy male donors with peripheral blood mononuclear cells (PBMCs) isolated by gradient density centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Briefly, samples were collected in EDTA and lithium heparin tubes, diluted 1:1 in complete RPMI 1640 (L-glutamine, 25 mM HEPES) (Invitrogen, Paisley, UK) supplemented with 10 % foetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Sigma-Aldrich, Arklow, Rep of Ireland), layered over 7.5 ml of Lymphoprep in 50 ml tubes, and centrifuged (1650 rpm, 25 min, 21 °C). The resultant buffy coat was transferred to a clean tube and washed twice in medium. An aliquot of cells were stained with Trypan Blue (Sigma-Aldrich) (0.4 % w/v; 1:5) for 5

min and counted using a Neubauer haemocytometer. Cell number per ml of culture medium was calculated using the equation specified previously in section 3.2.1.

5.2.2 Magnetic bead enrichment of $CD3^+$ T cells

T cells were enriched from PBMCs by staining with a FITC-conjugated CD3 mAb (Immunotools, Friesoythe, Germany) followed by positive selection using the Anti-Mouse IgG1 MicroBeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, PBMCs from each donor were resuspened in 1 ml of phosphate-buffered saline (PBS; containing $MgCl_2$ and $CaCl_2$) (Sigma-Aldrich) and fluorescently labelled with 10 μl of mouse IgG1-CD3 mAb for 15 mins in the dark at room temperature. Cells were then washed in 2 ml of an assay-specific buffer (PBS, pH 7.2; supplemented with 0.5 % w/v BSA; 2 mM EDTA), centrifuged (300 g, 10 min, 21 °C), and resuspended in 80 μl of this buffer. Anti-mouse IgG1 MicroBeads (20 μl) were added per tube, mixed by vortexing, and incubated at 4 °C for 15 min. Subsequently, cells were washed as before, resuspended in 500 μl of buffer, and passed through a MACS® column (Miltenyi Biotec). The magnetically labelled cells retained in the column were eluted with the aid of buffer under a column plunger. Purity of cell fractions was determined as 91 %, using flow cytometric analysis in which forward scatter was plotted against FITC-conjugated $CD3^+$ cells, using unstained cells as controls.

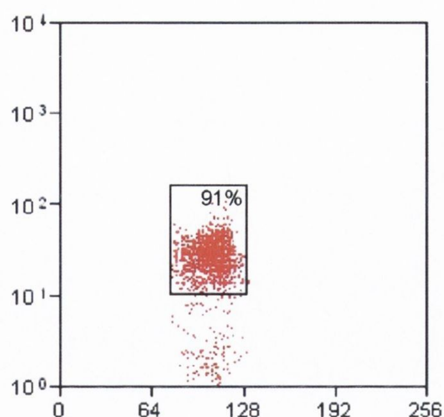


Figure 5.1 Electronically gated region within a representative flow cytometric dot plot indicating the purity of $CD3^+$ magnetic bead-enriched T cells.

5.2.3 Vγ9Vδ2 T cell expansion and purification

Lines of Vγ9Vδ2 T cells were generated by subjecting PBMCs to a single stimulation with HMB-PP (10 nM) (kindly donated by Drs. Hassan Jomaa and Armin Reichenberg, Jomaa Pharmaka GmbH, Giessen, Germany) in complete RPMI supplemented with IL-2 (50 U/ml), donated by the National Cancer Institute, Frederick Research Foundation Biological Resources Branch, USA. Cultures were fed every 3 days with fresh medium containing IL-2 and harvested on days 14-21. For phenotypic characterisation, cells were firstly washed with PBA buffer (PBS; 1 % w/v BSA), centrifuged (2000 rpm, 7 min, 21 °C), and then resuspended in 50 μl of buffer and stained with 10 μl of FITC-conjugated CD3 mAb and 10 μl of PE-conjugated Vδ2 mAb (BD Biosciences, Oxford, UK) / 1 x 10^6 cells for 30 min in the dark on ice. Cells were washed once again, combined in 1 ml of PBA buffer, and sorted using a MoFlo™ XDP Cell Sorter (Beckman Coulter, Miami, FL, USA). Purity was determined as 99 % by flow cytometric analysis, plotting FITC $CD3^+$ against PE $V\delta2^+$, using unstained cells as a control.

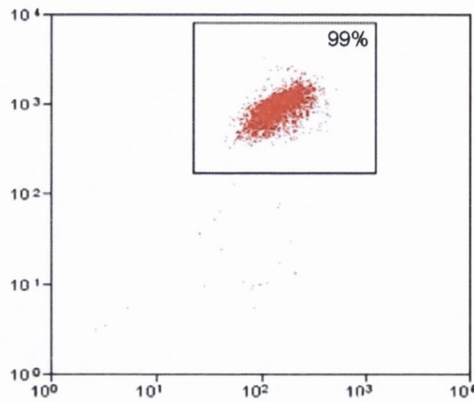


Figure 5.2 Electronically gated region within a representative flow cytometric dot plot indicating the purity of expanded Vγ9Vδ2$^+$ T cells.

5.2.4 Exposure of PBMCs, CD3$^+$ T cells, and Vγ9Vδ2 T cell lines to HIV-1 Tat clades

PBMCs were seeded in 24 well-plates at a density of 5 x 10^5 cells/ml in complete RPMI medium and exposed to HIV-1 Tat clades A, B, C, or D (200 ng/ml) for 24 hr. Samples were then stimulated for a further 24 hr with phorbol 12-myristate 13-acetate (PMA; 10 ng/ml)/ionomycin (I; 1 μg/ml) (Sigma-Aldrich), or (*E*)-4-hydroxy-3-methyl-but-2 enyl pyrophosphate (HMB-PP; 10nM) / interleukin-2 (IL-2; 50 U/ml), in the presence of Brefeldin A (10 μg/ml) (Sigma-Aldrich), an inhibitor of protein translocation from the endoplasmic reticulum to the Golgi apparatus. Enriched CD3$^+$ T cells were seeded in 48 well plates at a density of 1 x 10^5cells/well while Vγ9Vδ2 T cell lines were seeded in 96 well plates at a density of 5 x 10^3cells/well. Both cell fractions were treated identically to PBMCs, save for the addition of Brefeldin A.

HIV-1 Tat clades, HMB-PP, and IL-2 were prepared in sterile dH_2O (18 $M\Omega^{-cm}$). PMA, I, and Brefeldin A were prepared in DMSO (C_{final}= 0.01% v/v, 0.01% v/v, 0.13% v/v, respectively). All working stocks were prepared in medium.

5.2.5 Intracellular analysis of cytokine production in PBMC subsets

Following exposure to HIV-1 Tat clades and PMA/I activation, PBMCs were harvested, washed in PBA buffer containing sodium azide (1 % v/v BSA; 0.02 % w/v NaN_3; dissolved in PBS), centrifuged (600 g, 5 min, 21 °C), and resuspended in 50 µl of PBA. Using fluorescence-activated cell sorting (FACS) tubes, each sample was extracellularly labelled with 2 µl of Pacific Blue-conjugated CD3 mAb (BD Biosciences) and 3 µl of PE-conjugated Vδ2 mAb for 15 min in the dark. Cells were then washed with 2 ml of PBA / tube, centrifuged (800 g, 7 min, 21 °C), and supernatants discarded. In advance of intracellular staining, cells were fixed by resuspending in 500 µl of 2 % w/v paraformaldehyde (PFA) and incubated for 10 min in the dark. After washing, cells were then permeabilised in PBA containing 0.2 % w/v saponin for 10 min in the dark. Tubes were then centrifuged and cell pellets incubated with the anti-cytokine mAbs, APC-conjugated IFN-γ (1 µl) and FITC-conjugated TNF-α (1 µl) (both BD Biosciences) for 20 min in the dark. Cells were then washed, resuspended in PBS and analysed using a seven colour CyAn ADP (Beckman Coulter, High Wycombe, UK). Cell populations were defined by forward scatter, side scatter, and fluorescent channels utilising blue laser and red laser excitation at 488 nm and 635 nm wavelengths, respectively. Data was expressed as percentage of cells staining antigen positive from electronically gated dot plots.

5.2.6 Cytokine quantification by ELISA

Conditioned medium was reserved from $CD3^+$ T cells and Vγ9Vδ2 T cell subsets treated as described above (section 5.2.4). Cell debris was removed by centrifugation (5000 g, 5 min, 4 ºC) and samples aliquoted and stored at -80 ºC until required.

Stimulated T cell samples were diluted 1:3 in reagent diluent (PBS, pH 7.2; 1 % w/v BSA) prior to measurement of IFN-γ and TNF-α levels by ELISA (Human IFN-γ DuoSet and Human TNF-α DuoSet; R&D Systems) according to manufactures protocol. Optical density was read at 450 nm with a reference wavelength of 540 nm.

5.2.7 Data and statistical analysis

The percentage of cells positive for intracellular IFN-γ and TNF-α staining were derived from gated flow cytometry histograms using Summit© software (Dako, Fort Collins, USA). IFN-γ and TNF-α abundance were determined by interpolation from respective standard curves using KCjunior™ software and expressed as pg/ml.

Flow cytometry data for intracellular cytokines was derived from four independent experiments (n=4). ELISA samples were assayed in duplicate and experiments were repeated three independent times (n=3). Data was analysed by one-way ANOVA with *post hoc* analysis (*Bonferroni* or *Dunnetts*; GraphPad Prism, version 5.01) as appropriate and expressed as mean ± SEM. A value of $P<0.05$ was taken to indicate statistical significance.

5.3 Results

5.3.1 Effects of HIV-1 Tat clades on the percentage of $CD3^+$ T cells staining positive for intracellular IFN-γ and TNF-α

Using flow cytometry, we investigated intracellular IFN-γ and TNF-α production by human T cells. The percentage of electronically gated $CD3^+$ T cells that stained positive for IFN-γ were similar to background under non-stimulated conditions and HMB-PP/IL-2 (10 nM/50 U/ml)-stimulated conditions (0.4±0.1 and 1.3±0.5 %; Figure 5.3 A and B). Stimulation by PMA/I (10 ng/ml/1 μg/ml) significantly increased (P<0.05) the number of cells staining positive for IFN-γ (38±6.8 versus 0.4±0.1 %; Figure 5.3 C). Similarly, the percentage of TNF-α positive cells was minimal in unstimulated and HMB-PP/IL-2-stimulated cells (0.53±0.2 and 1.4±0.6 %; Figure 5.4 A and B) but increased substantially in cells exposed to PMA/I (41±9 %; P<0.05; Figure 5.4 C). When $CD3^+$ T cells were exposed to Tat clades A, B, C, and D (200 ng/ml) under either basal, or stimulated conditions, there was no effect on the percentage of cells producing IFN-γ or TNF-α.

5.3.2 Effects of HIV-1 Tat clades on the percentage of Vγ9Vδ2 T cells staining positive for intracellular IFN-γ and TNF-α

The mean percentage of Vγ9Vδ2 T cells staining positive for IFN-γ under basal conditions was 2.0±0.4 % and was not altered by exposure to any Tat clades (Figure 5.5 A). Upon re-stimulation with HMB-PP/IL-2, an ~24 fold increase in IFN-γ staining occured (48±14 versus 2.0±0.4 %; P<0.05; Figure 5.5 B). PMA/I re-stimulation caused an ~34 fold increase in IFN-γ staining (67±14 versus 2.0±0.4 %; P<0.05; Figure 5.5 C). As in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter IFN-γ positive cells at the concentration studied.

Mean percentage of Vγ9Vδ2 T cells staining positive for TNF-α under basal conditions was 2.9±0.6 % and was not altered by exposure to any of the Tat clades investigated (Figure 5.6 A). HMB-PP/IL-2 re-stimulation caused an ~14 fold increase in TNF-α staining (41±12 versus 2.9±0.6 %; $P<0.05$; Figure 5.6 B). PMA/I re-stimulation caused an ~24 fold increase in TNF-α staining (71±12 versus 2.9±0.6 %; $P<0.05$; Figure 5.6 C). As in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter the percentage of TNF-α positive cells at the concentration studied.

5.3.3 Effects of HIV-1 Tat clades on IFN-γ secretion by $CD3^+$ T cells

In unstimulated magnetic bead-enriched $CD3^+$ T cells, Tat clades A, B, C, and D did not alter IFN-γ production compared to untreated (medium) controls (49±1.6, 44±6.4, 46±3.5, and 35±2.9 versus 43±3.8 pg/ml respectively; Figure 5.7 A). Following stimulation by HMB-PP/IL-2, IFN-γ levels did not increase significantly (57±5.9 versus 43±3.8 pg/ml; Figure 5.7 B). While Tat clades A, C, and D did not alter IFN-γ secretion under these conditions, Tat clade B elicited an ~fold increase in IFN-γ levels relative to HMB-PP/IL-2 controls (105±4.2 versus 57±5.9 pg/ml; $P<0.05$; Figure 5.7 B). In samples stimulated with PMA/I, a massive induction of IFN-γ secretion occurred (734±17 versus 43±3.8 pg/ml; $P<0.05$; Figure 5.7 C). However, the inductive effect mediated by clade B under HMB-PP/IL-2 conditions was lost, and IFN-γ levels were not altered by any of the Tat clades investigated.

5.3.4 Effects of HIV-1 Tat clades on TNF-α secretion by $CD3^+$ T cells

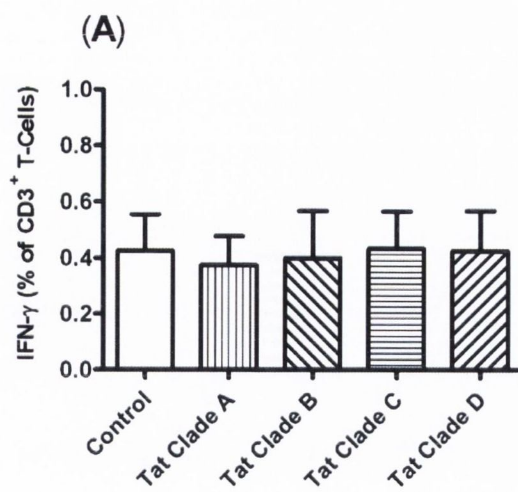
In culture medium supernatants from unstimulated $CD3^+$ T cells, TNF-α was undetectable. Exposure of these cells to Tat clades at the concentrations studied did not induce TNF-α to detectable levels. Stimulation of $CD3^+$ T cells with HMB-PP/IL-2 induced ($P<0.05$) TNF-α secretion (252±33 pg/ml; Figure 5.8 A). However, none of the Tat clades studied altered this response (Figure 5.8 A). Likewise, PMA/I also increased TNF-α production (752±13 pg/ml; $P<0.05$), and the response was not altered by the presence of Tat proteins investigated (Figure 5.8 B).

5.3.5 Effects of HIV-1 Tat clades on IFN-γ secretion by Vγ9Vδ2 T cells

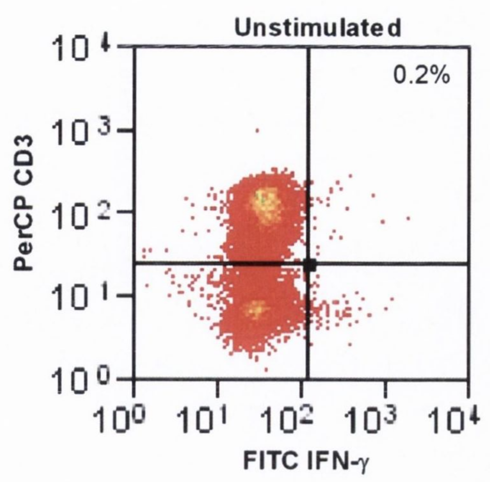
In view of the evidence that increased IFN-γ production resulted from Tat clade B exposure of phosphoantigen-stimulated $CD3^+$ T cells, we speculated that this may also occur in Vγ9Vδ2 T cell subsets as they are know responders to HMB-PP *in vitro* [394]. As TNF-α levels were unchanged however, we elected not to further investigate the effects of Tat clades on seretion of this cytokine by Vγ9Vδ2 T cells.

In expanded Vγ9Vδ2 T cell lines, generated by a single initial exposure of PBMCs to HMB-PP/IL-2 (10 nM/50 U/ml) and subsequent culture in the presence of IL-2 (50 U/ml), Tat clades A, B, C, and D significantly ($P<0.05$) increased IFN-γ secretion compared to untreated cells, with clade B generating the largest increase (26±1.5, 48±1.4, 24±1.2, and 22±2.7 versus 13±0.8 pg/ml; Figure 5.9 A). When re-stimulated with HMB-PP/IL-2, an ~4 fold increase in IFN-γ levels was noted (51±8.7 versus 13±0.8 pg/ml; $P<0.05$; Figure 5.9 B), and under these conditions, Tat clade B, but not A, C, or D, further increased secretion (117±5.7 versus 51±8.7 pg/ml; $P<0.05$; Figure 5.9 B). Using PMA/I as a stimulant also resulted in increases of IFN-γ secretion by these cells (80±20 versus 13±0.8 pg/ml;

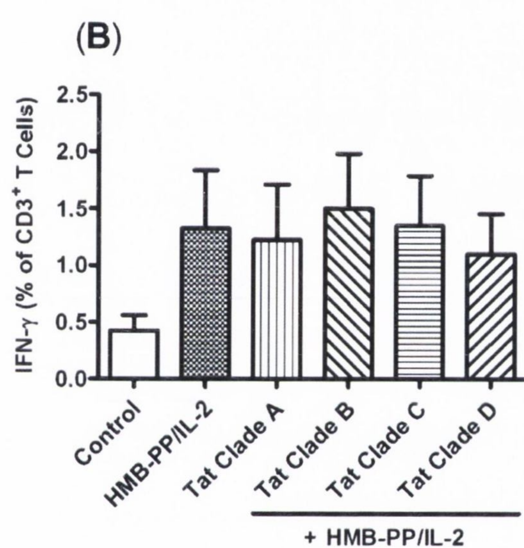
$P<0.05$), and again Tat clade B, but not A, C, or D, demonstrated an ~4 fold IFN-γ production increase compared to PMA/I controls (326±5.2 versus 80±20 pg/ml; $P<0.05$; Figure 5.9 C).



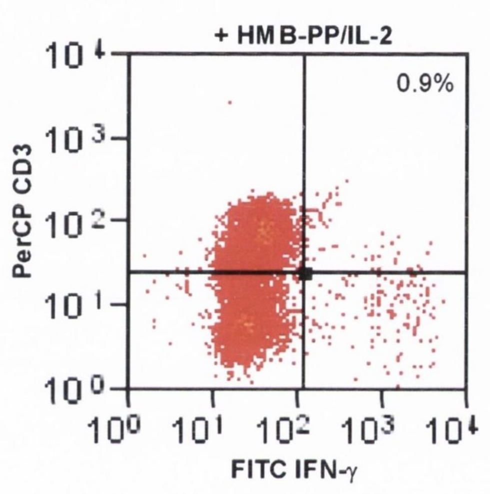
(A)
IFN-γ (% of CD3+ T-Cells)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D



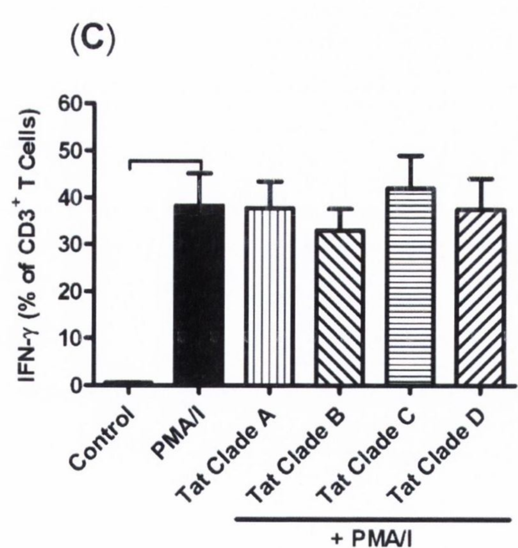
Unstimulated
0.2%
PerCP CD3
FITC IFN-γ



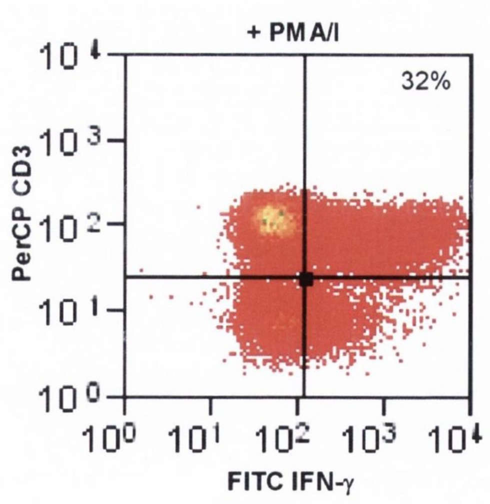
(B)
IFN-γ (% of CD3+ T Cells)
2.5
2.0
1.5
1.0
0.5
0.0
Control
HMB-PP/IL-2
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ HMB-PP/IL-2



+ HMB-PP/IL-2
0.9%
PerCP CD3
FITC IFN-γ

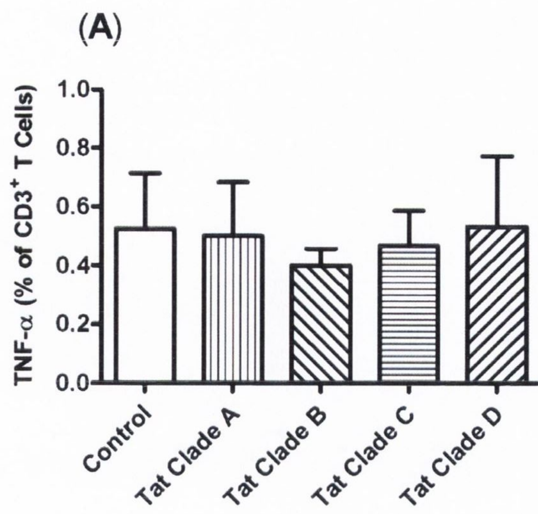


(C)
IFN-γ (% of CD3+ T Cells)
60
50
40
30
20
10
0
Control
PMA/I
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ PMA/I

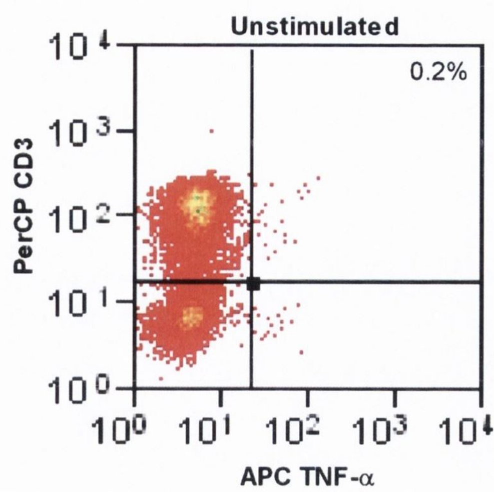


+ PMA/I
32%
PerCP CD3
FITC IFN-γ

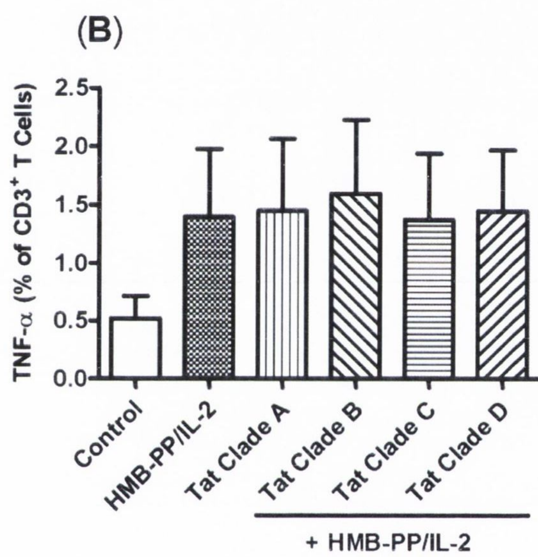
Figure 5.3 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on the percentage of $CD3^+$ T cells staining positive for intracellular IFN-γ. Column bar graphs and representative flow cytometric dot plots indicate the percentage of cells staining positive for IFN-γ under non-stimulated (A), HMB-PP/IL-2-stimulated (10 nM/50 U/ml) (B), and PMA/I-stimulated (10 ng/ml/1 μg/ml) (C) conditions after 48 hr incubations (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at $P < 0.05$). Representative flow cytometry dot plots contain numbers in upper right hand quadrants indicating the percentage of $CD3^+$ T cells positive for IFN-γ from an individual donor.



