An investigation of the mechanisms underlying HIV-1-mediated inflammasome activation

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Doctorate of Philosophy in Medicine

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Dedication

To my mother Judith, and to Jayson.

Summary

Around 35 million people are living with HIV-1 infection globally. Untreated infection results in progression to AIDS and mortality. Current antiretroviral treatment is not curative and does not treat the underlying inflammatory processes caused by the presence of HIV-1, a cytotoxic and tissue toxic virus. HIV-1 triggers overwhelming and dysregulated pro-inflammatory cytokine response during infection, including deranged interleukin-1 beta and interleukin-18 levels.

We investigate the mechanisms underlying how HIV-1 activates the inflammasome, a multimolecular complex involved in the production of potent inflammatory markers such as interleukin-1 beta and interleukin-18. The inflammasome is often activated in a two-signal process requiring initial sensing by Toll-like receptors to generate signal 1, priming inflammasome components; and signal 2 sensed by NOD-like receptors which recruit and activate the inflammasome.

Human leukocytes were stimulated with live HIV-1 or its cognate envelope proteins gp120 or gp41, or transfected with plasmids encoding HIV-1 viroporin Vpu or gp41. NLRP3 knock-down attenuated inflammasome activation and interleukin-1beta/interleukin-18 secretion following HIV-1, gp120 or gp41 stimulation. gp120 induced inflammasome activation also required the presence of TLR2 and TLR4, suggesting a degree of cooperation in effecting signal 1. Dual NLRP3/NLRC4 knockdown reduced the inflammasome response to HIV-1 gp41 stimulation which was found to be dependent on chloride anion flux across integral lipid rafts in the trans-Golgi network.

The results imply a critical role for TLR2 and TLR4 in recognising HIV-1 proteins providing signal 1 for inflammasome formation. Ion fluxes in the cell due to HIV-1 infection are the catalyst that triggers inflammasome activation via NLRP3 or NLRC4, and secretion of interleukin-1 beta and interleukin-18. The findings contribute to the general understanding of HIV-1 recognition by the human innate immune system and help to further clarify precisely how HIV-1 causes striking dysregulation in cytokine profiles in people living with HIV-1 infection.

Glossary of Abbreviations

-/-… Homozygous knock-out (of a gene).

%E…Efficiency of energy transfer between donor and acceptor.

A…Alanine (excluding DNA sequence figures where **A** represents Adenine).

AA…Amino acid.

AIM2…Absent in melanoma (protein) 2.

ALIX…ALG-2 (alpha-1,3-mannosyltransferase) -interacting protein X, commonly known as 'programmed cell death 6-interacting protein'.

AP-1…Activation protein 1.

APOBEC.'apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like' family proteins.

APS…ammonium persulfate.

Arg…Arginine.

ARV…Avian reovirus.

ASC…apoptotic speck-like protein.

Asp…Aspargine

Asn…Arginine.

ATP…Adenosine triphosphate.

ATCC…American Tissue Culture Collection.

AZT…Azidothymidine.

BAPTA-AM…1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxy methyl ester).

B.anthracis…Bacillus anthracis.

BD... Beckton Dickenson.

BIR…Baculovirus inhibitor of apoptosis repeat.

C'…Carboxyl terminus.

C…Cysteine.

Ca^{2+}…Calcium ion.

cAMP… Cyclic adenosine monophosphate.

CAPS1…Cryopyrin-associated periodic syndrome type-1.

CARD…Caspase activating and recruitment domain.

cART…Combination antiretroviral therapy.

CBC...Cap-binding complex.

CCR5…C-C (group of chemokine receptors) chemokine receptor type 5.

CD…Cluster of differentiation system of protein classification, e.g. 'CD4'.

CFP…Cyan-coloured fluorescence protein

CHR...C' (carboxyl) terminal heptad repeats.

Cl^-…Chloride ion.

CpG (DNA)…Single-stranded DNA containing cytosine triphosphate deoxynucleotide which precedes a guanine triphosphate deoxynucleotide.

C-Rel…V-rel avian reticuloendotheliosis viral oncogene homolog.

CREB… cAMP response element-binding protein.

CST…Cell Signalling Technologies.

CTX…Cholera toxin.

CVB...Coxsackie virus B.

CXCL10...C-X-C motif chemokine.

CXCR4…C-X-C motif chemokine receptor type 4.

CypA…Cyclophyllin A.

Cys…Cysteine.

D…Aspartic acid.

DAMP(s)…Damage associated molecular pattern(s).

DC-SIGN…Dendritic cell specific intracellular adhesion molecule 3 grabbing non-integrin.

DD…Death domain.

DMSO…Dimethyl sulfoxide.

DNA…Deoxyribonucleic acid.

dsDNA…double-stranded deoxyribonucleic acid.

dToll…Drosophila Toll.

E…Glutamic acid.

ECMV…Encephalomyocarditis virus.

E.coli…Escherichia coli.

ECL…Enhanced chemiluminescence.

EDTA…Ethylenediaminetetraacetic acid.

EIPA…5-(*N*-ethyl-*N*-isopropyl)amiloride.

EJC…Exon-exon junction complex.

ELISA…Enzyme-linked immunosorbance assay.

ELFO…Electrophoresis running buffer.

Env…Envelope protein.

ER…Endoplasmic reticulum.

ESCRT…Endosomal sorting complexes required for transport.

F…Phenylalanine.

FACS…Fluorescence Activated Cell Sorter

FACS4…Familial cold autoinflammatory syndrome 4.

FCS…Foetal calf serum.

FcγRIIIa…Fraction crystallisable gamma receptor IIIa.

FIIND…Function to find domain.

FITC… Fluorescein isothiocyanate.

FP…Fusion peptide.

FRET…Förster Resonance energy transfer.

FSC…Forward scatter.

G…Glutamic acid.

Gag…Group specific antigen protein.

GALT…Gut-associated lymphoid tissue.

GFP…Green fluorescent protein

Gln…Glutamine.

Glu…Glutamic acid.

Gly…Glycine.

GM-1…Monosialotetrahexosylganglioside.

gp41…Glycoprotein 41.

gp120…Glycoprotein 120.

GRR…Glycine rich regions.

GU-rich…Guanine uracil rich.

H…Histidine.

H^+…Hydrogen ion or proton.

H_2O_2…Hydrogen peroxide.

HAART…Highly active antiretroviral therapy.

HCV…Hepatitis C virus.

HDAC...Histidine deacetylase.

HEK293T…Human embryonic kidney 293 cells containing the large ‘T’-antigen.

HeLa…Eponymous immortal cervical cancer cell line acquired from a patient called 'Henrietta Lacks'.

His...Histidine.

HCl…Hydrochloric acid.

HIV-1…Human immunodeficiency virus type-1.

HPA…Health Protection Agency.

HRV…Human rhinovirus.

HRP…Horseradish peroxidase.

HSP…Heat shock protein.

hToll…Human Toll protein.

I…Isoleucine

IAVA…Influenza A virus.

ICAM-1…Intracellular adhesion molecule 1.

IFN(α/β)…Interferon (alpha/beta).

IFNAR…Interferon-alpha/beta receptor alpha chain.

IFNGR1…Interferon gamma receptor-1.

IκB…Inhibitor of (nuclear factor) kappa B.

IKK…I kappa B kinase complex.

IL-1β…Interleukin-1 beta.

IL-1R…Interleukin-1 receptor.

IL-6…Interleukin-6.

IL-18…Interleukin-18.

Ile…Isoleucine.

IMS…Industrial methylated spirit.

IPAF…ICE (interleukin-1 converting enzyme – caspase-1) protease activating factor.

IRAK4…IL-1R-associated kinase 4.

IRF…Interferon regulatory factor.

ISRE…Interferon stimulated response elements.

JAK…Janus kinase.

JCV…Eponymous virus first discovered in patient 'JC'.

K…Lysine.

K^+…Potassium ion.

L…Leucine.

L.acidophilus…Lactobacillus acidophilus

LAF…Lymphocyte activating factor commonly known as IL-1α (interleukin-1 alpha).

LEDGF/p75…Lens epithelium-derived growth factor p75.

LeTx…Lethal toxin.

Leu…Leucine.

LFA-1…Lymphocyte function-associated antigen 1.

LPS…Lipopolysaccharide.

LRR…Leucine rich receptor.

LTA…Lipoteichoic acid.

LTR…Long terminal repeat (sequence).

Lys…Lysine.

M…Methionine.

M2 protein…Matrix protein 2.

m^7G…7-methylguanosine.

MACS…(Miltenyi Biotech) magnetic cell separation technology.

MCMV…Murine cytomegalovirus.

Met…Methionine.

MD1…Lymphocyte antigen 86 (also commonly abbreviated to LY86).

MD2…Lymphocyte antigen 96 (also commonly abbreviated to LY96).

MHC…Major histocompatibility complex.

MQAE…N-(Ethoxycarbonylmethyl)-6-methoxyquinolinium bromide.

mRNA…Messenger ribonucleic acid.

myc-DKK…Polypeptide tags found in plasmid vectors comprising c-myc of the amino acid sequence N'-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-C' and DDK (Dickkopf related protein) of the amino acid sequence N'-Asp-Tyr-Asp-Asp-Asp-Asp-Lys-C'.

MyD88…Myeloid differentiation factor 88.

N…Aspargine.

N'…Amino terminus (of a polypeptide).

Na^+…Sodium ion.

NACHT…**NA**IP – neuronal apoptosis inhibitor protein, **C**2TA – MHC class 2 transcription activator, **H**ET-E – incompatibility locus protein from *Podospora anserine*, and **T**P1 – telomerase-associated protein.

NAIP…Neuronal apoptosis inhibitor protein.

NaN_3…Sodium azide.

NBD…Nucleotide binding domain, a synonym of NOD (nucleotide oligomerisation binding domain or NACHT).

NCM…Nitrocellulose membrane.

Nef…Negative factor

NEMO…Nuclear factor kappa B essential modulator.

NFκB…Nuclear factor kappa B.

NF-Y…Nuclear factor Y.

NHR…N' (nitrogen) terminal heptad repeats.

NIK…Nuclear factor kappa B inducing kinase.

NIBSC…National Institute for Biological Standards and Control.

NIHARP…National Institutes of Health AIDS Reagents Programme.

NK cell…Natural killer cell.

NKT cell…Natural killer T-cell.

NLR(s)…Nucleotide oligomerisation binding domain and leucine rich receptor domain containing receptor(s).

NLRC…NOD-like receptor family CARD domain containing (protein).

NLRC4…NOD-like receptor family CARD domain containing (protein) 4. NLRC5 as for NLRC4.

NLRP(s)…Nucleotide oligomerisation binding domain and leucine rich receptor domain containing receptor(s) which contain a pyrin domain, e.g., NLRP1b.

N.mucosa…Neisseria mucosa.

NRTI…Nucleoside reverse transcriptase inhibitor.

NNRTI…Non-nucleoside reverse transcriptase inhibitor.

NOD…Nucleotide oligomerisation-binding domain.

NXF1…Nuclear RNA export factor 1.

NXT1…Nuclear transport factor 2-related export protein 1.

P…Proline.

P2X7…P2X (designated) purinoceptor 7.

PAMP(s)…Pathogen associated molecular pattern(s)

PBMC…Peripheral blood mononuclear cells.

PBS…Phosphate-buffered saline.

pDC…Plasmacytoid dendritic cell.

PEP…Post-exposure prophylaxis.

PFA…Paraformaldehyde.

PFD…Pocket forming domain.

pH…Power of hydrogen.

Phe…Phenylalanine.

PhosphoIκB(α)/PIκB...Phosphorylated inhibitor of kappa B protein alpha subunit.

PI…Protease inhibitor.

PLWHIV…People living with HIV.

Pol…Polyprotein.

poly(I:C)…Polyinosinic-polycytidylic acid (synthetic dsRNA analogue).
PreP…Pre-exposure prophylaxis.

Pro…Proline.

Pro-Il-1β…Pro-interleukin-1 beta where pro designates the uncleaved immature form of IL-1β.

PRR…Pattern recognition receptor.

pshRNA…RNA plasmid possessing a small hairpin motif.

PV…Poliovirus.

PYD…Pyrin domain.

Q…Glutamine.

R…Arginine.

RANK…Receptor activator of nuclear factor kappa B.

Rel A…v-rel avian reticuloendotheliosis viral oncogene homolog A, also known as transcription factor p65.

Rel B…v-rel avian reticuloendotheliosis viral oncogene homolog B.

Rev…Anti-repression transactivator protein

RFX-5…Regulatory factor X-5.

RFX-ANK…Regulatory factor X-associated ankyrin containing protein.

RFX-AP…Regulatory factor X-associated protein.

RHD…Rel homology domain.

RLR(s)…Retinoic acid – inducible gene I-like receptor(s).

RP105…radioprotective 105kDa protein.

RPMI…Roswell Park Memorial Institute (culture medium).

RNAi…Ribonucleic acid interference.

ROS…Reactive oxygen species.

RSV…Respiratory syncytial virus.

RV…Rhinovirus.

S…Serine.

SALT…Skin-associated lymphoid tissue.

SARS-CoV…Severe acute respiratory syndrome coronavirus.

SBC…Santa Cruz Biotechnologies.

SCC…Side scatter.

SD…Standard deviation.

SDF-1…Stromal derived factor-1.

SDS PAGE…Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

SEAP…Serum embryonic alkaline phosphatase.

Ser…Serine.

SGT-1…Suppressor of G2 allele of SKP1 (S-phase kinase-associated protein 1).

SH protein…Small hydrophobic protein (channel).

SIV…Simian immunodeficiency virus.

SR…Serine arginine proteins.

shRNA…Small hairpin (motif-containing) ribonucleic acid.

ssRNA…Single stranded ribonucleic acid.

STAT…Signal transducer and activator of transcription.

STET…Sucrose, Tris HCl, EDTA, Triton.

SV…Sendai virus.

T…Threonine.

T-20…Enfuvirtide (HIV-1 fusion inhibitor).

TAD…Transactivation domain.

TABs…Transforming growth factor beta-activated kinase 1-binding protein family.

TANK…TRAF family member-associated NF-kappa-B activator.

TAK1…Transforming growth factor beta-activated kinase 1.

Tat…Transactivator of transcription.

T-cell…Thymocyte.

TEMED…Tetramethylethylenediamine

TF(s)…Transcription factor(s).

THP1…Human acute monocytic leukaemia cell line.

Thr…Threonine.

TIR…Toll-interleukin-1 receptor.

TIRAP…Toll-interleukin-1 receptor domain containing adaptor protein.

TLR(s)…Toll-like receptor(s).

TNF…Tumour necrosis factor.

Trp…Tryptophan.

TRITC…Tetramethylrhodamine.

TRIF…TIR-domain containing adaptor protein inducing IFNβ protein.

tRNA…Transfer ribonucleic acid.

tRNA(Lys)3…Where Lys = lysine, the tRNA primer for HIV-1 reverse transcription.

TRAM…TIRAP and TRIF-related adaptor molecule.

TRAF…TNF receptor-associated factor.

TBK-1…TANK-binding kinase-1.

TWEEN…Proprietary name for polysorbate 20 (a detergent).

TXNIP…Thioredoxin-interacting protein.

Tyr…Tyrosine

UTR…Untranslated region.

V…Valine.

Val…Valine.

Vif…Viral inhibitory factor

V.parvula…Veillonella parvula

Vpr…Viral protein 'R', also known as R ORF protein (R open reading frame protein).

Vpu…Viral protein 'unique'.

W…Tryptophan.

WT…Wild-type.

Y…Tyrosine.

Chapter 1: Introduction

1. Pattern recognition receptors: first line of defence against infection

1.1 Toll-like receptors: the first pattern recognition receptor family to be discovered

Pathogen recognition receptors (PRRs) are expressed by cells belonging to both of what was classically delineated as the innate and adaptive immune systems. The innate immune system classically comprises macrophages, natural killer cells, mast cells, basophils, eosinophils, neutrophils and the complement system. The adaptive immune system comprises the two super-classes of T-cells and B-cells. The innate immune system is responsible for the first-pass response to invading pathogens, whilst the main function of T and B cells is to specialise and memorise the response depending upon antigens presented to them. Several classes of dendritic cells and macrophages form the main antigen presenting link between the innate and adaptive immune systems. Molecular patterns belonging to pathogens commonly encountered throughout our evolution, pathogen-associated molecular patterns (PAMPS) are detected by PRRs which include Toll-like receptors (TLRs), RIG-I-like (retinoic acid inducible gene I -like) receptors (RLRs) NOD proteins, NOD-like receptors (NLRs), and C-type lectin receptors (Loiarro et al. 2010; Kawai & Akira 2011).

Toll-like receptors are type 1 transmembrane protein PRRs in possession of 24-29AA length extracellular leucine-rich repeats able to bind a plethora of PAMPS and signal downstream via cytosolic TIR domains to activate an immune response to a plethora of different pathogens. TLRs are markedly expressed in circulating white blood cells belonging to both branches of the immune system. TLRs not only detect and respond to PAMPs, but also sense and respond to endogenous molecules implicated in the pathogenesis of autoimmune and other disease processes (Loiarro et al. 2010; Kawai & Akira 2011; Mullen et al. 2015).

TLRs are, however, also expressed in cells and tissues belonging to points of contact between the outside environment and the body – mucosal lymphoid tissue of the gut and skin (gut associated lymphoid tissue/GALT and skin associated lymphoid tissue/SALT) being prime examples. Generally speaking, this feature places TLRs in the centre of the fully effective response against pathogens with some specific responses having been shown to be impotent in the absence of TLR mediated signalling (Kawai & Akira 2011; Takeuchi O & Akira S 2009).

Early work by Anderson's group on the *Drosophila* (fruit fly) genome hypothesised that a gene known as *Toll* belonging to the dorso-ventral regulatory region (a cassette of genes involved in embryonic development), encoded a transmembrane protein that was also highly conserved in mammalian and yeast tissues. A hydrophobic segment of 25 amino acids was thought to act as the transmembrane domain. This protein had an intracytoplasmic domain of 269 amino acids; and an extracytoplasmic domain of 802 amino acids, a significant proportion of these being leucine-rich repeat sequences. Given its similarity to the interleukin-1 receptor (IL-1R), the intracytoplasmic domain of what would be named TLR4 was termed a Toll/Interleukin 1 Receptor domain (TIR) (Hashimoto C et al. 1988).

Drosophila Toll (dToll) was thought to be exclusively involved in development of the *Drosophila* embryo. It then became evident that the pathway was still active in adult *Drosophila.* Since downstream pathways for *Drosophila Toll* and IL-1R both employ the transcription factor NFκB, it was considered curious that the gene was only involved in embryo development (Hashimoto C et al. 1988; Rosetto M et al. 1995).

Some years later, it was observed that *Drosophila* blood cells made to over-express a subtype of *Toll* increased expression of the gene *CecA1* which encodes an antibacterial peptide. MyD88, a protein originally thought to act exclusively in macrophage differentiation was

then found to be a member of the same protein family as *Toll a*nd IL-1R and hypothesised to be an important adaptor protein in mammalian signalling cascades. It was then found that *Drosophila* carrying mutations in *Toll* could not generate an antifungal response. These discoveries helped to support the hypothesis that *Toll* was indeed involved in the immune response, though evidence for a human or mammalian homologue was yet to be provided (Rosetto M et al. 1995; Lemaitre B et al. 1996).

It was shown that the cytoplasmic domain of the *Toll* receptor was very similar to that of a human transmembrane protein of previously hypothetical function. This protein was able to up-regulate genes involved in the immune response. The *hToll* (human *Toll*) protein showed conservation in *Drosophila* in terms of the leucine-rich domain as well as the cytoplasmic domain. It was then confirmed that *Toll/NFκB* signalling is present in humans in a similar fashion to *Drosophila*, with the transcription factor *Dorsal* being homologous to NFκB in humans. The TLR studied as the analogue to *Drosophila Toll* became known as TLR4, which was found to belong to a large family of TLRs, 1-9 being conserved across humans and mice whilst TLR10 has been shown to be redundant in the murine model and TLR11 absent in humans. A role for human TLR10 in the recognition of helicobacter pylori has recently been established. TLRs in specific cells have been shown to display tolerance upon re-stimulation with the same ligands (Hultmark D 1994; Rosetto M et al. 1995; Medzhitov R et al. 1997; Kawai & Akira 2011; Nagashima et al. 2015). Figure 1 overleaf summarises the broad PAMP repertoire of the TLR family in humans.

Human TLR	Ligand	Origin
TLR1	Triacyl lipopeptides (as TLR1/2 dimer)	Bacteria Mycobacteria
TLR2	HSP70 Lipopeptides Peptidoglycan, LTA Zymosan, βglycan Porins Envelope glycoproteins	Endogenous Numerous Gram +ve bacteria Fungi Neisseria Numerous viruses
TLR3	dsRNA	Viruses
TLR4	HSP 60 & 70 LPS Mannan Glycoinositolphospholipids Envelope glycoproteins F protein	Endogenous Gram –ve bacteria *Candida* *Trypanosoma* Numerous viruses RSV
TLR5	Flagellin	Flagellated bacteria
TLR6	Diacyl lipopeptides (as TLR2/6) LTA Zymosan	Mycoplasma Gram +ve bacteria Fungi
TLR7 TLR8	GU-rich ssRNA Imiquimod/resiquimod	RNA viruses Therapeutic
TLR9	CpG DNA Hemozoin	Bacteria, viruses *P. falciparum*
TLR10	?LPS (as TLR2/10)	*Helicobacter pylori*

Figure 1. ***Toll-like receptors recognise molecular patterns from a variety of pathogens, exogenous or host sources.*** **Adapted from Kawai and Akira 2011 (Kawai & Akira 2011). Abbreviations: LTA, lipoteichoic acid; HSP, heat shock protein; LPS, lipopolysaccharide. Recent evidence supports TLR2/10 heterodimer sensing of H. pylori with LPS postulated as the ligand (Nagashima et al. 2015).**

Toll-like receptors recognise a multitude of PAMPs from a wide array of pathogens (figure 1) either as homodimers or, to further increase the sensory repertoire, TLRs may heterodimerise to transduce downstream signalling in response to specific PAMPs. Though most commonly studied as cell surface PRRs, TLR2 and TLR4 may be expressed in endosomes or phagosomes respectively, thereby further increasing the repertoire of detectable molecular patterns (Loiarro et al. 2010; Kawai & Akira 2011).

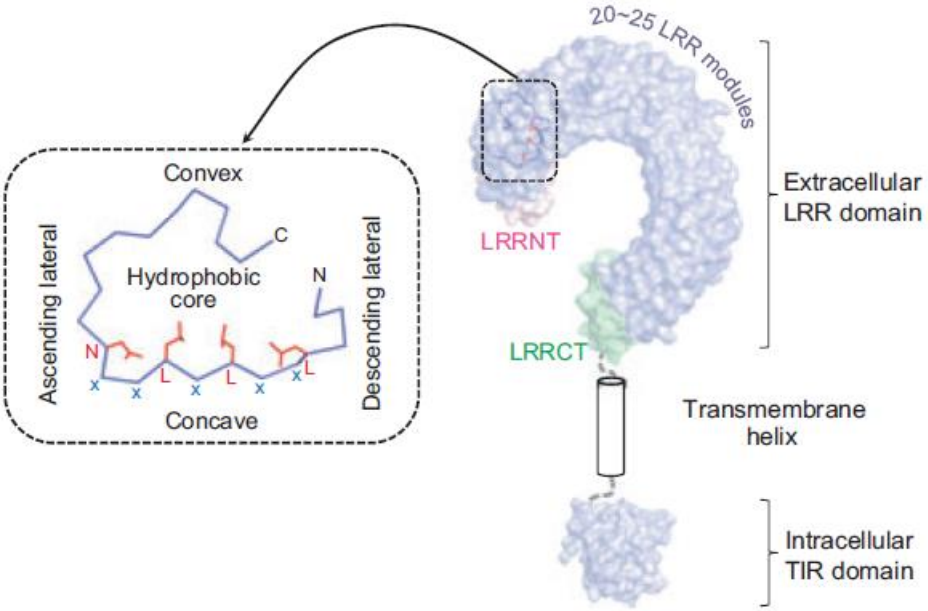


Figure 2. ***Representative schematic of a TLR monomer (TLR4 shown).*** **Abbreviations: LRRNT, Leucine-rich repeat N'terminal domains; LRRCT, leucine-rich repeat C' terminal domains. Adapted from Song and Lee 2012 (Song & Lee 2012).**

The structures of many TLRs have been well studied. Figure 2 above depicts the common structure of a TLR monomer exemplified by TLR4, with common features across TLRs including C-shaped extracellular leucine-rich repeat domain, transmembrane helix and intracellular TIR domain used for downstream signalling (pathways discussed in section 1.2). The ≈20-30AA leucine-rich repeat domain unit generally interacts with PAMPs via its own

side chains of variable residues which arise from within the C shape of the structure as can be seen in the break-out in figure 2 (Gay & Gangloff 2007). The convex surface of the LRR domain therefore contains multiple grooves and pockets for interaction with molecular patterns (Song & Lee 2012).

A single transmembrane α helix links the LRR domain to the intracellular TIR domain which is formed by five internal β helices encircled by five α helices. Although death domains are highly structurally conserved, the TIR domains are highly diverse conferring specificity of adaptor molecules (Dunne et al. 2003). The TIR domains are linked to downstream adaptor proteins via death domains, in particular those of the MyD88 and IRAK proteins (Gay & Gangloff 2007).

1.1.1 Toll-like receptor 2: structure and function

TLR2 has been identified in the recognition of various PAMPs from (mainly Gram stain positive) bacteria such as lipoteichoic acids, peptidoglycan, and lipopeptides notably Pam_2CSK_4 and Pam_3CSK_4 from *E. coli*. TLR2 also recognises viral PAMPs such as envelope glycoproteins (detailed in 3.1.1). A recent review (van Bergenhenegouwen et al. 2013) identified that TLR2 knock-out/knockdown influences a wide variety of immune functions such as cell proliferation by increasing susceptibility to inflammation-related colon cancers (Lowe et al. 2010); resistance to bacterial infection via TNFα dysregulation (Takeuchi et al. 2000); insulin resistance by increasing fatty tissue mobilisation for energy (Ehses et al. 2010); susceptibility to autoimmune disorders from impaired responses to necrotic cells (Kim et al. 2007), and the response to parasitic infection accounted for by disrupted Th1/Th2 balance in the adaptive immune response (Reyes et al. 2011).

Of particular interest is that a study showed the ability of TLR2 to recognise human β 2-glycoprotein-I in the pathogenesis of antiphospholipid syndrome (Alard et al. 2010). This

indicates that the innate immune system not only recognises exogenous glycoproteins but also self-glycoproteins. TLR2 is thus an important cell surface receptor sensing both pathogenic and significant non-pathogenic molecular patterns. The current understanding of TLR2 function has mainly come from studies using bacteria rather than viruses.

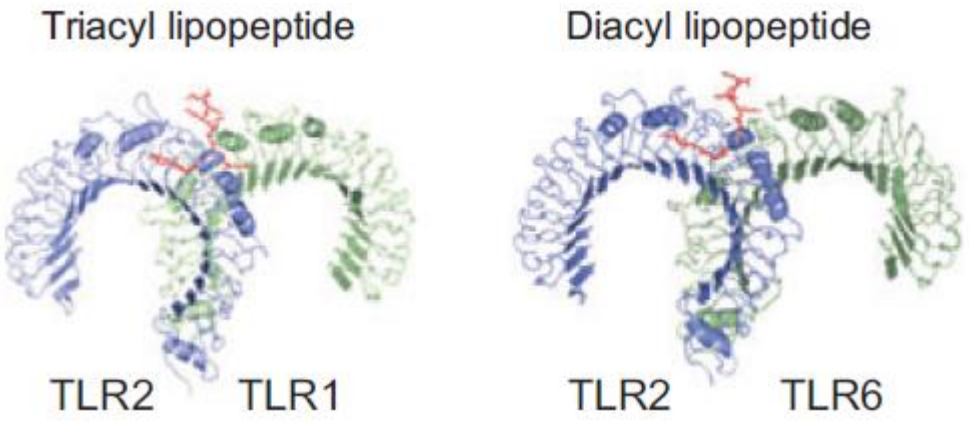


Figure 3. ***Extracellular domains of TLR2 heterodimerised with TLR1 and TLR6 to sense bacterial lipopeptides.*** **Adapted from Song & Lee 2012 (Song and Lee, 2012).**

As can be seen in figure 3 above, TLR2 activation requires the cooperation of other TLRs such as TLR1 or TLR6 which stabilise the interaction between bacterial lipopeptides and the heterodimer.(Ozinsky et al. 2000) TLR2 heterodimers can complex with other cell surface proteins to recognise other PAMPs. In the case of lipopeptide recognition, TLR2 complexes with CD14 along with TLR1 to initiate downstream signalling.(Manukyan et al. 2005) This observation has been well-studied in *P.gingivalis* models, (Hajishengallis et al. 2006) leading to an understanding that TLR2 requires not only the cooperation of other cell surface TLRs but also several other receptors including CD14 to form a large cell surface sensory protein complex. Indeed, the bioimaging analysis of TLR2 in peripheral blood monocytes confirmed that whilst TLR2 TLR1/TLR6 heterodimers exist at basal levels in the absence of PAMPs, they are formed largely in collaboration with other cell surface receptors in a PAMP-dependent manner.

Other evidence goes against the conventional understanding that TLR2 must heterodimerise to signal downstream: it was shown that diacylated lipopeptides were able to provoke a TLR2 mediated response in TLR1 or TLR6 -/- knockout mice.(Buwitt-Beckmann et al., 2006) Further study of *P.gingivalis* interactions with cell surface TLRs led to the notion that pathogens could recruit other cell surface receptors, such as CXCR4 (discussed in section 2.2.1 as an HIV-1 co-receptor) to inhibit TLR mediated downstream signalling, with therapeutic CXCR4 inhibition overcoming the evasion of the host immune system.(Hajishengallis G et al., 2008) In most cases, the binding of lipopeptides will cause TLR2 heterodimerisation. By example, bacterial triacyl lipopeptide recognition involves the insertion of two acyl chains in the TLR2 hydrophobic core, with the third acyl chain of the lipopeptide binding a TLR1 hydrophobic groove thereby bringing the two TLRs together.(Gay et al., 2014) So this perfectly demonstrates how the ligands effectively recruit their own TLR dimers for recognition, rather than TLR dimers being already present on the cell surface with monomers in association with each other.