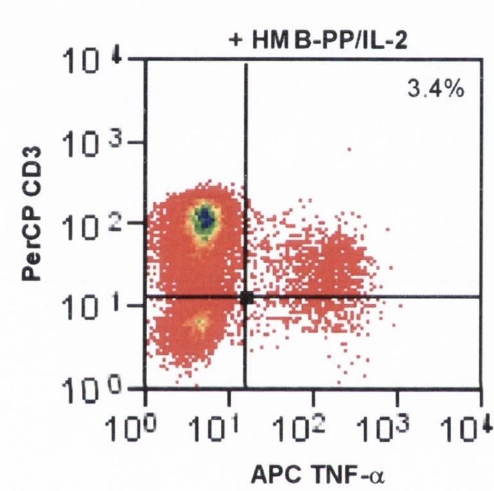
(A)
TNF-α (% of CD3+ T Cells)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D



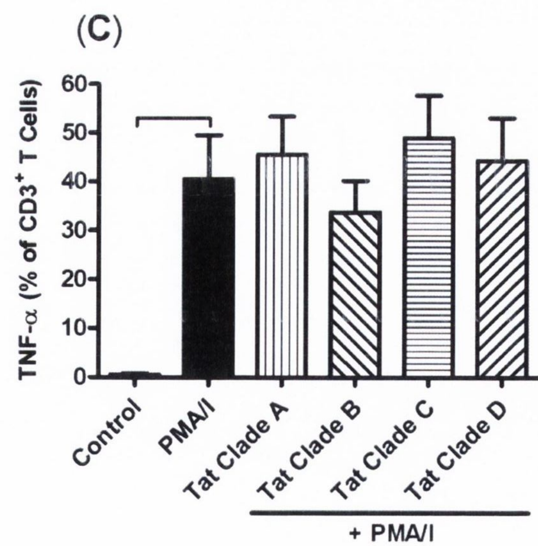
Unstimulated
0.2%
PerCP CD3
APC TNF-α



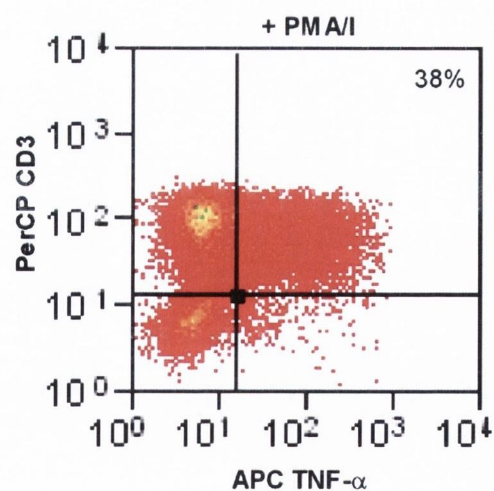
(B)
TNF-α (% of CD3+ T Cells)
2.5
2.0
1.5
1.0
0.5
0.0
Control
HMB-PP/IL-2
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ HMB-PP/IL-2



+ HMB-PP/IL-2
3.4%
PerCP CD3
APC TNF-α

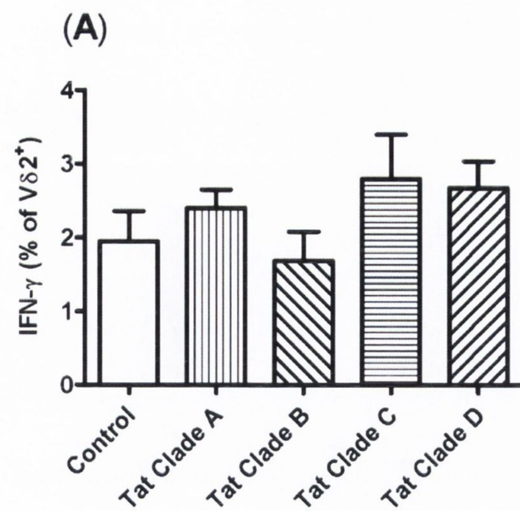


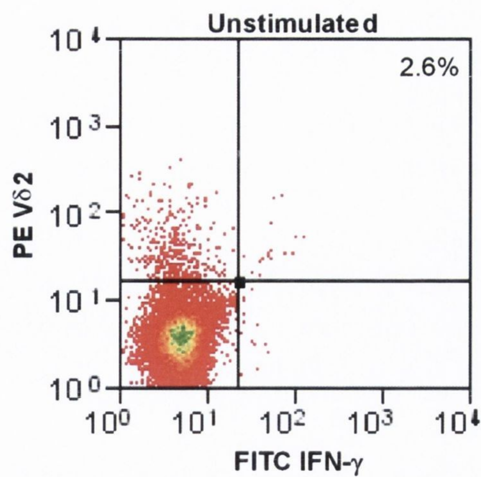
(C)
TNF-α (% of CD3+ T Cells)
60
50
40
30
20
10
0
Control
PMA/I
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ PMA/I

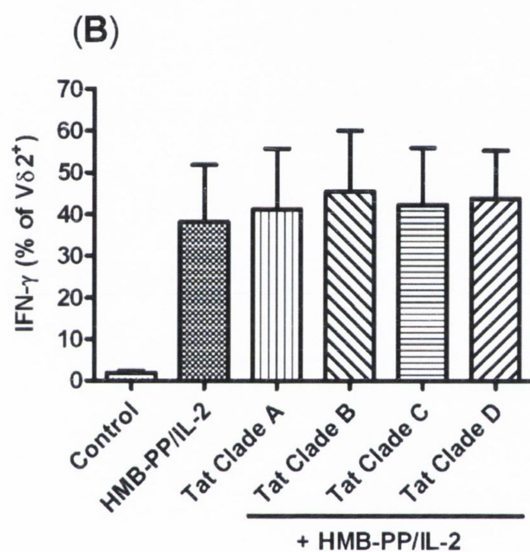


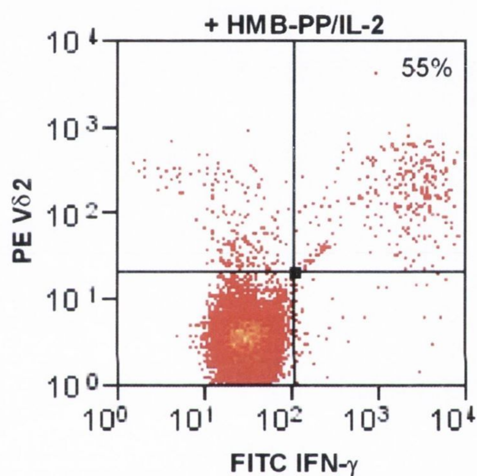
+ PMA/I
38%
PerCP CD3
APC TNF-α

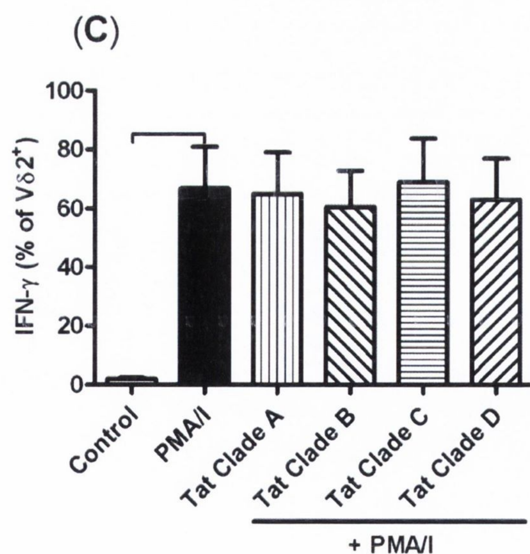
Figure 5.4 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on the percentage of $CD3^+$ T cells staining positive for intracellular TNF-α. Column bar graphs and representative flow cytometric dot plots indicate the percentage of cells staining positive for TNF-α under non-stimulated (A), HMB-PP/IL-2-stimulated (10 nM/ 50 U/ml) (B), and PMA/I-stimulated (10 ng/ml/1 μg/ml) (C) conditions after 48 hr incubations (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at P <0.05). Representative flow cytometry dot plots contain numbers in upper right hand quadrants indicating the percentage of $CD3^+$ T cells positive for TNF-α from an individual donor.


(A)
IFN-γ (% of Vδ2+)
0
1
2
3
4
Control
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D


Unstimulated
2.6%
PE Vδ2
FITC IFN-γ


(B)
IFN-γ (% of Vδ2+)
0
10
20
30
40
50
60
70
Control
HMB-PP/IL-2
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ HMB-PP/IL-2


+ HMB-PP/IL-2
55%
PE Vδ2
FITC IFN-γ


(C)
IFN-γ (% of Vδ2+)
0
20
40
60
80
100
Control
PMA/I
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ PMA/I

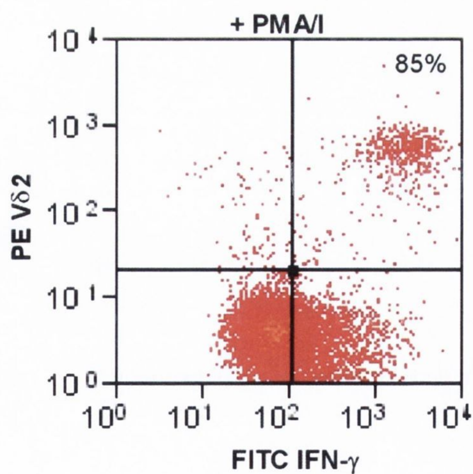
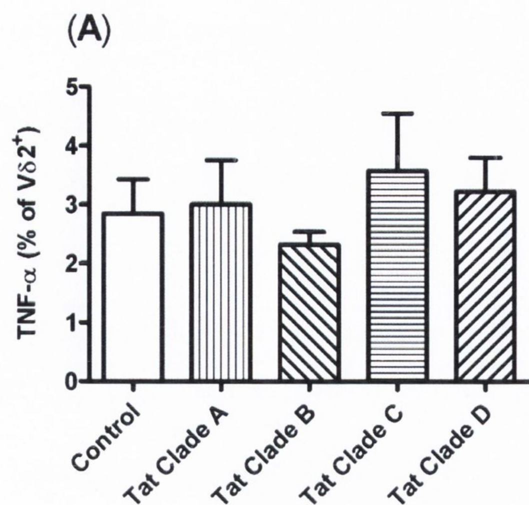
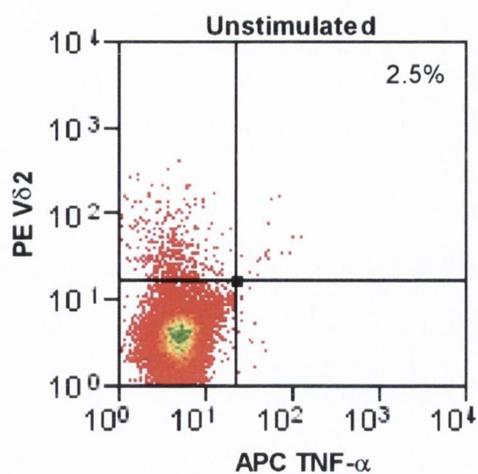
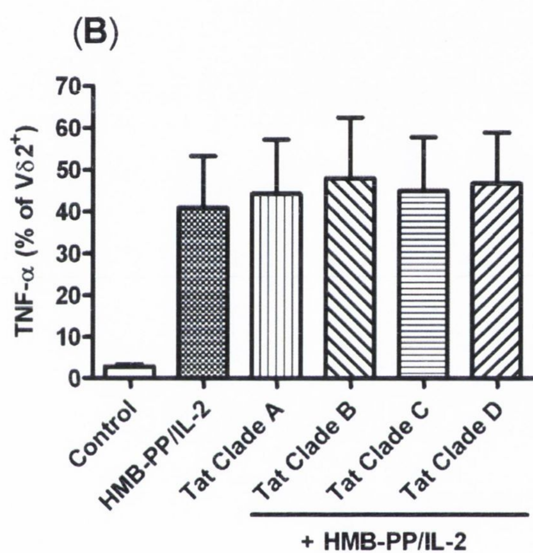
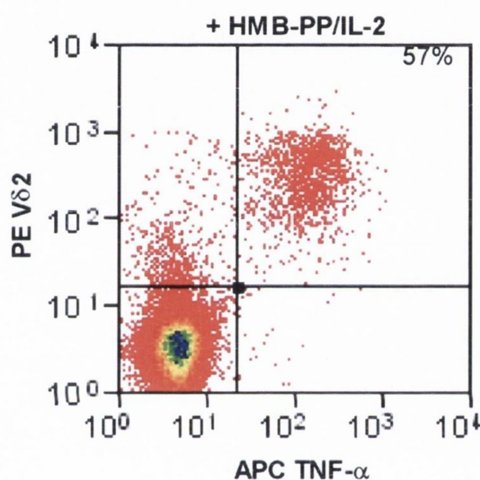

+ PMA/I
85%
PE Vδ2
FITC IFN-γ

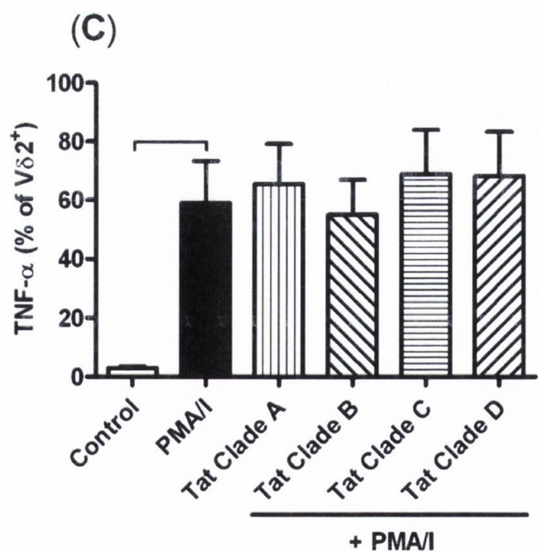
Figure 5.5 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on the percentage of $V\gamma9V\delta2^{+}$ T cells staining positive for intracellular IFN-γ. Column bar graphs and representative flow cytometric dot plots indicate the percentage of cells staining positive for IFN-y under non-stimulated (A), HMB-PP/IL-2-stimulated (10 nM/50 U/ml) (B), and PMA/I-stimulated (10 ng/ml/1 μg/ml) (C) conditions after 48 hr incubations (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at P <0.05). Representative flow cytometry dot plots contain numbers in upper right hand quadrants indicating the percentage of $CD3^{+}$ T cells positive for IFN-γ from an individual donor.


(A)
TNF-α (% of Vδ2+)
5
4
3
2
1
0
Control
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D


Unstimulated
2.5%
PE Vδ2
APC TNF-α


(B)
TNF-α (% of Vδ2+)
70
60
50
40
30
20
10
0
Control
HMB-PP/IL-2
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ HMB-PP/IL-2


+ HMB-PP/IL-2
57%
PE Vδ2
APC TNF-α


(C)
TNF-α (% of Vδ2+)
100
80
60
40
20
0
Control
PMA/I
Tat Clade A
Tat Clade B
Tat Clade C
Tat Clade D
+ PMA/I

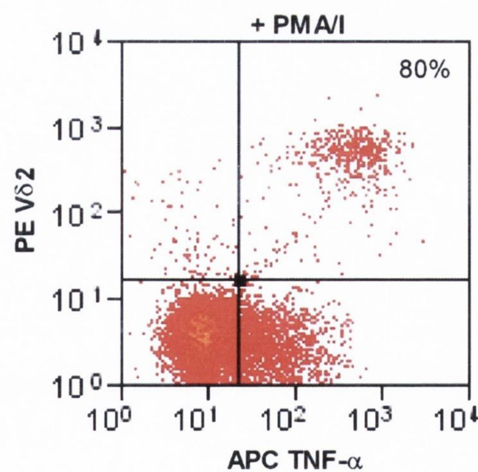

+ PMA/I
80%
PE Vδ2
APC TNF-α

Figure 5.6 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on the percentage of $V\gamma9V\delta2^+$ T cells staining positive for intracellular TNF-α. Column bar graphs and representative flow cytometric dot plots indicate the percentage of cells staining positive for TNF-α under non-stimulated (A), HMB-PP/IL-2-stimulated (10 nM/50 U/ml) (B), and PMA/I-stimulated (10 ng/ml/1 μg/ml) (C) conditions after 48 hr incubations (n=4; mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at $P < 0.05$). Representative flow cytometry dot plots contain numbers in upper right hand quadrants indicating the percentage of $CD3^+$ T cells positive for TNF-α from an individual donor.

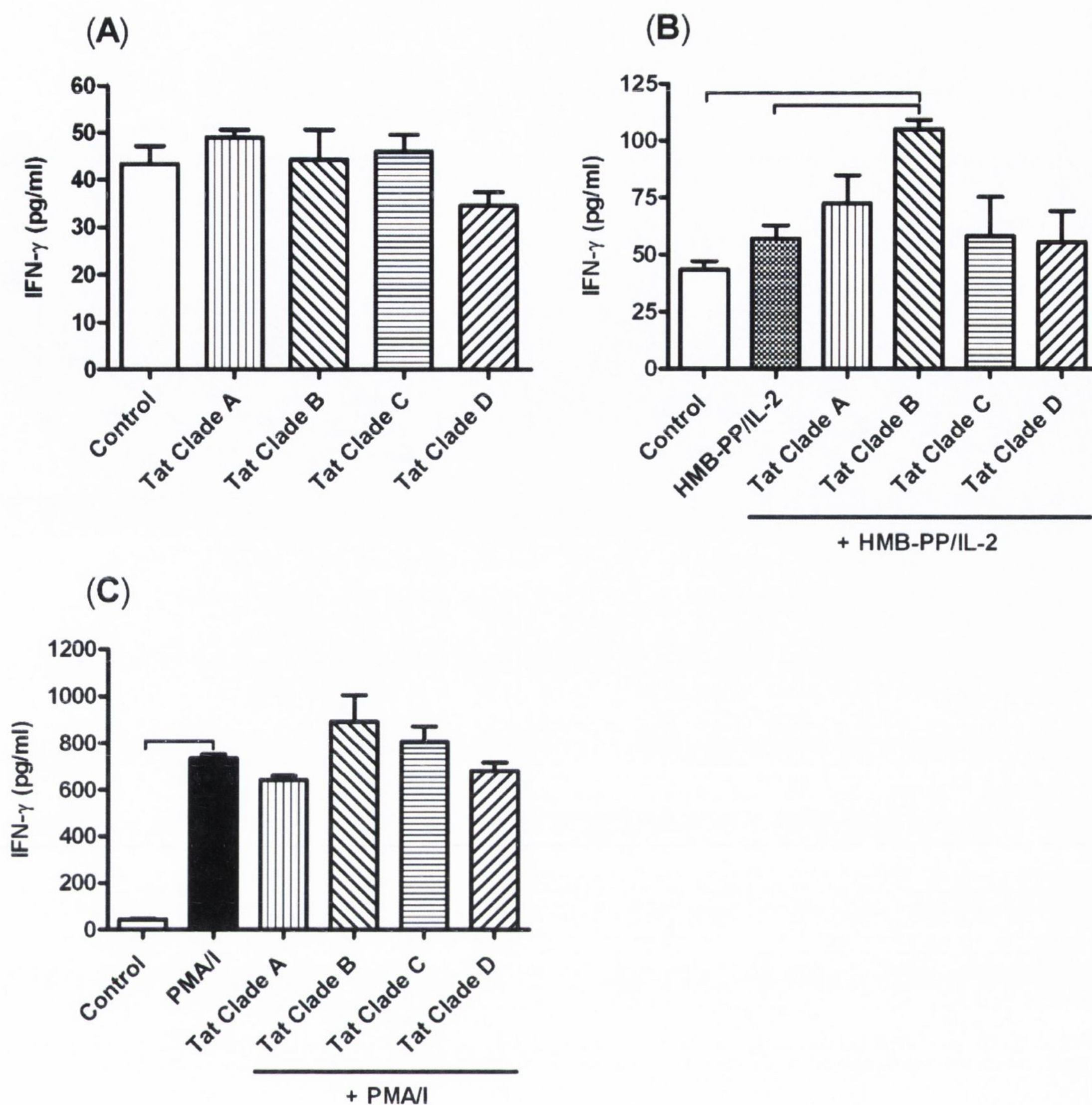


Figure 5.7 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on IFN-γ secretion after 48 hrs in $CD3^+$ T cells isolated from PBMCs of healthy donors. IFN-γ was measured in culture medium supernatants of un-stimulated (A), HMB-PP/IL-2-stimulated (10 nM/50 U/ml) (B), and PMA/I-stimulated (10 ng/ml/1 μg/ml) (C) cells. Column bar graphs indicate cytokine levels from 3 donors (mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at $P < 0.05$).

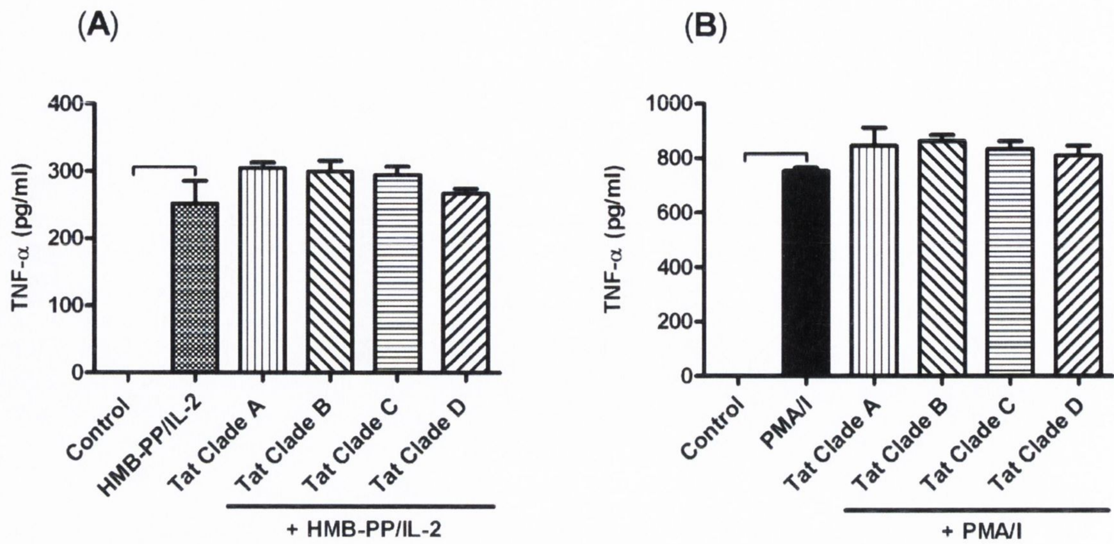


Figure 5.8 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on TNF-α secretion after 48 hrs in $CD3^+$ T cells isolated from PBMCs of healthy donors. TNF-α was measured in culture medium supernatants of HMB-PP/IL-2-stimulated (10 nM/50 U/ml) (A) and PMA/I-stimulated (10 ng/ml/1 μg/ml) (B) cells. Column bar graphs indicate cytokine levels from 3 donors (mean ± SEM; one-way ANOVA with *Dunnetts* post hoc test; horizontal capped bars indicate statistically significant differences from controls at P <0.05).

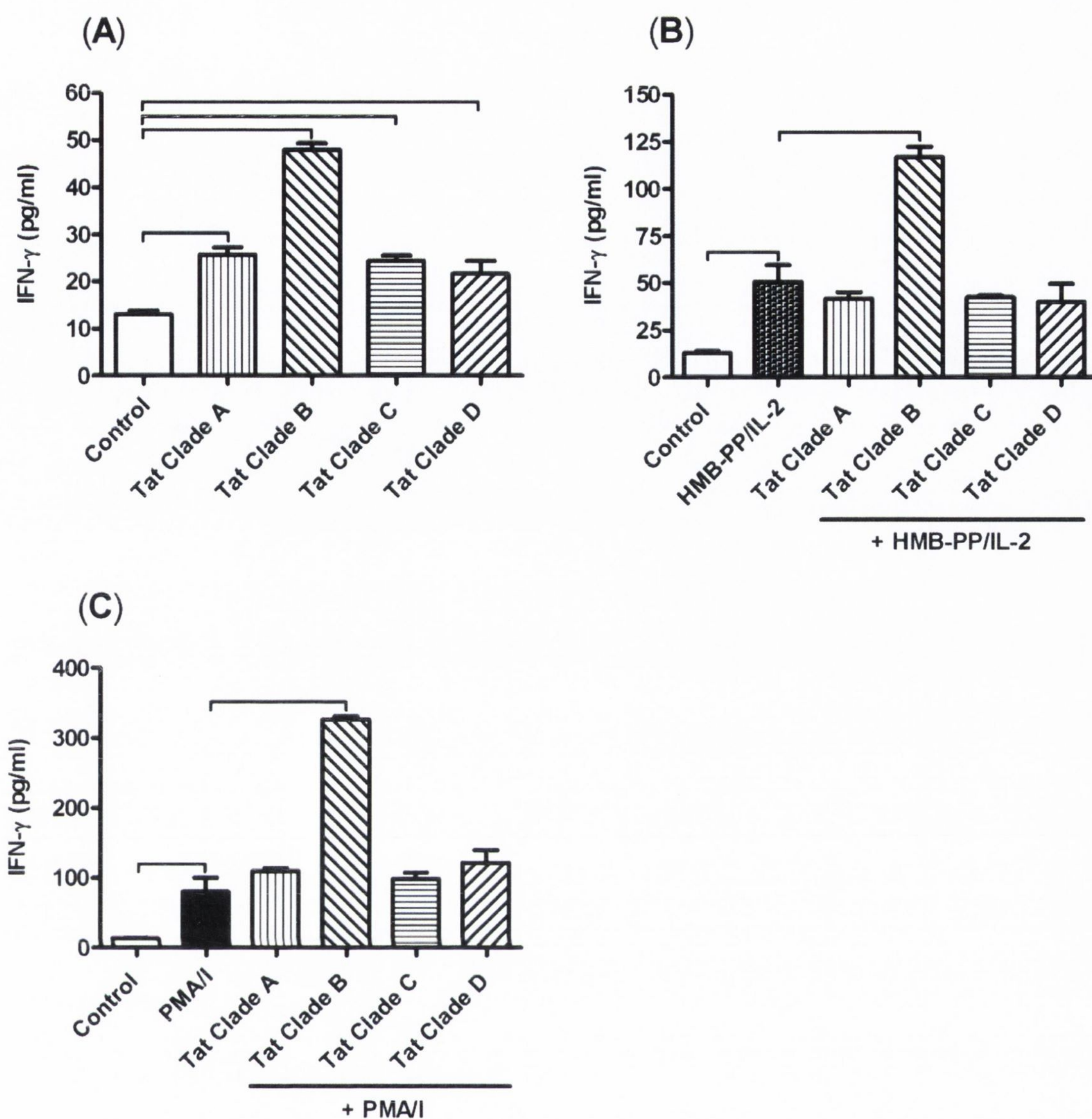


Figure 5.9 Effect of HIV-1 Tat clades A, B, C, and D (200 ng/ml) on IFN-γ secretion after 48 hrs in Vγ9Vδ2 T cells expanded from PBMCs of healthy donors under un-stimulated (A) and under re-stimulated conditions by HMB-PP/IL-2 (10 nM/50 U/ml) (B) and PMA/I (10 ng/ml/1 μg/ml) (C). Column bar graphs indicate cytokine levels from 3 donors (mean ± SEM; one-way ANOVA with *Bonferroni* or *Dunnetts* post hoc tests; horizontal capped bars indicate statistically significant differences from controls and between Tat clades at $P < 0.05$).

5.4 Discussion

In this study we demonstrate that exposure of human PBMCs *in vitro* to HIV-1 Tat clades A, B, C, and D does not alter the percentage of $CD3^+$ T cells or Vγ9Vδ2 T cells producing IFN-γ and TNF-α. However, the amount of secreted IFN-γ is significantly increased by Tat clade B in $CD3^+$ T cells under HMB-PP/IL-2-stimulated conditions. Furthermore, in Vγ9Vδ2 T cells, all clades increased IFN-γ secretion, with Tat clade B induction the most prominent. Under re-stimulted conditions, clade B maintained an inductive effect on IFN-γ secretion.

Due to its efficient cell membrane transduction properties, the HIV-1 regulatory protein Tat is released into the microenvironment and the circulation, and is readily taken up by surrounding cells [555]. Subsequently, Tat can initiate a number of kinase- and calcium-related signalling pathways resulting in downstream activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) [319, 556]. NF-κB is regarded as a major transcriptional regulator for the expression of cytokines involved in immune and inflammatory responses such as IFN-γ and TNF-α [557]. IFN-γ is a pleiotropic cytokine produced primarily by thymus-derived cells in response to viral infection. Although originally defined as an antiviral agent, it is involved in the regulation of nearly all phases of the immune and inflammatory responses, including the activation and differentiation of T cells, B cells, NK cells and macrophages, stimulation of antigen presentation through class I and II MHC molecules, and the orchestration of leukocyte-endothelium interactions, amongst many other functions [558]. In HIV-infected patients, the shift from a T helper type 1 (Th1) to a T helper type 2 (Th2) cytokine response may indicate enhanced disease progression [425-427]. Th1 cells are characterised by secretion of antiviral cytokines, IFN-γ and IL-2, and IFN-γ plays an important role in regulating the Th1/Th2 balance [559]. The pro-inflammatory cytokine TNF-α, also exerts a broad range of

biological activities, encompassing inflammatory and immune responses. Produced by a range of immune cells in response to various stimuli, including viral infection, TNF-α can mature and activate antigen presenting cells (APCs), induce cytokine production by monocytes, activate cytotoxic T lymphocytes, and induce apoptosis of mature T cells [560]. Owing to its ability to induce NF-κB, a transcriptional activator of the HIV-1 LTR, pro-viral effects have been attributed to TNF-α [440, 561-563].

Investigating the percentage of T cells staining positive for IFN-γ and TNF-α by flow cytometric analysis, we report that exposure to Tat clades A, B, C, and D does not induce any effects at the concentration studied in $CD3^+$, or $V\gamma9V\delta2^+$ subsets. We went on to investigate whether Tat A, B, C, or D influenced the amount of secreted IFN-γ and TNF-α by these cells. Exposure of $CD3^+$ T cells to Tat clades revealed that clade B induced the secretion of IFN-γ when cells were stimulated with the phosphoantigen HMB-PP, in combination with IL-2. Under non-stimulated and PMA/I-stimulted conditions there was no significant effect. Under both stimulated and non-stimulated conditions, TNF-α secretion was unaltered as a result of exposure to Tat clades. Whilst IL-2 is a broad T cell stimulant, HMB-PP has been shown to be a specific and potent γδ T cell activator [564]. We hypothesised therefore, that the induction of IFN-γ by Tat B in $CD3^+$ T cells stimulated thus may have been indicative of effects emanating from Vγ9Vδ2 subsets. Although Tat B also induced IFN-γ production under PMA/I-stimulated conditions, the increase failed to reach significance. Due to the massive upregulation of IFN-γ production by cells activated with these agents (734±17 versus 57±5.9 pg/ml), detectable differences between samples may have been obscured. Indeed, our subsequent experiments using HMB-PP/IL2 expanded Vγ9Vδ2 cells showed that all clades significantly induce IFN-γ production, but only Tat clade B maintains its effect when cells are re-stimulated with HMB-PP/IL2 or PMA/I.

The differential effects of HIV-1 Tat subtypes on cytokine activation was explored in a recent publication by Wong and colleagues [342]. Assessing the potential for Tat clades B and C to induce the immunosuppressive cytokine IL-10 in monocytes, the study reports that the cysteine to serine mutation at position 31, which is found in >90 % of clade C Tat proteins, results in a marked decrease in IL-10 production compared to clade B Tat. This C31S mutation disrupts the ^{30}C-C motif which is essential for the induction of an inward Ca^{2+} flux, and as previous studies have indicated, an increase in cytoplasmic Ca^{2+} concentration is critical to Tat-induced IL-10 and TNF-α production in monocytes [341, 565]. Whilst these studies provide important information regarding the mechanisms by which HIV-1 Tat subtypes might contribute to immunodeficiencies *in vivo*, the cell type of these and other investigations are almost entirely restricted to those of the monocyte/macrophage lineage. The current study is the first to demonstrate that differential cytokine modulation by Tat clades may also be cell type-specific. In the previous chapter we confirmed the findings of others with respect to Tat clade B induction of TNF-α in monocytes/macrophages [343] but report here that such effects may not apply to T cells, as Tat B did not alter TNF-α in $CD3^+$ cells. In these cells, and in Vγ9Vδ2 subsets, we have also shown that Tat B increases IFN-γ secretion, an effect that does not correspond with an increased number of cells producing this cytokine. Evidence suggests that Vγ9Vδ2 T cells possess substantive antiviral properties, exhibiting potent lytic activity against virally-infected cells [566-568], participating in antiviral immune surveillance [569, 570], and mediating immune cell maturation and activation [548-550]. During HIV infection, polyclonal decreases in absolute numbers of Vγ9Vδ2 T cells are indicated [408, 551, 571] and those that remain are typically anergic, unable to proliferate and/or express the IL-2 receptor [552, 553, 572]. Initiation of HAART however, appears restorative to both Vγ9Vδ2 functionality and number [412, 573].

Reports indicate that IFN-γ exerts an inhibitory role on HIV-1 replication in monocytes and macrophages [574-576] and facilitates immunological benefit *in vivo* when secreted by activated Vγ9Vδ2 T cells [577]. There are also studies to suggest an opposing stimulatory role. For example, exposure of chronically infected U1 monocytes to IFN-γ activates HIV replication enzyme activity [578], and treatment of U937 monocytes with IFN-γ modestly increases HIV expression, but when added with TNF-α, IFN-γ synergistically potentiates the stimulatory effect of TNF-α [290]. Furthermore, IFN-γ has been found to increase HIV-1-induced syncytium formation in PBMCs and $CD4^+$ T cells infected with the virus *in vitro* [579]. Our data, and that of others [394], demonstrates that activated Vγ9Vδ2 T cells produce significant amounts of IFN-γ and TNF-α. Dysregulated production of IFN-γ by Tat B as we have shown here, could suggest a pro-viral role for Vγ9Vδ2 T cells in circulation. Other studies indicate that IFN-γ augments HIV-1 Tat-induced neurotoxicity and so contributes to the development of HIV-associated dementia. Co-operative interaction between IFN-γ and Tat dramatically increases macrophage expression of CXCL10, a chemokine closely associated with CNS disease during HIV infection [580]. As the extravastion of lymphocytes, including γδ T cells, to sites of inflammation is also orchestrated by IFN-γ-mediated upregulation of CXCL10 [414, 581], Vγ9Vδ2 T cells secreting large amounts of IFN-γ could represent another avenue by which HIV subtype B induces neuroAIDS. Finally, depletion, and loss of functionality of Vγ9Vδ2 T cells soon after HIV-RNA rebound could be facilitated by increased secretion of IFN-γ. Pro-apoptotic molecules such as caspase 1 [582], protein kinase R (PKR) [583], TNF-α receptor (TNFR) [584], and Fas/Fas Ligand (Fas/Fas L) [585] are induced by IFN-γ, and a previous study has indicated that Vγ9Vδ2 cells are susceptible to activation-induced cell death triggered by Fas/Fas L interactions [586].

In conclusion, we have shown that exposure of $CD3^+$ T cells and Vγ9Vδ2 subsets to clades A, B, C and D, at a concentration anticipated to be reflective of *in vivo* levels [587], augments IFN-γ and TNF-α secretion differentially and that these effects are not associated with changes to the number of cells producing each cytokine. Changes in cytokine secretion levels pertained to IFN-γ and were most pronounced in Vγ9Vδ2 T cells exposed to Tat clade B. These results indicate that HIV-1 Tat clade B may influence the extensive immunoregulatory potential of Vγ9Vδ2 T cells by increasing IFN-γ secretion.

Chapter 6.

Conclusions

6.1 Conclusions

Dysregulated MMP/TIMP expression has been described in HIV- and HCV-infected patients and some evidence suggests that antiviral pharmacological agents modulate MMP abundance *in vitro* and *in vivo.* However, a lack of consensus pervades the data and no studies to date have assessed the MMP status of HIV/HCV co-infected patients or the effects of initiating RBV/PEG-IFN-α combination therapy on MMPs in these patients. Furthermore, data describing the effects of antiviral therapy on MMP expression in relevant cellular sources is limited. Therefore, the first study (Chapter 3) of this thesis assessed the effects of anti-HCV and anti-HIV pharmacological agents on MMP-9 production in human THP-1 monocytes/macrophages and LX-2 hepatic stellate cells *in vitro.* A follow-up clinical study sought to quantify circulatory MMP-2, MMP-9, and TIMP-2 abundance in HIV/HCV co-infected patients and to determine the effects of initiating RBV/PEG-IFN-α2b combination therapy on these MMPs.

In THP-1 cells, IFN-α2a dose-dependently decreased MMP-9 activity, an effect that was maintained when IFN-α2a was combined with RBV, using concentrations reflective of plasma levels for both drugs. RBV increased MMP-9 activity. At the mRNA level, RBV alone, and in combination with IFN-α2a, increased MMP-9 expression while IFN-α2a had no effect. Investigating the disparity between extracellular MMP-9 activity and intracellular MMP-9 mRNA, RBV was found to increase intracellular MMP-9 protein levels, while IFN-α2a had no effect. In combination with RBV however, IFN-α2a reduced the RBV-mediated increases. Co-treatment of THP-1 cells with a proteasome inhibitor increased MMP-9 activity but did not alter the effect of IFN-α2a on RBV-mediated increased MMP-9 activity. Assessment of temporal IFN-α2a effects on MMP-9 mRNA revealed reduced expression at 24 hr compared to controls, while no effects at 48 hr and 72 hr were recorded. In LX-2 cells, IFN-α2a alone, or in combination with RBV, did not alter

MMP-9 activity. However, RBV increased MMP-9 activity. RBV and IFN-α2a alone, or in combination, did not alter MMP-9 mRNA expression. The HIV protease inhibitors, saquinavir and lopinavir decreased MMP-9 activity in THP-1 and LX-2 cells, respectively. Abacavir, zidovudine, efavirenz, nevirapine, and atazanavir had no effect in either cell line.

In our clinical study, MMP-9 abundance was higher in HIV/HCV co-infected and HIV mono-infected patients compared to healthy controls. In HCV mono-infected patients MMP-9 levels were similar to that of healthy controls. MMP-2 activity was similar in all groups studied, as was TIMP-2 expression. In HIV/HCV co-infected patients, RBV/PEG-IFN-α2b decreased plasma MMP-9 abundance compared to baseline at 3 day and 14 day timepoints. MMP-2 activity and TIMP-2 expression was not altered following treatment in these patients. These findings indicate that *in vitro*, monocytes/macrophages and hepatic stellate cells are targets for MMP-9 modulation by antiviral agents. MMP-9 over-expression in HIV/HCV co-infected patients is most likely a consequence of HIV infection and is markedly decreased by the initiation of RBV/PEG-IFN-α2a therapy. The results of our *in vitro* investigations suggest that this effect may be mediated in immune cells rather than hepatic cells.

It has been demonstrated that HIV-1 and HCV viral proteins exert pleiotropic effects that modulate the expression of non-viral genes in uninfected cells. The HIV-1 transcription factor Tat has been shown to influence MMP and cytokine expression and recent data suggests that these effects are differentially modulated based on viral subtype, or clade. HCV non-structural proteins such as NS3 are evidenced to mediate dysregulation of cytokine production. Both viral proteins have been shown to exert these extravirological effects in immune cells, suggesting a role in HIV/HCV-related immunopathology. As no studies to date have assessed the effects of Tat subtypes or NS3 on MMP/TIMP

expression, we investigated whether these proteins alter MMP/TIMP abundance and pro-inflammatory cytokine expression in THP-1 monocytes/macrophages (Chapter 4).

HIV-1 Tat clade B increased MMP-9 and TIMP-1 expression. Tat B also increased the expression of TNF-α in a dose-dependent manner. Tat clade C did not alter MMP-9 abundance but caused a subtle dose-dependent increase in TIMP-1 expression. TNF-α production was not altered by Tat clade C. HCV NS3 protein increased MMP-9 and TIMP-1 expression. NS3 also induced TNF-α expression, and IL-1β expression was increased in a dose-dependent manner. These results are indicative of HIV and HCV viral proteins functioning as significant immunomodulators in which inflammatory pathways are activated. The differential effects in this respect observed for Tat clades B and C also suggest a possible mechanism for the enhanced pathogeneity of HIV-1B infection, particularly in relation to the development of neurological dysfunction where the activity of MMPs and pro-inflammatory cytokines are prominent. Overall, these data raise the possibility that the pleiotropic effects of Tat and NS3 during HIV/HCV infection might contribute to the chronic immune activation that persists in the majority of patients and could facilitate the accelerated disease progression characteristic of co-infected patients.

To further investigate the immunomodulatory potential of HIV-1 Tat with respect to its viral subtypes we conducted an *in vitro* study to determine the effects of exposing T lymphocytes to the four predominant global HIV-1 clades A, B, C, and D, on IFN-γ and TNF-α production (Chapter 5). To date, the effects of Tat clades on cytokine production have only been assessed in cells of the monocyte/macrophage lineage despite the significance of T cell responses for effective cell-mediated immunity during infection.