1.1.2 Toll-like receptor 4: structure and function

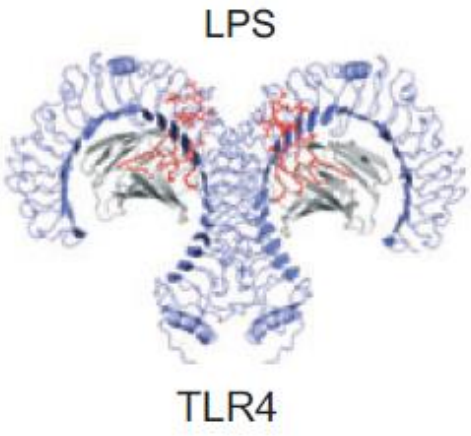


Figure 4. ***Extracellular domain of TLR4 homodimer with cognate lipopolysaccharide ligand*****. Adapted from Song & Lee 2012 (Song and Lee, 2012).**

TLR4 (partially depicted in figure 4 above), given its role in the pathogenesis of sepsis, has been highly studied in the context of bacterial lipopolysaccharide recognition (Gay et al. 2014). Like TLR2, TLR4 requires the association of other cell surface proteins to facilitate pattern recognition. An early study, making inferences from their findings in the *Drosophila* model and another LRR-containing receptor, RP105 (radioprotective 105kDa protein, which is not a PRR) in humans hypothesised that TLR4 would be found in association with an accessory protein similar to the situation of RP105 in association to MD1 (commonly known as lymphocyte antigen 86, LY86). TLR4 expressing cells significantly expressed MD2 but not MD1, implicating MD2 as the TLR4 accessory protein for pattern recognition (Shimazu et al. 1999). Soluble MD2 was found to highly increase TLR4 reporter cell responsiveness to

lipopolysaccharide, confirming the role of MD2 (commonly known as lymphocyte antigen 96, LY96) in TLR4 LPS sensing (Visintin et al. 2001).

Although TLR4 sensing of bacterial cell wall components also benefits from MD2 interaction, the therapeutic significance of MD2 is most apparent in the pathogenesis of sepsis and other diseases of TLR over-activation. Eritoran, a synthetic antagonist of MD2, prevents ligand binding and reduces excessive TLR mediated proinflammatory response in both sepsis and influenza murine models (Yeh et al. 2012; Shirey et al. 2013). A recent placebo-controlled trial of Eritoran in human sepsis suggested that MD2-TLR4 antagonism brought about by Eritoran was not protective against mortality (Opal et al. 2013). It is clear from this study that it is important to balance the need for TLR-mediated immune activation during host defence against infection, with the need to prevent excessive pro-inflammatory cytokine secretion.

MD2 is not the only accessory protein found in association with TLR4 during pattern recognition. In a cell-dependent manner, TLR4 may occur with a wide array of accessory proteins in association as a large activation cluster (Triantafilou & Triantafilou 2002). For example, Förster resonance energy transfer studies have clearly demonstrated monocyte sensing of LPS involving TLR4 associating with FcγRIIIa (antibody fraction crystallisable γ receptor IIIa), CD14 and 8 other CD cell surface proteins (Triantafilou et al. 2001; Shang et al. 2014).

Triantafilou and Triantafilou have demonstrated that membrane partitioning by lipid rafts augments the formation of these large TLR activation clusters. In nature, lipid rafts are not only localised to the plasma membrane, they may also occur in intracellular organelles such as the trans-Golgi network during protein handling (Resh 2004; Surma et al. 2012). Some accessory proteins are already present in proximity to TLR4, e.g. CD14 and heat shock

proteins 70 and 90 whilst others such as CXCR4 (discussed in section 2.2.1) arrive at the scene once pattern recognition has occurred (Triantafilou et al. 2001; Triantafilou et al. 2002; Triantafilou & Triantafilou 2002). Inhibition or absence of the TLR protein central to the activation cluster will prevent downstream signalling, i.e., the TLR is the receptor on which signalling is entirely dependent.

1.1.3 Toll-like receptors sense viral PAMPs

Many different viruses have been studied and well-characterised in both human and animal models in the context of TLR recognition, downstream signalling, and the contribution of this process to the pathogenesis of associated disease. The host's antiviral innate immune responses depend on TLR viral recognition, activation of signalling pathways and induction of cytokines and chemokines secretion. The cellular localisation of TLRs determines which PAMPs they can recognise (Loiarro et al. 2010; Kawai & Akira 2011). Intracellular TLRs recognise nucleic acid sequences to primarily drive a type-1 interferon antiviral response.

RECOGNITION OF DSRNA

TLR3 is a cellular receptor that recognises double stranded RNA sequences from many viruses such as murine cytomegalovirus and vesicular stomatitis virus (Edelmann et al. 2004). dsRNA is a replication intermediate for RNA or DNA viruses, and lysis of virus-infected cells is hypothesized to release dsRNA that can be detected by TLR3 molecules expressed on neighbouring cells.

RECOGNITION OF SSRNA.

The PAMP for TLR7 and TLR8 was identified as ssRNA. Influenza A virus, vesicular stomatitis virus (VSV), and human immunodeficiency virus type 1 (HIV) have been recognized by TLR7. (Diebold et al. 2004; Heil et al. 2004; Lund et al. 2004) A knockdown study of TLR7 and TLR8 showed reduced production of pro-inflammatory cytokines in the presence of human parechovirus-1 (HPEV-1) compared to wild-type target cells, implying that GU-rich (guanine uracil - rich) ssRNA from HPEV-1 is recognised by these intracellular PRRs (Triantafilou K et al. 2005).

RECOGNITION OF CPG DNA.

Herpes simplex virus type 1 (HSV-1), HSV-2, and murine cytomegalovirus (MCMV), all of which contain genomes rich in CpG DNA motifs, were shown to activate inflammatory cytokine and IFN-α secretion via TLR9. Mutations of TLR9 impaired the expression of type-1 interferons in response to viruses, implying that the unmethylated CpG DNA of DNA viruses is sensed by TLR9 (Tabeta et al. 2004).

RECOGNITION OF ENVELOPE GLYCOPROTEINS.

Viral envelope glycoproteins are another broad class of molecules that are emerging as the subject of TLR detection. One common theme among the viral glycoproteins implicated to date is that they play critical roles in the binding and/or entry of their respective viruses The F glycoprotein of RSV was shown to bind TLR2 resulting in the secretion of IL-6 (Kurt-Jones et al. 2000). Interestingly, TLR4 was additionally suggested to interact with RSV envelope proteins after it was found that TLR4 knock-out NK cells did not respond to infection. In their study, Bieback and colleagues showed that measles virus hemagglutinin is recognized by TLR2 inducing a potent IL-6 (an acute phase protein) response reflecting a potent pro-inflammatory response secretion (Bieback K et al. 2002). Furthermore, TLR4 senses viral capsid proteins of mouse mammillary tumour virus as well as those of Coxsackie virus B4. In both instances TLR4 is a major contributor to the host immune response (Bowie AG and Haga IR, 2005, Triantafilou K et al., 2005a).

1.2 Toll-like receptor signalling pathways

Medzhitov and Janeway (1997) demonstrated that cytokines were being expressed in response to downstream signalling via NFκB secondary to TLR binding (Medzhitov R et al. 1997). In all situations, TLR LRR binding with a PAMP results in the activation of NFκB and AP-1 (**a**ctivator **p**rotein **1**) transcription factors which stimulate production of cytokines and interferons. AP-1, a family of transcription factors including proto-oncogene c-Jun and p39, is also involved in pro-inflammatory signalling to the nucleus, though NFκB is the most highly studied and prominent second messenger in TLR activation.

NFκB and AP-1 transcription factors can be activated simultaneously by the same multitude of stimuli.(Abate et al. 1990; Karin et al. 2001) A number of reports have also shown that these transcription factors appear to be regulated by the same intracellular signal transduction cascades. The activation of JNK by inflammatory cytokines or by stress is often accompanied by the nuclear translocation of NFκB, and many genes require the concomitant activation of AP-1 and NFκB, suggesting that these transcription factors work cooperatively.(Verma et al. 1995)

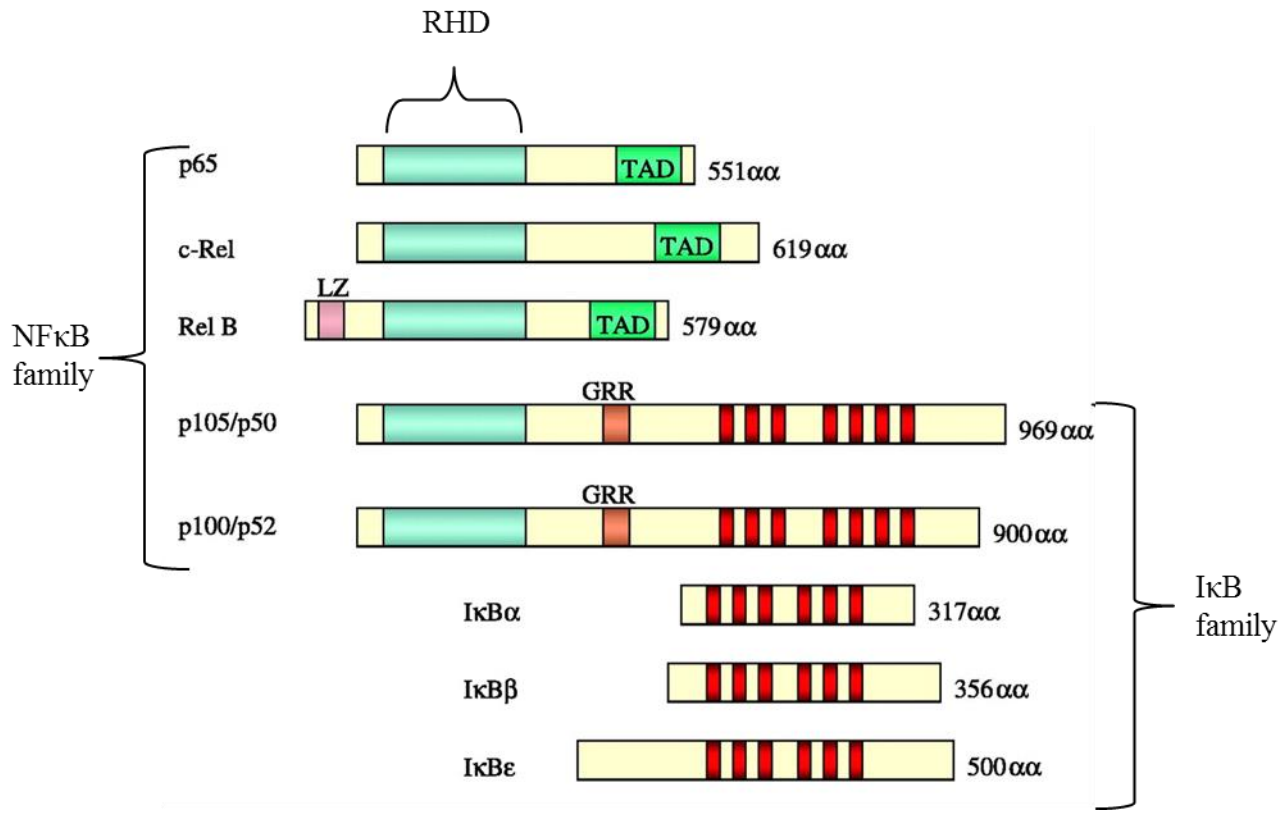


Figure 5. ***Structure and size of NFκB and IκB family proteins.*** **Abbreviations: RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper; GRR, glycine rich region; c Rel, V-rel avian reticuloendotheliosis viral oncogene homolog; Rel B, v-rel avian reticuloendotheliosis viral oncogene homolog B. Adapted from Lindstrom and Bennett 2005 (Lindstrom and Bennett, 2005).**

In the absence of TLR signalling, NFκB is retained in an inactive state by IκB (inhibitor of NFκB). TLR activation leads to a series of death domain interactions between various downstream adaptor molecules leading to the serine phosphorylation of IκB by the IKK (**Iκ**B **k**inase) complex consisting of the protein kinases IKKα and IKKβ along with a regulatory protein IKKγ. IκB is consequently destroyed by the 26S proteasome thereby removing the inhibition on NFκB which is now able to translocate to the nucleus to induce the expression of pro-inflammatory genes (Loiarro et al. 2010; Kawai & Akira 2011).

TLR adaptor molecules determine the specificity of the cytokine response. Many TLRs – 5, 7, 8, 9, 11– signal downstream via the MyD88 (myeloid differentiation factor 88) pathway. TLR3 uses TRIF (**TI**R-domain-containing adaptor protein inducing **IF**Nβ protein) instead. TLR 1, 2 and 6 recruit TIRAP (**T**oll-**I**nterleukin 1 **R**eceptor domain containing **A**daptor **P**rotein). TLR4 recruits TRIF and also MyD88 using TRAM (**T**IRAP and **TR**IF-related **A**daptor **M**olecule) (Loiarro et al. 2010; Kawai & Akira 2011).

Cytosolic TIR domains may bind TIRAP as well as MyD88. Homophillic death domain interactions with IRAK4 (**IL**-1**R**-**a**ssociated **k**inase 4) follow to regulate TRAF-6 (**T**NF **r**eceptor **a**ssociated **f**actor **6**). TRAF6 is an E3 ubiquitin ligase able to activate the TAK1 TABs complex which targets the IKK complex resulting in the phosphorylation of IκB. Once again NFκB is released from its inhibition to translocate to the nucleus and trigger expression of pro-inflammatory genes (Loiarro et al. 2010; Kawai & Akira 2011).

Non-canonical NFκB activation arises from NFκB-inducing kinase (NIK) activation which phosphorylates the IKKα subunit. NIK induced NFκB activation is a slower process relative to canonical activation, and becomes active following the K48 ubiquitin targeted degradation (mediated by E3 ubiquitin ligase) of an inhibitory TRAF complex. TRAF complex degradation arises from the binding of various ligands such as RANK (receptor activator of NFκB) to their upstream receptors (Ye et al. 2008; Sun 2012).

Ultimately TLR activation and downstream signalling may result in the secretion of pro-inflammatory cytokines including TNFα and IL-6. For example, these cytokines are secreted by macrophages in the acute phase response to infection, triggering local up-regulation of the innate immune system e.g., the complement system, as well as increasing the rate of antigen presentation. TNFα up-regulates migration of antigen presenting cells to the lymph nodes

where they are able to interact with T and B cells thereby informing the adaptive immune system of an invading pathogen.

TLR3 and TLR4, in the latter case with assistance from TRAM, may use the MyD88 independent pathway which is transduced by the TRIF adaptor molecule. Although NFκB and AP-1 are activated triggering the expression of pro-inflammatory cytokines, type-1 interferons are also induced. TRIF interaction with TRAF3 activates the inhibitory complex (IKK) of TRAF-associated NFκB activator **T**ANK-**b**inding **k**inase 1 (TBK1) as well as IKKε resulting in IRF3 and IRF7 phosphorylation. These two transcription factors then homodimerise or heterodimerise prior to entry in the nucleus and IFNβ enhancer binding. Thus, IRF-3 and IRF-7 may act alone or together to induce or regulate IFNα and IFNβ which, as type-1 interferons, promote a potent antiviral response(Loiarro et al. 2010; Kawai & Akira 2011).

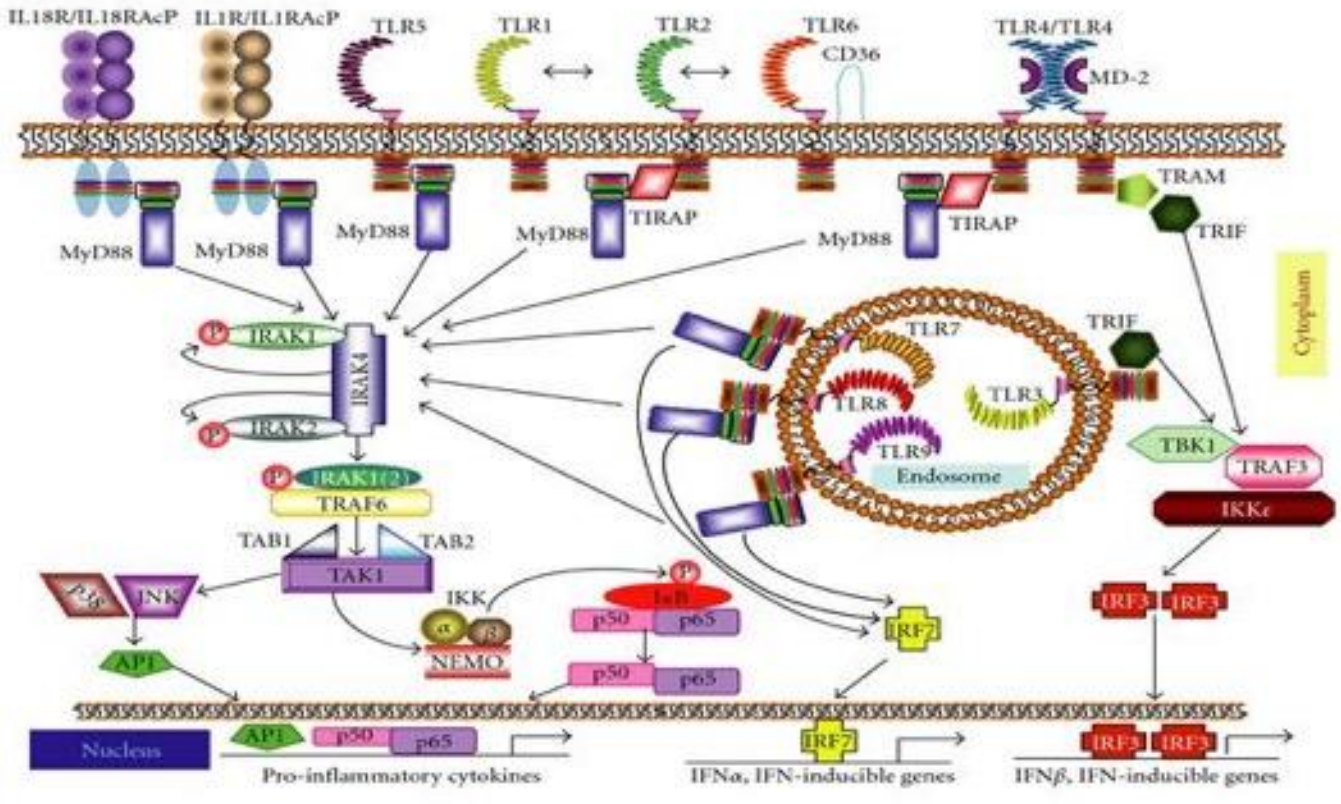


Figure 6. ***Stylised schematic diagram of TLR downstream signalling showing functional similarity with IL-1R & IL-18R signalling via MyD88*****. From (Loiarro et al., 2010). IRF3 homodimers shown: IRF7 homodimers may replace these, IRF3 may heterodimerise with IRF7 in the type-1 interferon activation pathway. TLR4 dimers may complex with CD14 (not shown) in addition to MD2. Abbreviations: TLR, toll-like receptor; MyD88, myeloid differentiation factor 88; IRAK, IL-1R-associated kinase; TRIF, TIR-domain-containing adaptor protein inducing IFNβ protein); TIRAP, Toll-Interleukin 1 Receptor domain containing adaptor protein; TRAM, TIRAP and TRIF-related adaptor molecule; IKK, inhibitor of nuclear factor κ B (IκB) kinase complex; NEMO, NFκB essential modulator otherwise termed IKKγ, IκB kinase subunit γ; NFκB, nuclear factor κ B; AP-1, activator protein 1.**

Due to complex cross-talk between second messengers at various points in the signalling cascade (as simplified in figure 6 above), a mixed response can potentially occur. This depends on which TLRs are being stimulated in synchrony or alone during an encounter with a PAMP or PAMPs. Indeed, TLR4 and TLR3 may act to cause expression of both type-1 interferons and caspase-1 recruitment, as well as pro-inflammatory cytokines via cross talk between TRIF and TRAF-6. Figure 7 overleaf summarises the complex picture of TLR signalling in a simplified model.

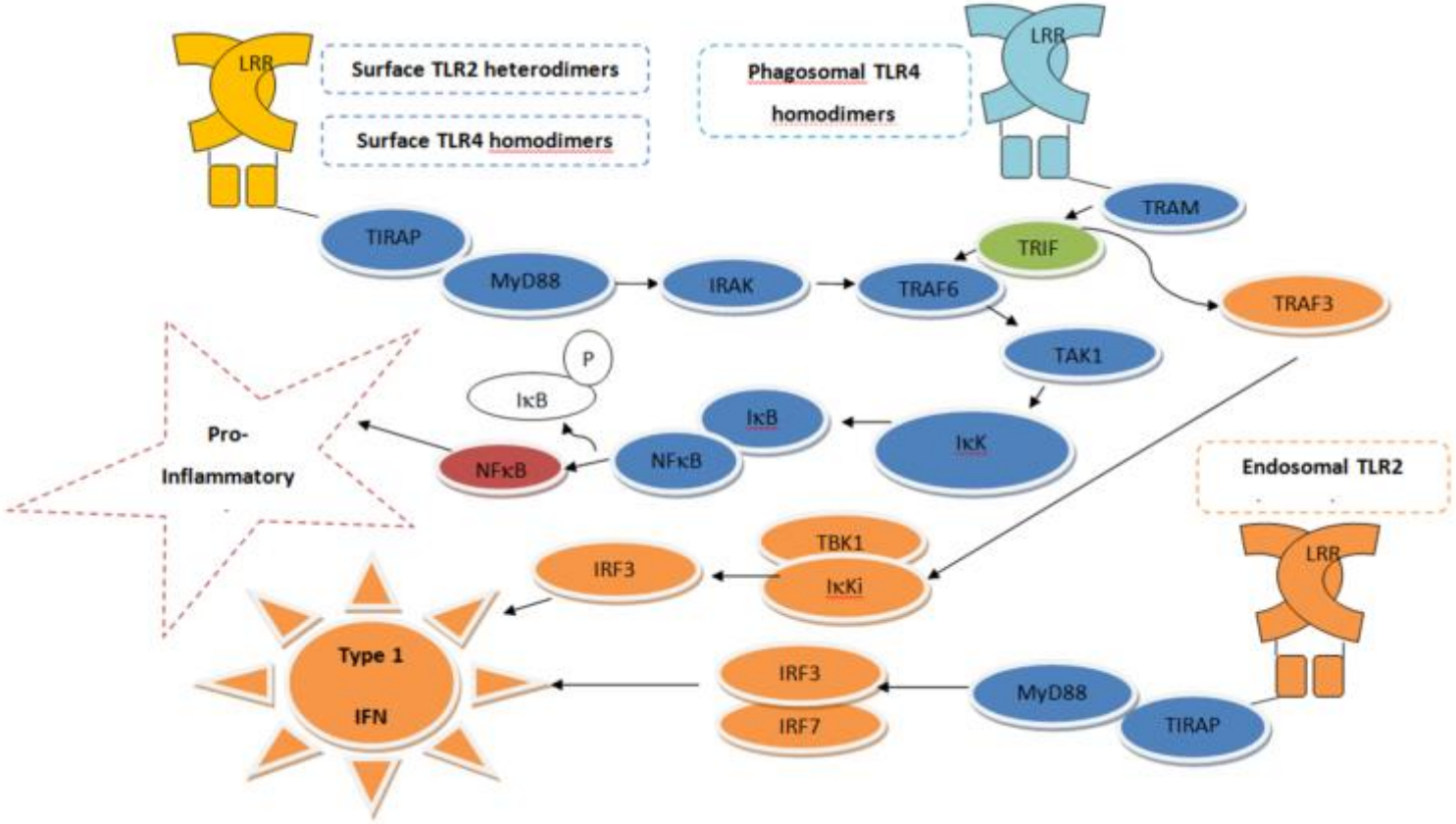


Figure 7. Simplified model summarising the complex picture of TLR downstream signalling pathways.

In an ideal world, putative therapeutic modulation of TLR activation levels to encourage the pro-inflammatory pathway over the antiviral interferon pathway or vice versa depending on the situation would lead to highly advantageous clinical outcomes. This principle has long been used therapeutically in the context of human papilloma virus infection, where imiquimod and similar compounds are used to induce TLR7/8 over-activation helping to clear the virus (Sauder DN 2003). Indeed, exploration of therapeutic targeting of TLR4 in the pathogenesis of bacterial sepsis has demonstrated that moderated TLR4 activation is necessary for directing the immune response against the bacterium, whilst too much TLR4 activation is commonly seen in sepsis-related mortality (Savva & Roger 2013). The question remains whether TLR activation could be successfully modulated in the context of severe viral infection to the advantage of the host.

1.3 Structure and function of the NLR inflammasome

Nucleotide oligomerisation-binding domain and **L**eucine-rich repeat domain containing **R**eceptors (NLRs) are cytoplasmic proteins, some of which recruit and activate caspase-1, forming the inflammasome, a large multi-protein complex which enacts the proteolytic cleavage of pro-interleukin 1 beta (pro-IL-1β) in to its active form (IL-1β) as well as similarly maturing interleukin-18 and -33. These three interleukins are secreted to affect a potent pro-inflammatory, antiviral and immunoregulatory response respectively (Martinon et al. 2002; Franchi et al. 2012; Rathinam et al. 2012; Vanaja et al. 2015). It is worth noting that receptors outside the NLR family are able to recruit and activate caspase-1 forming their own inflammasomes, these include the nucleic acid sensors AIM2 (absent in melanoma 2) and RIG-I-like (retinoic acid inducible gene-1) receptors (Vanaja et al. 2015; Lupfer et al. 2015).

IL-1β is an endogenous pyrogen understood to have potent pro-inflammatory effects during infection. IL-1β has multiple functions during the initial response to infection. This cytokine

increases the hypothalamic thermoregulatory set-point thereby contributing towards increasing body temperature over 37° Celsius; triggers expression of acute phase proteins, and potentiates TNFα and other pro-inflammatory cytokines (Netea et al. 2010; LaRock & Cookson 2013). IL-1β was discovered via observed structural homology to interleukin-1 alpha (IL-1α), a cytokine with comparable functions, which at the time was functionally termed LAF – lymphocyte activating factor (Van Damme et al. 1985).

IL-18 is a relatively more recently discovered (Ushio et al. 1996) pro-inflammatory cytokine known best for its ability to potentiate the interferon response in target cells via induction of T-helper cell secretion of interferon γ - which potentiates (type-1) interferon α/β pathways and activates macrophages (Nakanishi et al. 2001). IL-18 also upregulates NK cell activity in the spleen. The activities of IL-18 therefore bridge innate and adaptive mechanisms against infection (Okamura et al. 1998).

NLRs show structural homology to the caspase 9 activating APAF-1 (apoptotic protease-activating factor-1). The assembly of multiple NLRs, plus or minus adaptor proteins known as apoptotic speck-like protein (ASC) (Agostini et al. 2004) and caspase-1 proteins into a large macromolecular protein complex is known as the inflammasome by virtue of its ability to potentiate potent pro-inflammatory (and indeed) antiviral signals. Generally, NLRPs comprise a C' terminal LRR domain connected via a NOD domain to the N' terminal pyrin domain (PYD), (hence NLR*P*). NLRP1 and NLRP12 possess a FIIND (function to find) domain connecting NACHT (formed by **NA**IP – neuronal apoptosis inhibitor protein, **C**2TA – MHC class 2 transcription activator, **H**ET-E – incompatibility locus protein from *Podospora anserine*, and **T**P1 – telomerase-associated protein) and CARD (caspase activation and recruitment domain). NLRCs instead contain an N'terminal CARD domain (hence NLR*C*) to allow direct caspase-1 interaction. As distinct from the NLRPs and NLRCs,

NAIP NACHT domains connect the LRR to a BIR (baculovirus inhibitor of apoptosis repeat) domain (Martinon et al. 2002; Franchi et al. 2012; Rathinam et al. 2012; Vanaja et al. 2015).

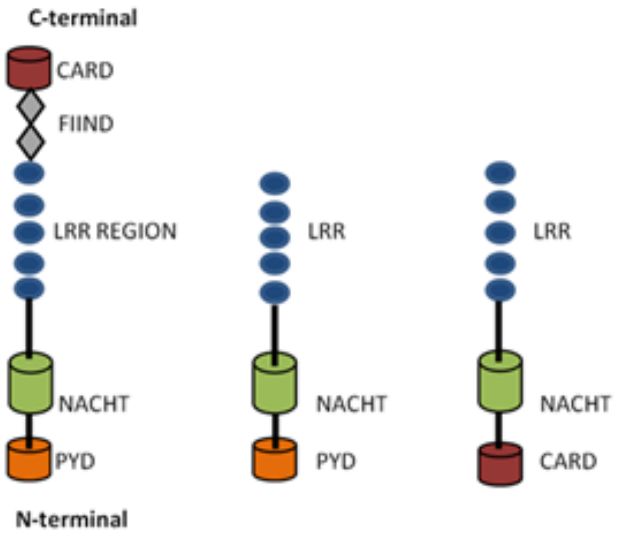


Figure 8. ***Structures of NLRP1, NLRP3 and NLRC4.*** **Abbreviations: LRR, leucine rich repeats; NACHT, see paragraph below; FIIND, function to find domain; PYD, pyrin domain. Adapted from Ranson and Eri 2013 (Ranson and Eri, 2013).**

The NACHT domain; though it may come as little surprise by virtue of its somewhat lengthy disambiguation, is a highly conserved protein throughout nature and is able to bind ribonucleotides allowing regulation of inflammasome component recruitment and activation (B K Davis et al. 2011).

Two common NLR domains, PYD and CARD are used for inflammasome activation and share an arrangement of six to seven antiparallel alpha helices with a hydrophobic core and an outer surface of charged residues. This arrangement is otherwise known as the death fold motif which is common to many protein domains involved in apoptotic and inflammatory responses. The PYD of NLRPs is a subclass of the death fold motif proteins. The PYD domains of particular downstream CARDs interact with the NLRP sensors thereby linking

LRR sensing to caspase-1 recruitment and activation. CARDs are highly conserved across many different protein types such as kinases, helicases, NLRs and caspases, to name a few. CARDs are known to interact with each other to link proteins in large functional complexes: the inflammasome CARDs are no different in their activity. CARDs possess PYDs which are able to interact with PYD domains of NLRPs which do not contain their own CARDs, thereby bridging caspase-1 CARDs and NLRP N' terminal PYDs permitting inflammasome recruitment (Martinon et al. 2002; Franchi et al. 2012; Rathinam et al. 2012; Vanaja et al. 2015).

1.4 Different NLRs recognise different pathogens

NLRs encompass a large subfamily of LRR containing proteins which include NLRP, NAIP and NLRC protein families. NLRP3 is by far the most studied protein in its subfamily for its interactions with various ligands from a plethora of pathogens and is not thought to require the use of a NAIP as an adaptor, as is the case for NLRC4 downstream signal transduction (Franchi et al. 2012; Vanaja et al. 2015).