Using flow cytometric analysis, it was determined that the percentage of $CD3^+$ T cells that stained positive for IFN-γ were similar to background under non-stimulated conditions and HMB-PP/IL-2-stimulated conditions. PMA/I stimulation increased the number of cells

staining positive for IFN-γ. The percentage of TNF-α positive cells was also minimal in unstimulated and HMB-PP/IL-2-stimulated cells but increased in cells exposed to PMA/I. $CD3^+$ T cells exposed to Tat clades A, B, C, and D under either basal, or stimulated conditions, showed no alteration to the percentage of cells producing IFN-γ or TNF-α.

The mean percentage of Vγ9Vδ2 T cells positive for IFN-γ under basal conditions was not altered by exposure to any Tat clades. Stimulation with HMB-PP/IL-2 or PMA/I induced IFN-γ staining. As in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter IFN-γ positive cells. Vγ9Vδ2 T cells staining positive for TNF-α under basal conditions was not altered by exposure to any of the Tat clades investigated. HMB-PP/IL-2 or PMA/I stimulation increased TNF-α staining and as in unstimulated cells, exposure to Tat clades A, B, C, and D did not alter the percentage of TNF-α positive cells.

Quantifying cytokine secretion by ELISA analysis, in unstimulated $CD3^+$ T cells, Tat clades A, B, C, and D did not alter IFN-γ secretion. Following stimulation by HMB-PP/IL-2, IFN-γ secretion did not increase significantly. Tat clades A, C, and D did not alter IFN-γ secretion, but Tat clade B increased IFN-γ levels relative to HMB-PP/IL-2 controls. PMA/I stimulation induced IFN-γ but this was not altered by any of the Tat clades investigated. TNF-α was undetectable under non-stimulated conditions and exposure of cells to Tat clades did not induce TNF-α to detectable levels. Stimulation with HMB-PP/IL-2 induced TNF-α secretion and none of the Tat clades studied altered this response. PMA/I also increased TNF-α production and again, the response was not altered by the presence of Tat clades. In Vγ9Vδ2 T cell lines, Tat clades A, B, C, and D increased IFN-γ secretion, with clade B generating the largest response. Re-stimulation with HMB-PP/IL-2 resulted in increased IFN-γ levels, and under these conditions, Tat clade B, but not A, C, or D, further increased secretion. PMA/I re-stimulation also resulted in increased IFN-γ secretion by

these cells, and again Tat clade B, but not A, C, or D, further induced production compared to PMA/I controls.

The results of this study relate the potential for HIV-1 Tat clades to modulate IFN-γ and TNF-α production by T lymphocyte subsets under stimulated and unstimulated conditions. We have demonstrated that exposure of PBMCs to Tat clades A, B, C, and D, does not alter the percentage of $CD3^+$ and $V\gamma9V\delta2^+$ T cells producing each cytokine. However, Tat clade B induced IFN-γ secretion in $CD3^+$ T cells under HMB-PP/IL-2-stimulated conditions and this effect was repeated in Vγ9Vδ2 subsets where all clades induced IFN-γ secretion, an effect maintained only by Tat B when cells were re-stimulated. As IFN-γ is a cytokine with extensive immunoregulatory potential, enhanced Tat B-mediated secretion by Vγ9Vδ2 T cells could mediate a series of divergent pro- and anti-viral/inflammatory effects *in vivo*.

The overall aims of this thesis were to assess the inflammatory response to HIV/HCV infection with particular interest to the expression of MMPs and their natural inhibitors, TIMPs, and cytokines possessing significant inflammatory/immunoregulatory functions, namely TNF-α, IL-1β, and IFN-γ. This objective was founded on the basis that MMP/TIMP and cytokine production is highly dysregulated in HIV and HCV patients and that aberrant expression of these factors mediates extensive immunopathology during the course of both infections. We aimed to delineate the effects of antiviral therapy on MMP/TIMP abundance *in vitro*, using immortalised cell lines, and *in vivo*, through a HIV/HCV co-infected patient cohort. We also investigated the potential for HIV and HCV viral proteins to regulate the expression of MMPs and TIMPs, and TNF-α, IL-1β and IFN-γ, in immortalised and/or primary leukocytes *in vitro*. The results of our studies relate that antiviral therapeutic agents modulate MMP expression *in vitro* and *in vivo* and that HIV-1 Tat and HCV NS3 proteins upregulate MMP/TIMP abundance and TNF-α, IL-1β and/or

IFN-γ production, Tat differentially based on viral subtype. The translational value of our *in vitro* findings may be mitigated by the lack of a viral cell model and the inherent artificiality of cell culture where cells exist in monolayers and are not subject to interactions with extracellular matrix components and the host of soluble factors encountered *in vivo*.

Taken together, the results of these studies contribute to our understanding of MMP/TIMP dysregulation during HIV/HCV infection and their modulation by antiviral therapies. They also indicate the potential for HIV and HCV viral proteins to orchestrate aberrant expression of MMPs, TIMPs, and the immunomodulatory cytokines TNF-α, IL-1β, and IFN-γ.

References

1. Barre-Sinoussi,F. *et al.* Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871 (1983).

2. Popovic,M. *et al.* Isolation and transmission of human retrovirus (human t-cell leukemia virus). *Science* **219**, 856-859 (1983).

3. Gilbert,M.T. *et al.* The emergence of HIV/AIDS in the Americas and beyond. *Proc. Natl. Acad. Sci. U. S. A* **104**, 18566-18570 (2007).

4. Hahn,B.H., Shaw,G.M., De Cock,K.M., & Sharp,P.M. AIDS as a zoonosis: scientific and public health implications. *Science* **287**, 607-614 (2000).

5. Grant,A.D., Djomand,G., & De Cock,K.M. Natural history and spectrum of disease in adults with HIV/AIDS in Africa. *AIDS* **11 Suppl B**, S43-S54 (1997).

6. Clapham,P.R. & McKnight,A. Cell surface receptors, virus entry and tropism of primate lentiviruses. *J. Gen. Virol.* **83**, 1809-1829 (2002).

7. Broder,C.C. & Berger,E.A. Fusogenic selectivity of the envelope glycoprotein is a major determinant of human immunodeficiency virus type 1 tropism for CD4+ T-cell lines vs. primary macrophages. *Proc. Natl. Acad. Sci. U. S. A* **92**, 9004-9008 (1995).

8. Coakley,E., Petropoulos,C.J., & Whitcomb,J.M. Assessing chemokine co-receptor usage in HIV. *Curr. Opin. Infect. Dis.* **18**, 9-15 (2005).

9. Harrich,D. & Hooker,B. Mechanistic aspects of HIV-1 reverse transcription initiation. *Rev. Med. Virol.* **12**, 31-45 (2002).

10. Van,M.B. & Debyser,Z. HIV-1 integration: an interplay between HIV-1 integrase, cellular and viral proteins. *AIDS Rev.* **7**, 26-43 (2005).

11. Emerman,M. & Malim,M.H. HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* **280**, 1880-1884 (1998).

12. James,C.O. *et al.* Extracellular Nef protein targets CD4+ T cells for apoptosis by interacting with CXCR4 surface receptors. *J. Virol.* **78**, 3099-3109 (2004).

13. Alexander,M., Bor,Y.C., Ravichandran,K.S., Hammarskjold,M.L., & Rekosh,D. Human immunodeficiency virus type 1 Nef associates with lipid rafts to downmodulate cell surface CD4 and class I major histocompatibility complex expression and to increase viral infectivity. *J. Virol.* **78**, 1685-1696 (2004).

14. Yu,X. *et al.* Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**, 1056-1060 (2003).

15. Sakai,K., Dimas,J., & Lenardo,M.J. The Vif and Vpr accessory proteins independently cause HIV-1-induced T cell cytopathicity and cell cycle arrest. *Proc. Natl. Acad. Sci. U. S. A* **103**, 3369-3374 (2006).

16. Neil,S.J., Zang,T., & Bieniasz,P.D. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451**, 425-430 (2008).

17. Zhu,P. *et al.* Distribution and three-dimensional structure of AIDS virus envelope spikes. *Nature* **441**, 847-852 (2006).

18. Watkins,D.I. The vaccine search goes on. *Sci. Am.* **299**, 69-74, 76 (2008).

19. Menendez-Arias,L. Targeting HIV: antiretroviral therapy and development of drug resistance. *Trends Pharmacol. Sci.* **23**, 381-388 (2002).

20. Sarafianos,S.G. *et al.* Structure and function of HIV-1 reverse transcriptase: molecular mechanisms of polymerization and inhibition. *J. Mol. Biol.* **385**, 693-713 (2009).

21. Ho,D.D. Perspectives series: host/pathogen interactions. Dynamics of HIV-1 replication in vivo. *J. Clin. Invest* **99**, 2565-2567 (1997).

22. Eigen,M. On the nature of virus quasispecies. *Trends Microbiol.* **4**, 216-218 (1996).

23. Spira,S., Wainberg,M.A., Loemba,H., Turner,D., & Brenner,B.G. Impact of clade diversity on HIV-1 virulence, antiretroviral drug sensitivity and drug resistance. *J. Antimicrob. Chemother.* **51**, 229-240 (2003).

24. Hemelaar,J., Gouws,E., Ghys,P.D., & Osmanov,S. Global trends in molecular epidemiology of HIV-1 during 2000-2007. *AIDS* **25**, 679-689 (2011).

25. Leitner,T. *et al.* Yet another subtype of HIV type 1? *AIDS Res. Hum. Retroviruses* **11**, 995-997 (1995).

26. Gao,F. *et al.* Evidence of two distinct subsubtypes within the HIV-1 subtype A radiation. *AIDS Res. Hum. Retroviruses* **17**, 675-688 (2001).

27. Triques,K. *et al.* Near-full-length genome sequencing of divergent African HIV type 1 subtype F viruses leads to the identification of a new HIV type 1 subtype designated K. *AIDS Res. Hum. Retroviruses* **16**, 139-151 (2000).

28. Robertson,D.L. *et al.* HIV-1 nomenclature proposal. *Science* **288**, 55-56 (2000).

29. McCutchan,F.E., Salminen,M.O., Carr,J.K., & Burke,D.S. HIV-1 genetic diversity. *AIDS* **10 Suppl 3**, S13-S20 (1996).

30. Taylor,B.S. & Hammer,S.M. The challenge of HIV-1 subtype diversity. *N. Engl. J. Med.* **359**, 1965-1966 (2008).

31. Suligoi,B., Raimondo,M., Fanales-Belasio,E., & Butto,S. The epidemic of HIV infection and AIDS, promotion of testing, and innovative strategies. *Ann. Ist. Super. Sanita* **46**, 15-23 (2010).

32. Fiebig,E.W. *et al.* Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* **17**, 1871-1879 (2003).

33. Kahn,J.O. & Walker,B.D. Acute human immunodeficiency virus type 1 infection. *N. Engl. J. Med.* **339**, 33-39 (1998).

34. Ford,E.S., Puronen,C.E., & Sereti,I. Immunopathogenesis of asymptomatic chronic HIV Infection: the calm before the storm. *Curr. Opin. HIV. AIDS* **4**, 206-214 (2009).

35. Goedert,J.J. The epidemiology of acquired immunodeficiency syndrome malignancies. *Semin. Oncol.* **27**, 390-401 (2000).

36. Baker,B.M., Block,B.L., Rothchild,A.C., & Walker,B.D. Elite control of HIV infection: implications for vaccine design. *Expert. Opin. Biol. Ther.* **9**, 55-69 (2009).

37. Freel,S.A., Saunders,K.O., & Tomaras,G.D. CD8(+)T-cell-mediated control of HIV-1 and SIV infection. *Immunol. Res.* **49**, 135-146 (2011).

38. Liu,R. *et al.* Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367-377 (1996).

39. Rana,S. *et al.* Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the delta ccr5 mutation. *J. Virol.* **71**, 3219-3227 (1997).

40. Miura,T. *et al.* HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte [corrected] recognition. *J. Virol.* **83**, 2743-2755 (2009).

41. Pereyra,F. *et al.* Genetic and immunologic heterogeneity among persons who control HIV infection in the absence of therapy. *J. Infect. Dis.* **197**, 563-571 (2008).

42. Kuppuswamy,M., Subramanian,T., Srinivasan,A., & Chinnadurai,G. Multiple functional domains of Tat, the trans-activator of HIV-1, defined by mutational analysis. *Nucleic Acids Res.* **17**, 3551-3561 (1989).

43. Garcia,J.A., Harrich,D., Pearson,L., Mitsuyasu,R., & Gaynor,R.B. Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat. *EMBO J.* **7**, 3143-3147 (1988).

44. Feinberg,M.B., Baltimore,D., & Frankel,A.D. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. U. S. A* **88**, 4045-4049 (1991).

45. Garber,M.E. *et al.* CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. *Mol. Cell Biol.* **20**, 6958-6969 (2000).

46. Ensoli,B. *et al.* Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J. Virol.* **67**, 277-287 (1993).

47. Xiao,H. *et al.* Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc. Natl. Acad. Sci. U. S. A* **97**, 11466-11471 (2000).

48. Tyagi,M., Rusnati,M., Presta,M., & Giacca,M. Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* **276**, 3254-3261 (2001).

49. Lin,X. *et al.* Transcriptional profiles of latent human immunodeficiency virus in infected individuals: effects of Tat on the host and reservoir. *J. Virol.* **77**, 8227-8236 (2003).

50. Buonaguro,L. *et al.* Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. *J. Virol.* **66**, 7159-7167 (1992).

51. Westendorp,M.O., Li-Weber,M., Frank,R.W., & Krammer,P.H. Human immunodeficiency virus type 1 Tat upregulates interleukin-2 secretion in activated T cells. *J. Virol.* **68**, 4177-4185 (1994).

52. Scala,G. *et al.* The expression of the interleukin 6 gene is induced by the human immunodeficiency virus 1 TAT protein. *J. Exp. Med.* **179**, 961-971 (1994).

53. Li,C.J. *et al.* Tat protein induces self-perpetuating permissivity for productive HIV-1 infection. *Proc. Natl. Acad. Sci. U. S. A* **94**, 8116-8120 (1997).

54. Huang,L., Li,C.J., & Pardee,A.B. Human immunodeficiency virus type 1 TAT protein activates B lymphocytes. *Biochem. Biophys. Res. Commun.* **237**, 461-464 (1997).

55. Zocchi,M.R., Rubartelli,A., Morgavi,P., & Poggi,A. HIV-1 Tat inhibits human natural killer cell function by blocking L-type calcium channels. *J. Immunol.* **161**, 2938-2943 (1998).

56. Huang,L., Bosch,I., Hofmann,W., Sodroski,J., & Pardee,A.B. Tat protein induces human immunodeficiency virus type 1 (HIV-1) coreceptors and promotes infection with both macrophage-tropic and T-lymphotropic HIV-1 strains. *J. Virol.* **72**, 8952-8960 (1998).

57. Vene,R., Benelli,R., Noonan,D.M., & Albini,A. HIV-Tat dependent chemotaxis and invasion, key aspects of tat mediated pathogenesis. *Clin. Exp. Metastasis* **18**, 533-538 (2000).

58. Ensoli,B. *et al.* Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. *Nature* **371**, 674-680 (1994).

59. Albini,A. *et al.* The angiogenesis induced by HIV-1 tat protein is mediated by the Flk-1/KDR receptor on vascular endothelial cells. *Nat. Med.* **2**, 1371-1375 (1996).

60. Sabatier,J.M. *et al.* Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J. Virol.* **65**, 961-967 (1991).

61. Shi,B., Raina,J., Lorenzo,A., Busciglio,J., & Gabuzda,D. Neuronal apoptosis induced by HIV-1 Tat protein and TNF-alpha: potentiation of neurotoxicity

mediated by oxidative stress and implications for HIV-1 dementia. *J. Neurovirol.* **4**, 281-290 (1998).

62. Jeang,K.T., Xiao,H., & Rich,E.A. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J. Biol. Chem.* **274**, 28837-28840 (1999).

63. Dienstag,J.L. & McHutchison,J.G. American Gastroenterological Association technical review on the management of hepatitis C. *Gastroenterology* **130**, 231-264 (2006).

64. Benson,C.A., Kaplan,J.E., Masur,H., Pau,A., & Holmes,K.K. Treating opportunistic infections among HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association/Infectious Diseases Society of America. *MMWR Recomm. Rep.* **53**, 1-112 (2004).

65. Cribier,B. *et al.* HIV increases hepatitis C viraemia irrespective of the hepatitis C virus genotype. *Res. Virol.* **148**, 267-271 (1997).

66. Dutoit,V., Ciuffreda,D., Comte,D., Gonvers,J.J., & Pantaleo,G. Differences in HCV-specific T cell responses between chronic HCV infection and HIV/HCV co-infection. *Eur. J. Immunol.* **35**, 3493-3504 (2005).

67. Capa,L. *et al.* Influence of HCV genotype and co-infection with human immunodeficiency virus on CD4(+) and CD8(+) T-cell responses to hepatitis C virus. *J. Med. Virol.* **79**, 503-510 (2007).

68. Mohsen,A.H. *et al.* Impact of human immunodeficiency virus (HIV) infection on the progression of liver fibrosis in hepatitis C virus infected patients. *Gut* **52**, 1035-1040 (2003).

69. Monga,H.K. *et al.* Hepatitis C virus infection-related morbidity and mortality among patients with human immunodeficiency virus infection. *Clin. Infect. Dis.* **33**, 240-247 (2001).

70. Tuyama,A.C. *et al.* Human immunodeficiency virus (HIV)-1 infects human hepatic stellate cells and promotes collagen I and monocyte chemoattractant protein-1 expression: implications for the pathogenesis of HIV/hepatitis C virus-induced liver fibrosis. *Hepatology* **52**, 612-622 (2010).

71. Marra,F. *et al.* Increased expression of monocyte chemotactic protein-1 during active hepatic fibrogenesis: correlation with monocyte infiltration. *Am. J. Pathol.* **152**, 423-430 (1998).

72. Bruno,R. *et al.* gp120 modulates the biology of human hepatic stellate cells: a link between HIV infection and liver fibrogenesis. *Gut* **59**, 513-520 (2010).

73. Lin,W. *et al.* HIV increases HCV replication in a TGF-beta1-dependent manner. *Gastroenterology* **134**, 803-811 (2008).

74. Karras,A. *et al.* Severe anoxic hepatic necrosis in an HIV-1-hepatitis C virus-co-infected patient starting antiretroviral triple combination therapy. *AIDS* **12**, 827-829 (1998).

75. Zylberberg,H. *et al.* Rapidly evolving hepatitis C virus-related cirrhosis in a human immunodeficiency virus-infected patient receiving triple antiretroviral therapy. *Clin. Infect. Dis.* **27**, 1255-1258 (1998).

76. Vergis,E., Paterson,D.L., & Singh,N. Indinavir-associated hepatitis in patients with advanced HIV infection. *Int. J. STD AIDS* **9**, 53 (1998).

77. Aranzabal,L. *et al.* Influence of liver fibrosis on highly active antiretroviral therapy-associated hepatotoxicity in patients with HIV and hepatitis C virus coinfection. *Clin. Infect. Dis.* **40**, 588-593 (2005).

78. Torti,C. *et al.* Influence of genotype 3 hepatitis C coinfection on liver enzyme elevation in HIV-1-positive patients after commencement of a new highly active antiretroviral regimen: results from the EPOKA-MASTER Cohort. *J. Acquir. Immune. Defic. Syndr.* **41**, 180-185 (2006).

79. Choo,Q.L. *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359-362 (1989).

80. Danta,M. *et al.* Recent epidemic of acute hepatitis C virus in HIV-positive men who have sex with men linked to high-risk sexual behaviours. *AIDS* **21**, 983-991 (2007).

81. Urbanus,A.T. *et al.* Hepatitis C virus infections among HIV-infected men who have sex with men: an expanding epidemic. *AIDS* **23**, F1-F7 (2009).

82. van de Laar,T.J. *et al.* Evidence of a large, international network of HCV transmission in HIV-positive men who have sex with men. *Gastroenterology* **136**, 1609-1617 (2009).

83. Perz,J.F., Armstrong,G.L., Farrington,L.A., Hutin,Y.J., & Bell,B.P. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J. Hepatol.* **45**, 529-538 (2006).

84. Seeff,L.B. & Hoofnagle,J.H. National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. *Hepatology* **36**, S1-S2 (2002).

85. Gravitz,L. Introduction: a smouldering public-health crisis. *Nature* **474**, S2-S4 (2011).

86. Simmonds,P. *et al.* Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* **42**, 962-973 (2005).

87. Penin,F., Dubuisson,J., Rey,F.A., Moradpour,D., & Pawlotsky,J.M. Structural biology of hepatitis C virus. *Hepatology* **39**, 5-19 (2004).

88. Santolini,E., Migliaccio,G., & La,M.N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J. Virol.* **68**, 3631-3641 (1994).

89. McLauchlan,J., Lemberg,M.K., Hope,G., & Martoglio,B. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* **21**, 3980-3988 (2002).

90. Kato,N. Molecular virology of hepatitis C virus. *Acta Med. Okayama* **55**, 133-159 (2001).

91. Op De,B.A. *et al.* Characterization of functional hepatitis C virus envelope glycoproteins. *J. Virol.* **78**, 2994-3002 (2004).

92. Bartosch,B. & Cosset,F.L. Cell entry of hepatitis C virus. *Virology* **348**, 1-12 (2006).

93. Stapleford,K.A. & Lindenbach,B.D. Hepatitis C virus NS2 coordinates virus particle assembly through physical interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. *J. Virol.* **85**, 1706-1717 (2011).

94. Heo,T.H., Lee,S.M., Bartosch,B., Cosset,F.L., & Kang,C.Y. Hepatitis C virus E2 links soluble human CD81 and SR-B1 protein. *Virus Res.* **121**, 58-64 (2006).

95. Wakita,T. *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* **11**, 791-796 (2005).

96. Blight,K.J., Kolykhalov,A.A., & Rice,C.M. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972-1974 (2000).

97. Pallaoro,M. *et al.* Characterization of the hepatitis C virus NS2/3 processing reaction by using a purified precursor protein. *J. Virol.* **75**, 9939-9946 (2001).