1.4.1 NLRP1b

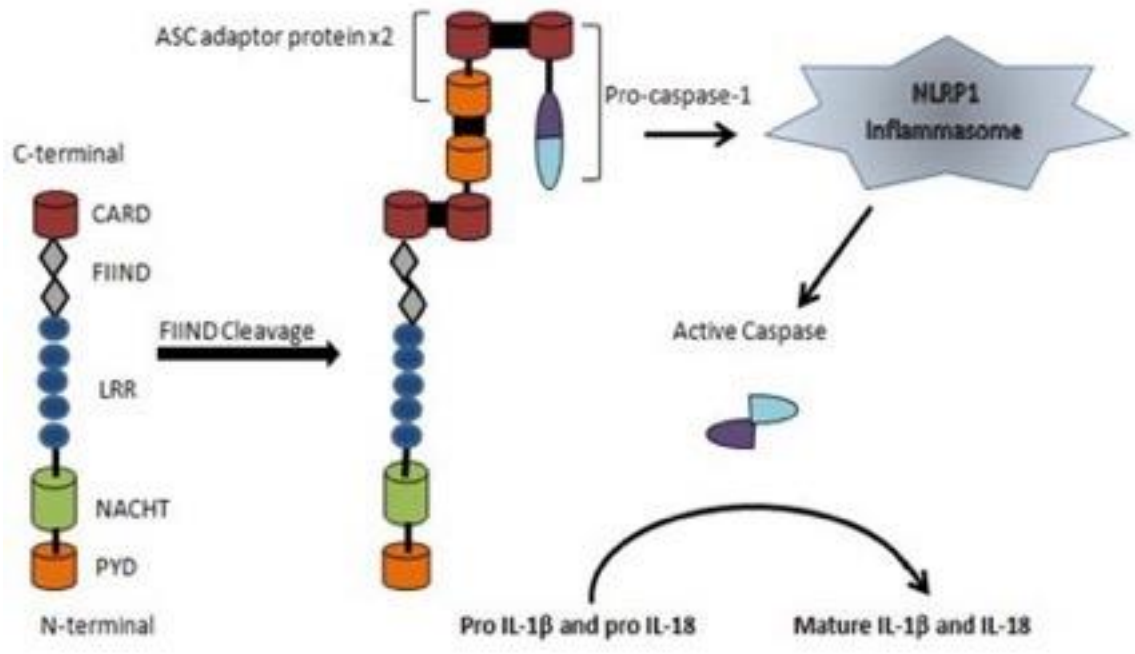


Figure 9. ***NLRP1b structure and downstream signalling*****. Somewhat uniquely, NLRP1b possesses a function to find (FIIND) domain which is autocleaved to bring the NLR into its active conformation. Adapted from Ranson and Eri (2013) (Ranson & Eri 2013).**

NLRP1b (around 1,443AA) has 7 identified isoforms occurring in nature. In humans, NLRP1 is expressed in cells of the adaptive immune system and cells derived from non-haematopoietic lineage. NLRP1b inflammasomes (figure 9) may comprise caspase-1 or

caspase-5. Much of the pathogen recognition activity of NLRP1b remains to be determined, initially limited by a lack of viable animal knockdown models to thoroughly investigate its function in this regard. It is known that anthrax lethal toxin (LeTx) can activate the NLRP1b inflammasome via LeTx mediated cleavage at the NLRP1b N' terminus and that this mechanism is crucial for murine defence against *B. anthracis* (Vanaja et al. 2015). NLRP1b has also been implicated in the pathogenesis of the autoimmune skin depigmentation disease known as vitiligo. Whether ASC is required for NLRP1 inflammasome activation remains controversial (Jin et al. 2007; Vanaja et al. 2015). Throughout the text, NLRP1b is shortened to NLRP1.

1.4.2 NLRP3

NLRP3 was first identified in studies of the cryopyrinopathies from which it took its initial name (cryopyrin). Mutations in NLRP3 were associated with a cryopyrinopathy, namely cryopyrin-associated periodic syndrome type 1 (CAPS1) which is a syndrome of four autoimmune disorders including familial cold autoinflammatory syndrome. NLRP3 has been demonstrated to be universally expressed in myeloid lineage cells and the potential for NLRP3 expression in lymphoid cells is studied in detail in Chapter 3. The interaction of NLRP3 and CARD8 remains under investigation (Martinon et al. 2002; Rathinam et al. 2012; Franchi et al. 2012). Without a functional NBD/NACHT domain, NLRP3 is unable to form an inflammasome (Ye et al. 2008).

NLRP3 is known to recognise and respond via inflammasome assembly (figure 10) and activation to a wide array of different signals arising from various pathogens and small particulate matter such as urate crystals. The structure of the NLRP3 inflammasome was initially proposed as a circular, Ferris Wheel-like arrangement of outer NLRP3 leucine-rich repeats around central caspase-1 proteins linked by internal NACHT then PYD to caspase-1 via ASC adaptor proteins (Horvath et al. 2011). More recently, a study of NLR sensing of salmonella in macrophages revealed through advanced imaging an entirely reversed, conical arrangement of the NLRP3 inflammasome, that being an "outer ring of apoptosis-associated speck-like protein containing a caspase activation and recruitment domain and an inner ring of NLRs" with caspase-1 proteins pointing inwards to the centre of the complex (Man et al. 2014). The precise structure of the NLRP3 inflammasome, however, remains to be determined.

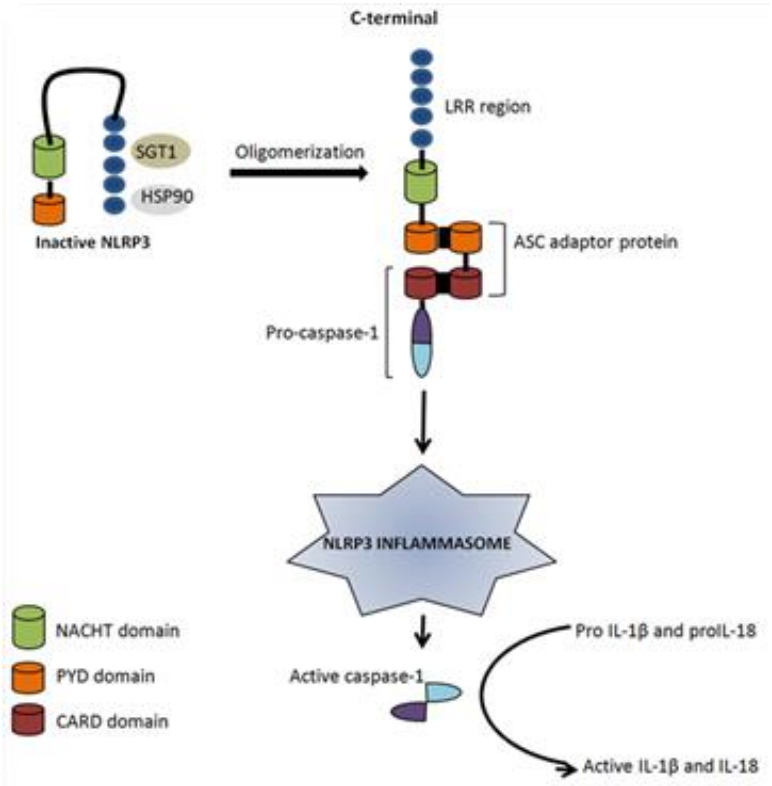


Figure 10. ***Structure of NLRP3 and downstream signalling.*** **Signal 2 results in the removal of NLRP3 inhibitory chaperone proteins SGT1 (suppressor of G2 allele of SKP1) and HSP90 (heat shock protein 90) allowing NLRP3 to oligomerise, recruiting pro-caspase-1 which undergoes**

autolytic cleavage to form the active inflammasome. Adapted from Ranson and Eri 2013 (Ranson & Eri 2013).

Overall, inflammasomes are thought to be activated in a two signal process requiring an initial priming 'signal 1' from other PRRs such as TLRs in response to a ligand; which via NFκB upregulates immature inflammasome components (pro-caspase-1 etc.) as well as the immature cytokines (e.g. pro-IL-1β) for later catalytic activation by the completed inflammasome (Bauernfeind et al. 2009; Latz E 2010). Signal 2 directs the NLR into an active conformation either by autoproteolysis of FIIND (NLRP1) or dissociation of inhibitory chaperones (NLRP3) to recruit ASC and caspase-1, which undergoes autoproteolysis into its active subunits, p20 (20kDa) and p10 (10kDa). Activated caspase-1 components induce proteolytic cleavage of the immature cytokines pro IL-1β and pro IL-18 into their active forms, IL-1β and IL-18 (Horvath et al. 2011; Vanaja et al. 2015).

The action of cytokines produced by caspase-1 downstream of NLR sensing results in a phenomenon known as pyroptosis, literally meaning 'falling with fire'. This type of cell-death is similar to apoptosis in that it is programmed; however it is distinct from apoptosis because the process involves the initial secretion of warning signals in the form of caspase-1 cytokines to provoke a 'fiery' pro-inflammatory response (Cookson & Brennan 2001).

Models of inflammasome activation (i.e., signal 2) have been studied in the context of NLRP3 sensing due to its ability to recognise and respond to a broad range of DAMPs. Putative models underlying signal 2 stimulation resulting in activation of NLRP3 to induce pyroptosis via inflammasome assembly and activation are numerous. Proposed models of signal 2 stimulation have historically included extracellular ATP, reactive oxygen species (ROS) via lysosomal destabilisation; or changes in intracellular cation concentration such as K^+ and Ca^{2+} (Horvath et al. 2011; Franchi et al. 2012; Triantafilou & Triantafilou 2014).

As cells become damaged by the actions of pathogens or particulate matter, reactive oxygen species (ROS) such as hydrogen peroxide may be generated. In an asbestos model, Hornung and colleagues demonstrated NLRP3 activation following induced phagosomal destabilisation and release of ROS into the cytosol (Hornung et al. 2008). Another potential source of ROS is from damaged mitochondria. It was found that blocking elements of the mitochondrial respiratory chain or mitochondrial degradation (mitophagy) generated ROS which activated the NLRP3 inflammasome (Zhou et al. 2011). Evidence underlying this process comes from investigation of TXNIP (**t**hiore**d**oxi**n-i**nteracting **p**rotein) a factor linked to insulin intolerance, which was shown to bind NLRP3 following stimulation with known NLRP3 activators in the presence of H_2O_2 (Zhou et al. 2010). It was later shown that TXNIP can link the NLRP3 inflammasome to ROS signalling in the context of the pathogenesis of diabetic retinopathy (Devi et al. 2012). Some evidence suggests that ROS acts upstream of the inflammasome and is involved in signal 1 – NFκB priming. It was shown that ROS scavenger treatment prior to stimulation with signal 1 ligands flagellin and LPS reduced in signal 1, a process required for successful inflammasome activation, which was the limiting factor for caspase-1 activation (Bauernfeind et al. 2011). This suggests that for common bacterial PAMPs such as flagellin and LPS, ROS does not necessarily act directly on the NLR.

Certain crystal structures such as silica or aluminium salts are able to activate the NLRP3 inflammasome in a cathepsin B dependent mechanism. Initial evidence of cathepsin B involvement in inflammasome activation arose when it was found that cathepsin B inhibition prevented nigericin-induced (i.e., K^+ ionophore induced) caspase-1 activation and IL-1β secretion (Hentze et al. 2003). Silica is not the DAMP which directly activates NLRP3, this process relies upon silica disrupting host lysosomal permeability. As the lysosome containing the crystal acidifies, the internal cysteine protease cathepsin B is activated along with other

lysosomal proteases which degrade their host lysosomes releasing danger signals to activate NLRP3 (Hornung et al. 2008).

Strong evidence also implicates Ca^{2+} as an additional source of signal 2. Early work suggested NLRP3 is activated by divalent calcium ion signalling as a consequence of ultraviolet irradiation (Feldmeyer et al. 2007). The NLRP3 inflammasome was prevented from activation by ATP via blockade of calcium mobilisation by another group some years later,(Murakami et al. 2012) sparking investigation into pathogen induced calcium mobilisation in the context of virus activity. Two recent studies identify calcium mobilisation by pore forming channels as an important signal in NLRP3 inflammasome activation. The calcium pore-forming membrane attack complex formed during complement responses to infection was shown to activate the NLRP3 inflammasome (Triantafilou, Hughes, et al. 2013), as were the HRV-associated calcium conducting membrane-integral pores (Triantafilou, Kar, van Kuppeveld, et al. 2013).

Franchi and colleagues (2007) defined a model of extracellular ATP-based signal 2 induction in the context of bacterial infections purported to stimulate the NLRP3 inflammasome (Franchi et al. 2007). Noting that K^+ efflux could be brought about by ATP binding the purine receptor P2X7 to bring about opening of K^+ selective channels via the pannexin-1 protein, it was found that inflammasome activation could not occur in ATP-pulsed cells previously incubated with pannexin-1 peptides. Thus it was suggested that extracellular ATP could be a DAMP produced by the activity of various bacteria inducing NLRP3 activation and subsequent inflammasome recruitment (Franchi et al., 2007). The role of K^+ efflux as a critical signal for NLRP3-inflammasome activation gained further strength when a group stimulated THP1 monocytes with various cognate NLRP3 activators such as monosodium urate, peptidoglycan and nigericin in the presence of high or low medium concentrations of

K^+. At high K^+ medium concentration, K^+ efflux could not occur: the absence of caspase-1 and IL-1β in subsequent immunoblot analysis was very clear in the data shown (Petrilli et al., 2007).

Years later, a landmark study evaluating the various models of NLRP3 activation showed K^+ efflux to be the central downstream process (Munoz-Planillo et al. 2013). The study first confirmed findings from Petrilli et al 2007 (Petrilli et al. 2007) that zero K^+ concentration medium was indeed able to activate the NLRP3 inflammasome by provoking cellular K^+ efflux. Four different mitochondria-toxic agents could not induce NLRP3, suggesting limitations in the earlier theory that signal 2 reflects ROS release from damaged mitochondria (Zhou et al. 2011). The other mechanisms involving increased extracellular calcium,(Feldmeyer et al. 2007) or particulate matter e.g. crystal phagocytosis (Hornung et al. 2008) were found to be acting upstream of K^+ efflux, with Na^+ influx correlating with, though was dispensable for NLRP3 activation in their model (Munoz-Planillo et al. 2013). Assuming the K^+ efflux theory of NLRP3 activation is correct, a major unknown therefore is that of is how K^+ efflux interacts directly (or indirectly via postulated upstream adaptor proteins) to transmit signal 2 to the NLRP3 sensor to recruit and activate the inflammasome. Regardless of the mechanism, it is clear that intracellular ion homeostasis somehow precisely regulates inflammasome activation (Triantafilou and Triantafilou, 2014).

1.4.3 NLRC4

NLRC4, initially termed IPAF (ICE – IL-β converting enzyme i.e., caspase-1, protease activating factor) before inflammasome pathways were fully understood, presents in nature as four isoforms. Isoform 1, reflecting the 'canonical sequence' (1024 AA in length) is expressed highly in the gut, lung, brain, monocytes and other leukocytes. Isoform 2 is ubiquitously expressed, though highly concentrated in the lung and spleen. NLRC4 activation

has been well studied in the context of bacterial flagellin. Flagellin appears to require the presence of upstream adaptor proteins NAIPs, particularly NAIP2 to activate the NLRC4 inflammasome (Vladimer et al. 2013). NLRC4 is to some extent compensated for the need of upstream adaptor proteins by the fact its CARD domains can directly interact with caspase-1 without the need for ASC linkage (Matusiak et al. 2015).

NLRC4 is implicated in sensing and responding via inflammasome recruitment to a range of gut bacteria. NLRC4 mutations are implicated in the development of autoimmune disorders such as FACS4 (Kitamura et al. 2014) and auto-inflammation with infantile enterocolitis,(Romberg et al. 2014) characterised by the development of enterocolitis shortly after birth and periodic fever which may reflect underlying periods of severe autoinflammation. Cooperation with other NLRs such as NLRP3 is possible under certain conditions (Man et al. 2014).

Little is known about the involvement of NLRC4 in sensing viruses, since it is clear that much substantive work has focused heavily on the gastrointestinal pathologies arising from bacteria, as described in this section. A recent murine model study implicated a bystander effect of NLRC4 sensing of bacterial flagellin in the presence of rotavirus infection, indicating that in the presence of different PAMPs, NLRC4 may aid and abet the immune response to pathogen types it is not classically understood to recognise (Zhang et al. 2014).

1.4.4 NLRC5

NLRC5 is an intriguing protein expressed in the spleen, thymus, lung, brain, tonsil, heart and prostate as well as lymphoid and myeloid cells.(Meissner et al. 2012) Whilst NLRC5 does not form an inflammasome, its activity has been shown to modulate the immune response downstream of inflammasome activation during antiviral responses. NLRC5, the largest protein of the NLR family,(Lamkanfi & Kanneganti 2012) translocates to the nucleus (Benko

et al. 2010) following putative interactions with upstream NLRs and RLRs, as well as the IκK complex as described in section 1.2 as an integral part of the MyD88 dependent TLR signalling pathway (Neerincx et al. 2013). Mechanisms of NLRC5 activation are summarised in figure 11 below.

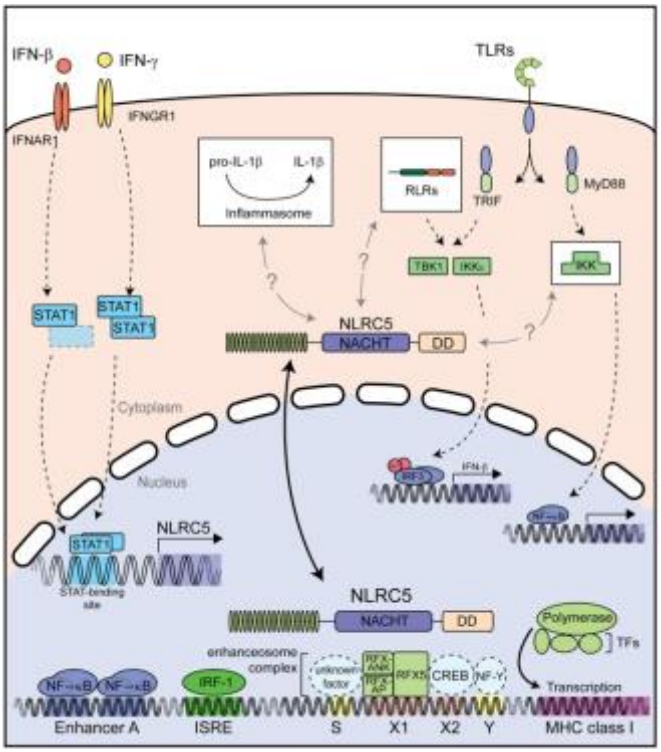


Figure 11. ***Overview of NLRC5 functions.*** **Induction by NLRC5 by the JAK/STAT pathway was first implied by cytomegalovirus experiments in primary human cells which also represents the first discovery of NLRC5 involvement in antiviral immune responses. Adapted from Neerincx et al 2013 (Neerincx et al. 2013).**

In the nucleus, NLRC5 interacts with various complexes to modulate transcription of immunogenic proteins such as NFκB, IRF-1 and MHC-I. A landmark NLRC5 over-expression study first determined that NFκB and IRF-1 expression was significantly attenuated compared to that of wild-type HEK293T cells. The same study finding that NLRC5 knockdown in a murine cell model led to large over-activation of the pro-

inflammatory cytokines these transcription factors upon stimulation with LPS or interferon γ (Benko et al. 2010).

Later, NLRC5 was found to be highly interferon γ inducible in THP1 and HeLa cells as well as other easily cultivable secondary cell lines in the human *in vitro* model. NLRC5 involvement regulating the antiviral response arose however from experiments addressing RLR-mediated responses to synthetic (polyI:C) and wild type (VSV) dsDNA (Cui et al. 2010). In anti-NLRC5 treated or NLRC5 knock-down cells it was found that dsDNA stimulation led to highly upregulated cytokine responses. Stimulation of male reproductive tract fibroblasts with dsDNA from cytomegalovirus provoked a strong paracrine interferon γ effect on NLRC5 activation, thereby implicating the JAK/STAT pathway in NLRC5 induction by *self* rather than pathogen signals to prime cells to respond to a pathogen they have not as yet sensed directly by whatever means (Kuenzel et al. 2010).

Studies by Cui et al 2010 and Kuenzel 2010 as described above therefore reflect conflicting models of NLRC5 involvement in the antiviral immune response. Cui et al 2010 suggests a negative regulatory model and Kuenzel 2010 suggests a positive one (Cui et al. 2010; Kuenzel et al. 2010). Further complexity arises by considering the relationship of NLRC5 with the upstream inflammasome, wherein experimentally induced absence of NLRC5 in primary monocytes prevented detection of key inflammasome components caspase-1, IL-1β and IL-18 (Beckley K Davis et al. 2011). Nevertheless, NLRC5 is now an important focus of study following the recent discovery of its ability to positively regulate MHC-class-I expression, making this NLR a critical regulator of the immune response to a vast array of pathogens (Kobayashi & van den Elsen 2012).

1.4.5 NLRP12

Otherwise known as Monarch 1, NLRP12 is expressed by peripheral blood leukocytes namely eosinophils and granulocytes, and at relatively lower levels in monocytes. This NLR was initially implicated in the recognition of mycobacterium tuberculosis and the pathogenesis of familial cold auto-inflammatory syndrome type 2 (FACS2) (Williams et al. 2005). In contrast to the inflammasome stimulatory actions of NLRP1, NLRP3 and NLRC4 amongst others, NLRP12 was postulated to play a negative regulatory role during pathogen sensing wherein it was thought to interact with the NIK-mediated non-canonical pathway of NFκB activation (Ye et al. 2008).

Currently, the question of whether NLRP12 can recruit and activate the inflammasome in response to various PAMPs or DAMPs or have an inhibitory role in immune responses remains under investigation. This question is important due to the emerging evidence that NLRP12 may play a role in the development of colon cancer,(Zaki et al. 2011) and more recently the immune response to malaria and *T. cruzii*: in the latter case NLRP12 was thought to be acting in concert with NLRP3 sensing (Pinheiro et al. 2011; Tuncer et al. 2014; Cunningham 2002; van de Veerdonk et al. 2011; Ataide et al. 2014). NLRP12 has also been proposed to form an inflammasome during Yersinia pestis infection, NLRP12 is reported to recognize acylated lipid A and Nlrp12−/− mice were more susceptible to infection and had reduced IL-18 levels (Vladimer et al. 2012).

It is therefore possible that NLRP12 may have a dual role in the innate immune response acting as a positive or negative regulator of such responses. This may depend upon on the various potential PAMPs or DAMPs that NLRP12 is encountering. Little is known about the involvement of NLRP12 in either recognising viruses or regulating the immune response to viruses.

1.4.5 Non-canonical inflammasome activation

It is also important to note that caspase-1 is not unique in its ability to form an inflammasome. Murine caspase-11 (considered to be orthologous with human caspase-4) forms an inflammasome to signal pyroptosis in response to non-canonical inflammasome stimuli such as cholera toxin B. A landmark paper (Kayagaki et al. 2011) compared the effects of canonical inflammasome activation versus cholera toxin B (CTX-B) and other stimuli not thought to activate NLRP3; in caspase-1 and/or caspase-11 knockout murine macrophages. The key finding was that cholera toxin B could not induce IL-1β in caspase-11 deficient murine macrophages, supporting a novel – non-canonical – model for upstream sensing. It was also found that in caspase-1 expressing mice, caspase-11 knockout was able to protect the model from a lethal dose of LPS.

Further investigation in the murine bacterial infection model implicated a role for ROS in the formation of non-canonical inflammasome activation (Lupfer et al. 2014). More recently, another murine model study investigating the innate immune response to enteric bacteria has revealed that inflammasome activation involves caspase-11 mediated cleavage of gasdermin D, which leads to pyroptosis (Kayagaki et al. 2015).

Non-canonical inflammasome activation has been well-studied in mice, though a recent study has made intriguing observations in the human model. *Ex vivo* human macrophages stimulated with various non-canonical Gram negative bacteria induced caspase-4 dependent pyroptosis mediated by IL-1α, rather than IL-1β (Casson et al. 2015). The concept of caspase-4 involvement in non-canonical inflammasome activation is also supported by the finding that LPS transfected THP1 cells undergo pyroptosis despite induced caspase-1, and therefore IL-1β deficiency. In this study, caspase-4 was observed to participate in the NLRP3 inflammasome (Schmid-Burgk et al. 2015). Ultimately, a degree of overlap between

the canonical and non-canonical inflammasomes suggests an evolutionary strategy to ensure pyroptosis when caspase-1 is potentially otherwise engaged with other DAMPs (Vanaja et al., 2015).

1.5 Unknowns in PRR sensing

Sensing of pathogens by TLRs and NLRs is a complex process which is not fully understood. Although the crystal structures of many PRRs have been characterised, particularly those of the TLRs, it is not known exactly how leucine-rich repeats belonging to TLRs and NLRs interact with PAMP and DAMP signals (Kawai & Akira 2011). In the case of NLR activation, K^+ efflux needs to be observed as a common denominator in all instances of pathogen recognition before it is accepted as the central process underlying inflammasome recruitment and activation. Many studies have investigated PRR sensing on the principle that pathogens commonly occurring in specific anatomical locations, e.g. the gut, will constitute the bulk of activators for PRRs highly expressed in those tissues. This is not always the case, as is exemplified by NLRP3 sensing of alum and other exogenous stimuli (Hornung et al. 2008).

Indeed, the 'million dollar question' in NLR research is that of how so many molecularly-distinct signals can cause inflammasome activation through a common NLR, such as NLRP3. Exogenous TLR7/8 over-activation can be utilised in the clearance of HPV infection therapeutically,(Sauder DN 2003) thus of particular interest is the exact mechanism which allows NLRs to sense and respond to viruses to provoke potent immune responses. Studying this mechanism may help the development of compounds able to potentiate the secretion of IL-1β, IL-18 to positively regulate an appropriate immune response in cases of insufficient viral clearance by the host immune system, such as in HIV-1 infection.

2. HIV-1 and Acquired Immune Deficiency Syndrome (AIDS)

2.1 The natural history of HIV-1 infection

An estimated 35 million people worldwide are living with HIV-1 infection. Untreated infection with human immunodeficiency virus type-1 (HIV-1) leads to progressive deterioration of the human immune system over time ultimately leading to mortality.((UNAIDS) 2013) The major cause of the deterioration of the immune system is a consequence of HIV-1 invading and ultimately destroying human CD4 T-helper cells (Levy JA 2009).

HIV-1 (human immunodeficiency virus type-1) was first identified as the causal factor of AIDS (acquired immune deficiency syndrome) by Gallo and Montangier. HIV-1 was isolated from the blood of donors with AIDS or at risk of developing AIDS (Chermann et al. 1983; Broder & Gallo 1984). Although CD4 T-cell infection and subsequent depletion is the primary cause of disease, HIV-1 may also successfully infect a plethora of cells classically grouped under the branch of the innate immune system such as dendritic cells, macrophages and monocytes. HIV-1 also interacts with a variety of non-immune cells during initial infection (McMichael et al. 2010; Kaushic 2011; Sundquist & Krausslich 2012; Levy JA 2009).

Initial infection is characterised by influenza-like symptoms in the majority (around eighty percent) of cases. These symptoms are reflected by immune activation in the acute phase of HIV-1 infection (4-8 weeks post-infection) characterised by the formation of antibodies against HIV-1 antigens, such as anti-gp120 – acute seroconversion. The subsequent cytotoxic CD8 T-cell driven response will be directed against cells expressing initial HIV-1 antigens, but unable to fully clear all HIV-1 infected cells. Following the acute phase immune

response, HIV-1 lies dormant for many years in resting memory T-cells and other reservoirs of latent infection. This is why HIV-1 belongs to the lentivirus (lenti = slow) family (McMichael et al. 2010; Ylisastigui et al. 2004).

HIV-1 induced dysregulation of innate immune signalling throughout infection is understood to contribute both to the pathogenesis of HIV-1 associated inflammatory diseases; as well as to the insufficient or inappropriate responses to invading pathogens. HIV-1 in the acute and chronic stages of infection is marked by dysregulated pro-inflammatory cytokine and interferon responses to varying extents, as is reflected in figure 12 below (Appay & Sauce 2008; McMichael et al. 2010; Miedema et al. 2013).

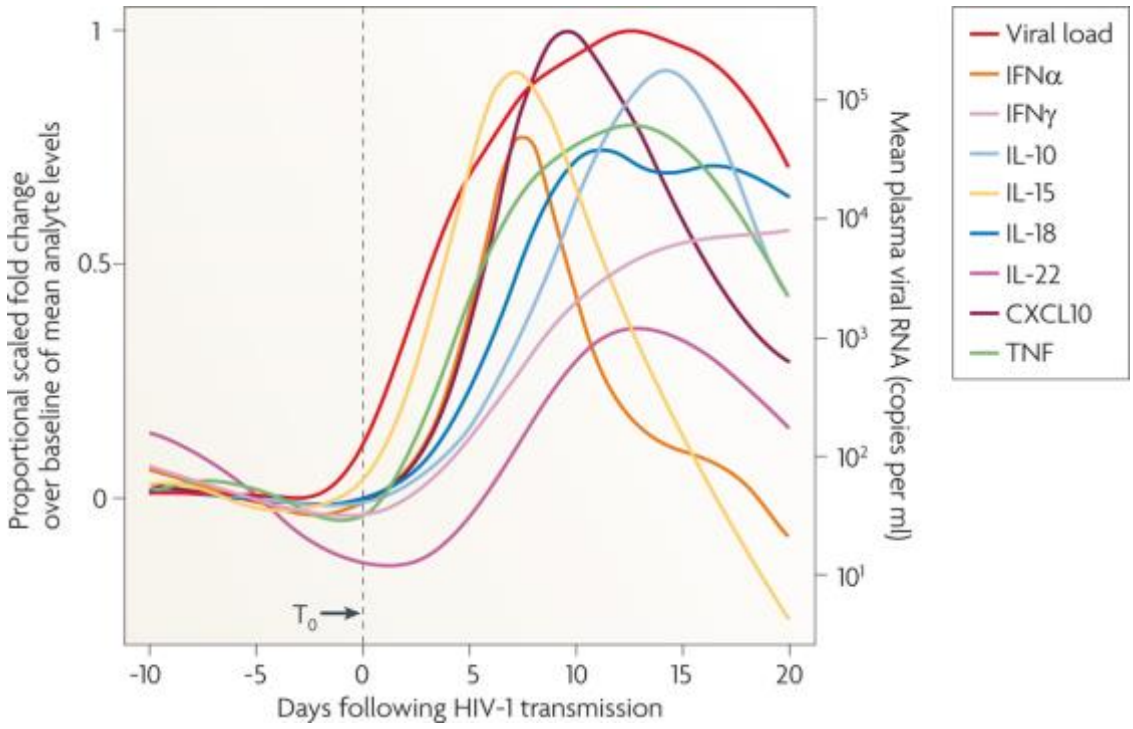


Figure 12. ***Overview of the cytokine storm in acute HIV-1 infection.*** **Interleukin-15 (IL-15) and interferon-α (IFNα) first peak around day 7, followed by tumour necrosis factor (TNFα), IL-18 and IL-10 peaking between days 10-15. Adapted from McMichael et al 2010 (McMichael et al. 2010).**

Innate immune system cytokines IFNα, IFNγ and IL-18 (as well as IL-1β) are upregulated during acute infection, as is reflected in figure 12 above. In genuine influenza infection, signs and symptoms include malaise, lethargy, coryza, myalgia, laryngitis, pyrexia and lymphadenopathy. Many of these symptoms are caused by type-1 interferon activity against the virus. HIV-1 acute seroconversion illness includes these features similarly caused by a potent (though non-sterilising) interferon response which sometimes is accompanied by a characteristic maculopapular rash (Appay & Sauce 2008; McMichael et al. 2010; Miedema et al. 2013). HIV-1 therefore provides a good model for studying PRR interactions as well as downstream pathways because there is a clinical prodrome with inflammatory features.