98. Love,R.A. *et al.* The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* **87**, 331-342 (1996).

99. Wolk,B. *et al.* Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J. Virol.* **74**, 2293-2304 (2000).

100. Kolykhalov,A.A., Mihalik,K., Feinstone,S.M., & Rice,C.M. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3' nontranslated region are essential for virus replication in vivo. *J. Virol.* **74**, 2046-2051 (2000).

101. Foy,E. *et al.* Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**, 1145-1148 (2003).

102. Dumont,S. *et al.* RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* **439**, 105-108 (2006).

103. Yu,G.Y., Lee,K.J., Gao,L., & Lai,M.M. Palmitoylation and polymerization of hepatitis C virus NS4B protein. *J. Virol.* **80**, 6013-6023 (2006).

104. Egger,D. *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**, 5974-5984 (2002).

105. Schmitz,U. & Tan,S.L. NS5A--from obscurity to new target for HCV therapy. *Recent Pat Antiinfect. Drug Discov.* **3**, 77-92 (2008).

106. Tan,S.L. & Katze,M.G. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. *Virology* **284**, 1-12 (2001).

107. Powdrill,M.H., Bernatchez,J.A., & Gotte,M. Inhibitors of the Hepatitis C Virus RNA-Dependent RNA Polymerase NS5B. *Viruses.* **2**, 2169-2195 (2010).

108. Carrere-Kremer,S. *et al.* Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J. Virol.* **76**, 3720-3730 (2002).

109. Sakai,A. *et al.* The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc. Natl. Acad. Sci. U. S. A* **100**, 11646-11651 (2003).

110. Lindenbach,B.D. & Rice,C.M. Unravelling hepatitis C virus replication from genome to function. *Nature* **436**, 933-938 (2005).

111. Barth,H., Liang,T.J., & Baumert,T.F. Hepatitis C virus entry: molecular biology and clinical implications. *Hepatology* **44**, 527-535 (2006).

112. von Hahn,T. & Rice,C.M. Hepatitis C virus entry. *J. Biol. Chem.* **283**, 3689-3693 (2008).

113. Evans,M.J. *et al.* Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* **446**, 801-805 (2007).

114. Blanchard,E. *et al.* Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J. Virol.* **80**, 6964-6972 (2006).

115. Barth,H. *et al.* Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J. Biol. Chem.* **278**, 41003-41012 (2003).

116. Wunschmann,S., Medh,J.D., Klinzmann,D., Schmidt,W.N., & Stapleton,J.T. Characterization of hepatitis C virus (HCV) and HCV E2 interactions with CD81 and the low-density lipoprotein receptor. *J. Virol.* **74**, 10055-10062 (2000).

117. Koutsoudakis,G. *et al.* Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J. Virol.* **80**, 5308-5320 (2006).

118. Bartosch,B., Dubuisson,J., & Cosset,F.L. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J. Exp. Med.* **197**, 633-642 (2003).

119. Ji,H., Fraser,C.S., Yu,Y., Leary,J., & Doudna,J.A. Coordinated assembly of human translation initiation complexes by the hepatitis C virus internal ribosome entry site RNA. *Proc. Natl. Acad. Sci. U. S. A* **101**, 16990-16995 (2004).

120. Otto,G.A. & Puglisi,J.D. The pathway of HCV IRES-mediated translation initiation. *Cell* **119**, 369-380 (2004).

121. Kapadia,S.B. & Chisari,F.V. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Natl. Acad. Sci. U. S. A* **102**, 2561-2566 (2005).

122. Okamoto,T. *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* **25**, 5015-5025 (2006).

123. Watashi,K. *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* **19**, 111-122 (2005).

124. Shimoike,T., Mimori,S., Tani,H., Matsuura,Y., & Miyamura,T. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. *J. Virol.* **73**, 9718-9725 (1999).

125. Bartenschlager,R. & Lohmann,V. Replication of hepatitis C virus. *J. Gen. Virol.* **81**, 1631-1648 (2000).

126. Racanelli,V. & Rehermann,B. Hepatitis C virus infection: when silence is deception. *Trends Immunol.* **24**, 456-464 (2003).

127. Su,A.I. *et al.* Genomic analysis of the host response to hepatitis C virus infection. *Proc. Natl. Acad. Sci. U. S. A* **99**, 15669-15674 (2002).

128. Li,K. *et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc. Natl. Acad. Sci. U. S. A* **102**, 2992-2997 (2005).

129. Lin,W. *et al.* Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. *J. Virol.* **80**, 9226-9235 (2006).

130. Polyak,S.J. *et al.* Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J. Virol.* **75**, 6095-6106 (2001).

131. Taylor,D.R., Shi,S.T., Romano,P.R., Barber,G.N., & Lai,M.M. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* **285**, 107-110 (1999).

132. Corado,J. *et al.* Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. *Clin. Exp. Immunol.* **109**, 451-457 (1997).

133. Meier,U.C. *et al.* Shared alterations in NK cell frequency, phenotype, and function in chronic human immunodeficiency virus and hepatitis C virus infections. *J. Virol.* **79**, 12365-12374 (2005).

134. Bain,C. *et al.* Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* **120**, 512-524 (2001).

135. Crotta,S. *et al.* Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J. Exp. Med.* **195**, 35-41 (2002).

136. Kanto,T. *et al.* Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J. Immunol.* **162**, 5584-5591 (1999).

137. Shata,M.T. *et al.* Characterization of the immune response against hepatitis C infection in recovered, and chronically infected chimpanzees. *J. Viral Hepat.* **9**, 400-410 (2002).

138. Woollard,D.J. *et al.* Characterization of HCV-specific Patr class II restricted CD4+ T cell responses in an acutely infected chimpanzee. *Hepatology* **38**, 1297-1306 (2003).

139. Lechmann,M. *et al.* T- and B-cell responses to different hepatitis C virus antigens in patients with chronic hepatitis C infection and in healthy anti-hepatitis C virus--positive blood donors without viremia. *Hepatology* **24**, 790-795 (1996).

140. Lancaster,T. *et al.* Quantitative and functional differences in CD8+ lymphocyte responses in resolved acute and chronic hepatitis C virus infection. *J. Viral Hepat.* **9**, 18-28 (2002).

141. Shoukry,N.H. *et al.* Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J. Exp. Med.* **197**, 1645-1655 (2003).

142. Farci,P. *et al.* Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. *Proc. Natl. Acad. Sci. U. S. A* **91**, 7792-7796 (1994).

143. Shimizu,Y.K. *et al.* Neutralizing antibodies against hepatitis C virus and the emergence of neutralization escape mutant viruses. *J. Virol.* **68**, 1494-1500 (1994).

144. von,H.T. *et al.* Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. *Gastroenterology* **132**, 667-678 (2007).

145. Alter,M.J. *et al.* The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N. Engl. J. Med.* **341**, 556-562 (1999).

146. Jeffers,L.J. Treating hepatitis C in African Americans. *Liver Int.* **27**, 313-322 (2007).

147. Yee,L.J. Host genetic determinants in hepatitis C virus infection. *Genes Immun.* **5**, 237-245 (2004).

148. Ge,D. *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461**, 399-401 (2009).

149. Thomas,D.L. *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461**, 798-801 (2009).

150. Tanaka,Y. *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* **41**, 1105-1109 (2009).

151. Eisenstein,M. Vaccines: a moving target. *Nature* **474**, S16-S17 (2011).

152. Grakoui,A., McCourt,D.W., Wychowski,C., Feinstone,S.M., & Rice,C.M. A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. U. S. A* **90**, 10583-10587 (1993).

153. Grakoui,A., McCourt,D.W., Wychowski,C., Feinstone,S.M., & Rice,C.M. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**, 2832-2843 (1993).

154. Yao,N. *et al.* Structure of the hepatitis C virus RNA helicase domain. *Nat. Struct. Biol.* **4**, 463-467 (1997).

155. Kim,J.L. *et al.* Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure.* **6**, 89-100 (1998).

156. Luo,D. *et al.* Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. *EMBO J.* **27**, 3209-3219 (2008).

157. Lam,A.M. & Frick,D.N. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. *J. Virol.* **80**, 404-411 (2006).

158. Jennings,T.A. *et al.* RNA unwinding activity of the hepatitis C virus NS3 helicase is modulated by the NS5B polymerase. *Biochemistry* **47**, 1126-1135 (2008).

159. Ma,Y., Yates,J., Liang,Y., Lemon,S.M., & Yi,M. NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly. *J. Virol.* **82**, 7624-7639 (2008).

160. Yamamoto,M. *et al.* Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* **169**, 6668-6672 (2002).

161. Seth,R.B., Sun,L., Ea,C.K., & Chen,Z.J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**, 669-682 (2005).

162. Loo,Y.M. *et al.* Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc. Natl. Acad. Sci. U. S. A* **103**, 6001-6006 (2006).

163. Bowen,D.G. & Walker,C.M. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* **436**, 946-952 (2005).

164. Lohmann,V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113 (1999).

165. Raney,K.D., Sharma,S.D., Moustafa,I.M., & Cameron,C.E. Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. *J. Biol. Chem.* **285**, 22725-22731 (2010).

166. Koziel,M.J. Cytokines in viral hepatitis. *Semin. Liver Dis.* **19**, 157-169 (1999).

167. Gressner,A.M., Weiskirchen,R., Breitkopf,K., & Dooley,S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci.* **7**, d793-d807 (2002).

168. Williams,E.J., Gaca,M.D., Brigstock,D.R., Arthur,M.J., & Benyon,R.C. Increased expression of connective tissue growth factor in fibrotic human liver and in activated hepatic stellate cells. *J. Hepatol.* **32**, 754-761 (2000).

169. Henderson,N.C. & Iredale,J.P. Liver fibrosis: cellular mechanisms of progression and resolution. *Clin. Sci. (Lond)* **112**, 265-280 (2007).

170. Hui,A.Y. & Friedman,S.L. Molecular basis of hepatic fibrosis. *Expert. Rev. Mol. Med.* **5**, 1-23 (2003).

171. Yang,C. *et al.* Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology* **124**, 147-159 (2003).

172. Neubauer,K., Saile,B., & Ramadori,G. Liver fibrosis and altered matrix synthesis. *Can. J. Gastroenterol.* **15**, 187-193 (2001).

173. Iredale,J.P., Murphy,G., Hembry,R.M., Friedman,S.L., & Arthur,M.J. Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinases-1. Implications for regulation of matrix degradation in liver. *J. Clin. Invest* **90**, 282-287 (1992).

174. Iredale,J.P. *et al.* Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J. Clin. Invest* **102**, 538-549 (1998).

175. Issa,R. *et al.* Apoptosis of hepatic stellate cells: involvement in resolution of biliary fibrosis and regulation by soluble growth factors. *Gut* **48**, 548-557 (2001).

176. Seeff,L.B. Natural history of chronic hepatitis C. *Hepatology* **36**, S35-S46 (2002).

177. Bataller,R. & Brenner,D.A. Liver fibrosis. *J. Clin. Invest* **115**, 209-218 (2005).

178. Mitsuya,H. *et al.* Suramin protection of T cells in vitro against infectivity and cytopathic effect of HTLV-III. *Science* **226**, 172-174 (1984).

179. Broder,S. *et al.* Effects of suramin on HTLV-III/LAV infection presenting as Kaposi's sarcoma or AIDS-related complex: clinical pharmacology and suppression of virus replication in vivo. *Lancet* **2**, 627-630 (1985).

180. Peter,K. & Gambertoglio,J.G. Intracellular phosphorylation of zidovudine (ZDV) and other nucleoside reverse transcriptase inhibitors (RTI) used for human immunodeficiency virus (HIV) infection. *Pharm. Res.* **15**, 819-825 (1998).

181. De,Clercq.E. Non-nucleoside reverse transcriptase inhibitors (NNRTIs): past, present, and future. *Chem. Biodivers.* **1**, 44-64 (2004).

182. De,Clercq.E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. *Int. J. Antimicrob. Agents* **33**, 307-320 (2009).

183. Matthews,T. *et al.* Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nat. Rev. Drug Discov.* **3**, 215-225 (2004).

184. Westby,M. & van der,R.E. CCR5 antagonists: host-targeted antivirals for the treatment of HIV infection. *Antivir. Chem. Chemother.* **16**, 339-354 (2005).

185. Taiwo,B., Murphy,R.L., & Katlama,C. Novel antiretroviral combinations in treatment-experienced patients with HIV infection: rationale and results. *Drugs* **70**, 1629-1642 (2010).

186. Keller,P.W., Adamson,C.S., Heymann,J.B., Freed,E.O., & Steven,A.C. HIV-1 maturation inhibitor bevirimat stabilizes the immature Gag lattice. *J. Virol.* **85**, 1420-1428 (2011).

187. European AIDS Clinical Society. Clinical Management and Treatment of HIV Infected Adults in Europe. 2010.
Ref Type: Pamphlet

188. Kitahata,M.M. *et al.* Effect of early versus deferred antiretroviral therapy for HIV on survival. *N. Engl. J. Med.* **360**, 1815-1826 (2009).

189. Sterne,J.A. *et al.* Timing of initiation of antiretroviral therapy in AIDS-free HIV-1-infected patients: a collaborative analysis of 18 HIV cohort studies. *Lancet* **373**, 1352-1363 (2009).

190. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *Lancet* **372**, 293-299 (2008).

191. Tozzi,V. *et al.* Drug-class-wide resistance to antiretrovirals in HIV-infected patients failing therapy: prevalence, risk factors and virological outcome. *Antivir. Ther.* **11**, 553-560 (2006).

192. Truong,H.M. *et al.* Routine surveillance for the detection of acute and recent HIV infections and transmission of antiretroviral resistance. *AIDS* **20**, 2193-2197 (2006).

193. Johnson,V.A. *et al.* Update of the drug resistance mutations in HIV-1: December 2009. *Top. HIV. Med.* **17**, 138-145 (2009).

194. Richman,D.D. *et al.* The prevalence of antiretroviral drug resistance in the United States. *AIDS* **18**, 1393-1401 (2004).

195. Maagaard,A. & Kvale,D. Long term adverse effects related to nucleoside reverse transcriptase inhibitors: clinical impact of mitochondrial toxicity. *Scand. J. Infect. Dis.* **41**, 808-817 (2009).

196. Hetherington,S. *et al.* Hypersensitivity reactions during therapy with the nucleoside reverse transcriptase inhibitor abacavir. *Clin. Ther.* **23**, 1603-1614 (2001).

197. Calza,L., Manfredi,R., & Chiodo,F. Dyslipidaemia associated with antiretroviral therapy in HIV-infected patients. *J. Antimicrob. Chemother.* **53**, 10-14 (2004).

198. Carr,A. HIV lipodystrophy: risk factors, pathogenesis, diagnosis and management. *AIDS* **17 Suppl 1**, S141-S148 (2003).

199. Walli,R. *et al.* Treatment with protease inhibitors associated with peripheral insulin resistance and impaired oral glucose tolerance in HIV-1-infected patients. *AIDS* **12**, F167-F173 (1998).

200. Wand,H. *et al.* Metabolic syndrome, cardiovascular disease and type 2 diabetes mellitus after initiation of antiretroviral therapy in HIV infection. *AIDS* **21**, 2445-2453 (2007).

201. Volberding,P.A. & Deeks,S.G. Antiretroviral therapy and management of HIV infection. *Lancet* **376**, 49-62 (2010).

202. Manns,M.P. *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**, 958-965 (2001).

203. Fried,M.W. *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* **347**, 975-982 (2002).

204. Zeuzem,S. *et al.* Peginterferon alfa-2a in patients with chronic hepatitis C. *N. Engl. J. Med.* **343**, 1666-1672 (2000).

205. Lindsay,K.L. *et al.* A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. *Hepatology* **34**, 395-403 (2001).

206. Feld,J.J. & Hoofnagle,J.H. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **436**, 967-972 (2005).

207. Martinot-Peignoux,M. *et al.* Predictors of sustained response to alpha interferon therapy in chronic hepatitis C. *J. Hepatol.* **29**, 214-223 (1998).

208. Hadziyannis,S.J. *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann. Intern. Med.* **140**, 346-355 (2004).

209. Hofmann,W.P. & Zeuzem,S. A new standard of care for the treatment of chronic HCV infection. *Nat. Rev. Gastroenterol. Hepatol.* **8**, 257-264 (2011).

210. Pol,S. & Soriano,V. Management of chronic hepatitis C virus infection in HIV-infected patients. *Clin. Infect. Dis.* **47**, 94-101 (2008).

211. Ridruejo,E., Adrover,R., Cocozzella,D., Fernandez,N., & Reggiardo,M.V. Efficacy, tolerability and safety in the treatment of chronic hepatitis C with combination of PEG-Interferon - Ribavirin in daily practice. *Ann. Hepatol.* **9**, 46-51 (2010).

212. Witthoft,T. *et al.* Safety, tolerability and efficacy of peginterferon alpha-2a and ribavirin in chronic hepatitis C in clinical practice: The German Open Safety Trial. *J. Viral Hepat.* **14**, 788-796 (2007).

213. Manns,M.P. *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**, 958-965 (2001).

214. Fried,M.W. Side effects of therapy of hepatitis C and their management. *Hepatology* **36**, S237-S244 (2002).

215. Perni,R.B. *et al.* Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3-4A serine protease. *Antimicrob. Agents Chemother.* **50**, 899-909 (2006).

216. Lin,K., Perni,R.B., Kwong,A.D., & Lin,C. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCv replicon cells. *Antimicrob. Agents Chemother.* **50**, 1813-1822 (2006).

217. Malcolm,B.A. *et al.* SCH 503034, a mechanism-based inhibitor of hepatitis C virus NS3 protease, suppresses polyprotein maturation and enhances the antiviral activity of alpha interferon in replicon cells. *Antimicrob. Agents Chemother.* **50**, 1013-1020 (2006).

218. Zeuzem,S. *et al.* Telaprevir for retreatment of HCV infection. *N. Engl. J. Med.* **364**, 2417-2428 (2011).

219. Bacon,B.R. *et al.* Boceprevir for previously treated chronic HCV genotype 1 infection. *N. Engl. J. Med.* **364**, 1207-1217 (2011).

220. Schlutter,J. Therapeutics: new drugs hit the target. *Nature* **474**, S5-S7 (2011).

221. Burrage,P.S., Mix,K.S., & Brinckerhoff,C.E. Matrix metalloproteinases: role in arthritis. *Front Biosci.* **11**, 529-543 (2006).

222. Sternlicht,M.D. *et al.* The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* **98**, 137-146 (1999).

223. Newby,A.C. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev.* **85**, 1-31 (2005).

224. Van,L.P. & Libert,C. Chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. *J. Leukoc. Biol.* **82**, 1375-1381 (2007).

225. Parks,W.C., Wilson,C.L., & Lopez-Boado,Y.S. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat. Rev. Immunol.* **4**, 617-629 (2004).

226. Wilson,C.L. *et al.* Differential Processing of {alpha}- and {beta}-Defensin Precursors by Matrix Metalloproteinase-7 (MMP-7). *J. Biol. Chem.* **284**, 8301-8311 (2009).

227. Conant,K. *et al.* Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *Proc. Natl. Acad. Sci. U. S. A* **95**, 3117-3121 (1998).

228. Liuzzi,G.M. *et al.* Increased activity of matrix metalloproteinases in the cerebrospinal fluid of patients with HIV-associated neurological diseases. *J. Neurovirol.* **6**, 156-163 (2000).

229. Hemmann,S., Graf,J., Roderfeld,M., & Roeb,E. Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J. Hepatol.* **46**, 955-975 (2007).

230. Visse,R. & Nagase,H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ. Res.* **92**, 827-839 (2003).

231. Page-McCaw,A., Ewald,A.J., & Werb,Z. Matrix metalloproteinases and the regulation of tissue remodelling. *Nat. Rev. Mol. Cell Biol.* **8**, 221-233 (2007).

232. Yan,C. & Boyd,D.D. Regulation of matrix metalloproteinase gene expression. *J. Cell Physiol* **211**, 19-26 (2007).

233. Mancini,A. & Di Battista,J.A. Transcriptional regulation of matrix metalloprotease gene expression in health and disease. *Front Biosci.* **11**, 423-446 (2006).

234. Van Wart,H.E. & Birkedal-Hansen,H. The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. U. S. A* **87**, 5578-5582 (1990).

235. Pei,D. & Weiss,S.J. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature* **375**, 244-247 (1995).

236. Brew,K., Dinakarpandian,D., & Nagase,H. Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim. Biophys. Acta* **1477**, 267-283 (2000).

237. Baker,A.H., Edwards,D.R., & Murphy,G. Metalloproteinase inhibitors: biological actions and therapeutic opportunities. *J. Cell Sci.* **115**, 3719-3727 (2002).

238. Wang,Z., Juttermann,R., & Soloway,P.D. TIMP-2 is required for efficient activation of proMMP-2 in vivo. *J. Biol. Chem.* **275**, 26411-26415 (2000).

239. Strickland,D.K. *et al.* Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this molecule is a multifunctional receptor. *J. Biol. Chem.* **265**, 17401-17404 (1990).

240. Murphy,G. & Nagase,H. Progress in matrix metalloproteinase research. *Mol. Aspects Med.* **29**, 290-308 (2008).

241. Tsai,H.C. *et al.* Expression of matrix metalloproteinases and their tissue inhibitors in the serum and cerebrospinal fluid of patients with HIV-1 infection and syphilis or neurosyphilis. *Cytokine* **54**, 109-116 (2011).

242. Ahuja,T.S., Gopalani,A., Davies,P., & Ahuja,H. Matrix metalloproteinase-9 expression in renal biopsies of patients with HIV-associated nephropathy. *Nephron Clin. Pract.* **95**, c100-c104 (2003).

243. Diaz,A. *et al.* Lymphoid tissue collagen deposition in HIV-infected patients correlates with the imbalance between matrix metalloproteinases and their inhibitors. *J. Infect. Dis.* **203**, 810-813 (2011).

244. Zhou,X. *et al.* Expression of matrix metalloproteinase-2 and -14 persists during early resolution of experimental liver fibrosis and might contribute to fibrolysis. *Liver Int.* **24**, 492-501 (2004).

245. Takahara,T. *et al.* Dual expression of matrix metalloproteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers. *Hepatology* **26**, 1521-1529 (1997).

246. Lichtinghagen,R. *et al.* Expression and coordinated regulation of matrix metalloproteinases in chronic hepatitis C and hepatitis C virus-induced liver cirrhosis. *Clin. Sci. (Lond)* **105**, 373-382 (2003).

247. Reif,S. *et al.* Matrix metalloproteinases 2 and 9 are markers of inflammation but not of the degree of fibrosis in chronic hepatitis C. *Digestion* **71**, 124-130 (2005).

248. Leroy,V. *et al.* Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. *Am. J. Gastroenterol.* **99**, 271-279 (2004).

249. Mastroianni,C.M. *et al.* Matrix metalloproteinase-9 and tissue inhibitors of matrix metalloproteinase-1 in plasma of patients co-infected with HCV and HIV. *HIV. Clin. Trials* **3**, 310-315 (2002).

250. Larrousse,M. *et al.* Noninvasive diagnosis of hepatic fibrosis in HIV/HCV-coinfected patients. *J. Acquir. Immune. Defic. Syndr.* **46**, 304-311 (2007).

251. Dhawan,S., Toro,L.A., Jones,B.E., & Meltzer,M.S. Interactions between HIV-infected monocytes and the extracellular matrix: HIV-infected monocytes secrete neutral metalloproteases that degrade basement membrane protein matrices. *J. Leukoc. Biol.* **52**, 244-248 (1992).

252. Dhawan,S. *et al.* HIV-1 infection alters monocyte interactions with human microvascular endothelial cells. *J. Immunol.* **154**, 422-432 (1995).

253. Toborek,M. *et al.* Mechanisms of the blood-brain barrier disruption in HIV-1 infection. *Cell Mol. Neurobiol.* **25**, 181-199 (2005).

254. Conant,K. *et al.* Cerebrospinal fluid levels of MMP-2, 7, and 9 are elevated in association with human immunodeficiency virus dementia. *Ann. Neurol.* **46**, 391-398 (1999).

255. Liuzzi,G.M. *et al.* Myelin degrading activity in the CSF of HIV-1-infected patients with neurological diseases. *Neuroreport* **6**, 157-160 (1994).

256. Zhang,K. *et al.* HIV-induced metalloproteinase processing of the chemokine stromal cell derived factor-1 causes neurodegeneration. *Nat. Neurosci.* **6**, 1064-1071 (2003).

257. Toschi,E. *et al.* Activation of matrix-metalloproteinase-2 and membrane-type-1-matrix-metalloproteinase in endothelial cells and induction of vascular permeability in vivo by human immunodeficiency virus-1 Tat protein and basic fibroblast growth factor. *Mol. Biol. Cell* **12**, 2934-2946 (2001).

258. Pantanowitz,L., Dezube,B.J., Hernandez-Barrantes,S., Tahan,S.R., & Dabbous,M.K. Matrix metalloproteinases in the progression and regression of Kaposi's sarcoma. *J. Cutan. Pathol.* **33**, 793-798 (2006).

259. Benelli,R. *et al.* Inhibition of AIDS-Kaposi's sarcoma cell induced endothelial cell invasion by TIMP-2 and a synthetic peptide from the metalloproteinase propeptide: implications for an anti-angiogenic therapy. *Oncol. Res.* **6**, 251-257 (1994).

260. Cianfrocca,M. *et al.* Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi's sarcoma: a phase I AIDS malignancy consortium study. *J. Clin. Oncol.* **20**, 153-159 (2002).

261. Mellanen,L. *et al.* 72-kDa and 92-kDa gelatinases in saliva of patients with human immunodeficiency virus infection. *Acta Odontol. Scand.* **56**, 135-142 (1998).

262. Mellanen,L., Lahdevirta,J., Tervahartiala,T., Meurman,J.H., & Sorsa,T. Matrix metalloproteinase-7, -8, -9, -25, and -26 and CD43, -45, and -68 cell-markers in HIV-infected patients' saliva and gingival tissue. *J. Oral Pathol. Med.* **35**, 530-539 (2006).

263. Takahara,T. *et al.* Increased expression of matrix metalloproteinase-II in experimental liver fibrosis in rats. *Hepatology* **21**, 787-795 (1995).

264. Ikeda,K. *et al.* In vitro migratory potential of rat quiescent hepatic stellate cells and its augmentation by cell activation. *Hepatology* **29**, 1760-1767 (1999).

265. Benyon,R.C. *et al.* Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. *Hepatology* **30**, 977-986 (1999).

266. Knittel,T. *et al.* Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1. *J. Hepatol.* **30**, 48-60 (1999).

267. Roderfeld,M. *et al.* Cytokine blockade inhibits hepatic tissue inhibitor of metalloproteinase-1 expression and up-regulates matrix metalloproteinase-9 in toxic liver injury. *Liver Int.* **26**, 579-586 (2006).

268. Han,Y.P. *et al.* Essential role of matrix metalloproteinases in interleukin-1-induced myofibroblastic activation of hepatic stellate cell in collagen. *J. Biol. Chem.* **279**, 4820-4828 (2004).

269. Reif,S. *et al.* Matrix metalloproteinases 2 and 9 are markers of inflammation but not of the degree of fibrosis in chronic hepatitis C. *Digestion* **71**, 124-130 (2005).

270. Yu,Q. & Stamenkovic,I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev.* **14**, 163-176 (2000).

271. Boeker,K.H. *et al.* Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis C. *Clin. Chim. Acta* **316**, 71-81 (2002).

272. Yata,Y. *et al.* Spatial distribution of tissue inhibitor of metalloproteinase-1 mRNA in chronic liver disease. *J. Hepatol.* **30**, 425-432 (1999).

273. Iredale,J.P. *et al.* Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. *Hepatology* **24**, 176-184 (1996).

274. Benyon,R.C., Iredale,J.P., Goddard,S., Winwood,P.J., & Arthur,M.J. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver. *Gastroenterology* **110**, 821-831 (1996).

275. Duffield,J.S. *et al.* Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J. Clin. Invest* **115**, 56-65 (2005).

276. Fischer,R., Cariers,A., Reinehr,R., & Haussinger,D. Caspase 9-dependent killing of hepatic stellate cells by activated Kupffer cells. *Gastroenterology* **123**, 845-861 (2002).

277. Friedman,S.L. Mac the knife? Macrophages- the double-edged sword of hepatic fibrosis. *J. Clin. Invest* **115**, 29-32 (2005).

278. Harty,M.W. *et al.* Hepatic macrophages promote the neutrophil-dependent resolution of fibrosis in repairing cholestatic rat livers. *Surgery* **143**, 667-678 (2008).

279. Webster,N.L. & Crowe,S.M. Matrix metalloproteinases, their production by monocytes and macrophages and their potential role in HIV-related diseases. *J. Leukoc. Biol.* **80**, 1052-1066 (2006).

280. Zhou,H., Bernhard,E.J., Fox,F.E., & Billings,P.C. Induction of metalloproteinase activity in human T-lymphocytes. *Biochim. Biophys. Acta* **1177**, 174-178 (1993).

281. Montgomery,A.M., Sabzevari,H., & Reisfeld,R.A. Production and regulation of gelatinase B by human T-cells. *Biochim. Biophys. Acta* **1176**, 265-268 (1993).

282. Weiss,S.J. Tissue destruction by neutrophils. *N. Engl. J. Med.* **320**, 365-376 (1989).

283. Opdenakker,G. *et al.* Gelatinase B functions as regulator and effector in leukocyte biology. *J. Leukoc. Biol.* **69**, 851-859 (2001).

284. Merrill,J.E., Koyanagi,Y., & Chen,I.S. Interleukin-1 and tumor necrosis factor alpha can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. *J. Virol.* **63**, 4404-4408 (1989).

285. Wahl,L.M. *et al.* Human immunodeficiency virus glycoprotein (gp120) induction of monocyte arachidonic acid metabolites and interleukin 1. *Proc. Natl. Acad. Sci. U. S. A* **86**, 621-625 (1989).

286. Molina,J.M., Scadden,D.T., Byrn,R., Dinarello,C.A., & Groopman,J.E. Production of tumor necrosis factor alpha and interleukin 1 beta by monocytic cells infected with human immunodeficiency virus. *J. Clin. Invest* **84**, 733-737 (1989).

287. Roux-Lombard,P., Modoux,C., Cruchaud,A., & Dayer,J.M. Purified blood monocytes from HIV 1-infected patients produce high levels of TNF alpha and IL-1. *Clin. Immunol. Immunopathol.* **50**, 374-384 (1989).

288. Granowitz,E.V., Saget,B.M., Wang,M.Z., Dinarello,C.A., & Skolnik,P.R. Interleukin 1 induces HIV-1 expression in chronically infected U1 cells: blockade by interleukin 1 receptor antagonist and tumor necrosis factor binding protein type 1. *Mol. Med.* **1**, 667-677 (1995).

289. Poli,G., Kinter,A.L., & Fauci,A.S. Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc. Natl. Acad. Sci. U. S. A* **91**, 108-112 (1994).

290. Han,X., Becker,K., Degen,H.J., Jablonowski,H., & Strohmeyer,G. Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on

replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. *Eur. J. Clin. Invest* **26**, 286-292 (1996).

291. Huang,Y.S. *et al.* Serum levels of cytokines in hepatitis C-related liver disease: a longitudinal study. *Zhonghua Yi. Xue. Za Zhi. (Taipei)* **62**, 327-333 (1999).

292. Tiggelman,A.M., Boers,W., Linthorst,C., Sala,M., & Chamuleau,R.A. Collagen synthesis by human liver (myo)fibroblasts in culture: evidence for a regulatory role of IL-1 beta, IL-4, TGF beta and IFN gamma. *J. Hepatol.* **23**, 307-317 (1995).

293. Mancini,R., Benedetti,A., & Jezequel,A.M. An interleukin-1 receptor antagonist decreases fibrosis induced by dimethylnitrosamine in rat liver. *Virchows Arch.* **424**, 25-31 (1994).

294. Saren,P., Welgus,H.G., & Kovanen,P.T. TNF-alpha and IL-1beta selectively induce expression of 92-kDa gelatinase by human macrophages. *J. Immunol.* **157**, 4159-4165 (1996).

295. Leber,T.M. & Balkwill,F.R. Regulation of monocyte MMP-9 production by TNF-alpha and a tumour-derived soluble factor (MMPSF). *Br. J. Cancer* **78**, 724-732 (1998).

296. Vaday,G.G. *et al.* Fibronectin-bound TNF-alpha stimulates monocyte matrix metalloproteinase-9 expression and regulates chemotaxis. *J. Leukoc. Biol.* **68**, 737-747 (2000).

297. Nguyen,J., Knapnougel,P., Lesavre,P., & Bauvois,B. Inhibition of matrix metalloproteinase-9 by interferons and TGF-beta1 through distinct signalings accounts for reduced monocyte invasiveness. *FEBS Lett.* **579**, 5487-5493 (2005).

298. Zhang,Y., McCluskey,K., Fujii,K., & Wahl,L.M. Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-alpha, granulocyte-macrophage CSF, and IL-1 beta through prostaglandin-dependent and -independent mechanisms. *J. Immunol.* **161**, 3071-3076 (1998).

299. Lacraz,S., Isler,P., Vey,E., Welgus,H.G., & Dayer,J.M. Direct contact between T lymphocytes and monocytes is a major pathway for induction of metalloproteinase expression. *J. Biol. Chem.* **269**, 22027-22033 (1994).

300. Baram,D. *et al.* Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrine regulation by TNF-alpha. *J. Immunol.* **167**, 4008-4016 (2001).

301. Gearing,A.J. *et al.* Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* **370**, 555-557 (1994).

302. Gallea-Robache,S. *et al.* A metalloproteinase inhibitor blocks the shedding of soluble cytokine receptors and processing of transmembrane cytokine precursors in human monocytic cells. *Cytokine* **9**, 340-346 (1997).

303. Ito,A. *et al.* Degradation of interleukin 1beta by matrix metalloproteinases. *J. Biol. Chem.* **271**, 14657-14660 (1996).

304. Taub,D.D. & Oppenheim,J.J. Chemokines, inflammation and the immune system. *Ther. Immunol.* **1**, 229-246 (1994).

305. Overall,C.M., McQuibban,G.A., & Clark-Lewis,I. Discovery of chemokine substrates for matrix metalloproteinases by exosite scanning: a new tool for degradomics. *Biol. Chem.* **383**, 1059-1066 (2002).

306. Van den Steen,P.E., Proost,P., Wuyts,A., Van,D.J., & Opdenakker,G. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* **96**, 2673-2681 (2000).

307. D'Haese,A. *et al.* In vivo neutrophil recruitment by granulocyte chemotactic protein-2 is assisted by gelatinase B/MMP-9 in the mouse. *J. Interferon Cytokine Res.* **20**, 667-674 (2000).

308. Galon,J. *et al.* Identification of the cleavage site involved in production of plasma soluble Fc gamma receptor type III (CD16). *Eur. J. Immunol.* **28**, 2101-2107 (1998).

309. Bazil,V. & Strominger,J.L. Metalloprotease and serine protease are involved in cleavage of CD43, CD44, and CD16 from stimulated human granulocytes. Induction of cleavage of L-selectin via CD16. *J. Immunol.* **152**, 1314-1322 (1994).

310. Matias-Roman,S. *et al.* Membrane type 1-matrix metalloproteinase is involved in migration of human monocytes and is regulated through their interaction with fibronectin or endothelium. *Blood* **105**, 3956-3964 (2005).

311. Lafrenie,R.M. *et al.* HIV-1-Tat modulates the function of monocytes and alters their interactions with microvessel endothelial cells. A mechanism of HIV pathogenesis. *J. Immunol.* **156**, 1638-1645 (1996).

312. Johnston,J.B. *et al.* HIV-1 Tat neurotoxicity is prevented by matrix metalloproteinase inhibitors. *Ann. Neurol.* **49**, 230-241 (2001).

313. Ju,S.M. *et al.* Extracellular HIV-1 Tat up-regulates expression of matrix metalloproteinase-9 via a MAPK-NF-kappaB dependent pathway in human astrocytes. *Exp. Mol. Med.* **41**, 86-93 (2009).

314. Barillari,G. *et al.* Inflammatory cytokines synergize with the HIV-1 Tat protein to promote angiogenesis and Kaposi's sarcoma via induction of basic fibroblast growth factor and the alpha v beta 3 integrin. *J. Immunol.* **163**, 1929-1935 (1999).

315. Yim,H.C., Li,J.C., Lau,J.S., & Lau,A.S. HIV-1 Tat dysregulation of lipopolysaccharide-induced cytokine responses: microbial interactions in HIV infection. *AIDS* **23**, 1473-1484 (2009).

316. Fiorentino,D.F., Bond,M.W., & Mosmann,T.R. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170**, 2081-2095 (1989).

317. Badou,A. *et al.* Tat protein of human immunodeficiency virus type 1 induces interleukin-10 in human peripheral blood monocytes: implication of protein kinase C-dependent pathway. *J. Virol.* **74**, 10551-10562 (2000).

318. Gee,K. *et al.* Intracellular HIV-Tat expression induces IL-10 synthesis by the CREB-1 transcription factor through Ser133 phosphorylation and its regulation by the ERK1/2 MAPK in human monocytic cells. *J. Biol. Chem.* **281**, 31647-31658 (2006).

319. Leghmari,K., Bennasser,Y., & Bahraoui,E. HIV-1 Tat protein induces IL-10 production in monocytes by classical and alternative NF-kappaB pathways. *Eur. J. Cell Biol.* **87**, 947-962 (2008).

320. Ambrosino,C. *et al.* HIV-1 Tat induces the expression of the interleukin-6 (IL6) gene by binding to the IL6 leader RNA and by interacting with CAAT enhancer-binding protein beta (NF-IL6) transcription factors. *J. Biol. Chem.* **272**, 14883-14892 (1997).

321. Mahieux,R. *et al.* Cell cycle regulation of human interleukin-8 gene expression by the human immunodeficiency virus type 1 Tat protein. *J. Virol.* **75**, 1736-1743 (2001).

322. Esser,R. *et al.* Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization. *Blood* **91**, 4752-4760 (1998).

323. Foli,A., Saville,M.W., May,L.T., Webb,D.S., & Yarchoan,R. Effects of human immunodeficiency virus and colony-stimulating factors on the production of interleukin 6 and tumor necrosis factor alpha by monocyte/macrophages. *AIDS Res. Hum. Retroviruses* **13**, 829-839 (1997).

324. Vyakarnam,A., McKeating,J., Meager,A., & Beverley,P.C. Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. *AIDS* **4**, 21-27 (1990).

325. Lahdevirta,J., Maury,C.P., Teppo,A.M., & Repo,H. Elevated levels of circulating cachectin/tumor necrosis factor in patients with acquired immunodeficiency syndrome. *Am. J. Med.* **85**, 289-291 (1988).

326. Baqui,A.A. *et al.* Enhanced interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha production by LPS stimulated human monocytes isolated from HIV+ patients. *Immunopharmacol. Immunotoxicol.* **22**, 401-421 (2000).

327. Leghmari,K., Contreras,X., Moureau,C., & Bahraoui,E. HIV-1 Tat protein induces TNF-alpha and IL-10 production by human macrophages: differential implication of PKC-betaII and -delta isozymes and MAP kinases ERK1/2 and p38. *Cell Immunol.* **254**, 46-55 (2008).

328. Li,J.C., Lee,D.C., Cheung,B.K., & Lau,A.S. Mechanisms for HIV Tat upregulation of IL-10 and other cytokine expression: kinase signaling and PKR-mediated immune response. *FEBS Lett.* **579**, 3055-3062 (2005).

329. Mayne,M., Holden,C.P., Nath,A., & Geiger,J.D. Release of calcium from inositol 1,4,5-trisphosphate receptor-regulated stores by HIV-1 Tat regulates TNF-alpha production in human macrophages. *J. Immunol.* **164**, 6538-6542 (2000).

330. Sui,Z. *et al.* Functional synergy between CD40 ligand and HIV-1 Tat contributes to inflammation: implications in HIV type 1 dementia. *J. Immunol.* **178**, 3226-3236 (2007).

331. Esser,R. *et al.* Individual cell analysis of the cytokine repertoire in human immunodeficiency virus-1-infected monocytes/macrophages by a combination of immunocytochemistry and in situ hybridization. *Blood* **91**, 4752-4760 (1998).

332. Baqui,A.A. *et al.* Enhanced interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha production by LPS stimulated human monocytes isolated from HIV+ patients. *Immunopharmacol. Immunotoxicol.* **22**, 401-421 (2000).

333. Buonaguro,L. *et al.* Effects of the human immunodeficiency virus type 1 Tat protein on the expression of inflammatory cytokines. *J. Virol.* **66**, 7159-7167 (1992).

334. Nath,A., Conant,K., Chen,P., Scott,C., & Major,E.O. Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J. Biol. Chem.* **274**, 17098-17102 (1999).

335. Kornbluth,R.S., Oh,P.S., Munis,J.R., Cleveland,P.H., & Richman,D.D. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. *J. Exp. Med.* **169**, 1137-1151 (1989).

336. Dhawan,S. *et al.* Interferon-gamma-induced downregulation of CD4 inhibits the entry of human immunodeficiency virus type-1 in primary monocytes. *Pathobiology* **63**, 93-99 (1995).

337. Koyanagi,Y. *et al.* Cytokines alter production of HIV-1 from primary mononuclear phagocytes. *Science* **241**, 1673-1675 (1988).

338. Meyaard,L. *et al.* Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. *J. Immunol.* **157**, 2712-2718 (1996).

339. Dhillon,N. *et al.* Molecular mechanism(s) involved in the synergistic induction of CXCL10 by human immunodeficiency virus type 1 Tat and interferon-gamma in macrophages. *J. Neurovirol.* **14**, 196-204 (2008).

340. Giunta,B. *et al.* EGCG mitigates neurotoxicity mediated by HIV-1 proteins gp120 and Tat in the presence of IFN-gamma: role of JAK/STAT1 signaling and implications for HIV-associated dementia. *Brain Res.* **1123**, 216-225 (2006).

341. Campbell,G.R., Watkins,J.D., Singh,K.K., Loret,E.P., & Spector,S.A. Human immunodeficiency virus type 1 subtype C Tat fails to induce intracellular calcium flux and induces reduced tumor necrosis factor production from monocytes. *J. Virol.* **81**, 5919-5928 (2007).

342. Wong,J.K., Campbell,G.R., & Spector,S.A. Differential induction of interleukin-10 in monocytes by HIV-1 clade B and clade C Tat proteins. *J. Biol. Chem.* **285**, 18319-18325 (2010).

343. Gandhi,N. *et al.* Differential effects of HIV type 1 clade B and clade C Tat protein on expression of proinflammatory and antiinflammatory cytokines by primary monocytes. *AIDS Res. Hum. Retroviruses* **25**, 691-699 (2009).

344. Nunez,O. *et al.* Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut* **53**, 1665-1672 (2004).

345. Mazzocca,A. *et al.* Binding of hepatitis C virus envelope protein E2 to CD81 up-regulates matrix metalloproteinase-2 in human hepatic stellate cells. *J. Biol. Chem.* **280**, 11329-11339 (2005).

346. Krishnadas,D.K., Ahn,J.S., Han,J., Kumar,R., & Agrawal,B. Immunomodulation by hepatitis C virus-derived proteins: targeting human dendritic cells by multiple mechanisms. *Int. Immunol.* **22**, 491-502 (2010).

347. Eisen-Vandervelde,A.L. *et al.* Hepatitis C virus core selectively suppresses interleukin-12 synthesis in human macrophages by interfering with AP-1 activation. *J. Biol. Chem.* **279**, 43479-43486 (2004).

348. Brady,M.T., MacDonald,A.J., Rowan,A.G., & Mills,K.H. Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur. J. Immunol.* **33**, 3448-3457 (2003).