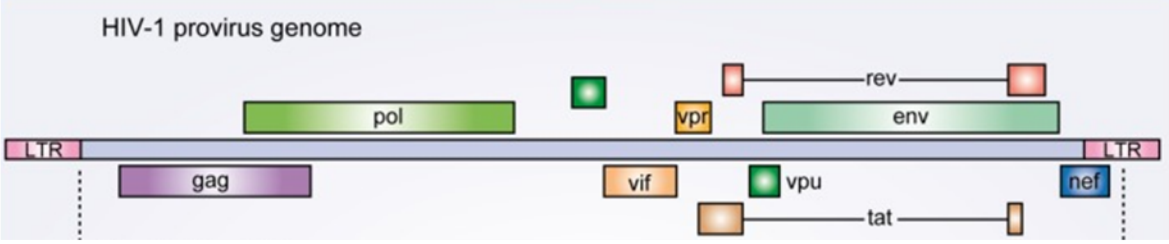
Following a latent period, reactivation of HIV-1 replication causes CD4 T-helper cell counts to decline and the human body becomes susceptible to opportunistic infection and cancers otherwise rare in healthy individuals. The types of potential opportunistic infections can be predicted at particular inadequate CD4 T-helper cell count thresholds. Beyond this, the human body may develop a constellation of diseases known as 'AIDS-defining illnesses', ranging from severe opportunistic infections such as *Pneumocystis* pneumonia, cancers such as Kaposi sarcoma, and conditions caused directly by the toxic inflammatory effects of HIV-1 e.g. HIV wasting syndrome. Figure 13 below lists AIDS-defining illnesses an adult patient may develop in the final stages of disease (WHO 2006; Levy JA 2009).

Clinical stage 4[ii]
HIV wasting syndrome
Pneumocystis pneumonia
Recurrent severe bacterial pneumonia
Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration or visceral at any site)
Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)
Extrapulmonary tuberculosis
Kaposi's sarcoma
Cytomegalovirus infection (retinitis or infection of other organs)
Central nervous system toxoplasmosis
HIV encephalopathy
Extrapulmonary cryptococcosis including meningitis
Disseminated non-tuberculous mycobacterial infection
Progressive multifocal leukoencephalopathy
Chronic cryptosporidiosis (with diarrhoed)
Chronic isosporiasis
Disseminated mycosis (coccidiomycosis or histoplasmosis)
Recurrent non-typhoidal Salmonella bacteraemia
Lymphoma (cerebral or B-cell non-Hodgkin) or other solid HIV-associated tumours
Invasive cervical carcinoma
Atypical disseminated leishmaniasis
Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

Figure 13. ***AIDS-defining illnesses representing World Health Organisation Stage 4 HIV-1 infection.*** **(WHO 2006)**

2.2 Virology and lifecycle of HIV-1

HIV-1 is a single-stranded negative sense RNA 100nm-sized blood-borne lentivirus belonging to the subfamily of *orthoretrovirinae*, by virtue of its ability to employ constitutive enzymes to reverse-transcribe its RNA into linear double stranded DNA, using reverse transcriptase. During unchecked active HIV-1 genome expression, up to ten billion new virions can be produced every 24 hours. Figure 14 below introduces the HIV-1 genome and outlines general functions of the HIV-1 proteome: proteins discussed throughout this section which contribute in varying ways to the viral life-cycle and ultimately the pathogenesis of HIV-1-related morbidity (WHO 2006).



HIV-1 Gene	Protein/Function
Gag	Core and matrix proteins
Pol	Reverse transcriptase (ssRNA→dsDNA), integrase (dsDNA nuclear translocation) protease (maturation)
Env	Glycoproteins gp120 & gp41
Tat	Stimulates transcription of HIV-1
Rev	Export of HIV-1 nuclear transcripts
Vif	Aids and abets virion infectivity
Vpr	Multiple – HIV-1 dsDNA→ host nucleus, ↑ viral production, arrest of cell cycle
Vpu	Viral escape from cell
Nef	↑viral replication, ↓CD4 and MHC I/II expression

Figure 14. ***Introduction of HIV-1 genome and protein products by general function.*** **HIV-1 encodes several accessory proteins which augment virion replication and escape from the host immune system. Abbreviations:** ***gag*****, group specific antigen;** ***pol*****, polyprotein;** ***env*****, envelope;** ***tat*****, transactivator;** ***rev*****, regulator of viral expression;** ***vif*****, viral infectivity;** ***vpr*****, viral protein R;** ***vpu*****, viral protein U;** ***nef*****, negative-regulation factor.**

2.2.1 Tissue entry and cell fusion

Persistent HIV-1 infection relies on successful penetration of the human body. Penetration may occur via damaged mucosa, transcytosis across columnar epithelia, and direct subdermal injection (in the case of intravenous drug use or nosocomial transmission routes). HIV-1 penetration of damaged mucosal surfaces may occur at the vaginal, penile or anal epithelia. Alternatively, HIV-1 virions may undergo transcytosis through reproductive tract epithelia, e.g. columnar epithelial cells in the ectocervix (Bobardt et al. 2007).

Regardless of the route of transmission, successful penetration will lead to persistent infection in the absence of post-exposure prophylaxis. Though $CD4^+$ T-cells are preferentially infected and are the major site of HIV-1 replication, dendritic cells (DC) and macrophages are also $CD4^+$ and are reservoirs for HIV-1. HIV-1 is also thought to infect peripheral blood monocytes, thus peripheral blood monocytes can be used to study the virus (Chattergoon et al. 2014). Following initial HIV-1 penetration through mucosal barriers, envelope glycoproteins facilitate virion attachment and fusion with the target cell with HIV-1 *Env* proteins and host CD4 plus co-receptors CCR5 or CXCR4 (in macrophage-tropic and T-lymphocyte tropic clades respectively) (Grivel JC & Margolis LB 1999).

HIV-1 *Env* encodes the fusion apparatus comprising gp120 and gp41. The precursor gp160 is converted to gp120 and gp41 respectively. ‘gp140’ is a term to describe a section of the fusion apparatus, rather than an active product. The term ‘gp140’ refers to the area of gp120+gp41 (i.e. gp160) that lies outside the virion lipid membrane as is depicted in figure 15. HIV-1 gp120 heterodimers non-covalently associate to form a trimer bundle raised from the viral membrane by gp41 (Vivès RR et al. 2005).

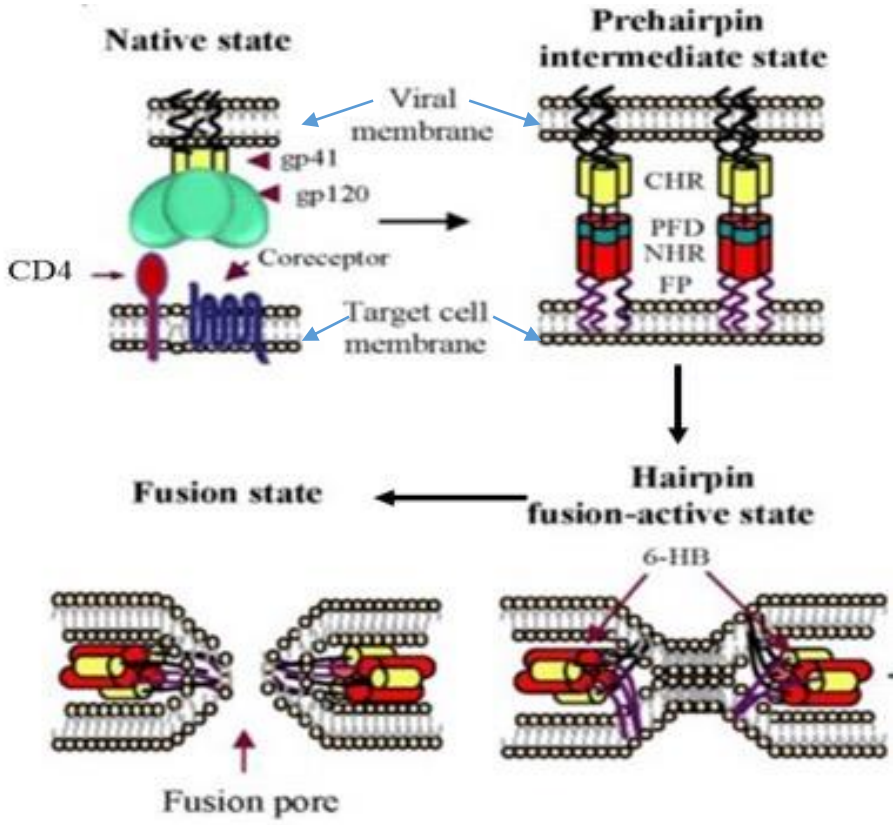


Figure 15. ***Stages of HIV-1 fusion with host cell membrane.*** **Abbreviations: CHR, C' terminal heptad repeats; PFD, pocket forming domain; NHR, N'terminal heptad repeat; FP, fusion peptide. Adapted from Merk and Subramaniam, 2013 (Merk & Subramaniam 2013).**

HIV-1 gp120 binds CD4 and the chemokine co-receptor CCR5 or CXCR4 (see figure 15 above), causing gp120 monomer rotation to place the trimer in its 'open state' from its pre-fusion 'closed' conformation. This exposes the gp41 fusion peptide to the host cell membrane, with proximal gp41 C' terminal heptad repeats of the core N-peptide, N36, and C-peptide bringing the virion closer to the membrane. The gp41 then forms as a six helix bundle of antiparallel CHRs invested into the pockets of the NHR trimers at the hairpin state for fusion. The N'terminal heptad repeats contain deep hydrophobic pockets – pocket forming domains - which have been studied as a target of therapeutic inhibition (IM Enfuvirtide/T20 plus more in development). These actions of gp41 lead to self-virion lipid rearrangement and ultimate release of virion contents into the cytosol (Merk & Subramaniam

2013; Bartesaghi et al. 2013). It is thought that these events occur around cholesterol-rich lipid domains on the cell membrane (Yang et al. 2015).

Although the HIV-1 genome is highly unstable, HIV-1 occurs as specific types (M-'**m**ain'-type dominates globally, O-'**o**utlier'-type is less common and N-'**n**either M nor O'-type is rare) and subtypes otherwise known as clades. Certain types and clades of HIV-1 may be T-lymphotropic or macrophage-tropic, i.e. they preferentially infect these cell types. T-lymphotropic HIV-1 primarily uses the chemokine co-receptor CXCR4 to penetrate the target cell whilst macrophage-tropic HIV-1 uses the CCR5 co-receptor. Based upon their chemokine co-receptor tropism, HIV-1-Bal is known as the R5 clade and HIV-1-IIIB is known as the X4 clade (McMichael et al. 2010; Vivès RR et al. 2005; Lederman MM et al. 2006).

Individuals with mutations in co-receptors such as CCR5 or CXCR4 display resistance to infection, as HIV-1 cannot bind these co-receptors and then go on to infect the cell. Outside the context of HIV-1 infection, C-terminal serine residue phosphorylation is important for CCR5 chemokine signal transduction. The protein kinase C mediated recruitment of protein mediators of endocytosis following this process is exploited by HIV-1(Vivès RR et al. 2005; Raport CJ et al. 1996). The Δ32 base-pair deletion in *ccr5* leads to a non-functional receptor that HIV-1 gp120 cannot bind. Monocytes in humans homozygous for the *ccr5* Δ32 cannot become infected; thus progression of the natural history of HIV-1 infection will be delayed. Such patients are known as long-term 'non-progressors' (Piacentini L et al. 2009). In health, the SDF1 (stromal derived factor-1) ligand of CXCR4 functions to stimulate processes involved in cell division and survival. Much like CCR5 polymorphisms, *Cxcr4* polymorphisms affect HIV-1 infectivity. CXCR1-Ha haplotype transfection was shown to reduce CXCR4 (and CD4) expression; thus reducing the rate of CXCR4-tropic HIV-1

infection in T-cell subsets. *SDF-1* polymorphisms may confer a protective effect against HIV-1 infection by increasing CXCR4-SDF-1 binding (Winkler et al. 1998; Mazzucchelli et al. 2001).

Other than the fusion model described above, HIV-1 may enter cells such as dendritic cells via a mechanism involving clathrin-dependent endocytosis mediated by the C-type lectin receptor DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin). Whether productive infection (infection leading to the production and release of new HIV-1 molecules) occurs in some cells that HIV-1 fuses with remains controversial (Beignon AS et al. 2005; Gringhuis et al. 2010; Melikyan 2014). The observation that gp120 monomer rotation, an essential step in fusion, may be induced by interactions with proteins other than CD4 explains how HIV-1 may occasionally penetrate cells understood to express very low levels of, or no CD4 at all.

2.2.2 Reverse transcription and integration to host genome

Now that the HIV-1 virion has entered the target cell, capsid uncoating releases the RNA genome and accessory proteins into the cytosol. The HIV-1 genome is first reverse transcribed by the action of reverse transcriptase using the positive sense ssRNA to form linear proviral dsDNA bilaterally flanked by the long terminal repeat sequences. To assist the activity of reverse transcriptase, accessory protein Vif binds HIV-1 nucleic acids and targets APOBEC ('apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like' family proteins) for proteasomal destruction. APOBEC proteins are cytidine deaminases which prevent successful reverse transcription of the initial mRNA transcript: the absence of APOBEC permits unabated creation of provirus (Ellegard et al. 2011). The completed 9kb HIV-1 (DNA) provirus complexes with reverse transcriptase, integrase, nucleocapsid, Vpr and matrix proteins. This is known as the pre-integration complex which, in an ATP-dependent manner is imported into the host cell nucleus (Guerrero et al. 2015).

At the nucleus, HIV-1 integrase integrates the dsDNA HIV-1 genome into the genome of the host cell. Once this process is completed, the DNA cannot be removed (Guerrero et al. 2015). Integration of HIV-1 dsDNA is a two-step process involving initial cytosolic long terminal repeat sequence processing by integrase dimers to render each end of the linear strands compatible with the host genome; and secondly integrase generates 3' terminal oxygen atoms to cut the DNA of the chromosome. The second phase is mediated by the bilateral dimers joining and interacting with host protein LEDGF/p75 (lens epithelium-derived growth factor/p75) to link the 'double dimer' to the chromatin. Usurped host nuclear repair proteins then complete the integration process, resulting in HIV-1 provirus integration into the human genome. The integration step of HIV-1 DNA is thought to target genes undergoing active transcription to enhance HIV-1 survival (Krishnan & Engelman 2012).

Various integrase host restriction factors acting upon HIV-1 integrase have been described. One host strategy is to prevent the oligomerisation of integrase at the site of chromatin attachment (Busschots et al. 2007). Studies where LEDFGp75 was inhibited by a group of small molecules as explored by Christ and colleagues have shown promising results (Christ et al. 2012). These small molecules able to inhibit integrase oligomer-chromatin interaction, are consequently termed ‘LEDGINs’ and have generated significant interest within the pharmaceutical industry (Desimmie et al. 2013).

2.2.3 Transcription and translation

In the host cell nucleus, HIV-1 provirus DNA is transcribed to mRNA by host cell RNA polymerase II which binds a promoter sequence on the 5' long terminal repeat sequence (LTR). HIV-1 mRNA transcripts undergo 5' untranslated region (UTR) 7-methylguanosine (m^7G) capping and 3' polyadenylation. The m^7G cap marks the mRNA transcript for nuclear export into the cytosol which is mediated by a macromolecular complex consisting of cap binding proteins 80/20 heterodimers, mRNA export effectors EJC and SR (**e**xon-exon **j**unction **c**omplex and **s**erine-**a**rginine proteins respectively) and finally the NXF1/NXT1 (nuclear RNA export factor 1/nuclear transport factor 2-related export protein 1) heterodimer, this last subcomplex specifically initiating the process of nuclear export (Guerrero et al. 2015).

Translation occurs first as what should be considered a 'test run' mediated by the CBC (cap-binding complex) which is then removed from the 5'UTR m^7G cap for final translation to occur through the usual host apparatus. Interestingly, HIV-1 protease and Vpr (discussed later in section 4.3) are thought to partially negatively regulate translation of HIV-1 mRNA to free host cell ATP for use in the latter stages of the viral life cycle as HIV-1 mRNA translation is an ATP-dependent process (Guerrero et al. 2015).

HIV-1 *Tat* and *Rev* translation is permitted by multiple splicing of HIV-1 mRNA transcripts, producing spliced *Tat* and *Rev* mRNA. Tat acts in two ways to amplify HIV-1 mRNA processing: firstly, by enhancing proviral transcription and secondly by directly binding HIV-1 mRNA transcripts to ensure stability for translation. More recently, HIV-1 Tat is also proposed as a potential negative regulator of HIV-1 mRNA translation under certain conditions. *Rev* assists in mRNA transcript nuclear export to the cytosol. In what is effectively a positive feedback loop, increasing *Rev* levels result in increased concentration of

singly or unspliced mRNA transcripts in the cytosol. Singly spliced transcripts are translated into core, matrix and envelope proteins as well as reverse transcriptase, integrase, protease. Unspliced transcripts are not translated and are instead packaged in the mature virion (figure 16) forming its genome (Guerrero et al. 2015).

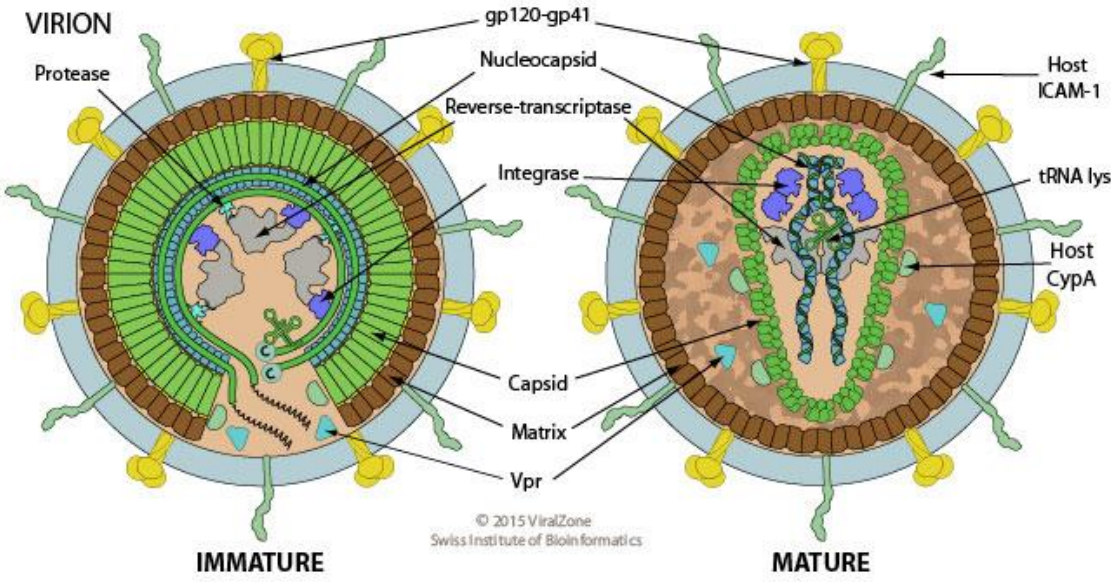


Figure 16. ***Schematic diagram of HIV-1 immature and mature virion.*** **Mature HIV-1 takes on host proteins to further infectivity in the host systems.(ViralZone 2014)**

During processing, new immature HIV-1 virions incorporate a number of host molecules such as ICAM-1, tRNA lys and CypA as depicted in figure 16 above. ICAM-1 (intracellular adhesion molecule 1) is a ligand for leukocyte adhesion protein LFA-1, which in health is primarily involved in the migration of leukocytes across endothelia. Clearly this function presents an advantage for HIV-1 dissemination (Caughman et al. 1992). tRNA(Lys)3, one of three tRNA(Lys) isoacceptors active during translation, is attached to 5' terminal sequences of the HIV-1 RNA genome by a complex of both HIV-1 and host factors. tRNA(Lys)3 is used by mature HIV-1 virions to further prime the reverse transcription process in newly infected cells. The nature of the tRNA(Lys3) annealing complex remains under investigation

though models of activity have been proposed (Saadatmand & Kleiman 2012). The conferral of a survival advantage upon HIV-1 of cyclophyllin A (CypA) remains under investigation, though it was recently proposed that CypA may aid and abet both capsid assembly and capsid homo-oligomerisation (Cortines et al. 2015).

2.2.4 Trafficking of mature HIV-1 and release

Nearly all events occurring during HIV-1 virion assembly are managed by the Gag-Pro-Pol polyprotein, created by translating ribosome shift into the −1 reading frame at a site on the 3′ end of the *gag* open reading frame, the ribosome then translates the *pol* gene. The Gag-Pro-Pol polyprotein binds the plasma membrane, creates spherical particles, concentrates the trimeric fusion apparatus gp120+gp41, and packages two copies of the genomic single stranded RNA resulting from the previously described *Rev* dependent transcription of unspliced mRNA (Sundquist & Krausslich 2012).

HIV-1 protease (Pro) is encoded by HIV-1 *pol* and cleaves the Gag-Pro-Pol polyprotein to their mature components, those being matrix protein, capsid protein, nucleocapsid protein, p6, protease, reverse transcriptase and integrase. HIV-1 protease dimerises to form its active state, though the underlying mechanism of protease dimerization is unknown and remains under investigation. It was suggested that transient dimerization of Gag-Pro-Pol protease and non-processed protease could result in autocatalytic activation, but given the weak dimerization potential of non-processed protease is unlikely to be the sole activation mechanism (Sundquist & Krausslich 2012).

The cleavage of gp160 into mature gp120 and gp41 is mediated by host endoproteolytic cleavage via convertase proteins. The major host convertase demonstrated in this process *in vitro* is furin, (FES – feline sarcoma oncogene homolog – upstream region). In health, furin

processes multiple substrates such as pro-parathyroid hormone and proalbumin (Moulard & Decroly 2000).

Viral budding of mature HIV-1 however is mediated by the host endosomal sorting complexes required for transport (ESCRT) pathway. In health, the ESCRT pathway performs various major functions. Of particular relevance is ESCRTs role in cytokinesis whereby multiple ESCRT subunits form a spiral shaped fibril array across the cell with one subunit recruiting spastin to cleave the microtubules across the midpoint. HIV-1 coercion of ESCRT to enable budding occurs via a process not-dissimilar to that of the role of ESCRT in cytokinesis but only initially requires Gag binding of two subunits, ESCRT1 and ALIX, their actions bilaterally pinching off the virion bud from the cell membrane (Sundquist & Krausslich 2012; Morita et al. 2011). Vpr induction of cell cycle arrest at G2 (i.e., pre-cytokinesis) probably facilitates this process by preventing the recruitment of ESCRT apparatus to genuine cytokinesis or other host cell processes (Kogan & Rappaport 2011).

Accessory proteins Nef, Vpr and Vpu assist in viral release. HIV-1 Nef down-regulates MHC-I and II as well as CD4 thereby preventing immune recognition as well as secondary binding of new HIV-1 to host cell CD4 upon release. Vpr has an additional role to downregulate various host cell proteins post-HIV-1 translation, though the significance is as yet only partially known (Pawlak & Dikeakos 2015). HIV-1 Vpu plays a number of roles in the release of HIV-1 from host cells: the actions of Vpu during the HIV-1 life-cycle are explored in section 4.3.

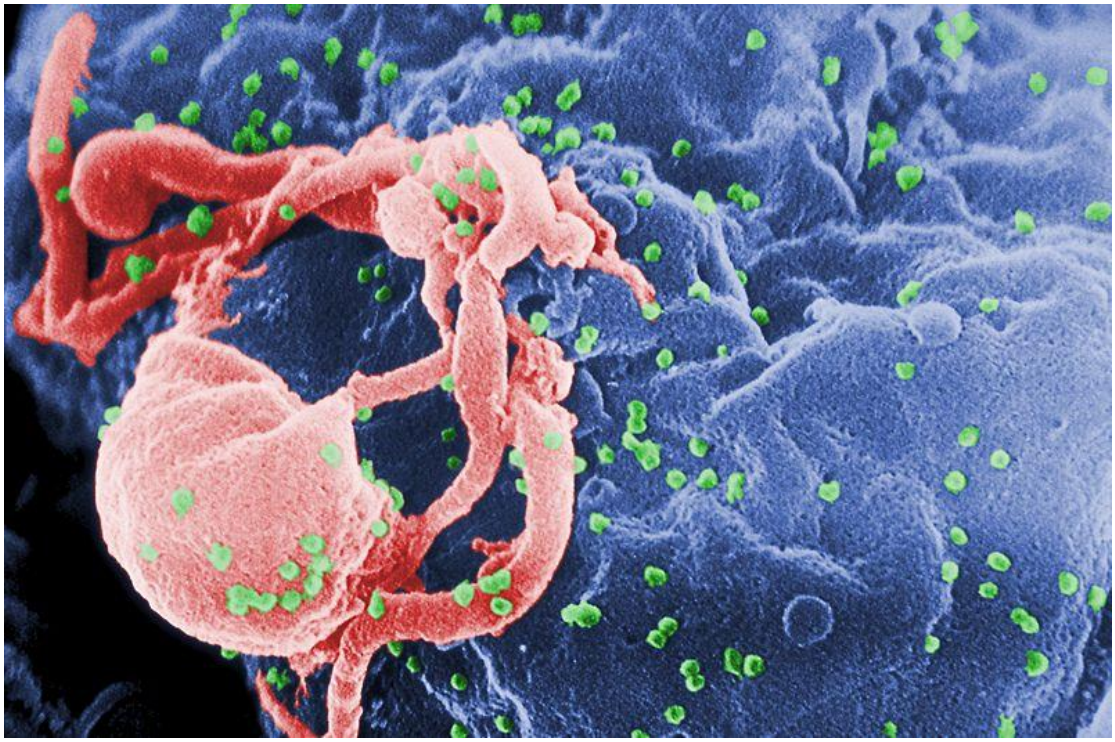


Figure 17. ***Scanning colour electron micrograph of HIV-1 virions (in green) budding from a cultured lymphocyte (pink).*** **Image credit Goldsmith, C (CDC, USA) used under common licence (Goldsmith C 2005).**

The end result of the highly studied HIV-1 life cycle is the release of a new HIV-1 virion (see figure 17 above). Following successful establishment in initial target cells, HIV-1 is transported to the lymph nodes via the circulatory system and infection persists. Clinical virologists are currently investigating ways in which HIV-1 could be therapeutically 'kicked', i.e., reactivated, in resting cell reservoirs to allow the action of conventional and novel therapies to destroy the cell and the HIV-1 genome with it. Exactly how HIV-1 switches from its dormant to an active state is unknown, though studies using this 'kick and kill' approach are isolating candidate exogenous signals which may shed further light on the endogenous process (Ward, 2014).

2.3 Treatments for HIV-1 infection

A surge of investment in AIDS research led to the discovery of nucleoside reverse transcriptase inhibitors. The first treatment was azidothymidine (otherwise known as zidovudine or AZT), a former cancer chemotherapy candidate. AZT monotherapy was the mainstay of early treatment, though unfortunately most patients developed drug resistance and side effects (Mitsuya et al. 1985; Levy JA 2009).

It was then discovered that triple therapy using HIV-1 protease inhibitors in combination with nucleoside reverse transcriptase inhibitors produced a stronger inhibition of HIV-1 replication and survival. Patients fortunate enough to be selected to the therapeutic arm of randomised controlled trials designed to investigate this hypothesis began to exhibit lower HIV-1 viral load and higher CD4 T-cell counts. The advent of triple antiretroviral therapy or as it is now termed HAART (highly active antiretroviral therapy) or cART (combination antiretroviral therapy) transformed HIV-1 infection from a universally terminal illness to a disease that people could live with, rather than die from (Hammer et al. 1997; Gulick et al. 1997; Levy JA 2009).

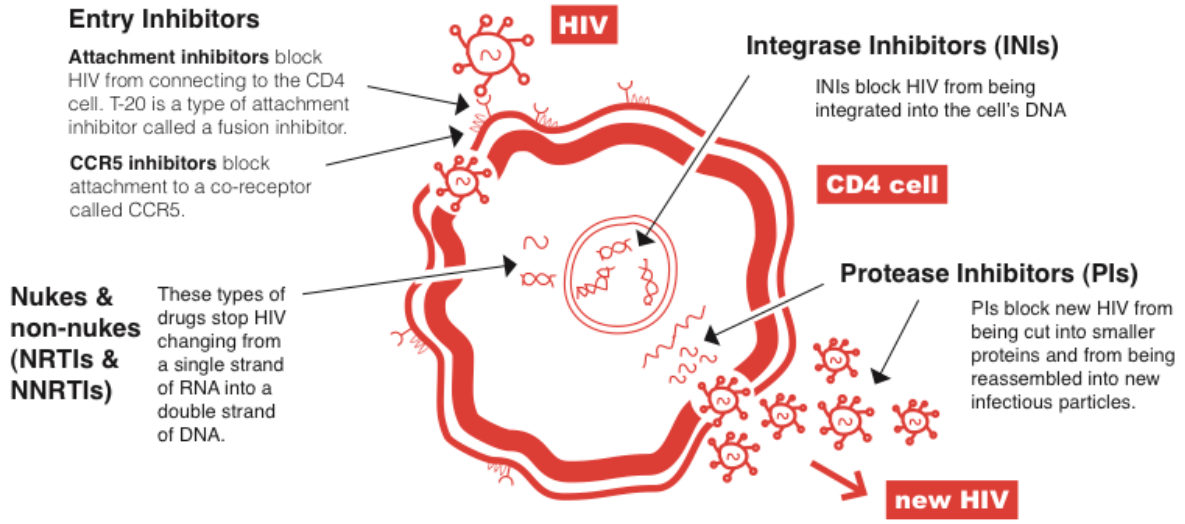


Figure 18. ***Antiretroviral therapies active at stages of HIV-1 lifecycle*****. Reprinted with express permission. Image credit: Simon Collins, iBase, London UK (Collins S 2014b).**

As therapeutic research advanced, other molecules were found to inhibit HIV-1 entry into cells or the integration of HIV-1 DNA into that of the host cell genome. Figure 18 above summarises the current types of antiretroviral drugs prescribed to people living with HIV to prevent further progression of immune deterioration and protect CD4 T-cells from infection. Newer types of antiretroviral therapy include maraviroc, the CCR5 inhibitor; T-20 the attachment inhibitor and the integrase inhibitors raltegravir or elvitegravir. Integrase inhibitors show (in comparison to other ARTs) less severe adverse effect profiles and are listed along with other commonly prescribed ARTs in figure 19 overleaf.

Drug	Mode of Action
Lamivudine	NRTI
Abacavir	NRTI
Emtricitabine	NRTI
Tenofovir	NRTI
Efavirenz	NNRTI
Nevirapine	NNRTI
Etravirine	NNRTI
Rilpivirine	NNRTI
Raltegravir	Integrase inhibitor
Elvitegravir	Integrase inhibitor
Dolutegravir	Integrase inhibitor
Atazanavir	Boosted PI
Darunavir	Boosted PI

Drug	Mode of Action
Cobicistat	Pharmacokinetic booster
Ritonavir	Pharmacokinetic booster

Proprietary FDCs	Constituents
Atripla	Efavirenz, Emtricitabine, Tenofovir
Eviplera	Rilpivirine, Emtricitabine, Tenofovir
Stribild	Elvitegravir, Cobicistat, Emtricitabine
Truvada	Tenofovir, Emtricitabine
Kivexa	Abacavir, Lamivudine

Figure 19. ***Current commonly prescribed HIV-1 antiretroviral therapies.*** **Abbreviations: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor, FDCs: Fixed Dose Combinations. Adapted with permission from iBase (2014) (Collins S 2014a).**

Pre-and post-exposure oral prophylaxis (PrEP and PEP) are relatively new developments in the field of therapeutic HIV-1 prevention. These oral interventions respectively comprise daily *Truvada* fixed dose combination; and higher dose (relative to normal dosing in PLWHIV) antiretroviral triple therapy. In some high risk populations, these interventions have been shown to be safe, effective (at reducing infection rates/risk) and tolerable in the short term (McCormack S 2014).

For some time, HIV treatment was withheld at initial diagnosis, often justified by the need for counselling and lifestyle changes for the patient to incorporate the lifelong antiretroviral therapy into their daily routine and totally adhere to the prescription for the rest of their lives. Recently however, a ground-breaking clinical trial (START) of 685 HIV positive people at 211 sites in 35 different countries assessing the optimal time to begin antiretroviral therapy has been suspended in advance of its expected endpoint (2016). It was found that early treatment group participants benefited so greatly in comparison to the treatment delay group by way of reduced frequency of AIDS and mortality; that the study had to be stopped and findings reported to the international community ((NAID) 2015). It is likely therefore that HIV-1 will no longer be considered a special case for treatment initiation and, like any other serious infection, rapid intervention to prevent the cytotoxic and inflammatory effects of HIV-1 will be indicated.

There remain several challenges in HIV therapeutics. Firstly, the need for total adherence to HAART regimens is paramount, as treatment interruption, even at low levels, may give rise to HIV-1 genome mutations rendering initial HAART regimens ineffective, i.e., HIV-1 drug resistance. Indeed, a large scale multi-centre global trial of planned treatment interruption (the ‘SMART’ study) confirmed that treatment discontinuation removed the suppression of HIV-1 and resulted in lower CD4 T-cell counts (Group 2006). Secondly, the number of

serious adverse events with specific antiretrovirals, particularly the older drugs, such as reproductive teratogenicity or even psychosis seen in efavirenz, means that some antiretrovirals must be restricted from specific populations. Importantly, current drug therapies are unable to completely remove the virus from the human body as a consequence of the existence of latent reservoirs of HIV infection, largely accounted for by resting CD4 T-cells (Chun et al. 1997).

The need to reserve more sophisticated antiretrovirals for patients for whom older first-line antiretrovirals may be unsuitable due to serious adverse events or the development of drug resistance adds a further degree of complexity to prescribing. Many seropositive patients whose HIV has been sufficiently suppressed by HAART over decades of treatment are now beginning to experience adverse events thought to be caused by the effects of HAART rather than HIV-1 toxicity. These include cardiotoxicity, renal impairment and (often subclinical) osteopenia. These adverse events are considered to be a consequence of being on HAART, rather than that of HIV-1 infection (Levy JA 2009).

The absence of an effective vaccine against HIV-1 is a further challenge in the response to HIV-1. Several recent HIV-1 vaccine clinical trials have demonstrated variable degrees of efficacy, yet in real world settings, (i.e. Phase 3 clinical trials and beyond), effectiveness has not been demonstrable (Burton & Mascola 2015). Many potential curative interventions, such as HDAC inhibitors to reactivate HIV-1 replication in resting cells exposing latent HIV-1 to the action of HAART (the 'kick and kill' approach), as of 2014 have been proposed and remain under early phase investigation (Wei et al. 2014; Ward 2014).

While people continue to live with HIV without immediate prospect of cure, the investigation of HIV-1 mediated immune dysfunction remains important. The questions of which HIV components and activities precipitate the immune dysfunction, and, which are the key

immune system mechanisms that constitute the antiviral response require further study. ‘Sterilising’ cure of HIV-1 infection would assume that the immune system, as reflected in cytokine profile, is able to return to the state resembling that of an uninfected individual, yet with investigations of ‘functional’ cure, wherein very low HIV-1 provirus concentrations are tolerable in the absence of disease progression, are now seen as more realistic. The short and long term effects on the human immune system of this approach is unknown (Ward 2014).

3. HIV-1-pattern recognition receptor interactions

3.1 HIV-1 interacts with TLRs

HIV-1 has been shown to interact with pattern recognition receptors, especially intracellular TLRs (Beignon AS et al. 2005). An early *in vitro* study showed IL-1β release from neuroblastoma cells upon stimulation with HIV-1 gp120, implying gp120 was either being sensed by cell surface TLRs to trigger downstream activation of other inflammatory pathways or that gp120 was being taken up to be sensed by intracellular PRRs (Corasaniti MT et al. 2001). Interestingly, certain HIV-1 protease inhibitors are able to downregulate TLR2 and TLR4 expression upon treatment *in vitro* (Equils O et al. 2004). This suggests that chronically untreated HIV-1 infection results in TLR over-expression. HIV-1 replication in TLR2, 4, and 9 tolerised transgenic mice is up-regulated in macrophages suggesting that these TLRs have a role in the suppression of HIV-1 replication (Chang JJ & Altfeld M 2009).

TLR4 polymorphisms (1063A/G [D299G] and 1363C/T [T399I]) were also shown to be associated with high viral load in 201 antiretroviral naïve patients, implicating a role for cell surface TLRs in the sensing and host response to HIV-1 (Pine SO et al. 2009). The mechanism by which D299G or T399I may influence HIV viral load is currently unknown. LPS in the bloodstream of these patients is indicative of microbial translocation across the gut mucosa while levels of serum LPS and systemic immune activation are correlatively lower in HIV-1 patients that do not display these polymorphisms. Another genome-wide association study of 276 North Americans identified 2 single-nucleotide polymorphisms in TLR4 significantly associated with seropositive HIV status (Jurevic et al. 2014). Taken together, these studies imply that TLR4 could have a role in initial infection as well as long term host suppression of HIV-1.

Furthermore, TLR4 stimulating mucosal commensals such as *E. coli*, *N. mucosa* and *V. parvula*, appeared to have an anti-HIV-1 effect in a macrophage model. In the same study, *L. acidophilus* stimulation of TLR2 appeared to enhance macrophage HIV-1 replication (Ahmed et al. 2010). Type-1 interferon secretion from HIV infected macrophages was induced by TLR4 stimulation with an endogenous ligand, prothymosin α which is secreted by CD8 T-cells. The action of prothymosin α appeared to suppress HIV in the infected macrophages (Mosoian et al. 2010).

Ex vivo, TLR2 and TLR4 expression levels have been found to be modulated in monocyte-derived macrophages (Chang JJ & Altfeld M 2009) and peripheral blood monocytes (Heggelund L et al. 2004) respectively from HIV-1 seropositive subjects. More recent work suggests dual recognition of HIV-1 gp120 in the female reproductive tract via TLR2 and TLR4 with NFκB signalling occurring most frequently between 30 minutes to two hours. The observed increase in proinflammatory cytokines mediated by TLR2 and TLR4 gp120 recognition exists as a potential mechanism of HIV-1 entry through the mucosal epithelia(Nazli et al. 2013).

Intracellular TLRs also sense HIV-1. Plasmacytoid dendritic cells (pDC) are able to drive a cell-mediated innate immune response via type-1 IFNs after HIV-1 RNA sensing by TLR7 (Beignon et al 2005). Unfortunately, this sensing is insufficient to control HIV-1 pDC infection leading to further dissemination of HIV-1 by effective hijacking of pDC translocation to lymph nodes for antigen presentation (Beignon AS et al. 2005). Certain TLR9 polymorphisms were found to be associated with rapid disease progression in HIV-1 infection(Bochud PY et al. 2007). In advanced disease, peripheral blood monocyte TLR6, 7 and 8 were found to be transcriptionally elevated along with TLR2 and 4, adding further weight to their recognition of various HIV-1 PAMPs (Lester RT et al. 2008). Blockade of

TLR8 could inhibit HIV-1 replication and infectivity in myeloid dendritic cells. Some earlier studies appeared to suggest that stimulation of intracellular TLRs, such as TLR7 or TLR9 abetted HIV replication and infectivity (Trifonova et al. 2009; Fraietta et al. 2010). Recently however, TLR7 ligands were trialled in a simian SIV model showing showed promising transient reduction in HIV-1 viral load (Whitney J Osuna C, Sansisety S, Barnes T, Cihlar T, Geleziunas R, Hesselgesser J, Hraber P 2015) which could potentially explored in human *ex vivo* models.

There remain many questions to be answered in TLR-HIV-1 interaction. Specifically, whether a gp120 cytokine response depends upon TLR2, TLR4 or both; whether gp41 can be sensed by, and activate cell surface TLRs; and more generally that of how TLR signalling may influence the activation of intracellular PRRs downstream. Ultimately it would be advantageous to know whether stimulating or inhibiting TLR activity in the context of HIV-1 infection potentiates a host-protective immune response *in vivo*, one study suggested the approach of tailoring TLR-mediated responses at the genital mucosa constitutes a potential early therapeutic strategy against HIV-1 (Ellegard et al. 2011).

3.2 HIV-1 interacts with NLRs

Compared to HIV-1 PAMP sensing by TLRs, NLR-HIV-1 interaction has been so far relatively understudied. Before the mechanisms of NLR sensing and inflammasome-mediated IL-1β secretion were discovered, an early study identified that HIV-1 was able to increase IL-1β production in stimulated cells. Yamato and colleagues (1990) observed increased IL-1β RNA in monocytes from PLWHIV donors compared to healthy controls (Yamato K et al. 1990). Subsequently, in a prospective study of 35 children it was observed that there was a chronic increase in serum IL-1β as HIV-1 infection progressed(Arditi et al. 1991). A larger study (N=44) of both adults and children living with HIV-1 also found that the previously

observed increased IL-1β levels were also accompanied by increased IL-18 levels in HIV positive patients (Torre et al. 2000). Ten years following the initial studies and shortly before the characterisation of the inflammasome, Corasaniti and colleagues (2001) reported that HIV-1 gp120 stimulated IL-1β secretion suggesting a role in programmed cell death during HIV-1 infection. With a high degree of prescience, admitting that the underlying mechanism was unknown, their study suggested that changes in calcium ion concentrations, now considered a signal 2 candidate for inflammasome activation, were likely responsible (Corasaniti MT et al. 2001).

Specific NLR-HIV-1 interaction was first shown by a group of studies led by one research team in seropositive Brazilian populations. Pontillo and colleagues first investigated inflammasome component polymorphisms and identified some polymorphisms in NLRP3 and caspase-1 which correlated with increased progression of HIV-1 in infected individuals (Pontillo, Oshiro et al. 2012). The group then went on to show that CARD8 polymorphism also influenced susceptibility to mycobacterium tuberculosis infection in people living with HIV-1 (Pontillo, Silva et al. 2012). Lastly, this group showed that 1: HIV-1 stimulation of innate immune cells from HIV negative subjects could activate the inflammasome; and, 2: attempts to stimulate dendritic cell inflammasomes in people living with chronic HIV-1 infection were unsuccessful (Pontillo et al 2013). They hypothesised that long-term HIV-1 infection could render the NLRP sensor apparatus refractory to stimulation in the context of well-established HIV-1 induced long term immune dysfunction (Pontillo, Oshiro, et al. 2012; Pontillo, Silva, et al. 2012; Pontillo et al. 2013).

In another study, monocyte derived macrophages were stimulated with HIV-1 and shown via western blotting to demonstrate NFκB induction as the priming signal 1. ELISA assay quantification of IL-1β comparing WT and HIV-1 env knock-out strains suggested that the

trimeric fusion apparatus was dispensable for inflammasome activation and that only addition of ATP in the presence of HIV-1 was able to provoke IL-1β secretion. Indeed, if K^+ efflux is truly the common denominator for signal 2 induction, it is curious that the addition of Alum, nigericin and monosodium urate crystals did not increase IL-β secretion in the presence of HIV-1, as these signals have been linked to the induction of K^+ efflux (Hernandez et al. 2014).

So far, therefore, investigation of NLRP3 inflammasome activation by HIV-1 led to two conflicting models. One group implied that the virus HIV-1 was enough to stimulate both signal 1 and signal 2 for inflammasome activation;(Guo et al. 2014) whereas another study showed that HIV-1 could not induce signal 2 stimulation and that priming was also required (Hernandez et al. 2014). To add a further level of complexity, TLR8 sensing of HIV-1 RNA was suggested to trigger signal 1, which was necessary for signal 2 to progress (Guo et al. 2014). The early work of Corasaniti and colleagues (Corasaniti MT et al. 2001) taken together with that of Latz's group (Hernandez et al., 2014) suggests that while HIV-1 gp120 is dispensable for inflammasome activation, gp120 may independently activate this pathway and during virion infection, contribute to the stimulation of the NLR-inflammasome and its rapid recruitment (Walsh et al. 2014). Therefore, while many unknowns surrounding NLR-HIV-1 interaction persist, particularly around the ability of components of the HIV-1 proteome to activate NLRP3 independently of the rest of the virion, further study is indicated.

4.0 Viroporins interact with the innate immune system

4.1 Structure and function of viroporins

Viroporins are small (generally <100AA) hydrophobic accessory proteins involved in the latter stages of the viral life cycle and are a main focus of this study. They are encoded by many viruses such as respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza virus A (IAVA), encephalomyocarditis virus (ECMV), hepatitis C virus (HCV) as well as HIV-1 (explored in section 4.3) (Nieva et al., 2012). Many viroporins are involved in the budding process, oligomerising to create pores from which mature virions may escape the cell. In many instances, transmembrane conductance of cations such as Na^+, K^+, Ca^{2+}, Cl^- and H^+/protons disrupts membrane potential leading to membrane depolarisation which helps virion release. The change in membrane permeability induced by the ion channel action of viroporins is termed 'membrane leakiness'(Nieva et al. 2012; Wang et al. 2011; Triantafilou & Triantafilou 2014). A proposed classification model of viroporins is based firstly on the transmembrane folding properties and secondly by the transmembrane location of C' and N' terminal domains as visualised in figure 20 overleaf (Nieva et al. 2012).

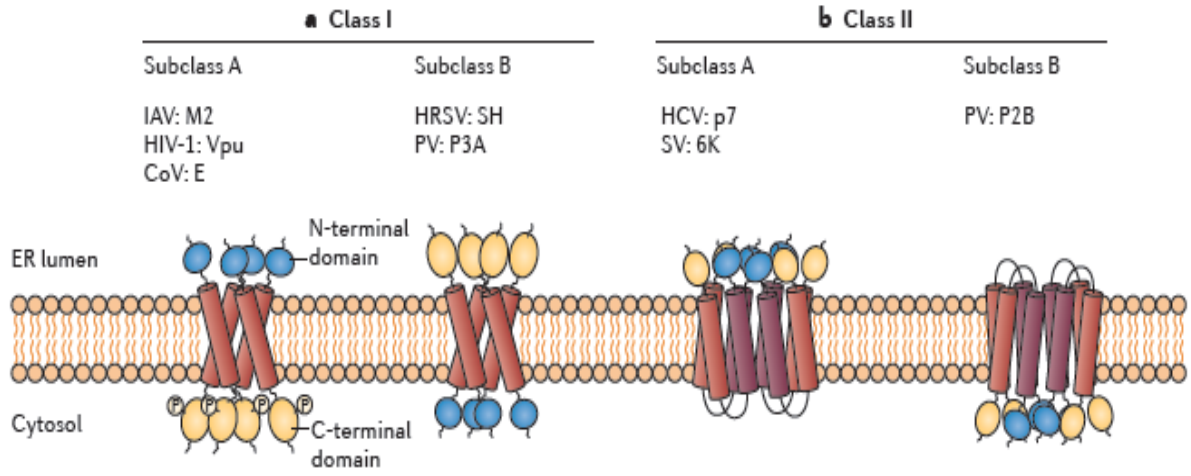


Figure 20. ***Viroporins: proposed classification by structure.*** **Adapted from Nieva et al 2012 (Nieva et al. 2012).**

Some viroporins are also thought to perform other roles which indirectly contribute to viral escape from host cell defences as the mature virion leaves the cell such as the downregulation of certain cell surface receptors or viral restriction factors. By definition, viroporins are "not *essential* for viral replication", but their presence has been shown to "assist viral growth and their presence is nearly always *essential* for viral release" (Nieva et al. 2012).

4.2 Viroporins activate the NLRP inflammasome

Viroporins have been known to stimulate the NLRP3 inflammasome pathway. Viroporins from IAVA, RV and RSV have been shown to activate the NLRP3 inflammasome. The underlying mechanism is the viroporin mediated alteration of specific intracellular cation concentrations (Nieva et al. 2012; Triantafilou & Triantafilou 2014).

The M2 protein is encoded by a small genomic IAVA RNA fragment. M2 allows the entry of protons into virions, promoting virus uncoating in endosomes (Mould et al. 2000). In addition, the ion channel activity of M2 might lead to a pH balance in IAV infected cells, between that of the acid lumen of the TGN and the pH of the cytoplasm (Sakaguchi et al. 1996). M2 viroporin was shown to activate the NLRP3 inflammasome via its actions upon H^+ which promotes endosomal IAVA uncoating in the viral life cycle and appeared to trigger K^+ efflux as signal 2 (Ichinohe et al. 2010).

SH is a small hydrophobic protein encoded by the RSV. Its absence leads to viral attenuation and prevention of apoptosis in infected cells. SH protein has a single α-helical transmembrane domain and forms homopentamers in several detergents. The SH protein forms oligomers that behave as ion channels when activated at low pH (Gan et al. 2012). A study using deletion mutants of RSV proteins has found that SH viroporin activates the NLRP3 inflammasome in lung epithelial cells by disturbing the Na^+/K^+ cell homeostasis in

infected cells. SH was found to localise in the Golgi membranes (Triantafilou, Kar, Vakakis, et al. 2013).

Enteroviruses (such as poliovirus or enterovirus 71) encode 2B which is a small hydrophobic protein that is localized at the rough ER membrane (Bienz et al. 1987). The ability of protein 2B to enhance membrane permeability serves to facilitate virus release (van Kuppeveld et al. 2005). 2B forms membrane-embedded pores of extracellular Ca^{2+} and also facilitates release of Ca^{2+} from intracellular stores, thus causing disturbances in the intracellular Ca^{2+} homeostasis (van Kuppeveld et al. 1997). These disturbances are recognised by NLRP3 thereby activating the inflammasome (M Ito et al. 2012).

Similarly, the HRV 2B protein shunts Ca^{2+} ions out of the ER and Golgi apparatus thereby increasing cytosolic Ca^{2+}concentration, thus triggering inflammasome activation. Calcium channel inhibitors such as verapamil were able to decrease IL-β secretion (Triantafilou, Kar, van Kuppeveld, et al. 2013). Additionally, ECMV viroporin 2B was studied in a murine model using dendritic cells and monocytes, and it was shown that ECMV, like HRV 2B signals to the NLRP3 via alterations in Ca^{2+} concentration (M Ito et al. 2012).

HCV (hepatitis C virus) p7 is a small hydrophobic protein that is involved in the production and release of infectious HCV particles from infected cells. HCV p7 has the ability to insert into membranes and assemble into homo-oligomeric complexes that function as ion channels (Steinmann et al. 2007). HCV p7 activates NLRP3 by causing fluxes of endoplasmic reticulum Ca^{2+} to the mitochondria via the cytosol resulting in apoptosis promoting HCV dissemination. This is the mechanism by which HCV is observed to activate the NLRP3 inflammasome in monocytes and hepatic macrophages (Chattergoon et al. 2014; Negash et al. 2013). Interestingly, HCV also causes fluxes in intracellular chloride concentrations during its life cycle but this effect is not mediated by p7 (Igloi et al. 2015).

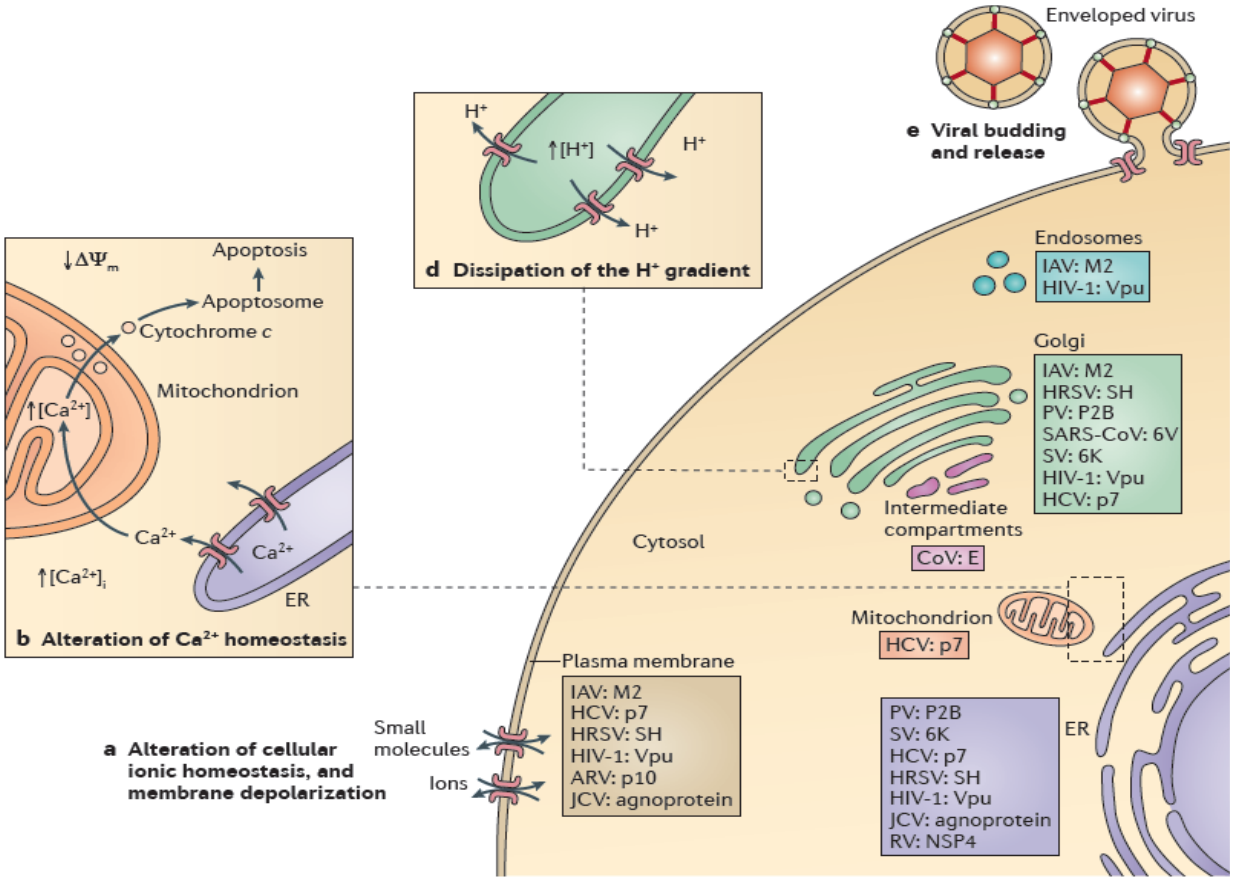


Figure 21. ***Modelling potential mechanisms of viroporin induced inflammasome activation.*** **Various viroporin activities have been suggested as candidates for signal 2, including changes in cell membrane depolarisation, derangement of cytosolic, mitochondrial, or ER and Golgi apparatus cation homeostasis and processes involved in viral budding. Adapted from Nieva et al 2012 (Nieva et al. 2012).**

4.3 HIV-1 Vpu viroporin

HIV-1 virus protein unique (Vpu) is an 81 AA length class Ia viroporin (by virtue of its protein folding and cytosolic C' terminal domain/cell surface N'terminal domain, see figure 22 below). HIV-1 Vpu is translated late in the viral replication cycle from bicistronic *vpu-env* mRNA as a consequence of 'leaky' ribosome scanning across a common *vpu-env* open reading frame in an HIV-1 Tat dependent manner (Schwartz et al. 1990). Vpu is processed to putatively form a structure comprising five alpha helices arranged around a central ion transducing pore (Padhi et al. 2013; Nieva et al. 2012). HeLa cells infected with HIV-1 Vpu_{del} were prevented from releasing new virions after Vpu was transfected, implying the critical role for this viroporin during HIV-1 infection (Varthakavi et al. 2003). Curiously, the transmembrane domain of fusion protein gp41, by virtue of the membrane perturbing activities of its lentiviral lytic peptide domains has been classified a viroporin, though it is Vpu which behaves as the true viroporin of HIV-1 (Costin et al. 2007).

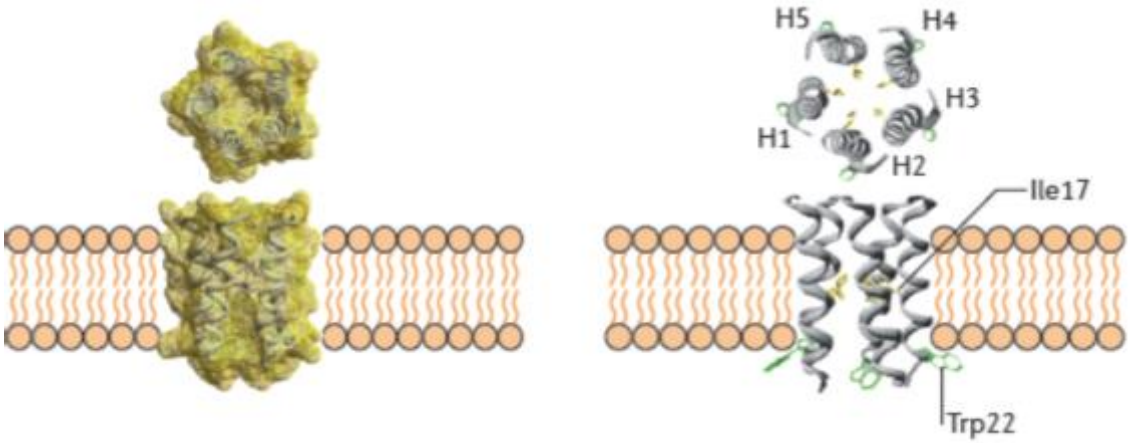


Figure 22. ***Proposed structure of HIV-1 Vpu.*** **Biophysical studies suggest the active Vpu oligomer is composed of 5 alpha helix motifs forming a putative central ion channel with a cytosolic C' terminal and extracellular N' terminal domain (Padhi et al. 2013; Nieva et al. 2012).**

During viral release, Vpu marks for degradation or inhibits various host defence proteins to favour HIV-1 survival. Vpu down-regulates CD4 (to both mask the cell from the immune system and to prevent departing virion *Env* proteins from attaching to their parent host cell (Willey et al. 1992). M-type Vpu is also thought to mark the viral restriction factor tetherin for destruction by the E3 ubiquitin/proteasome system (Mangeat B et al. 2009; Blanchet et al. 2012).Vpu also down-regulates signalling lymphocyte activation molecule family member 6 (SLAMF6) which is a candidate inducer of NK cell killing as well as downregulating CD1d, a cell surface protein involved in NKT-cell interactions (Richard & Cohen 2010; Roy et al. 2014).

Vpu exhibits weak bidirectional monovalent cation conduction activity. This Vpu ion channel activity was first confirmed using a patch-clamp method with *Xenopus* oocytes, which ideally lend themselves to exogenous ion channel exploration as they contain no *self*-ion channels. It was also shown that Vpu has some permeability to divalent cations such as Ca^{2+} (Schubert U et al. 1996; Ewart et al. 1996). Later, more sophisticated biophysical studies confirmed Vpu

cation conductance characteristics as well as confirming that Vpu may also weakly conduct anions at a ratio of 1 anion to 20-30 cations (Mehnert et al. 2008; Li et al. 2013).