349. Dolganiuc,A. *et al.* Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J. Immunol.* **170**, 5615-5624 (2003).

350. Liuzzi,G.M. *et al.* Anti-HIV drugs decrease the expression of matrix metalloproteinases in astrocytes and microglia. *Brain* **127**, 398-407 (2004).

351. Latronico,T. *et al.* Antiretroviral therapy inhibits matrix metalloproteinase-9 from blood mononuclear cells of HIV-infected patients. *AIDS* **21**, 677-684 (2007).

352. Bourlier,V. *et al.* Protease inhibitor treatments reveal specific involvement of matrix metalloproteinase-9 in human adipocyte differentiation. *J. Pharmacol. Exp. Ther.* **312**, 1272-1279 (2005).

353. De,B.S., Zakaroff-Girard,A., Lafontan,M., Galitzky,J., & Bourlier,V. Inhibition of human preadipocyte proteasomal activity by HIV protease inhibitors or specific inhibitor lactacystin leads to a defect in adipogenesis, which involves matrix metalloproteinase-9. *J. Pharmacol. Exp. Ther.* **320**, 291-299 (2007).

354. Dhawan,S. *et al.* Interferon-gamma inhibits HIV-induced invasiveness of monocytes. *J. Leukoc. Biol.* **58**, 713-716 (1995).

355. Slaton,J.W., Perrotte,P., Inoue,K., Dinney,C.P., & Fidler,I.J. Interferon-alpha-mediated down-regulation of angiogenesis-related genes and therapy of bladder cancer are dependent on optimization of biological dose and schedule. *Clin. Cancer Res.* **5**, 2726-2734 (1999).

356. Slaton,J.W. *et al.* Treatment with low-dose interferon-alpha restores the balance between matrix metalloproteinase-9 and E-cadherin expression in human transitional cell carcinoma of the bladder. *Clin. Cancer Res.* **7**, 2840-2853 (2001).

357. Sanceau,J., Boyd,D.D., Seiki,M., & Bauvois,B. Interferons inhibit tumor necrosis factor-alpha-mediated matrix metalloproteinase-9 activation via interferon regulatory factor-1 binding competition with NF-kappa B. *J. Biol. Chem.* **277**, 35766-35775 (2002).

358. Giannelli,G. *et al.* Antifibrogenic effect of IFN-alpha2b on hepatic stellate cell activation by human hepatocytes. *J. Interferon Cytokine Res.* **26**, 301-308 (2006).

359. Diaz-Sanjuan,T. *et al.* Interferon alpha increases metalloproteinase-13 gene expression through a polyomavirus enhancer activator 3-dependent pathway in hepatic stellate cells. *J. Hepatol.* **50**, 128-139 (2009).

360. Harris,J.E. *et al.* IFNgamma synergizes with IL-1beta to up-regulate MMP-9 secretion in a cellular model of central nervous system tuberculosis. *FASEB J.* **21**, 356-365 (2007).

361. Ninomiya,T. *et al.* Significance of serum matrix metalloproteinases and their inhibitors on the antifibrogenetic effect of interferon-alfa in chronic hepatitis C patients. *Intervirology* **44**, 227-231 (2001).

362. Mitsuda,A., Suou,T., Ikuta,Y., & Kawasaki,H. Changes in serum tissue inhibitor of matrix metalloproteinase-1 after interferon alpha treatment in chronic hepatitis C. *J. Hepatol.* **32**, 666-672 (2000).

363. Flisiak,R., Jaroszewicz,J., Lapinski,T.W., Flisiak,I., & Prokopowiczi,D. Effect of pegylated interferon alpha 2b plus ribavirin treatment on plasma transforming growth factor-beta1, metalloproteinase-1, and tissue metalloproteinase inhibitor-1 in patients with chronic hepatitis C. *World J. Gastroenterol.* **11**, 6833-6838 (2005).

364. Marinosci,F. *et al.* Clinical role of serum and tissue matrix metalloprotease-9 expression in chronic HCV patients treated with pegylated IFN-alpha2b and ribavirin. *J. Interferon Cytokine Res.* **25**, 453-458 (2005).

365. Collman,R.G., Perno,C.F., Crowe,S.M., Stevenson,M., & Montaner,L.J. HIV and cells of macrophage/dendritic lineage and other non-T cell reservoirs: new answers yield new questions. *J. Leukoc. Biol.* **74**, 631-634 (2003).

366. Henderson,A.J. & Calame,K.L. CCAAT/enhancer binding protein (C/EBP) sites are required for HIV-1 replication in primary macrophages but not CD4(+) T cells. *Proc. Natl. Acad. Sci. U. S. A* **94**, 8714-8719 (1997).

367. Peng,G., Lei,K.J., Jin,W., Greenwell-Wild,T., & Wahl,S.M. Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. *J. Exp. Med.* **203**, 41-46 (2006).

368. Kornbluth,R.S., Oh,P.S., Munis,J.R., Cleveland,P.H., & Richman,D.D. Interferons and bacterial lipopolysaccharide protect macrophages from productive infection by human immunodeficiency virus in vitro. *J. Exp. Med.* **169**, 1137-1151 (1989).

369. Han,X., Becker,K., Degen,H.J., Jablonowski,H., & Strohmeyer,G. Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. *Eur. J. Clin. Invest* **26**, 286-292 (1996).

370. Diebold,S.S., Kaisho,T., Hemmi,H., Akira,S., & Reis e Sousa Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**, 1529-1531 (2004).

371. Kadowaki,N. *et al.* Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* **194**, 863-869 (2001).

372. Mandl,J.N. *et al.* Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat. Med.* **14**, 1077-1087 (2008).

373. Gondois-Rey,F. *et al.* Hepatitis C virus is a weak inducer of interferon alpha in plasmacytoid dendritic cells in comparison with influenza and human herpesvirus type-1. *PLoS. One.* **4**, e4319 (2009).

374. Geijtenbeek,T.B. *et al.* DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**, 587-597 (2000).

375. Kawamura,T. *et al.* R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. *Proc. Natl. Acad. Sci. U. S. A* **100**, 8401-8406 (2003).

376. Dieu,M.C. *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* **188**, 373-386 (1998).

377. Vecchi,A. *et al.* Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells. *J. Leukoc. Biol.* **66**, 489-494 (1999).

378. Granucci,F., Vizzardelli,C., Virzi,E., Rescigno,M., & Ricciardi-Castagnoli,P. Transcriptional reprogramming of dendritic cells by differentiation stimuli. *Eur. J. Immunol.* **31**, 2539-2546 (2001).

379. Granucci,F., Feau,S., Angeli,V., Trottein,F., & Ricciardi-Castagnoli,P. Early IL-2 production by mouse dendritic cells is the result of microbial-induced priming. *J. Immunol.* **170**, 5075-5081 (2003).

380. Fortis,C., Tasca,S., Capiluppi,B., & Tambussi,G. Natural killer cell function in HIV-1 infected patients. *J. Biol. Regul. Homeost. Agents* **16**, 30-32 (2002).

381. Ferlazzo,G. *et al.* The interaction between NK cells and dendritic cells in bacterial infections results in rapid induction of NK cell activation and in the lysis of uninfected dendritic cells. *Eur. J. Immunol.* **33**, 306-313 (2003).

382. Biron,C.A., Nguyen,K.B., Pien,G.C., Cousens,L.P., & Salazar-Mather,T.P. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**, 189-220 (1999).

383. Henkart,P.A. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity.* **1**, 343-346 (1994).

384. Zamai,L. *et al.* Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J. Exp. Med.* **188**, 2375-2380 (1998).

385. Robertson,M.J. Role of chemokines in the biology of natural killer cells. *J. Leukoc. Biol.* **71**, 173-183 (2002).

386. Fehniger,T.A. *et al.* Natural killer cells from HIV-1+ patients produce C-C chemokines and inhibit HIV-1 infection. *J. Immunol.* **161**, 6433-6438 (1998).

387. Brenner,M.B. *et al.* Identification of a putative second T-cell receptor. *Nature* **322**, 145-149 (1986).

388. Lanier,L.L. *et al.* Structural and serological heterogeneity of gamma/delta T cell antigen receptor expression in thymus and peripheral blood. *Eur. J. Immunol.* **18**, 1985-1992 (1988).

389. Tanaka,Y. *et al.* Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* **375**, 155-158 (1995).

390. Morita,C.T. *et al.* Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity.* **3**, 495-507 (1995).

391. Bukowski,J.F., Morita,C.T., & Brenner,M.B. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity.* **11**, 57-65 (1999).

392. Kunzmann,V., Bauer,E., & Wilhelm,M. Gamma/delta T-cell stimulation by pamidronate. *N. Engl. J. Med.* **340**, 737-738 (1999).

393. Morita,C.T. *et al.* Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity.* **3**, 495-507 (1995).

394. Dunne,M.R., Mangan,B.A., Madrigal-Estebas,L., & Doherty,D.G. Preferential Th1 cytokine profile of phosphoantigen-stimulated human Vgamma9Vdelta2 T cells. *Mediators. Inflamm.* **2010**, 704941 (2010).

395. Wesch,D., Glatzel,A., & Kabelitz,D. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. *Cell Immunol.* **212**, 110-117 (2001).

396. Caccamo,N. *et al.* CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J. Immunol.* **177**, 5290-5295 (2006).

397. Ness-Schwickerath,K.J., Jin,C., & Morita,C.T. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vgamma2Vdelta2 T cells. *J. Immunol.* **184**, 7268-7280 (2010).

398. Poccia,F. *et al.* Phosphoantigen-reactive Vgamma9Vdelta2 T lymphocytes suppress in vitro human immunodeficiency virus type 1 replication by cell-released antiviral factors including CC chemokines. *J. Infect. Dis.* **180**, 858-861 (1999).

399. Cipriani,B. *et al.* Activation of C-C beta-chemokines in human peripheral blood gammadelta T cells by isopentenyl pyrophosphate and regulation by cytokines. *Blood* **95**, 39-47 (2000).

400. Malkovsky,M. *et al.* Are gamma delta T cells important for the elimination of virus-infected cells? *J. Med. Primatol.* **21**, 113-118 (1992).

401. Biswas,P. *et al.* Double-edged effect of Vgamma9/Vdelta2 T lymphocytes on viral expression in an in vitro model of HIV-1/mycobacteria co-infection. *Eur. J. Immunol.* **33**, 252-263 (2003).

402. Jason,J. *et al.* Natural T, gammadelta, and NK cells in mycobacterial, Salmonella, and human immunodeficiency virus infections. *J. Infect. Dis.* **182**, 474-481 (2000).

403. Gan,Y.H. & Malkovsky,M. Mechanisms of simian gamma delta T cell cytotoxicity against tumor and immunodeficiency virus-infected cells. *Immunol. Lett.* **49**, 191-196 (1996).

404. Bellan,C. *et al.* Burkitt's lymphoma: new insights into molecular pathogenesis. *J. Clin. Pathol.* **56**, 188-192 (2003).

405. Fisch,P. *et al.* Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science* **250**, 1269-1273 (1990).

406. Spits,H., Paliard,X., Vandekerckhove,Y., van,V.P., & de Vries,J.E. Functional and phenotypic differences between CD4+ and CD4- T cell receptor-gamma delta clones from peripheral blood. *J. Immunol.* **147**, 1180-1188 (1991).

407. De,P.P. *et al.* A subset of gamma delta lymphocytes is increased during HIV-1 infection. *Clin. Exp. Immunol.* **83**, 187-191 (1991).

408. De,M.A. *et al.* Selective increase of a subset of T cell receptor gamma delta T lymphocytes in the peripheral blood of patients with human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **165**, 917-919 (1992).

409. Autran,B. *et al.* T cell receptor gamma/delta+ lymphocyte subsets during HIV infection. *Clin. Exp. Immunol.* **75**, 206-210 (1989).

410. Boullier,S., Cochet,M., Poccia,F., & Gougeon,M.L. CDR3-independent gamma delta V delta 1+ T cell expansion in the peripheral blood of HIV-infected persons. *J. Immunol.* **154**, 1418-1431 (1995).

411. Bordon,J. *et al.* Association between longer duration of HIV-suppressive therapy and partial recovery of the V gamma 2 T cell receptor repertoire. *J. Infect. Dis.* **189**, 1482-1486 (2004).

412. Martini,F. *et al.* gammadelta T-cell anergy in human immunodeficiency virus-infected persons with opportunistic infections and recovery after highly active antiretroviral therapy. *Immunology* **100**, 481-486 (2000).

413. Poles,M.A. *et al.* Human immunodeficiency virus type 1 induces persistent changes in mucosal and blood gammadelta T cells despite suppressive therapy. *J. Virol.* **77**, 10456-10467 (2003).

414. Poggi,A. *et al.* Migration of V delta 1 and V delta 2 T cells in response to CXCR3 and CXCR4 ligands in healthy donors and HIV-1-infected patients: competition by HIV-1 Tat. *Blood* **103**, 2205-2213 (2004).

415. Wheelock,E.F. Interferon-like virus-inhibitor induced in human leukocytes by phytohemagglutinin. *Science* **149**, 310-311 (1965).

416. Perussia,B. Lymphokine-activated killer cells, natural killer cells and cytokines. *Curr. Opin. Immunol.* **3**, 49-55 (1991).

417. Mosmann,T.R. & Coffman,R.L. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**, 145-173 (1989).

418. Sad,S., Marcotte,R., & Mosmann,T.R. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity.* **2**, 271-279 (1995).

419. Darnell,J.E., Jr., Kerr,I.M., & Stark,G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415-1421 (1994).

420. Groettrup,M., Khan,S., Schwarz,K., & Schmidtke,G. Interferon-gamma inducible exchanges of 20S proteasome active site subunits: why? *Biochimie* **83**, 367-372 (2001).

421. Steimle,V., Siegrist,C.A., Mottet,A., Lisowska-Grospierre,B., & Mach,B. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* **265**, 106-109 (1994).

422. Mach,B., Steimle,V., Martinez-Soria,E., & Reith,W. Regulation of MHC class II genes: lessons from a disease. *Annu. Rev. Immunol.* **14**, 301-331 (1996).

423. Tamaki,K. & Nakamura,K. Differential enhancement of interferon-gamma-induced MHC class II expression of HEp-2 cells by 1,25-dihydroxyvitamin D3. *Br. J. Dermatol.* **123**, 333-338 (1990).

424. Ljunggren,G. & Anderson,D.J. Cytokine induced modulation of MHC class I and class II molecules on human cervical epithelial cells. *J. Reprod. Immunol.* **38**, 123-138 (1998).

425. Clerici,M. & Shearer,G.M. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol. Today* **14**, 107-111 (1993).

426. Clerici,M. & Shearer,G.M. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol. Today* **15**, 575-581 (1994).

427. Klein,S.A. *et al.* Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry. *AIDS* **11**, 1111-1118 (1997).

428. Flesch,I.E. *et al.* Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon gamma and tumor necrosis factor alpha. *J. Exp. Med.* **181**, 1615-1621 (1995).

429. Hsieh,C.S. *et al.* Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* **260**, 547-549 (1993).

430. Trinchieri,G. The two faces of interleukin 12: a pro-inflammatory cytokine and a key immunoregulatory molecule produced by antigen-presenting cells. *Ciba Found. Symp.* **195**, 203-214 (1995).

431. Lederer,J.A. *et al.* Cytokine transcriptional events during helper T cell subset differentiation. *J. Exp. Med.* **184**, 397-406 (1996).

432. Trinchieri,G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* **13**, 251-276 (1995).

433. Fehniger,T.A. *et al.* Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* **162**, 4511-4520 (1999).

434. Snapper,C.M. *et al.* Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J. Exp. Med.* **175**, 1367-1371 (1992).

435. Snapper,C.M. *et al.* IFN-gamma is a potent inducer of Ig secretion by sort-purified murine B cells activated through the mIg, but not the CD40, signaling pathway. *Int. Immunol.* **8**, 877-885 (1996).

436. Locksley,R.M., Killeen,N., & Lenardo,M.J. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501 (2001).

437. Ware,C.F., VanArsdale,T.L., Crowe,P.D., & Browning,J.L. The ligands and receptors of the lymphotoxin system. *Curr. Top. Microbiol. Immunol.* **198**, 175-218 (1995).

438. Mauri,D.N. *et al.* LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity.* **8**, 21-30 (1998).

439. Baud,V. & Karin,M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11**, 372-377 (2001).

440. Osborn,L., Kunkel,S., & Nabel,G.J. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. U. S. A* **86**, 2336-2340 (1989).

441. Folks,T.M. *et al.* Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. U. S. A* **86**, 2365-2368 (1989).

442. Duh,E.J., Maury,W.J., Folks,T.M., Fauci,A.S., & Rabson,A.B. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc. Natl. Acad. Sci. U. S. A* **86**, 5974-5978 (1989).

443. Griffin,G.E., Leung,K., Folks,T.M., Kunkel,S., & Nabel,G.J. Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature* **339**, 70-73 (1989).

444. Herbein,G., Montaner,L.J., & Gordon,S. Tumor necrosis factor alpha inhibits entry of human immunodeficiency virus type 1 into primary human macrophages: a selective role for the 75-kilodalton receptor. *J. Virol.* **70**, 7388-7397 (1996).

445. Herbein,G. & Gordon,S. 55- and 75-kilodalton tumor necrosis factor receptors mediate distinct actions in regard to human immunodeficiency virus type 1 replication in primary human macrophages. *J. Virol.* **71**, 4150-4156 (1997).

446. Cocchi,F. *et al.* Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8+ T cells. *Science* **270**, 1811-1815 (1995).

447. Verani,A. *et al.* C-C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells. *J. Exp. Med.* **185**, 805-816 (1997).

448. Lane,B.R. *et al.* TNF-alpha inhibits HIV-1 replication in peripheral blood monocytes and alveolar macrophages by inducing the production of RANTES and decreasing C-C chemokine receptor 5 (CCR5) expression. *J. Immunol.* **163**, 3653-3661 (1999).

449. Biron,C.A., Nguyen,K.B., Pien,G.C., Cousens,L.P., & Salazar-Mather,T.P. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu. Rev. Immunol.* **17**, 189-220 (1999).

450. Oliva,A. *et al.* Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *J. Clin. Invest* **102**, 223-231 (1998).

451. Moore,K.W., de Waal,M.R., Coffman,R.L., & O'Garra,A. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* **19**, 683-765 (2001).

452. Ancuta,P. *et al.* Opposite effects of IL-10 on the ability of dendritic cells and macrophages to replicate primary CXCR4-dependent HIV-1 strains. *J. Immunol.* **166**, 4244-4253 (2001).

453. Weissman,D., Poli,G., & Fauci,A.S. Interleukin 10 blocks HIV replication in macrophages by inhibiting the autocrine loop of tumor necrosis factor alpha and interleukin 6 induction of virus. *AIDS Res. Hum. Retroviruses* **10**, 1199-1206 (1994).

454. Weissman,D., Poli,G., & Fauci,A.S. IL-10 synergizes with multiple cytokines in enhancing HIV production in cells of monocytic lineage. *J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol.* **9**, 442-449 (1995).

455. Hunter,C.A., Subauste,C.S., Van,C., V, & Remington,J.S. Production of gamma interferon by natural killer cells from Toxoplasma gondii-infected SCID mice:

regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infect. Immun.* **62**, 2818-2824 (1994).

456. Tripp,C.S., Wolf,S.F., & Unanue,E.R. Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. U. S. A* **90**, 3725-3729 (1993).

457. Tsuchiya,S. *et al.* Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer* **26**, 171-176 (1980).

458. Tsuchiya,S. *et al.* Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* **42**, 1530-1536 (1982).

459. Xu,L. *et al.* Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* **54**, 142-151 (2005).

460. Yeni,P. Update on HAART in HIV. *J. Hepatol.* **44**, S100-S103 (2006).

461. Singal,A.K. & Anand,B.S. Management of hepatitis C virus infection in HIV/HCV co-infected patients: clinical review. *World J. Gastroenterol.* **15**, 3713-3724 (2009).

462. Mastroianni,C.M. & Liuzzi,G.M. Matrix metalloproteinase dysregulation in HIV infection: implications for therapeutic strategies. *Trends Mol. Med.* **13**, 449-459 (2007).

463. Tsukada,S., Parsons,C.J., & Rippe,R.A. Mechanisms of liver fibrosis. *Clin. Chim. Acta* **364**, 33-60 (2006).

464. Khan,F., Peltekian,K.M., & Peterson,T.C. Effect of interferon-alpha, ribavirin, pentoxifylline, and interleukin-18 antibody on hepatitis C sera-stimulated hepatic stellate cell proliferation. *J. Interferon Cytokine Res.* **28**, 643-651 (2008).

465. Knittel,T. *et al.* Expression of matrix metalloproteinases and their inhibitors during hepatic tissue repair in the rat. *Histochem. Cell Biol.* **113**, 443-453 (2000).

466. Parola,M. & Robino,G. Oxidative stress-related molecules and liver fibrosis. *J. Hepatol.* **35**, 297-306 (2001).

467. Cao,Q., Mak,K.M., & Lieber,C.S. Dilinoleoylphosphatidylcholine prevents transforming growth factor-beta1-mediated collagen accumulation in cultured rat hepatic stellate cells. *J. Lab Clin. Med.* **139**, 202-210 (2002).

468. Roderfeld,M. *et al.* Inhibition of hepatic fibrogenesis by matrix metalloproteinase-9 mutants in mice. *FASEB J.* **20**, 444-454 (2006).

469. Sundstrom,J.B. *et al.* Effects of norepinephrine, HIV type 1 infection, and leukocyte interactions with endothelial cells on the expression of matrix metalloproteinases. *AIDS Res. Hum. Retroviruses* **17**, 1605-1614 (2001).

470. Koulentaki,M. *et al.* Matrix metalloproteinases and their inhibitors in acute viral hepatitis. *J. Viral Hepat.* **9**, 189-193 (2002).

471. Hopkins,S. *et al.* Role of individualization of hepatitis C virus (HCV) therapy duration in HIV/HCV-coinfected individuals. *HIV. Med.* **7**, 248-254 (2006).