Structurally, Vpu has been shown to exhibit striking homology with that of the M2 viroporin of IAVA (Pinto et al. 1992; Ichinohe et al. 2010). It is not known whether HIV-1 Vpu is able to activate the NLRP3 inflammasome in a similar manner to that of the M2 viroporin encoded by IAVA where signal 2 primarily comprises M2 induced changes in proton concentration (Ichinohe et al. 2010). Vpu conducts the monovalent cations Na^+ and K^+ as well as the divalent cation Ca^{2+} (marginally) (Schubert U et al. 1996). The minimal threshold of ion efflux required for signal 2 NLR activation and recruitment of the inflammasome is unknown. In an *S. cerevisiae* model, Vpu was thought to indirectly modify intracellular K^+ concentrations, which is significant as this cation is thought to be very important as an upstream factor for NLRP3 activation (Herrero et al. 2013; Munoz-Planillo et al. 2013).

Taken together, studies of viroporin cation channel activity on the inflammasome appear to imply that their activity on cation homeostasis, if not initially K^+, could trigger downstream physiological changes resulting in K^+ efflux. This explanation could explain why a plethora of DAMPs are able to activate the same NLR signalling pathways.

5. Aims

Pathogen sensing by the innate immune system results in pro-inflammatory cytokine secretion; which provides host defence against microbial pathogens. HIV-1 infection is known to trigger an inflammatory response and dysregulation of the normal profiles of IL-1β and IL-18 secretion both acutely and chronically. This study aims to explore how HIV-1 is sensed by the innate immune system and the pattern recognition receptors (PRRs) involved in HIV-1 host defence and inflammasome activation.

Aims:

- To determine the PRRs involved in sensing HIV-1 proteins such as gp120 in THP1 monocytes and MOLT4 CD4 T-cells.
- To elucidate the mechanism of inflammasome recognition of HIV-1 proteins such as gp120, gp41 and Vpu in THP1 cells and primary monocytes and determine how they trigger IL-1β secretion and IL-18 secretion.
- Furthermore, to try to inhibit inflammasome activation by using different inflammasome as well as ion channel or ROS inhibitors.
- To investigate using confocal microscopy as well as Förster resonance energy transfer (FRET) the entry pathway of HIV-1 virions in primary human monocytes to explore how HIV-1 interacts with different cellular components and determine whether HIV-1 cellular location is critical for triggering cytokine secretion.
- To determine the mechanism of inflammasome activation by HIV-1 in primary human monocytes and investigate whether lipid rafts, which play an important role in the life cycles of most enveloped viruses, are critical in HIV-1 induced inflammasome activation.

Chapter 2: Methods & Materials

1. Tissue Culture

To prevent contamination, cells underwent regular tissue culture in a Microflow Class 2 laminar flow hood in a sterile facility. Industrial methylated spirit was used to clean the work area, equipment and hood cabinet surface prior to use. Disposable gloves and shoes were worn along with lab coats exclusive to the tissue culture room to prevent cross-contamination with the main lab area.

1.1 Cell Types

THP1 monocytes are acute monocytic leukaemia cells,(Tsuchiya et al., 1980) which proliferate in suspension in culture medium. In this project, THP1 cells were obtained from the American Tissue Culture Collection (ATCC), MD, USA. THP1 cells were propagated in culture flasks using cell culture medium comprising GIBCO RPMI 1640 GlutaMAX™-I, 10% (v/v) heat-inactivated foetal calf serum, 1% (v/v) non-essential amino acids, (all of which obtained from Life Technologies Ltd, UK) and incubated at 37ºC 5% CO_2. Every three days, the cells were centrifuged at 200G using a benchtop Hettich centrifuge, the supernatant was aspirated and replaced with new medium to prevent the build-up of toxic metabolites, then cells were transferred in new medium to a new 75 cm^2 flask. THP1 cells were passaged to new culture flasks when they became over approximately 90% confluent.

MOLT4 T-lymphoblasts are acute lymphoblastic leukaemia cells which are semi-adherent in nature (CRL-1582, obtained from American Tissue Culture Collection, USA). MOLT4 cells were also propagated in GIBCO RPMI 1640 GlutaMax-I™, 10% (v/v) FCS at 37ºC 5% CO_2. Every three days, supernatant was aspirated and replaced with new medium to prevent the build-up of toxic metabolites as described for THP1 cells above.

1.2 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (referred to as primary monocytes throughout the text to avoid confusion with secondary cell lines), were isolated from buffy coat by Ficoll-Paque Plus (Sigma Aldrich). Primary monocytes were isolated using the Miltenyi Biotec (Miltenyi Biotec Ltd, Almac House, Bisley, Woking GU24 9DR Tel. 01483 799800) MACS magnetic cell separation system in order to isolate the $CD14^+$ fraction from the primary monocytes. Primary monocytes were maintained in GIBCO RPMI 1640 GlutaMAX™-I, 10% (v/v) heat-inactivated foetal calf serum, 1% (v/v) non-essential amino acids.

1.3 Freezing and thawing cells

Cells were frozen in 10% (v/v) dimethyl sulfoxide (DMSO) in foetal calf serum (FCS) at -80°C. Upon thawing, cells were immediately diluted with the GIBCO RPMI 1640 GlutaMAX™-I, 10% (v/v) heat-inactivated foetal calf serum and centrifuged at 200G using a benchtop Hettich centrifuge. Supernatant was rapidly aspirated to prevent DMSO induced cell toxicity. Cells were then resuspended in a small volume of GIBCO RPMI 1640 GlutaMAX™-I, 10% (v/v) heat-inactivated foetal calf serum and passaged at 90% confluency.

1.4 Cell counting & viability

The Neuerbauer haemocytometer is a tool used to manually count cells to accurately estimate the concentration of cells in a sample prior to use in experiments. A flask of cells is resuspended in cell culture medium (adherent cell lines must be first separated from each-other and the flask using trypsin) and placed directly on to the haemocytometer ‘slide’. The haemocytometer slide contains a fine lattice of trenches to enable accurate counting under light microscopy. Between counts, the slide is washed using liberal amounts of industrial

methylated spirit and autoclaved distilled water then allowed to dry. light microscopy. Between counts, the slide is washed using liberal amounts of industrial methylated spirit and autoclaved distilled water then allowed to dry. The number of cells per millilitre is calculated by dividing the number of cells in each large square by five and then multiplying by 10,000.

To estimate cell viability throughout stimulation experiments, cells were counted before and after stimulations. There are various ways to determine cell viability such as probing with markers of cell necrosis or apoptosis. The Trypan Blue Live-Dead assay is a quick method of determining cell viability. Dead or dying cells become permeable to the Trypan Blue stain, and these may be counted under light microscopy using a Neuerbauer haemocytometer (Strober, 2001).

Trypan Blue 0.4% (w/v) solution was obtained from Sigma (catalogue number -T8154). Equal volumes of 0.4% (w/v) trypan blue stain and a well-mixed cell suspension (not too vigorous) were mixed (e.g. mix 100µl trypan blue stain with 100 µl cell suspension). The trypan blue/cell mix was visualised with the haemocytometer grid under the microscope. Trypan Blue is a "vital stain", so it is excluded from live cells. Live cells appear colourless and bright (refractile) under phase contrast. Dead cells stain blue and are non-refractile. The cells viable (live) and dead cells in one or more large corner squares were counted. The formula used to calculate cell concentration per ml was as follows: Average number of cells in one large square x dilution factor* x 10^4, where dilution factor is usually 2 (1:1 dilution with Trypan Blue), but may need to further dilute (or concentrate) cell suspensions. The formula used to calculate cell viability was: No. of Viable Cells Counted / Total Cells Counted (viable and dead) x 100 = % viable cells.

2 Antibodies, markers, reagents

2.1 Antibodies & markers

Target	Species	Source	Cat. No.
TLR2	Goat polyclonal	SCB	sc-8694
TLR3	Rabbit polyclonal	SCB	sc-28999
TLR4	Goat polyclonal	SCB	sc-8690
TLR7	Mouse monoclonal	SCB	sc-57463
TLR8	Mouse monoclonal	SCB	sc-373760
NLRP1	Mouse monoclonal	SCB	sc-166368
NLRP1	Goat monoclonal	SCB	sc-123579
NLRP3	Rabbit polyclonal	SCB	sc-66846
NLRP3	Goat polyclonal	SCB	sc-34410
NLRC4	Rabbit polyclonal	SCB	sc-99175
NLRC5	Goat polyclonal	SCB	sc-248094
NLRP12	Rabbit polyclonal	SCB	sc-99056
Caspase-1 p10	**Mouse monoclonal**	**SCB**	**sc-22166**
ASC	**Goat monoclonal**	**SCB**	**sc-33958**
Pro-IL-1β	**Rabbit polyclonal**	**AbCam**	**ab2105**
Phospho-Iκbα	**Rabbit monoclonal**	**CST**	**2859L**
β-actin	**Mouse monoclonal**	**SCB**	**sc-47778**
HIV-1 Vpu	**Rabbit polyclonal**	**NIHARP**	**969**
HIV-1 Vpu	**Rabbit polyclonal**	**AbCam**	**ab81532**
HIV-1 gp41	**Rabbit polyclonal**	**AbCam**	**ab30755**
HIV-1 gp41(7HR)	**Mouse monoclonal**	**NIHARP**	**12295**

Figure 23. ***Primary antibodies used according to manufacturer's instruction for Western Blotting, Flow Cytometry and Imaging studies.*** **Rabbit polyclonal anti-HIV-1 Vpu from NIH donated by Dr. Stephan Bour and Dr. Klaus Strebel (Nguyen et al., 2004). NIH: HIV-1 anti-gp41 mAb (7H6), from Dr. Mark Connors via NIBSC, UK.**

Target	**Species/Fluorophore**	**Source**	**Cat No.**
Goat	Rabbit IgG-FITC Fc fragment specific	Jackson ImmunoResearch	305-095-046-JIR
Mouse	Goat IgG-FITC Fc fragment specific	Jackson ImmunoResearch	305-095-046-JIR
Mouse	Goat IgG-FITC	DAKO, Denmark	F0479
Rabbit	Goat IgG FITC	DAKO, Denmark	F0205
Goat	Donkey IgG-TRITC	Santa Cruz	SC 3855
Mouse	Rabbit HRP	DAKO	P 0260
Rabbit	Swine HRP	DAKO	P 0399
Biotinylated SDS PAGE Stain Broad Range	N/A	BioRad	1610319
Intracellular Cl^-	MQAE	Invitrogen	E-3101
Lipid Rafts	Cholera toxin B-TRITC	List Biological Laboratories, 540 Division Street Campbell California USA	107

Figure 24. Table of secondary antibodies and other markers used in Western Blotting, agarose gel electrophoresis, anion tagging or cell organelle markers. All markers were used according to the manufacturer's instructions. Abbreviations: FITC, fluorescin isothiocyanate; TRITC, tetramethylrhodamine; HRP, horseradish peroxidase; MQAE, N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide. Further company details are described overleaf.

Company/Institution	Physical Address	Contact No./Web Address for Further Contact Details
DAKO	Dako Denmark A/S Produktionsvej 42 DK-2600 Glostrup Denmark	Tel. 0845 712 5292 www.dako.com/uk/index/aboutdako/contact.htm
Sigma Aldrich	Sigma Aldrich 328/329 Cambridge Science Park Cambridge CB4 0WE	Tel. 0800 717181 www.sigmaaldrich.com/united-kingdom.html/
Invitrogen & Invivogen (ThermoFischer)	Life Technologies Ltd 3 Fountain Drive Inchinnan Business Park Paisley PA4 9RF	Tel. 0800 269 210 www.thermofisher.com/uk/en/home/technical-resources/contact-us.html?cid=fl-contactus
NIH AIDS Reagents Programme 'NIHARP'	20301 Century Boulevard Building 6, Suite 200 Germantown, MD 20874	Tel. 1-240-686-4740 www.aidsreagent.org/contact_us.cfm
The National Institute for Biological Standards and Control 'NIBSC'	Blanche Ln, South Mimms, Potters Bar EN6 3QG	Tel. 01707 641000 www.nibsc.org/about_us/contact_us.aspx
Santa Cruz Biotechnology 'SCB'	Bergheimer Str. 89-2 69115 Heidelberg, Germany	Tel. 1-800-457-3801 www.scbt.com/contact.html
AbCam	330, Cambridge Science Park Milton Road, Cambridge Cambridgeshire, CB4 0FL	Tel. 01223 696000 www.abcam.com/index.html?pageconfig=contactus
Jackson ImmunoResearch distributed by Stratech Scientific	Stratech Scientific Ltd. Unit 7, Acorn Business Centre Oaks Drive , Newmarket, Suffolk, CB8 7SY	Tel. 01638 782600 www.stratech.co.uk
Cell Signalling Technologies distributed by New England Biolabs	75-77 Knowl Piece Wilbury Way Hitchin, Herts SG4 0TY	Tel. 0800 318486 www.neb.uk.com/customerService/index.asp
Beckton Dickenson	The Danby Building Edmund Halley Road Oxford Science Park, Oxford OX4 4DQ	Tel. 01865 748844 www.bd.com/uk/contact/
List Biological Laboratories distributed by QuadraTech Diagnostics	PO Box 167 Epsom, Surrey KT18 7YL	Tel. 0333 321 2371 www.quadratech.co.uk/contactus

Figure 25. ***Table of Main Company/Institutional Contact Details.*** **Distributor telephone number/physical address/website is shown where appropriate.**

2.2 Standard laboratory chemicals

All standard laboratory chemicals were obtained from Sigma Aldrich UK, Gillingham, Dorset SP8 4XT, unless stated otherwise.

2.3 Inhibitors

Name	Target/Function	Abbreviation	Cat. No.
Amantadine hydrochloride	Proton channel inhibition	N/A	A1260
Rimantadine	Proton channel inhibition	N/A	R9151
Verapamil hydrochloride	Calcium channel inhibition	N/A	V4629
1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester)	Intracellular calcium chelator	BAPTA-AM	A1076
N-acetylcysteine	Reactive oxygen species inhibitor	NAC	A7250
Diphenyliodonium hexafluorophosphate	Reactive oxygen species inhibitor	DPI	548014
Tetramethylammonium chloride	Potassium channel inhibitor	TEA	T19526
Cyclohexamethylene amiloride	Vpu channel inhibitor	HMA	A9561
5-(*N*-ethyl-*N*-isopropyl)amiloride	Proton or sodium channel inhibitor	EIPA	A3085
5-nitro-2-(3-phenylpropyl-amino) benzoic acid	Chloride channel inhibitor	NPPB	N4779
R(+)-[(6,7-Dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid	Chloride channel inhibitor	IAA-94	I117

Figure 26. Table of inhibitors used in this study. All inhibitors were sourced from Sigma Aldrich (address in section 2.2).

3 Plasmids

3.1 Transformation

3.1.2 Principles

The competence of bacteria to take up an engineered 'designed for purpose' plasmid may be natural or artificially induced. The induction of artificial competence may involve the use of solutions containing cations to induce the passive permeability of bacterial cell walls to take-up the plasmid. *E. coli* for example has a negatively charged cell wall – the negatively charged DNA is thereby bridged allowing DNA-cell wall interaction.

3.1.3 Transformation method

In this project, competent cells (*E. coli* GT116 strain from Invitrogen) were used. 20ng of the desired plasmid was mixed with 100µl *E. coli* GT116 then incubated on ice for 30 minutes, followed by a 45 second heat shock at 42°C. The sample was then incubated on ice for 2 minutes before incubation in 500µl Luria broth (86mM NaCl, 10% (w/v) bactopeptone, 5% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and $MgSO_4$)) for 1 hour at 37°C, 225rpm on an incubator-shaker. Using a sterilised glass spreader, colonies were spread on an agar plate containing ampicillin (100µg/mL) for antibiotic selection pressure and incubated overnight at 37°C in a Genlab general purpose incubator. As a control this step was repeated with 20ng of pUC19 control plasmid in a separate tube.

The following morning the plates were checked for colonies. Individual colonies were selected with a sterile pipette tip or toothpick and immerse in a sterile culture tube containing

Luria broth (10ml) supplemented with Ampicillin (10µl from 100µg/mL stock). Tubes were shaken in an orbital shaker at 37ºC overnight.

3.2 Isolation and electrophoresis

3.2.1 Principles

Phenol-chloroform extraction of DNA relies upon the localisation of nucleic acids to the aqueous phase of a water-saturated mixture of phenol, chloroform and isoamyl alcohol. Chloroform stabilises an unclear phenol interface, and isoamyl alcohol prevents foaming of the sample. After successive centrifugations, one can be confident that undesired molecules such as proteins have localised in the organic phase and that DNA remains in the aqueous phase (Wallace 1987a).

Ethanol is used to precipitate DNA in the presence of a cation by virtue of its comparatively lower dialectric constant relative to water, which would disrupt the potential of DNA phosphate groups to undergo ionic bonding. The addition of large volumes of ethanol to the aqueous phase allows ionic bonds between DNA phosphate groups and cations such as sodium to form. The sample is then frozen to increase efficiency of precipitation, then immediately centrifuged to precipitate DNA-cation complexes out of the solution. Following rapid aspiration of aqueous solvent, precipitated DNA is re-suspended in autoclaved distilled water (Wallace 1987b). For quality control, an agarose gel is cast. The negatively charged DNA runs down the gel and is separated according to mass by the agarose gel matrix – shorter bands travel faster along the voltage gradient.

3.2.2 Isolation of plasmid DNA & quality control

To isolate plasmid DNA from competent cells, Luria broths containing transformed *E. Coli,* grown to a cell density of approximately 3–4 x 10^9 cells per ml were centrifuged and lysed after re-suspension in 400µl STET buffer (233mM sucrose, 1M Tris HCl 500mM EDTA, 1.7M Triton X-100, pH 8.0) by boiling with 10µl (50mg/ml) lysozyme for 5 minutes. Samples were then incubated on dry ice for 1 minute, centrifuged for 30 minutes after which the pellet (cell debris) was removed. Samples then underwent 30 minute incubation with 5µl (20µg/ml) RNAase at 37°C to remove unwanted RNA. Samples were then vortexed in phenol-chloroform-isoamyl alcohol (ratio 45:45:1) between two successive 15 minute centrifugations at 200G using a Hettich MIKRO centrifuge in new autoclaved Eppendorf tubes containing isolated aqueous phases of each sample to increase purity. Phenol-chloroform-isoamyl alcohol stages were performed in a fume cupboard. Phenol-chloroform extraction removes remaining contaminant proteins and RNase A from the DNA sample. When phenol is mixed with the aqueous solution containing DNA, proteins will move into the phenol phase and will be separated from the aqueous DNA.

The aqueous phase of each sample was transferred once again to a new autoclaved Eppendorf tube, to which 20µl 2M sodium acetate (pH=6.5) then 1ml 95% (v/v) ethanol was added prior to freezing at <0°C. Samples were taken from -80°C conditions and immediately centrifuged for 20 minutes at 200G using a Hettich MIKRO centrifuge and aspirated with autoclaved 1ml tips. 80µl autoclaved distilled water was used to re-suspend the pellet of precipitated DNA. The suspension of DNA was then transferred to a new autoclaved tube and stored at -20°C.

To confirm the presence of DNA, agarose electrophoresis was used. A 1% (w/v) agarose gel was cast using 1.0g type II agarose in 100ml ELFO buffer (2M Tris base, 50mM EDTA, pH 7.7) and 15µl Gel Red (Biotium, Cat No. 41003) in 100µl distilled H_2O. Samples in addition

to a 1kb DNA ladder (BioRad, 161-0391) were loaded to the gel and electrophoresed at 100V for 45 minutes prior to visualisation using a Stratagene Eagle Eye UV imaging system.

3.2.3 RNA interference

Plasmids typically contain a promoter region for RNA polymerase III to optimise expression of the desired protein, and an antibiotic resistance gene to ensure that the only cells surviving and replicating are those which take up the plasmid during transfection. Silencing plasmids contain the complementary RNA sequence against the endogenous RNA sequence of the protein of interest. In general, this is known as RNAi (RNA interference). As part of our analyses, we used small hairpin RNA (shRHA) sequences which make a tight hairpin turn and have a comparably lower degradation rate to RNAi which does not contain a small hairpin turn.

In this study, complementary sequences against the RNA sequence for the protein of interest were generated from the 'NUCLEOTIDE' online database. Plasmids containing the complementary RNA sequence were generated by Invivogen using the psh7SK vector (Invitrogen), see figure 27.

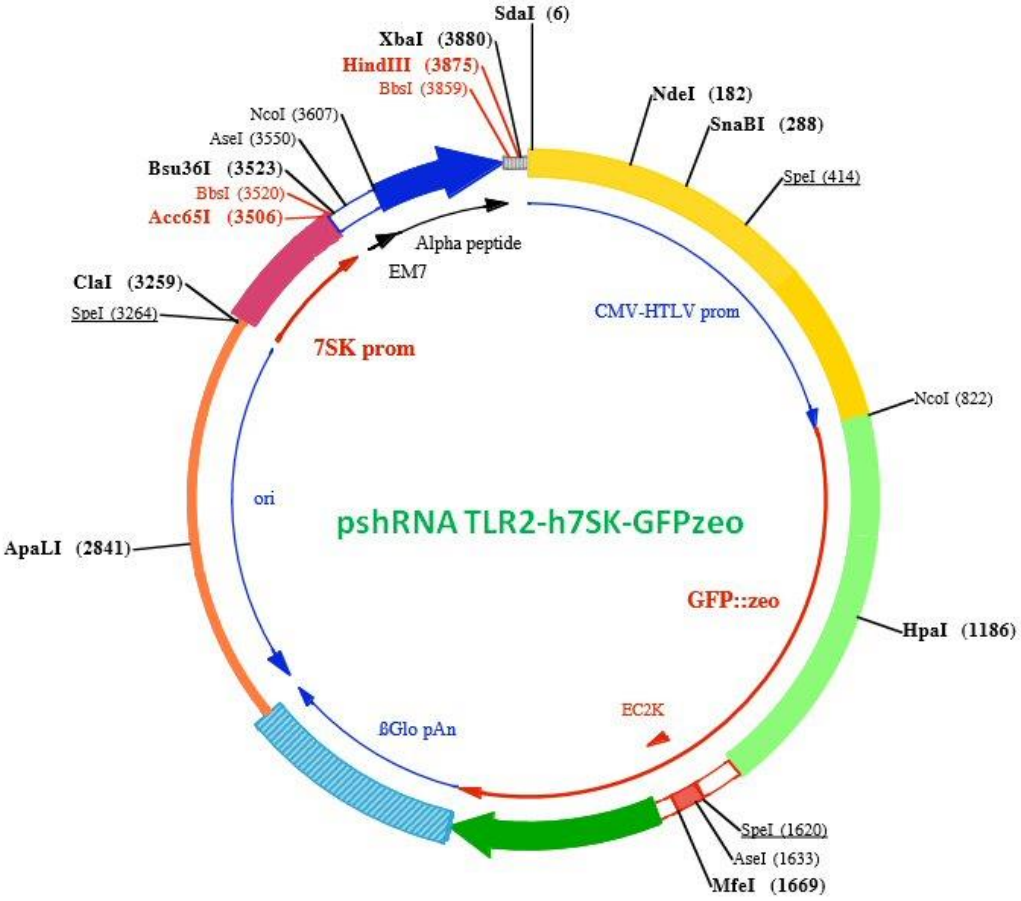


Figure 27. ***TLR2 pshRNA plasmid.*** **This is a schematic representation of the pshRNA-7SKGFP::Zeo plasmid from the human 7SK RNA pol II promoter. The shRNA region (red) encodes TLR2 interfering RNA. Image supplied by Invivogen.**

Different pshRNA clones were generated using the psh7SK vector from Invitrogen. The same protocol was used for TLR2, TLR4, TLR7, TLR8, NLRP1, NLRC4 and NLRC5: RNA interference was used in order to silence the listed genes. The most efficient clones were those against the sequences described in the table (figure 28) overleaf.

Target	Target Sequence
TLR2	GTCAATTCAGAACGTAAGTCA
TLR4	GCCAGGAGAACTACTACGTGTGAA
TLR7	GGGTATCAGCGTCTAATACA
TLR8	GACCAACTTCGATACCTAAA
NLRP1	GAAGGAGGAGCTGAAGGAGTT GGCCTGATTATGTGGAGGAGA
NLRP3	GGAAGTGGACTGCGAGAAGTT
NLRC4	GGATGCTGCTAGAGGGATCAT GACAACTGGGCTCCTCTGTAA
NLRC5	GAACCTGTGGAGCTGTCTTGT GCAACAGCATCTGCGTGTCAA
Clathrin heavy chain target sequences	AAGCUGGGAAAACUCUUCAGA (chc-1) UAAUCCAAUUCGAAGACCAAU (chc-2)

Figure 28. ***Table of RNAi sequences.***

TRANSFECTION OF PLASMID DNA

THP1 or primary monocytes ($1x10^5$) were seeded in 24 well plates. 1µg of plasmid siRNA were re-suspended in 30µl GIBCO Opti-MEM (31985-062, Life Technologies Ltd. UK), complexed with 10µl Lipofectamine 2000 (11668027, Life Technologies Ltd. UK) + 40µl Opti-MEM. The cells ($1x10^5$ cells per well) were resuspended in 800µl of Opti-MEM. After the addition of Lipofectamine 2000-complexed DNA, cells were incubated overnight in a 24 well plate. The following day the medium was removed and cells were re-suspended in GIBCO RPMI 1640 GlutaMAX™-I + 100ug/ml Zeocin to apply antibiotic selection pressure. Control plates with untransfected cells, cells with just the psh7SK vector and psh7SK with

scrambled sequence were also used. Efficiency of transfection was determined by flow cytometry, since the plasmids expressed GFP, as well as western blotting for the receptor of interest (figure 29). The procedure was repeated until 80% knockdown efficiency was achieved and then the cells were used for stimulations.

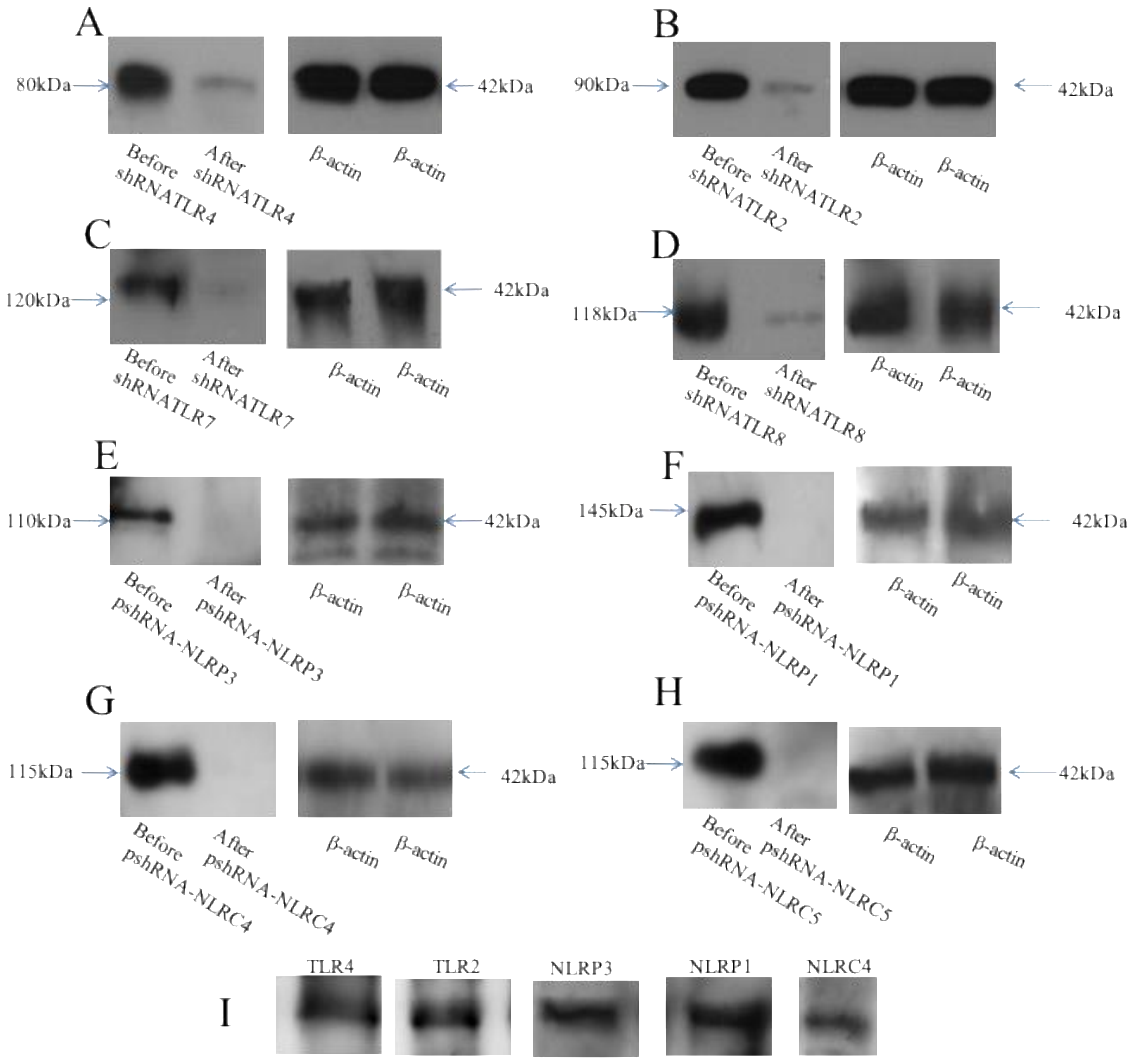


Figure 29. ***THP1 cells silenced for TLR4, TLR2, TLR7, TLR8, NLRP3, NLRP1 and NLRC5.*** **THP1 cells were silenced for (A) TLR4, (B) TLR2, (C) TLR73, (D) TLR8, (E) NLRP3, (F) NLRP1, (G) NLRC4, (H) NLRC5 and percentage of knockdown was compared to untransfected THP1 cells. Panel I: different gels showing the expression of TLR4, TLR2, NLRP3, NLRP1 and NLRC4 when pshRNA with scrambled sequence was used. The data is a representative of four independent experiments.**

4. HIV-1 Reagents & Protocols

4.1 HIV-1 envelope glycoproteins

Recombinant HIV-1 IIIB (MN) CXCR4-tropic glycoprotein (gp) 120 (cat.no. EVA646), (HIV-1 gp120) was obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project. Recombinant HIV-1 gp41 (cat. no. ARP6005) was similarly obtained from the Centre for AIDS Reagents.

4.2 HIV-1 Protein Plasmids

The HIV-1 Vpu plasmid (for map see figure 30 overleaf) was kindly donated by Dr. Fabien Blanchett, Cardiff University and transformed to competent DH10Bac *E. coli* (10360-014, Life Technologies Ltd, UK). HIV-1 Vpu DNA plasmid was originally sourced through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pcDNA-Vphu from Dr. Stephan Bour and Dr. Klaus Strebel. pcDNA-Vphu anti-Vpu antiserum originally from NIH AIDS Reagent programme (Nguyen et al. 2004; Maldarelli et al. 1993). The Vpu plasmid was transfected into THP1 or primary monocytes.

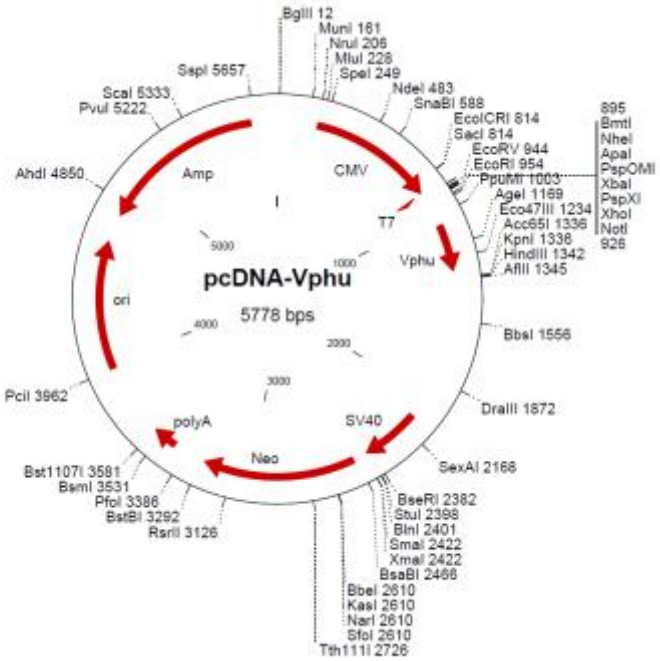


Figure 30. ***Plasmid map of HIV-1 Vpu plasmid pcDNA-Vphu.***

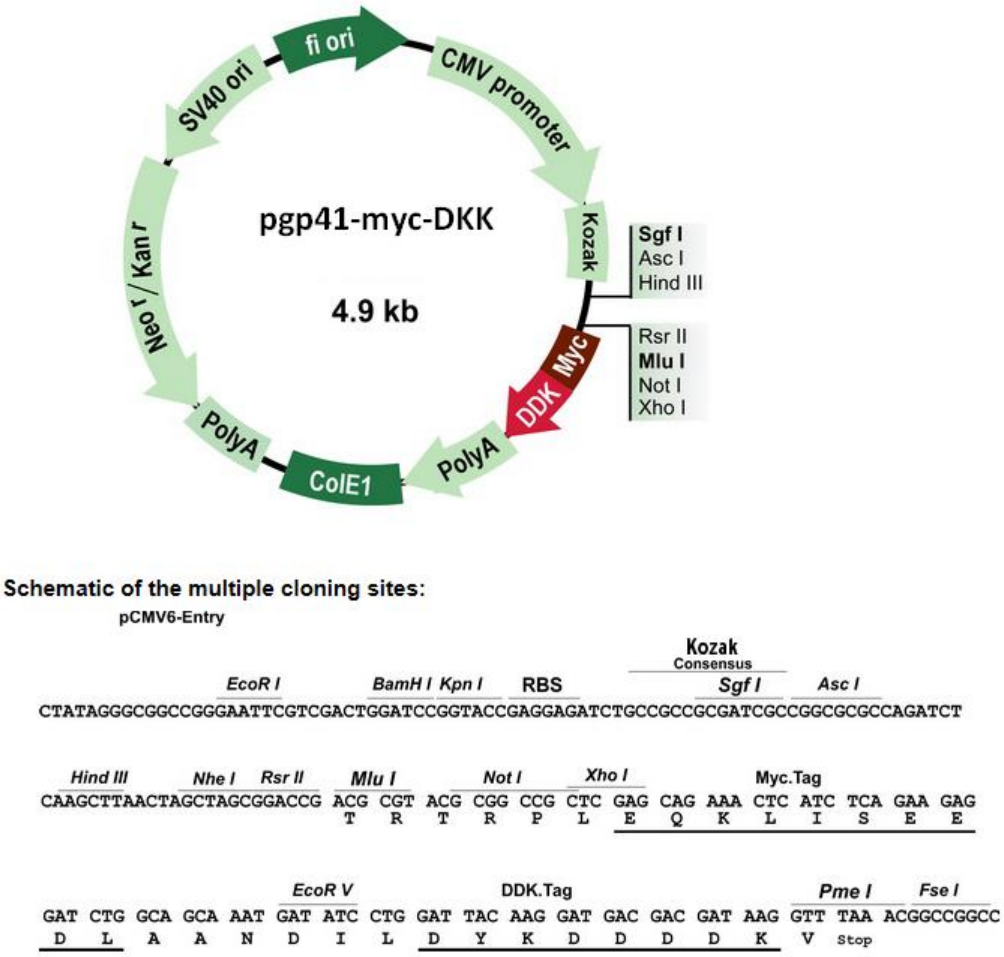


Figure 31. Plasmid map of HIV-1 gp41 plasmid pg41-myc-DKK.

The HIV-1 gp41 (pgp41-myc-DKK) is a plasmid encoding gp41 with a C terminal myc-DKK tag which was obtained from Origene (9620 Medical Center Dr # 200, Rockville, MD 20850, United States) distributed by Cambridge Bioscience (Munro House, Trafalgar Way, Bar Hill, Cambridge CB23 8SQ, Tel. 01223 316855). The gp41 plasmid map is shown in fig. 31 above. The vector was customised by Origene to express the gp41 NCBI Reference Sequence: NP_579895.1. catalogue number VC101730.

4.3 Virus production and infectivity assay

All HIV-1 production and infectivity assays were performed under category III biosafety conditions. HIV_{NL4-3} (X4-tropic) and HIV-1 $_{R8Bal}$ (R5-tropic) plasmids (kindly processed by Magdalena Czubala) were prepared by transfection of 1×10^6 293T cells per well in a 24-well plate with 1μg of the appropriate full-length infectious HIV-1 plasmid (figure 32 overleaf) using the FuGene6 transfection reagent (catalogue number E2693) from Promega UK (Delta House, Southampton Science Park, Southampton SO16 7NS, Tel. 023 80760225).

Virus-containing supernatants were passed through a through 0.45μm pore-size nitrocellulose membrane (Spin-X; Corning) filter to remove cellular debris and precipitated in 20% (w/v) polyethylene glycol at 4°C. Precipitated virus was then centrifuged at 14,000G using a SW28 rotor of Beckman centrifuge for 20 minutes, re-suspended in PBS, and frozen at −80°C until use. The level of HIV-1 p24 (capsid) protein in cell culture supernatants was determined by HIV-1 p24-specific ELISA assay, performed by HIV-1 $p24^{CA}$ Antigen Capture Assay kit from the AIDS & Cancer Virus Program (Frederick National Laboratory for Cancer Research, NCI at Frederick, Bldg. 427, Rm. 1 ,P.O. Box B, Frederick, MD, USA, Tel. 1-301-846-6829) which measures the amount of viral capsid protein in the supernatant.

Primary or THP1 monocytes ($1x10^6$) were incubated with 50ng HIV-1 p24: X4 or R5 tropic. For pharmacological inhibition studies, cells were treated with inhibitors described in section 2.3 for 1 hour at 37°C before HIV-1 infection and then cultured in the presence of drugs. HIV pNL4-3X4 was sourced through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: p83-2 from Dr. Ronald Desrosiers (Gibbs et al. 1995). Samples were centrifuged at 200G using a Hettich MIKRO centrifuge, then lysates were prepared at each time-point by vortexing the pellet in SDS PAGE reducing buffer and freezing at -20°C. The author thanks

and acknowledges Magdalena Czubala for her expertise and assistance in this component of the project.

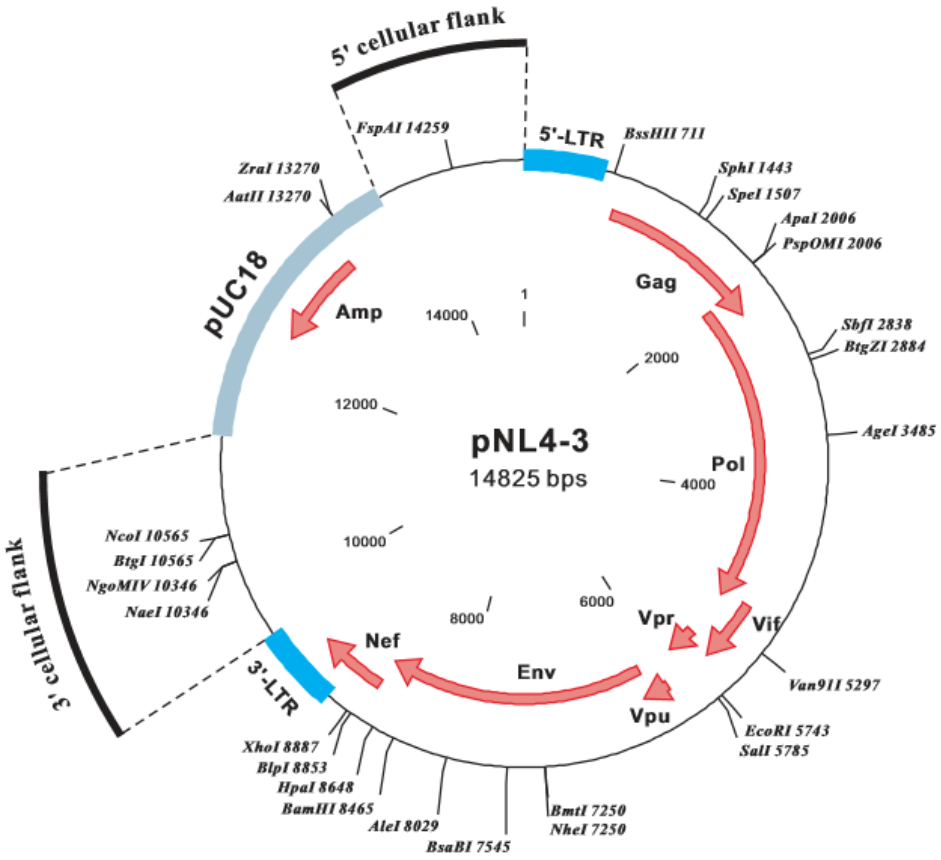


Figure 32. ***Full length HIV-1 plasmid pNL4-3.***

Upon transfection this clone directed the production of infectious virus particles in a wide variety of cells.

4.4 Limulus amoebocyte lysate assay

To exclude the presence of confounding bacteria/bacterial endotoxin contaminants, the Pierce LAL Chromogenic Endotoxin Quantitation kit (Cat. No. 88282) from ThermoFischer Scientific was employed in accordance with the proprietary protocol. Underlying principles of the assay are explained in figure 33.

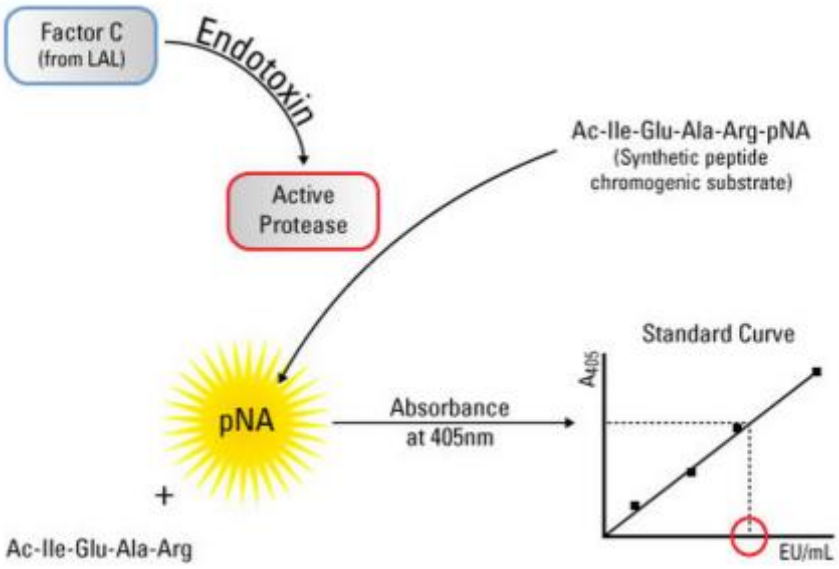


Figure 33. ***Limulus amoebocyte lysate assay principles.*** **The sample is mixed with the limulus amoebocyte lysate; the proteolytic activity of Factor C is activated by any endotoxins present in the sample. A chromogenic substrate is added which is cleaved by Factor C. The absorbance of light at 405nm can be measured and plotted against a standard curve. Adapted from (Life Technologies, 2014)**

7. Flow cytometry

7.1 Principles

Flow cytometry is a single cell quantification technique. Interestingly, developments in flow cytometry were rapid during the HIV epidemic of the 1980s, during which early research necessitated the quantification and analysis of CD4 T cell counts to define the natural history of infection. Cells of interest rapidly flow through laser beams in single file causing the emission of light which is then detected. This emission of light is known as 'scattering', with the forward scatter (FSC) reflecting the size of the cell and side scatter (SSC) reflecting the cell's granularity. Specific proteins can be detected by the addition of fluorophores conjugated with antibodies against the protein of interest. Luminescence of selected fluorophores is at 90° to the direction of cell flow and is thus received by the SCC detector.

To detect intracellular proteins, both primary and secondary antibody must be able to cross the membrane of the fixed cell: saponin is used to ensure adequate cell permeability. NaN_3 is used as a preservative. Whether probing for intracellular or cell-surface proteins, bovine serum albumin is used in washing stages to remove excess fluorescent antibodies thereby reducing background fluorescence to a minimum.

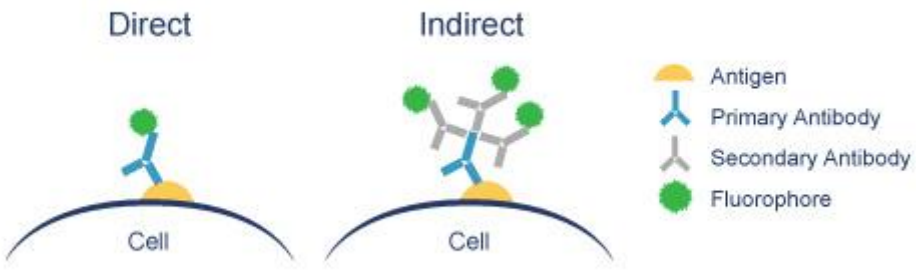


Figure 34. Direct vs. indirect immunofluorescence. Adapted from Vancells 2015. (Vancells JC 2015)

To detect proteins of interest, cells are tagged with antibodies raised against the specific proteins. Figure 34 above shows the difference between two common methods of probing cells for specific proteins. Direct immunofluorescence describes the use of an antibody directly conjugated to a fluorophore a compound which absorbs and then emits light i.e., fluoresces, at specific wavelengths for detection, wherein the antibody has been raised against the specific protein of interest. Indirect immunofluorescence, the technique used in this project for flow cytometry and enzyme-linked immunosorbance assay utilises two antibodies – the first (primary antibody) is raised against the protein of interest, and the second (secondary antibody) is raised against the primary antibody and conjugated to a fluorophore.

7.2 Analysing PRR expression via flow cytometry

Cell samples containing $1x10^6$ cells/ml, (both stimulated and unstimulated), were washed in 500µl phosphate-buffered saline (PBS) then fixed in 300µl 4% (w/v) paraformaldehyde for 15 minutes. Samples were then washed with PBS twice and then re-suspended in 200µl PBS/0.02% (w/v) bovine serum albumin /0.02% (w/v) NaN_3 /saponin prior to the addition of 2µg of primary antibody targeted against the PRR of interest. All primary antibodies were used at the same mass of 2µg and the same concentration of 2µg/ml.

Following 60 minute incubation at room temperature, samples were washed with 500µl PBS/0.02% (w/v) bovine serum albumin/0.02% (w/v) NaN_3 prior to incubation with 1µg of appropriate secondary antibody-FITC conjugate in 200µl PBS/0.02% (w/v) bovine serum albumin/0.02% (w/v) NaN_3/saponin for 45 minutes. All secondary antibodies were used at the same mass of 1µg and the same concentration of 1µg/ml. Samples were then washed twice in 500µl PBS/0.02% (w/v) bovine serum albumin/ 0.02% (w/v) NaN_3/saponin to remove excess secondary antibody-FITC, re-suspended and analysed with the Becton Dickinson FACS Calibur via Cell Quest software (BD Biosciences, California, USA) counting 10,000 cells under cell-specific settings. Unstimulated isotype samples as were used as negative controls and incubated with both primary and secondary antibodies. Low-level background fluorescence was determined by incubating unstimulated isotype controls with secondary antibody-FITC conjugate only for each individual experiment to allow full analysis of data.

8. Western Blotting

8.1 Principles

Western blotting of protein samples following sodium dodecyl sulphate (SDS) polyacrylamide electrophoresis represents the gold standard in confirming the presence of particular proteins in a cell lysate and therefore demonstrating the activation (or lack of) a particular signalling cascade. SDS disrupts protein secondary and tertiary structures and applies a mass-proportional negative charge to each protein thereby forming linearised negatively charged proteins. β-mercaptoethanol reduces disulphide bonds, and boiling of samples adds a further level of protein denaturation.(Shapiro et al. 1967) A biotinylated streptavidin ladder containing proteins of known mass in kDa is also run down the gel to permit later analysis of the resulting Western blot.

All proteins in the gel are then transferred to a specialised membrane (known as Western Blotting). The membrane is blocked using milk protein to permit exclusive binding of selected antibodies. The membrane is then probed with the primary antibody raised against the protein of interest. The membrane is then incubated with a secondary antibody which has been raised against the primary antibody and conjugated to horseradish peroxidase. A series of washes in large volumes of a detergent such as PBS 0.1% (v/v) TWEEN removes nonspecific binding of secondary antibody.

As a high resolution western blot was desired, equally charged proteins were electrophoresed through a polyacrylamide gel made using two pH-discontinuous buffers (known as discontinuous SDS PAGE) which first *stack* the proteins to a single, well demarcated band. The proteins were then *resolved* according to mass through narrower pores (Ornstein 1964; Davis 1964). Proteins move towards the anode and are separated by mass, which can be

visually compared against a protein standard (containing proteins of known molecular weights) (Raymond & Weintraub 1959). Proteins in the gel then undergo electrophoretic transfer to a nitrocellulose membrane (NCM) in a specialised cassette "sandwich" which is placed transversely, relative to the electrical field, allowing current to attract proteins to the NCM (Towbin et al. 1979).

Enhanced chemiluminescence describes the process of treating the membrane with peroxide compounds to activate the horseradish peroxidase conjugated to the secondary antibody. As the secondary antibody-HRP is bound to the primary antibody, chemiluminescence produced by the reaction will reveal the protein of interest on the membrane, the mass in kDa is then confirmed against a standard protein marker. To detect the protein ladder, HRP-conjugated reagents are added. Specialised photographic film is used to capture the luminescence.

8.2 Detection of pattern recognition receptor activation

To confirm PRR activation, it is necessary to analyse for the presence of crucial proteins involved in 'committed steps' of their downstream signalling. To detect phospho-Iκb (TLR), caspase-1 and ASC (NLR-inflammasome) in THP1 monocytes, CD4 T-cells, or primary monocytes following stimulation experiments, lysates including un-stimulated/non-transfected isotype controls were defrosted on ice, before boiling at 100°C for 5 minutes along with a protein standard prior to loading to polyacrylamide gel (Protogel 30%, Cat. No. EC-890, National Diagnostics, Atlanta, USA) and running at 200V in (25mM Tris-192mM glycine, 0.1% (w/v) SDS, pH 8.8 running buffer).

In order to cast four resolving polyacrylamide gels, 5ml 1.5M Tris HCl, 200µl 10% (w/v) SDS, 6.66ml polyacrylamide (Protogel 30%), 100µl APS – ammonium persulfate, and 10.0µl TEMED – tetramethylethylenediamine; was mixed by pipetting in 8.04ml autoclaved

distilled water prior to transfer to the BioRad gel casting system. Stacking gels were prepared by mixing 5ml 0.5M Tris HCl, 200µl 10% (w/v) SDS, 2.6ml polyacrylamide (30% Protogel), 100µl APS and 20µl TEMED in 12.2ml autoclaved distilled water and added to each gel cast with a BioRad mould for ten samples of 30µl lysate. The volumes of reagents used in this protocol were found to be appropriate for the proteins of interest.

Protein bands were transferred (20mM Tris, 0.1% (v/v): 10% (w/v) SDS in solution, 20% (v/v) propan-2-ol, pH 8.3 transfer buffer)) to a nitrocellulose membrane (Whatman, Dassel, DE) at 210mA for 60 minutes in a transfer box containing an ice block to keep the transfer box cool thereby preventing denaturing of the proteins. The membrane then was blocked with blocking solution (1% (w/v) dried milk in PBS)) followed by 2 washes in PBS 0.1% (v/v) Tween and incubated overnight with primary antibody diluted (in µg/ml) to 1/1000 (for anti-caspase-1 p10 and anti-ASC), 1/8000 dilution (for PhosphoIκbα) and 1/6000 (for pro-IL-1β) in PBS 0.1% (v/v) Tween.

The nitrocellulose membranes (Whatman, Dassel, DE) were washed with PBS 0.1% (v/v) Tween then incubated for 45 minutes at room temperature with secondary antibody conjugated to HRP- (Jackson ImmunoResearch, West Baltimore, USA) (1/6000 dilution). Nitrocellulose membranes were washed several times in PBS 0.1% (v/v) Tween. Proteins were detected via enhanced chemiluminescence in a photographic light environment using Amersham ECL Western Blotting Detection Reagent (Amersham, RPN2106) and Amersham Hyperfilm ECL (GE Healthcare Life Sciences, 28-9068-36). GE Healthcare Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA UK.

9 Cytokine analysis

9.1 Principles

HEK293 (human embryonic kidney 293) or THP1 reporter cells are modified immortal cell lines purchased from Invivogen. The reporter cells are engineered to express serum embryonic alkaline phosphatase (SEAP) in response to the activation of downstream signalling cascades induced by particular cytokines (e.g. IL-1β) binding to their cognate receptor (e.g. IL-1R), as is shown in figure 35 below depicting IL-1β as a worked example.

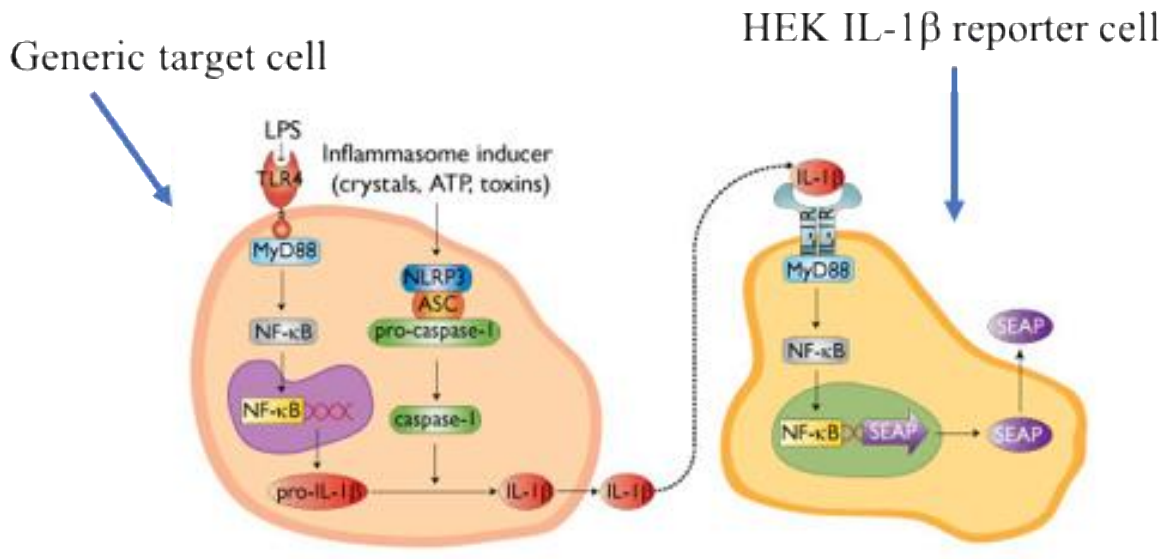


Figure 35. Mechanism of cytokine detection with reporter cells (Image adapted from Invivogen).

Initial incubation of an appropriate population of reporter cells in the presence of supernatant from samples of interest will result in the generation of SEAP proportional to the concentration of cytokine in the assay. If the particular cytokine is absent in the supernatant of interest, no SEAP will be produced by the reporter cells. Incubation of the SEAP-containing supernatant with alkaline detection reagent results in a light absorbance shift in the

supernatant which may be quantified by photospectrometry analysis (Berger et al. 1988; Yang et al. 1997).

9.2 Detection of specific cytokines using reporter cells

Reporter cells THP1 (cat. no. hkb-ila) or HEK293 (cat. no. hkb-il1b) and detection reagent were obtained from Invivogen. $1x10^4$ reporter cells (per analysed sample) specific for IL-6 (THP1) or IL-1β (HEK) respectively were incubated overnight with duplicate supernatants reflecting each experiment and associated time-points. The following day supernatant was transferred to Eppendorfs containing 150µl QuantiBlue alkaline phosphatase detection reagent (catalogue number: rep-qb1), incubated for 60 minutes prior to analysis using a Shimadzu Biospec-1601 DNA/Protein/Enzyme Analyzer (Tokyo, JP) photospectrometer (at 655nm) in order to determine cytokine expression at different time points compared to un-stimulated supernatants.

9.3 Detection of specific cytokines using enzyme-linked immunosorbance assay

The enzyme-linked immunosorbance assay (ELISA) operates on the principle of indirect immunofluorescence as previously explained. Cell supernatants were collected after each stimulation and frozen until the assays were performed. The IL-18 and human IL-1β (catalogue number: BMS224, BMS267 respectively both from eBiosciences) antibodies were used according to the manufacturer's directions.

To obtain optimal precision, 150µL of prepared standards and samples were pipetted into a 96-well polyvinyl plate in the same order as the assay is to be run. Once all samples and standards had been prearranged in the polyvinyl plate, 100µL of each sample were transferred to the antibody coated wells simultaneously using a multi-channel pipette. The reaction starts as soon as the samples are pipetted into the antibody coated wells; therefore,

pipetting should be completed as quickly as possible. The samples were incubated for 60 minutes at room temperature, followed by the aspiration and discard of the contents of each well. Each well was filled with Wash Solution. and washed for 3 additional times. After the washing procedure, residual liquid was removed by inverting the plate and tapping on absorbent tissue. It is important that the wells are not allowed to dry. The Wash Solution was used at room temperature.

100µL of the Conjugate Reagent were pipette into each well with a multi-channel pipette and incubate 60 minutes at room temperature. At the end of the incubation period the contents of the wells were aspirated and discarded followed by 3 washes with the Wash Solution. Then 100µL of Substrate Reagent were added into each well and incubated at room temperature for 30 minutes. A blue colour appeared in IL-18 or IL-1β containing wells. The Stop Solution was poured in a clean reservoir and 100µL of the Stop Solution were pipetted into each well. Upon addition of Stop Solution, the colour within the wells changes from blue to yellow. The absorbance of each well was measured at 450 nm. The measurement should be made within 30 minutes of adding Stop Solution. The plate reader can be blanked using a well containing 100 µL Substrate Reagent plus 100µL Stop Solution.

The human IL-18 or IL-1β concentration of the samples was interpolated from the standard curve. If the samples measured were diluted, the dilution factor was multiplied to the concentrations from interpolation to obtain the concentration before dilution.

10. Confocal microscopy

10.1 Principles

Confocal laser scanning microscopy is regarded as a gold standard for the visualisation of the presence and location of proteins of interest relative to cell organelles. Laser beams pass through a pinhole aperture in order to exclude unwanted non-focused light towards a dichromatic mirror which permits beams of a specific wavelength to exclusively reflect toward the focal plane of the sample. Between the focal plane and the dichromatic mirror, an objective lens further directs the beam to focus in the plane of the sample.

Laser-induced fluorescence of fluorophores conjugated to proteins of interest within or on the surface of a cell will direct an emission beam back through the objective and dichromatic mirror towards a photomultiplier tube (Nwaneshiudu et al. 2012). The process of probing cells with fluorophores directed against a protein of interest comparable that of the indirect immunofluorescence process used in preparing cells for flow cytometry, wherein cells are incubated with a primary antibody which is then complexed with a secondary antibody-fluorophore-conjugate.

The emission beam must be further focused by a detector pinhole aperture prior to photomultiplier tube detection. The detector pinhole effectively excludes unwanted light arising from unavoidable emission from fluorophores outside the focal plane (i.e. unwanted "background"), meaning that the detector pinhole must be conjugate to the focal point of the objective lens, becoming a confocal pinhole (Nwaneshiudu et al. 2012).

To prepare the slides for imaging 30,000 cells were used per sample. They were stimulated as required at certain time point and then were fixed using 4% (w/v) PFA to prevent potential re-organisation of the proteins during the course of the experiment. Cells were permeabilised

using PBS/0.02% (w/v) BSA/0.02% (w/v) saponin and labelled with antibodies for NLRP3, NLRC4, gp41, Vpu, TLR4 etc. (2µg) Followed by an appropriate species specific secondary antibody (2µg) conjugated to Alexa 488, Alexa546 or Alexa 647/ We have validated our confocal experiments with isotype control antibodies as well as with only secondary antibody. In the instance of dual labelling, secondary antibodies conjugated to Alexa488 and Alexa546 were used and Topro-3 (1/10,000 dilution) was the selected nuclear stain since it has far-red fluorescence (Excitation⁄Emission :642⁄661nm) similar to Alexa 647.

Labelled cells were mounted on coated polylysine slides (ThermoFischer, UK). To reduce photobleaching we used Slowfade antifade reagent (cat. No. S36967) also from ThermoFischer; or Vectashield (Cat. No. H-1000) from Vector Laboratories (3 Accent Park, Bakewell Road, Peterborough, PE2 6XS, Tel. 01733 237999). Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63x Zeiss objective. The images were analysed using LSM 2.5 image analysis software (Carl Zeiss, Inc.).

11. Förster Resonance energy transfer (FRET)

FRET is a non-invasive imaging technique used to determine molecular proximity. FRET can occur over 1-10 nm distances, and effectively increases the resolution of light microscopy to the molecular level. FRET was measured in terms of dequenching of donor fluorescence after complete photo-bleaching of the acceptor fluorophore. Increased donor fluorescence after complete destruction of the acceptor indicates association between the two molecules of interest (Ishikawa-Ankerhold et al. 2012).