472. Davidson,F. *et al.* Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. *J. Gen. Virol.* **76 (Pt 5)**, 1197-1204 (1995).

473. Rodriguez-Torres,M. *et al.* Effect of ribavirin on intracellular and plasma pharmacokinetics of nucleoside reverse transcriptase inhibitors in patients with human immunodeficiency virus-hepatitis C virus coinfection: results of a randomized clinical study. *Antimicrob. Agents Chemother.* **49**, 3997-4008 (2005).

474. Dahari,H. *et al.* Pharmacodynamics of PEG-IFN-alpha-2a in HIV/HCV co-infected patients: Implications for treatment outcomes. *J. Hepatol.* **53**, 460-467 (2010).

475. von Hentig,N. *et al.* The steady-state pharmacokinetics of atazanavir/ritonavir in HIV-1-infected adult outpatients is not affected by gender-related co-factors. *J. Antimicrob. Chemother.* **62**, 579-582 (2008).

476. Klein,C.E. *et al.* Effects of acid-reducing agents on the pharmacokinetics of lopinavir/ritonavir and ritonavir-boosted atazanavir. *J. Clin. Pharmacol.* **48**, 553-562 (2008).

477. Bittner,B., Riek,M., Holmes,B., & Grange,S. Saquinavir 500 mg film-coated tablets demonstrate bioequivalence to saquinavir 200 mg hard capsules when boosted with twice-daily ritonavir in healthy volunteers. *Antivir. Ther.* **10**, 803-810 (2005).

478. Yuen,G.J., Weller,S., & Pakes,G.E. A review of the pharmacokinetics of abacavir. *Clin. Pharmacokinet.* **47**, 351-371 (2008).

479. Marier,J.F. *et al.* Comparative bioavailability study of zidovudine administered as two different tablet formulations in healthy adult subjects. *Int. J. Clin. Pharmacol. Ther.* **44**, 240-246 (2006).

480. Liu,P., Foster,G., LaBadie,R.R., Gutierrez,M.J., & Sharma,A. Pharmacokinetic interaction between voriconazole and efavirenz at steady state in healthy male subjects. *J. Clin. Pharmacol.* **48**, 73-84 (2008).

481. Tarinas,A. *et al.* Bioequivalence study of two nevirapine tablet formulations in human-immunodeficiency-virus-infected patients. *Farm. Hosp.* **31**, 165-168 (2007).

482. Schmittgen,T.D. & Livak,K.J. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**, 1101-1108 (2008).

483. Elkington,P.T., O'Kane,C.M., & Friedland,J.S. The paradox of matrix metalloproteinases in infectious disease. *Clin. Exp. Immunol.* **142**, 12-20 (2005).

484. Kuo,W.H., Chou,F.P., Lu,S.C., Chu,S.C., & Hsieh,Y.S. Significant differences in serum activities of matrix metalloproteinase-2 and -9 between HCV- and HBV-infected patients and carriers. *Clin. Chim. Acta* **294**, 157-168 (2000).

485. Larrat,S. *et al.* Ribavirin quantification in combination treatment of chronic hepatitis C. *Antimicrob. Agents Chemother.* **47**, 124-129 (2003).

486. Poynard,T., McHutchison,J., Manns,M., Myers,R.P., & Albrecht,J. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. *Hepatology* **38**, 481-492 (2003).

487. Pineda,J.A. *et al.* HAART and the liver: friend or foe? *Eur. J. Med. Res.* **15**, 93-96 (2010).

488. Marine-Barjoan,E. *et al.* Impact of antiretroviral treatment on progression of hepatic fibrosis in HIV/hepatitis C virus co-infected patients. *AIDS* **18**, 2163-2170 (2004).

489. Hazenberg,M.D. *et al.* Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* **17**, 1881-1888 (2003).

490. Deeks,S.G. *et al.* Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* **104**, 942-947 (2004).

491. Gonzalez,V.D. *et al.* High levels of chronic immune activation in the T-cell compartments of patients coinfected with hepatitis C virus and human immunodeficiency virus type 1 and on highly active antiretroviral therapy are

reverted by alpha interferon and ribavirin treatment. *J. Virol.* **83**, 11407-11411 (2009).

492. Roe,B. *et al.* Phenotypic characterization of lymphocytes in HCV/HIV co-infected patients. *Viral Immunol.* **22**, 39-48 (2009).

493. Soto,B. *et al.* Human immunodeficiency virus infection modifies the natural history of chronic parenterally-acquired hepatitis C with an unusually rapid progression to cirrhosis. *J. Hepatol.* **26**, 1-5 (1997).

494. Jeang,K.T., Xiao,H., & Rich,E.A. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. *J. Biol. Chem.* **274**, 28837-28840 (1999).

495. Westendorp,M.O. *et al.* Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375**, 497-500 (1995).

496. Frankel,A.D. & Pabo,C.O. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **55**, 1189-1193 (1988).

497. Kumar,A., Dhawan,S., Mukhopadhyay,A., & Aggarwal,B.B. Human immunodeficiency virus-1-tat induces matrix metalloproteinase-9 in monocytes through protein tyrosine phosphatase-mediated activation of nuclear transcription factor NF-kappaB. *FEBS Lett.* **462**, 140-144 (1999).

498. Lafrenie,R.M., Wahl,L.M., Epstein,J.S., Yamada,K.M., & Dhawan,S. Activation of monocytes by HIV-Tat treatment is mediated by cytokine expression. *J. Immunol.* **159**, 4077-4083 (1997).

499. Buonaguro,L., Tornesello,M.L., & Buonaguro,F.M. Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications. *J. Virol.* **81**, 10209-10219 (2007).

500. Kim,J.L. *et al.* Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* **87**, 343-355 (1996).

501. Lohmann,V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113 (1999).

502. Grakoui,A., McCourt,D.W., Wychowski,C., Feinstone,S.M., & Rice,C.M. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**, 2832-2843 (1993).

503. Grakoui,A., McCourt,D.W., Wychowski,C., Feinstone,S.M., & Rice,C.M. A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. U. S. A* **90**, 10583-10587 (1993).

504. Rajkowitsch,L. *et al.* RNA chaperones, RNA annealers and RNA helicases. *RNA. Biol.* **4**, 118-130 (2007).

505. Jarvis,T.C., Newport,J.W., & von Hippel,P.H. Stimulation of the processivity of the DNA polymerase of bacteriophage T4 by the polymerase accessory proteins. The role of ATP hydrolysis. *J. Biol. Chem.* **266**, 1830-1840 (1991).

506. Sun,S., Rao,V.B., & Rossmann,M.G. Genome packaging in viruses. *Curr. Opin. Struct. Biol.* **20**, 114-120 (2010).

507. Brady,M.T., MacDonald,A.J., Rowan,A.G., & Mills,K.H. Hepatitis C virus non-structural protein 4 suppresses Th1 responses by stimulating IL-10 production from monocytes. *Eur. J. Immunol.* **33**, 3448-3457 (2003).

508. Fiorentino,D.F., Zlotnik,A., Mosmann,T.R., Howard,M., & O'Garra,A. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**, 3815-3822 (1991).

509. de Waal,M.R. *et al.* Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* **174**, 915-924 (1991).

510. Tsai,S.L., Liaw,Y.F., Chen,M.H., Huang,C.Y., & Kuo,G.C. Detection of type 2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. *Hepatology* **25**, 449-458 (1997).

511. Poli,G. *et al.* Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. *J. Exp. Med.* **172**, 151-158 (1990).

512. Zaitseva,M. *et al.* Interferon gamma and interleukin 6 modulate the susceptibility of macrophages to human immunodeficiency virus type 1 infection. *Blood* **96**, 3109-3117 (2000).

513. Ma,X. & Montaner,L.J. Proinflammatory response and IL-12 expression in HIV-1 infection. *J. Leukoc. Biol.* **68**, 383-390 (2000).

514. Gearing,A.J. *et al.* Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* **370**, 555-557 (1994).

515. Ito,A. *et al.* Degradation of interleukin 1beta by matrix metalloproteinases. *J. Biol. Chem.* **271**, 14657-14660 (1996).

516. Connolly,N.C., Riddler,S.A., & Rinaldo,C.R. Proinflammatory cytokines in HIV disease-a review and rationale for new therapeutic approaches. *AIDS Rev.* **7**, 168-180 (2005).

517. Neuman,M.G. *et al.* Cytokines as predictors for sustained response and as markers for immunomodulation in patients with chronic hepatitis C. *Clin. Biochem.* **34**, 173-182 (2001).

518. Huigen,M.C., Kamp,W., & Nottet,H.S. Multiple effects of HIV-1 trans-activator protein on the pathogenesis of HIV-1 infection. *Eur. J. Clin. Invest* **34**, 57-66 (2004).

519. Cheng,P.L., Chang,M.H., Chao,C.H., & Lee,Y.H. Hepatitis C viral proteins interact with Smad3 and differentially regulate TGF-beta/Smad3-mediated transcriptional activation. *Oncogene* **23**, 7821-7838 (2004).

520. Kaleebu,P. *et al.* Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. *J. Infect. Dis.* **185**, 1244-1250 (2002).

521. Vasan,A. *et al.* Different rates of disease progression of HIV type 1 infection in Tanzania based on infecting subtype. *Clin. Infect. Dis.* **42**, 843-852 (2006).

522. Rao,V.R. *et al.* HIV-1 clade-specific differences in the induction of neuropathogenesis. *J. Neurosci.* **28**, 10010-10016 (2008).

523. Riedel,D. *et al.* Screening for human immunodeficiency virus (HIV) dementia in an HIV clade C-infected population in India. *J. Neurovirol.* **12**, 34-38 (2006).

524. Tardieu,M., Hery,C., Peudenier,S., Boespflug,O., & Montagnier,L. Human immunodeficiency virus type 1-infected monocytic cells can destroy human neural cells after cell-to-cell adhesion. *Ann. Neurol.* **32**, 11-17 (1992).

525. Cheng,J. *et al.* Neuronal excitatory properties of human immunodeficiency virus type 1 Tat protein. *Neuroscience* **82**, 97-106 (1998).

526. Nath,A. & Geiger,J. Neurobiological aspects of human immunodeficiency virus infection: neurotoxic mechanisms. *Prog. Neurobiol.* **54**, 19-33 (1998).

527. New,D.R., Maggirwar,S.B., Epstein,L.G., Dewhurst,S., & Gelbard,H.A. HIV-1 Tat induces neuronal death via tumor necrosis factor-alpha and activation of non-N-methyl-D-aspartate receptors by a NFkappaB-independent mechanism. *J. Biol. Chem.* **273**, 17852-17858 (1998).

528. Shi,B., Raina,J., Lorenzo,A., Busciglio,J., & Gabuzda,D. Neuronal apoptosis induced by HIV-1 Tat protein and TNF-alpha: potentiation of neurotoxicity mediated by oxidative stress and implications for HIV-1 dementia. *J. Neurovirol.* **4**, 281-290 (1998).

529. Williams,R., Yao,H., Dhillon,N.K., & Buch,S.J. HIV-1 Tat co-operates with IFN-gamma and TNF-alpha to increase CXCL10 in human astrocytes. *PLoS. One.* **4**, e5709 (2009).

530. Johnston,J.B. *et al.* HIV-1 Tat neurotoxicity is prevented by matrix metalloproteinase inhibitors. *Ann. Neurol.* **49**, 230-241 (2001).

531. Beuche,W. *et al.* Matrix metalloproteinase-9 is elevated in serum of patients with amyotrophic lateral sclerosis. *Neuroreport* **11**, 3419-3422 (2000).

532. Kolb,S.A. *et al.* Matrix metalloproteinases and tissue inhibitors of metalloproteinases in viral meningitis: upregulation of MMP-9 and TIMP-1 in cerebrospinal fluid. *J. Neuroimmunol.* **84**, 143-150 (1998).

533. Pagenstecher,A., Stalder,A.K., Kincaid,C.L., Shapiro,S.D., & Campbell,I.L. Differential expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase genes in the mouse central nervous system in normal and inflammatory states. *Am. J. Pathol.* **152**, 729-741 (1998).

534. Tan,H.K. *et al.* Tissue inhibitor of metalloproteinase 1 inhibits excitotoxic cell death in neurons. *Mol. Cell Neurosci.* **22**, 98-106 (2003).

535. Zylberberg,H. *et al.* Soluble tumor necrosis factor receptors in chronic hepatitis C: a correlation with histological fibrosis and activity. *J. Hepatol.* **30**, 185-191 (1999).

536. Powell,E.E. *et al.* Host genetic factors influence disease progression in chronic hepatitis C. *Hepatology* **31**, 828-833 (2000).

537. Sakaki,H. *et al.* Interleukin-1beta induces matrix metalloproteinase-1 expression in cultured human gingival fibroblasts: role of cyclooxygenase-2 and prostaglandin E2. *Oral Dis.* **10**, 87-93 (2004).

538. Yokoo,T. & Kitamura,M. Dual regulation of IL-1 beta-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-kappa B and AP-1. *Am. J. Physiol* **270**, F123-F130 (1996).

539. Kiebala,M. & Maggirwar,S.B. Ibudilast, a pharmacologic phosphodiesterase inhibitor, prevents human immunodeficiency virus-1 Tat-mediated activation of microglial cells. *PLoS. One.* **6**, e18633 (2011).

540. Dolganiuc,A. *et al.* Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* **127**, 1513-1524 (2004).

541. Becker,Y. The changes in the T helper 1 (Th1) and T helper 2 (Th2) cytokine balance during HIV-1 infection are indicative of an allergic response to viral proteins that may be reversed by Th2 cytokine inhibitors and immune response modifiers--a review and hypothesis. *Virus Genes* **28**, 5-18 (2004).

542. Guidotti,L.G. & Chisari,F.V. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* **19**, 65-91 (2001).

543. Sher,A. *et al.* Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. *Immunol. Rev.* **127**, 183-204 (1992).

544. Imami,N. *et al.* A balanced type 1/type 2 response is associated with long-term nonprogressive human immunodeficiency virus type 1 infection. *J. Virol.* **76**, 9011-9023 (2002).

545. Brandes,M., Willimann,K., & Moser,B. Professional antigen-presentation function by human gammadelta T Cells. *Science* **309**, 264-268 (2005).

546. Poccia,F. *et al.* CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive V gamma 9V delta 2 T lymphocytes. *J. Immunol.* **159**, 6009-6017 (1997).

547. Wallace,M. *et al.* Gamma delta T lymphocyte responses to HIV. *Clin. Exp. Immunol.* **103**, 177-184 (1996).

548. Devilder,M.C. *et al.* Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. *J. Immunol.* **176**, 1386-1393 (2006).

549. Brandes,M. *et al.* Cross-presenting human gammadelta T cells induce robust CD8+ alphabeta T cell responses. *Proc. Natl. Acad. Sci. U. S. A* **106**, 2307-2312 (2009).

550. Caccamo,N. *et al.* CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J. Immunol.* **177**, 5290-5295 (2006).

551. Poccia,F. *et al.* Peripheral V gamma 9/V delta 2 T cell deletion and anergy to nonpeptidic mycobacterial antigens in asymptomatic HIV-1-infected persons. *J. Immunol.* **157**, 449-461 (1996).

552. Wesch,D., Kabelitz,D., Friese,K., & Pechhold,K. Mycobacteria-reactive gamma delta T cells in HIV-infected individuals: lack of V gamma 9 cell responsiveness is due to deficiency of antigen-specific CD4 T helper type 1 cells. *Eur. J. Immunol.* **26**, 557-562 (1996).

553. Wallace,M. *et al.* Functional gamma delta T-lymphocyte defect associated with human immunodeficiency virus infections. *Mol. Med.* **3**, 60-71 (1997).

554. Poccia,F., Wallace,M., Colizzi,V., & Malkovsky,M. Possible protective and pathogenic roles of gamma delta T lymphocytes in HIV-infections (Review). *Int. J. Mol. Med.* **1**, 409-413 (1998).

555. Morris,M.C., Depollier,J., Mery,J., Heitz,F., & Divita,G. A peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat. Biotechnol.* **19**, 1173-1176 (2001).

556. Gonzalez,E., Punzon,C., Gonzalez,M., & Fresno,M. HIV-1 Tat inhibits IL-2 gene transcription through qualitative and quantitative alterations of the cooperative Rel/AP1 complex bound to the CD28RE/AP1 composite element of the IL-2 promoter. *J. Immunol.* **166**, 4560-4569 (2001).

557. Beinke,S. & Ley,S.C. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. *Biochem. J.* **382**, 393-409 (2004).

558. Le,P.C., Genin,P., Baines,M.G., & Hiscott,J. Interferon activation and innate immunity. *Rev. Immunogenet.* **2**, 374-386 (2000).

559. Flesch,I.E. *et al.* Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon gamma and tumor necrosis factor alpha. *J. Exp. Med.* **181**, 1615-1621 (1995).

560. McDermott,M.F. TNF and TNFR biology in health and disease. *Cell Mol. Biol. (Noisy. -le-grand)* **47**, 619-635 (2001).

561. Folks,T.M. *et al.* Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. U. S. A* **86**, 2365-2368 (1989).

562. Duh,E.J., Maury,W.J., Folks,T.M., Fauci,A.S., & Rabson,A.B. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc. Natl. Acad. Sci. U. S. A* **86**, 5974-5978 (1989).

563. Griffin,G.E., Leung,K., Folks,T.M., Kunkel,S., & Nabel,G.J. Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature* **339**, 70-73 (1989).

564. Hintz,M. *et al.* Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli. *FEBS Lett.* **509**, 317-322 (2001).

565. Gee,K., Angel,J.B., Mishra,S., Blahoianu,M.A., & Kumar,A. IL-10 regulation by HIV-Tat in primary human monocytic cells: involvement of calmodulin/calmodulin-dependent protein kinase-activated p38 MAPK and Sp-1 and CREB-1 transcription factors. *J. Immunol.* **178**, 798-807 (2007).

566. Bukowski,J.F., Morita,C.T., & Brenner,M.B. Recognition and destruction of virus-infected cells by human gamma delta CTL. *J. Immunol.* **153**, 5133-5140 (1994).

567. Maccario,R. *et al.* HLA-unrestricted killing of HSV-1-infected mononuclear cells. Involvement of either gamma/delta+ or alpha/beta+ human cytotoxic T lymphocytes. *J. Immunol.* **150**, 1437-1445 (1993).

568. Wallace,M., Gan,Y.H., Pauza,C.D., & Malkovsky,M. Antiviral activity of primate gamma delta T lymphocytes isolated by magnetic cell sorting. *J. Med. Primatol.* **23**, 131-135 (1994).

569. Poccia,F. *et al.* Innate T-cell immunity in HIV infection: the role of Vgamma9Vdelta2 T lymphocytes. *Curr. Mol. Med.* **2**, 769-781 (2002).

570. Poccia,F., Wallace,M., Colizzi,V., & Malkovsky,M. Possible protective and pathogenic roles of gamma delta T lymphocytes in HIV-infections (Review). *Int. J. Mol. Med.* **1**, 409-413 (1998).

571. Autran,B. *et al.* T cell receptor gamma/delta+ lymphocyte subsets during HIV infection. *Clin. Exp. Immunol.* **75**, 206-210 (1989).

572. Wallace,M. *et al.* Gamma delta T lymphocyte responses to HIV. *Clin. Exp. Immunol.* **103**, 177-184 (1996).

573. Bordon,J. *et al.* Association between longer duration of HIV-suppressive therapy and partial recovery of the V gamma 2 T cell receptor repertoire. *J. Infect. Dis.* **189**, 1482-1486 (2004).

574. Sarol,L.C. *et al.* Inhibitory effects of IFN-gamma on HIV-1 replication in latently infected cells. *Biochem. Biophys. Res. Commun.* **291**, 890-896 (2002).

575. Poli,G. & Fauci,A.S. The effect of cytokines and pharmacologic agents on chronic HIV infection. *AIDS Res. Hum. Retroviruses* **8**, 191-197 (1992).

576. Poli,G., Biswas,P., & Fauci,A.S. Interferons in the pathogenesis and treatment of human immunodeficiency virus infection. *Antiviral Res.* **24**, 221-233 (1994).

577. Ali,Z. *et al.* Gammadelta T cell immune manipulation during chronic phase of simian-human immunodeficiency virus infection [corrected] confers immunological benefits. *J. Immunol.* **183**, 5407-5417 (2009).

578. Biswas,P. *et al.* Interferon gamma induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells. *J. Exp. Med.* **176**, 739-750 (1992).

579. Vyakarnam,A., McKeating,J., Meager,A., & Beverley,P.C. Tumour necrosis factors (alpha, beta) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. *AIDS* **4**, 21-27 (1990).

580. Dhillon,N. *et al.* Molecular mechanism(s) involved in the synergistic induction of CXCL10 by human immunodeficiency virus type 1 Tat and interferon-gamma in macrophages. *J. Neurovirol.* **14**, 196-204 (2008).

581. Gil,M.P. *et al.* Biologic consequences of Stat1-independent IFN signaling. *Proc. Natl. Acad. Sci. U. S. A* **98**, 6680-6685 (2001).

582. Chin,Y.E., Kitagawa,M., Kuida,K., Flavell,R.A., & Fu,X.Y. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. *Mol. Cell Biol.* **17**, 5328-5337 (1997).

583. Lee,S.B. & Esteban,M. The interferon-induced double-stranded RNA-activated protein kinase induces apoptosis. *Virology* **199**, 491-496 (1994).

584. Tsujimoto,M., Yip,Y.K., & Vilcek,J. Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. *J. Immunol.* **136**, 2441-2444 (1986).

585. Xu,X., Fu,X.Y., Plate,J., & Chong,A.S. IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res.* **58**, 2832-2837 (1998).

586. Gan,Y.H., Wallace,M., & Malkovsky,M. Fas-dependent, activation-induced cell death of gammadelta cells. *J. Biol. Regul. Homeost. Agents* **15**, 277-285 (2001).

587. Hayashi,K. *et al.* HIV-TAT protein upregulates expression of multidrug resistance protein 1 in the blood-brain barrier. *J. Cereb. Blood Flow Metab* **26**, 1052-1065 (2006).