FRET involves a nonradiative transfer of energy from an excited state donor fluorophore to a nearby acceptor. The energy transfer efficiency, FRETeff, is directly related to the distance r separating a given donor and acceptor pair by: FRETeff=1/[1+(r/R0)6].

The resolution of FRET is thus defined by R0 which is typically < 10-70 Å. R0 depend of the extent of overlap between the donor emission and the acceptor spectra (figure 36 overleaf). FRET Acceptor Bleaching involves measuring the donor "de-quenching" in the presence of an acceptor. This can be done by comparing donor fluorescence intensity in the same sample before and after destroying the acceptor by photobleaching. If FRET was initially present, a resultant increase in donor fluorescence will occur on photobleaching of the acceptor.

The energy transfer efficiency can be quantified as: FRET eff= (Dpost-Dpre)/Dpost. Dpost is the fluorescence intensity of the Donor after acceptor photobleaching, and Dpre the fluorescence intensity of the Donor before acceptor photobleaching. The FRETeff is considered positive when Dpost > Dpre.

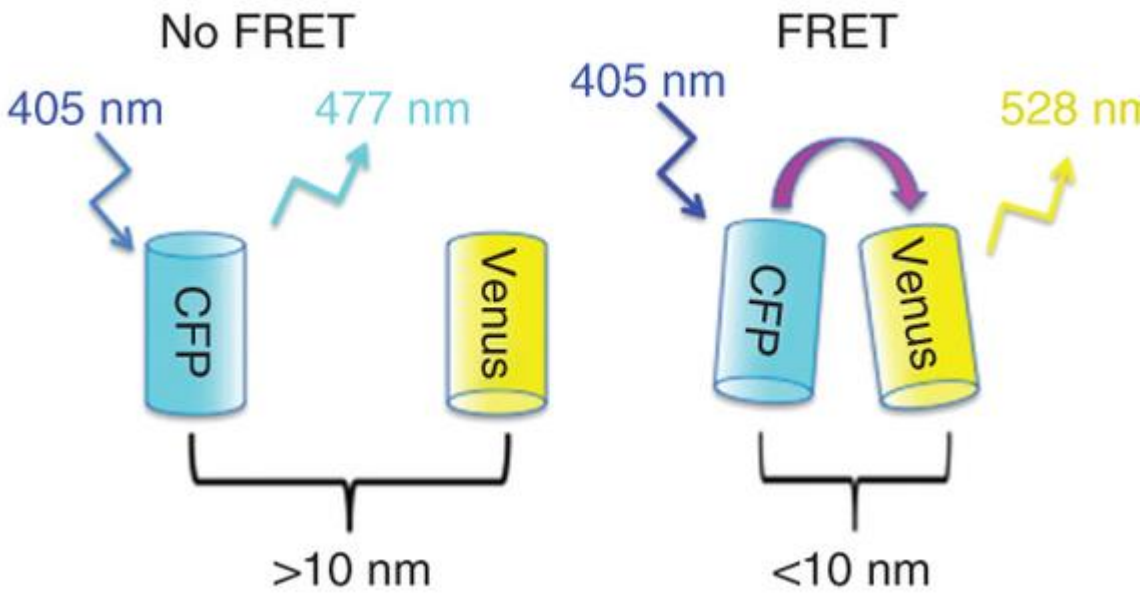


Figure 36. ***The principles of fluorescence resonance energy transfer (FRET).*** **CFP, cyan-coloured fluorescence protein (donor); Venus, yellow-coloured (acceptor). Where there is overlap of donor and acceptor (distance less than 10nm) there is FRET. In distances more than 10nm there is no overlap (No FRET). Image adapted from Broussard et al, 2013 (Broussard et al. 2013).**

In the present study, FRET was quantified as previously described (Kenworthy and Edidin 1999). Briefly, the change in donor (FITC) or (Alexa488) emission following the acceptor (TRITC) or Alexa (546) bleaching was measured. Bleaching of the acceptor eliminates the light excitation of the fluorophore and FRET would not occur between donor-acceptor, therefore the donor emission will not be sequestered. If the donor/acceptor pair is ≤10nm apart the donor emission will increase after bleaching of the acceptor. Scaling factor of 10,000 was used in order to expand E to the scale of the 12-bit images.

THP1 cells or primary monocytes were seeded on polylysine coated glass slides (ThermoFischer, UK). TRITC was bleached by continuous excitation with an arc lamp using a TRITC filter set for 10 minutes. Under these conditions, FITC was not bleached. FRET images were calculated from the increase in donor fluorescence subsequent to the acceptor photobleaching.

Cells were labelled with a mixture of 1μg donor conjugated antibody (FITC/Alexa 488) and 1μg acceptor conjugated antibody (TRITC/Alexa 546). The cells were then rinsed twice in PBS/BSA/NaN_3, prior to fixation with 4% (w/v) PFA for 15 min. Cells were imaged on a Carl Zeiss LSM510 confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63x Zeiss objective (Carl Zeiss, Inc.).

The images were analysed using the LSM5 image analysis software (available for download from Zeiss at https://www.zeiss.com/microscopy/int/home.html), and FITC (Excitation/emission: 490/525nm) TRITC (Excitation/emission: 548/561nm) were detected using the appropriate filter sets. Using typical exposure times for image acquisition (<5 sec), no fluorescence was observed from the FITC-labelled specimen using the TRITC/Alexa546 filters, nor was TRITC fluorescence detected using the FITC/Alexa488 filter sets.

13. Statistics & Software

Data was analysed using GraphPad Prism version 6.00 for Mac, GraphPad Software, San Diego California USA, www.graphpad.com. For all graphs displaying data for immunofluorescence and cytokine studies, error bars display standard deviation (SD) from the mean of three independent experiments unless otherwise stated in the legend. The data was normally (Gaussian) distributed thus to establish the significance of difference between data from samples (for example) comparing unstimulated/untransfected isotype controls versus stimulated/transfected samples the Student's T-test for paired samples was performed. The results are depicted in bar graphs where: ns = no significant difference, * $p < 0.05$, ** represents $p \leq 0.01$, *** represents $p \leq 0.001$. Significant difference between analysed data was established when $p < 0.05$. The Zeiss LSM5 program was used to analyse confocal microscopy and FRET data.

Chapter 3: HIV-1 envelope glycoprotein triggers an innate immune response

1.1 Introduction

TLR2 and TLR4 are known to interact with a variety of different viral envelope glycoproteins, including HIV-1, to trigger downstream activation of pro-inflammatory pathways (Bowie AG & Haga IR 2005). Blood from 49 people living with HIV-1 exhibited increased TLR2 expression (Heggelund L et al. 2004). HIV-1 gp120 is known to induce IL-1β in certain circumstances, although the precise mechanism remains unknown. Early studies suggested involvement of HIV-1 gp120, putatively acting via calcium signalling, to activate IL-1β secretion and apoptotic signalling in neuronal cells (Corasaniti MT et al. 2001). Since NLR sensing leading to caspase-1 inflammasome activation was defined as the major cause of IL-1β secretion, gp120 may be contributing to inflammasome activation. It is unknown whether or which cell surface PRRs may contribute to the well-defined 'signal 1' for inflammasome activation in the context of HIV-1.

In this study we tried to determine how gp120 is recognised by the host and which PRRs become up-regulated in the presence of HIV-1 gp120. THP1 monocytes which are known to highly express all TLRs, were used as a model as well as MOLT4 CD4 T cells since they are both susceptible to HIV-1 infection. THP1 monocytes and MOLT4 CD4 T-cells were stimulated with HIV-1 gp120 over a 12 hour period for 1,2,4,6 or 12 hours. PRR expression levels were investigated using indirect immunofluorescence and flow cytometry and the consequent signalling cascades triggered were investigated using Western Blotting and cytokine reporter cell assays.

1.2 HIV-1 envelope glycoprotein gp120 activates the innate immune system

The HIV envelope glycoprotein gp120 binds with high affinity to CD4 and is responsible for the tropism of HIV for CD4+ T cells and peripheral blood monocytes. Thus we assessed the host's innate immune response against recombinant gp120 by trying to determine the PRRs involved in gp120 detection. Therefore, using indirect immunofluorescence and flow cytometry, we analysed PRRs upregulation in the presence of gp120 envelope protein as compared to gp120 unstimulated cells. THP1 monocytes were stimulated (or left unstimulated in fresh cell culture medium) with two different concentrations of gp120 over 1,2,4,6 or 12 hours beginning with a saturation concentration (Heggelund L et al. 2004) and analysed for PRR expression levels (figures 37-43).

1.3 HIV-1 gp120 upregulates TLRs and NLRs

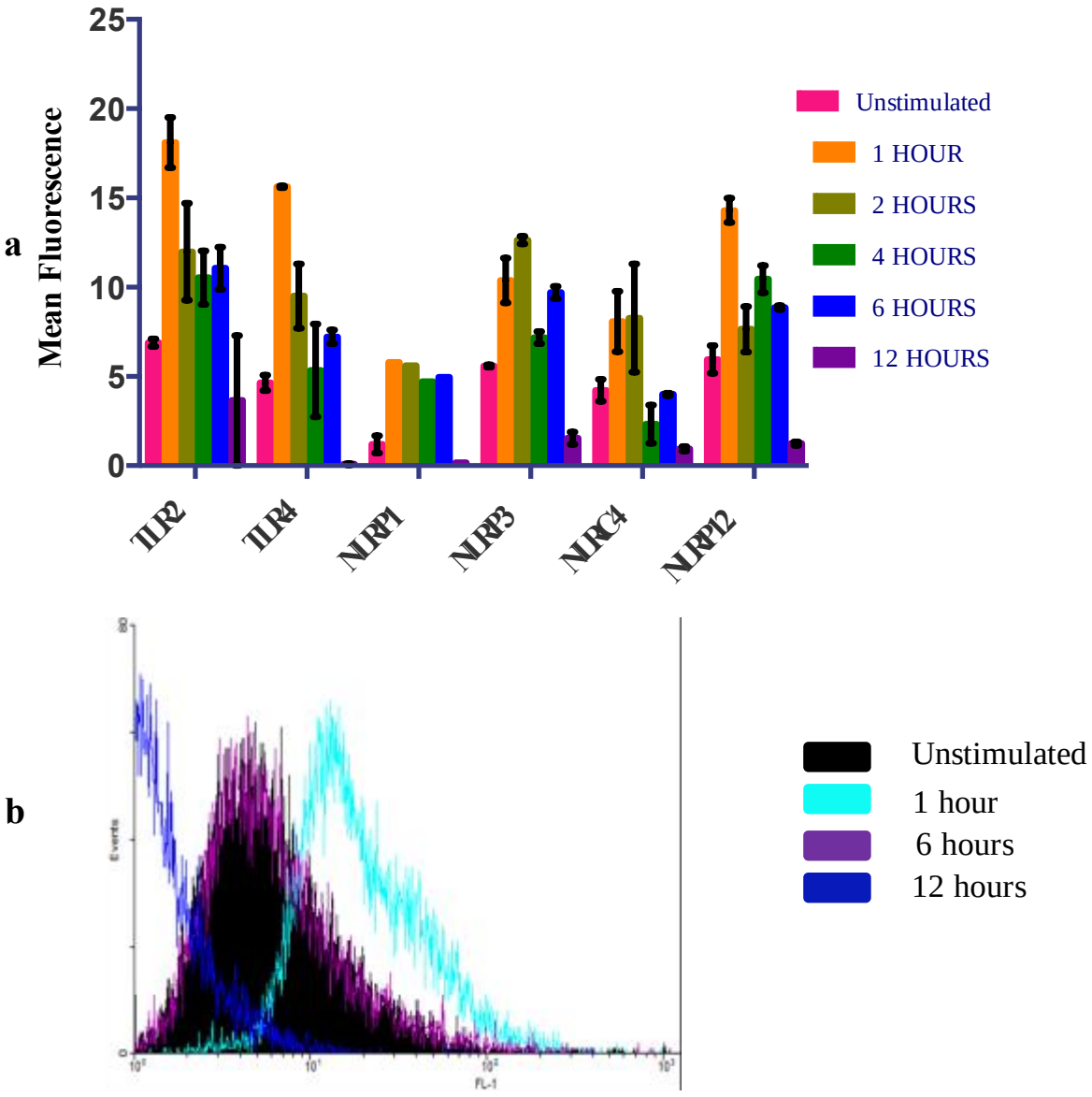


Figure 37. ***PRR expression levels in THP1 monocytes stimulated with HIV-1 gp120.*** **(a) 1x10^6 THP1 monocytes were stimulated with 1µg/ml HIV1 gp120 fixed, and probed for the depicted PRRs with the appropriate FITC-conjugated antibody. Fluorescence intensity was detected using a FACSCalibur (Becton Dickinson) counting 10,000 cells gated on THP1 settings subtracting for low background fluorescence. Mean background fluorescence for series = 1.58 (N=6, 95% CI=1.32-1.84, SD=0.25). Histogram (b) shows WinMDI analysis of 1µg/ml HIV-1 gp120 stimulation series for TLR4 expression.**

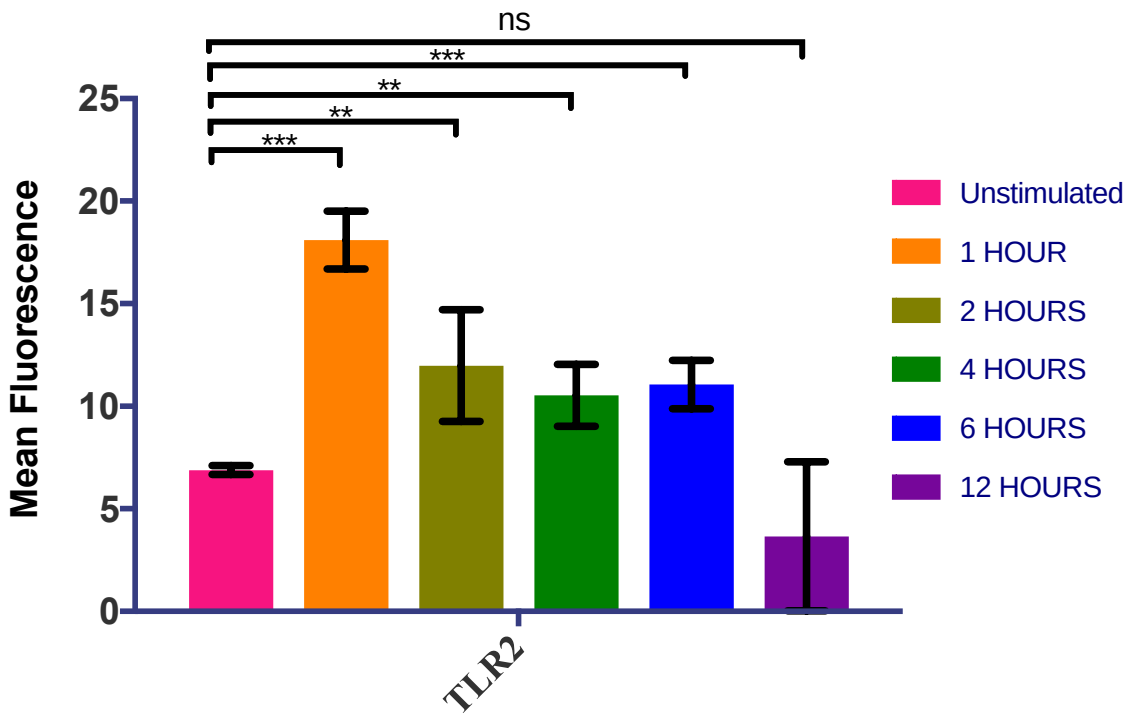


Figure 38.*TLR2 expression in THP1 monocytes stimulated with 1µg gp120.* For US, 1, 2, 4, 6 and 12 hours the SD = 0.29, 1.98, 3.83, 2.12, 1.66 and 5.14 respectively.

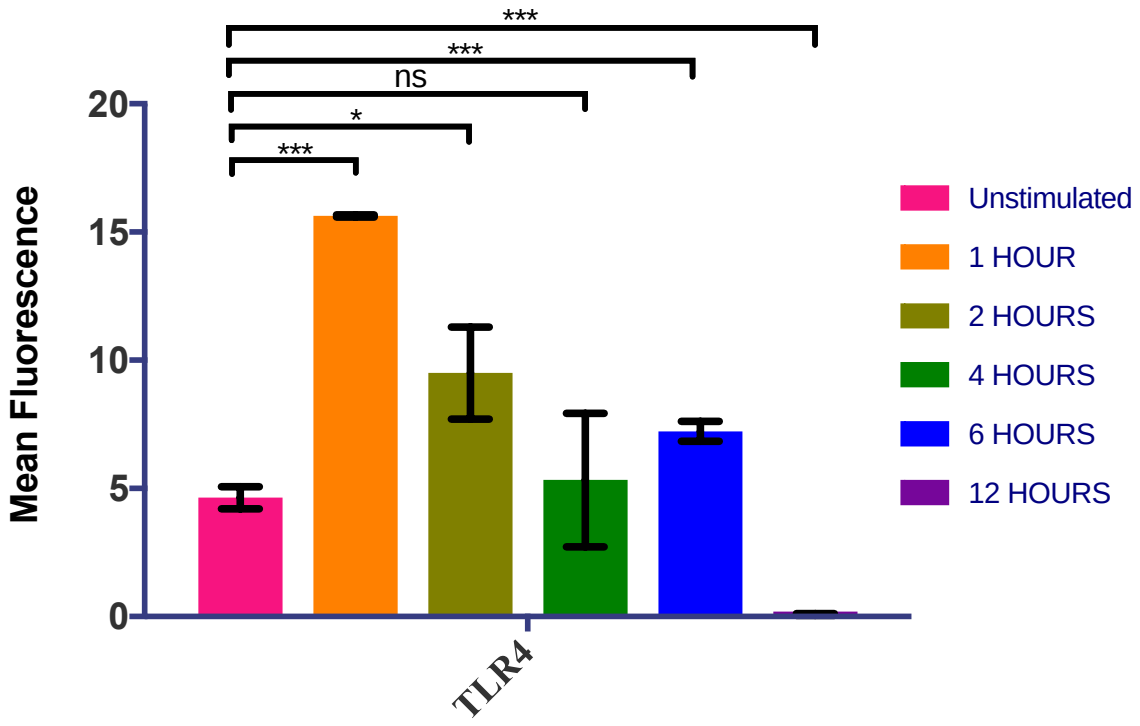


Figure 39. ***TLR4 expression in THP1 monocytes stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.62, 0.06, 2.53, 3.67, 0.55 and 0.05 respectively.**

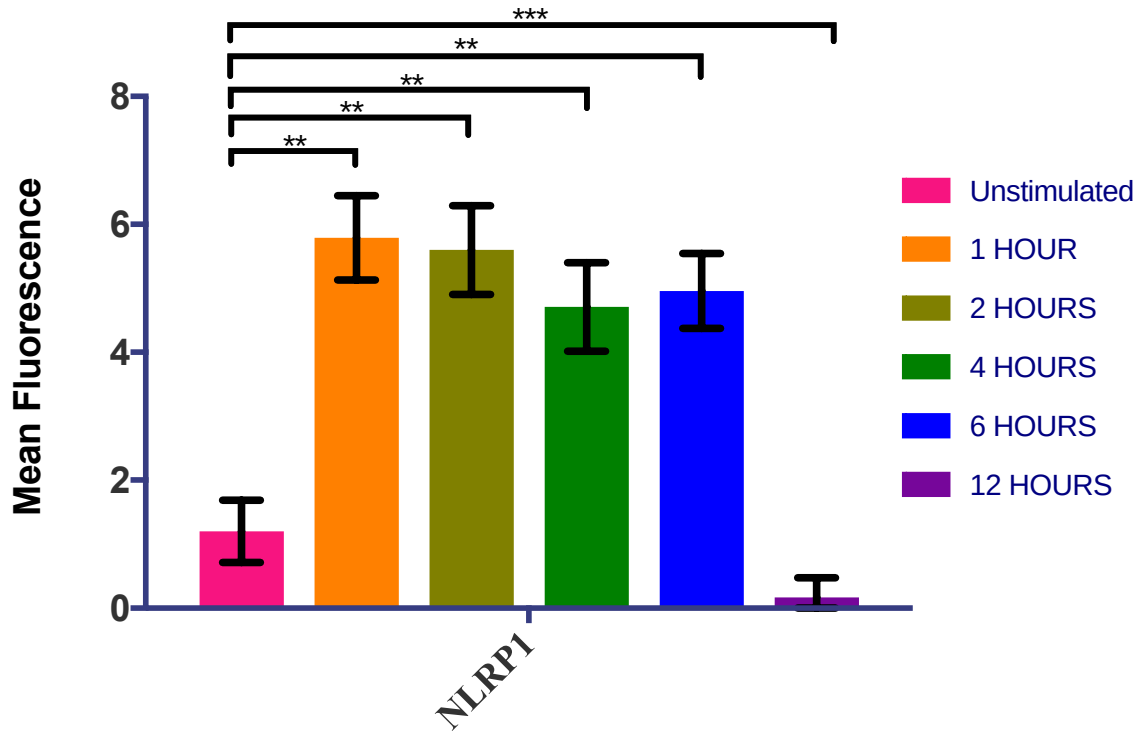


Figure 40. ***NLRP1 expression in THP1 monocytes stimulated with 1µg gp120 stimulated.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 1.29, 1.93, 1.98, 1.98, 1.83 and 0.94 respectively.**

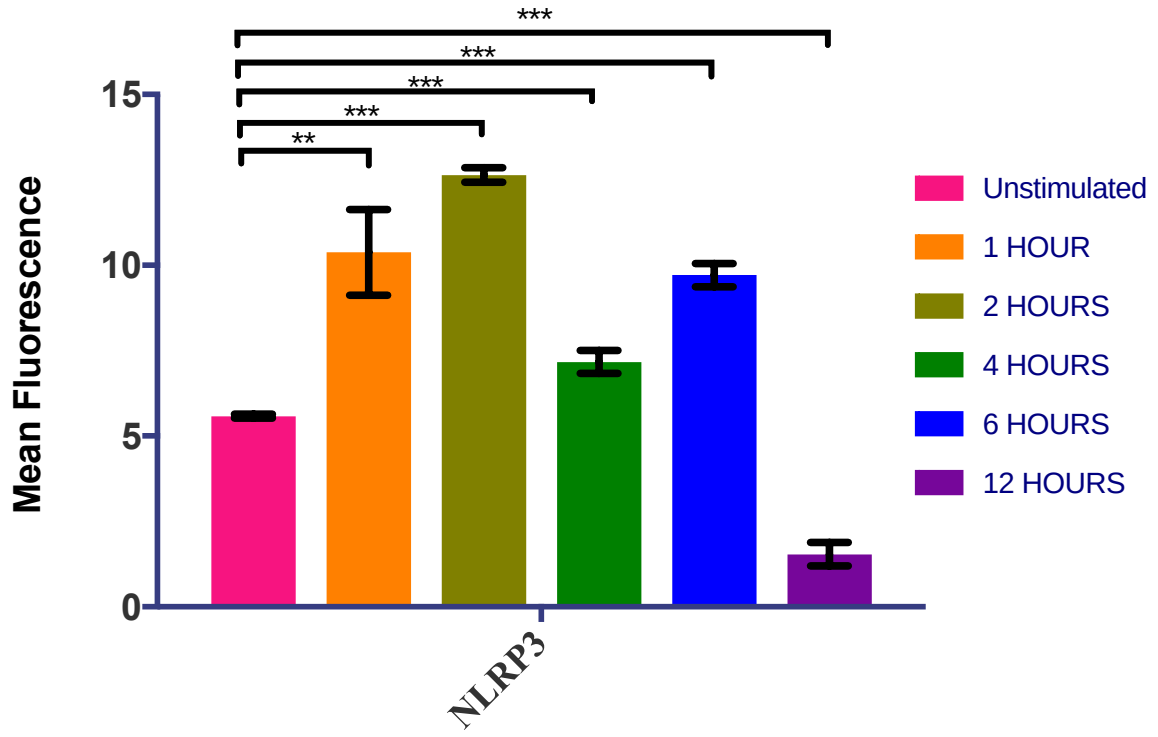


Figure 41. ***NLRP3 expression in THP1 monocytes stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.07, 3.74, 0.47, 0.72 and 0.79.**

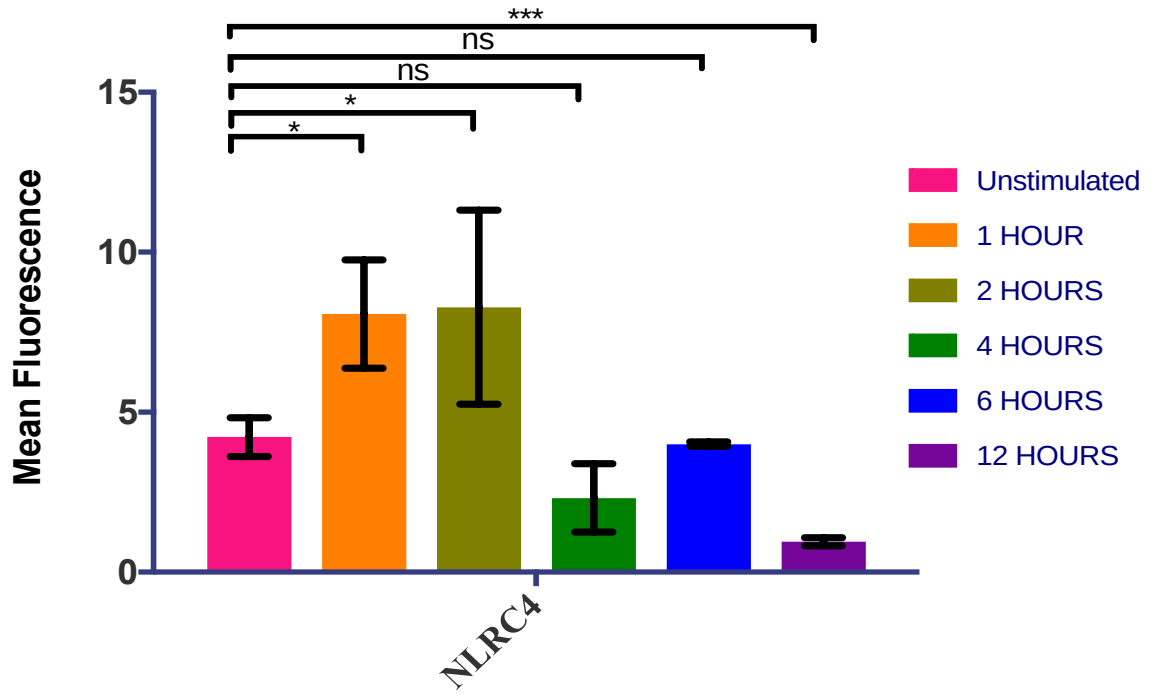


Figure 42. ***NLRC4 expression in THP1 monocytes stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.86, 2.39, 4.28, 1.51, 0.98, 0.18 respectively.**

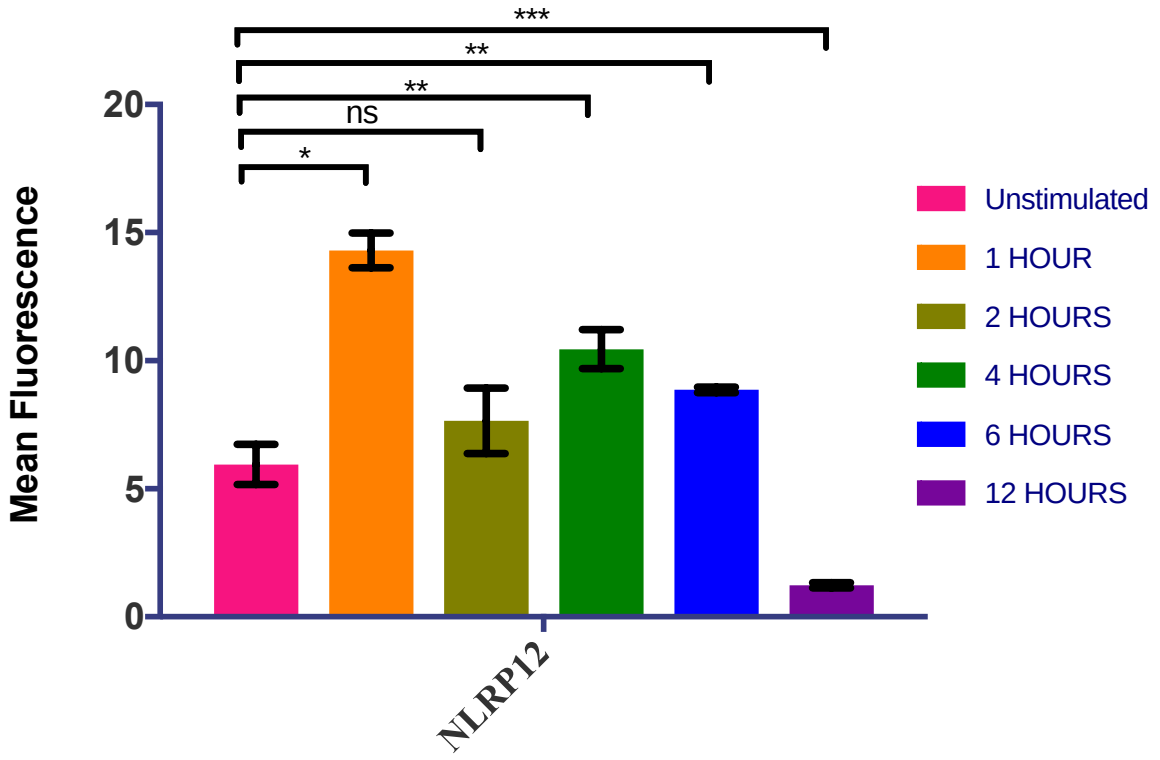


Figure 43. ***NLRP12 expression in THP1 monocytes stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 1.10, 0.95, 1.07, 0.16, and 0.14 respectively.**

The data showed an initial upregulation in the expression of most pattern recognition receptors followed by an overall picture of downregulation (with some exceptions, e.g. NLRP3 expression at 6 hours) across the 1µg gp120 stimulation time points. Some pattern recognition receptors such as TLR2, TLR4 and NLRC4 showed wide variation in expression levels as reflected by the standard deviation at specific time points: TLR2 at 4, 6 and 12 hours; TLR4, at 2 and 4 hours; and NLRC4 at 2 and 4 hours. Consequently, the dose of gp120 was reduced and the stimulations at 1,2,4,6 and 12 hours were repeated using 500ng gp120 along with unstimulated isotype controls (fig. 44-50 overleaf).

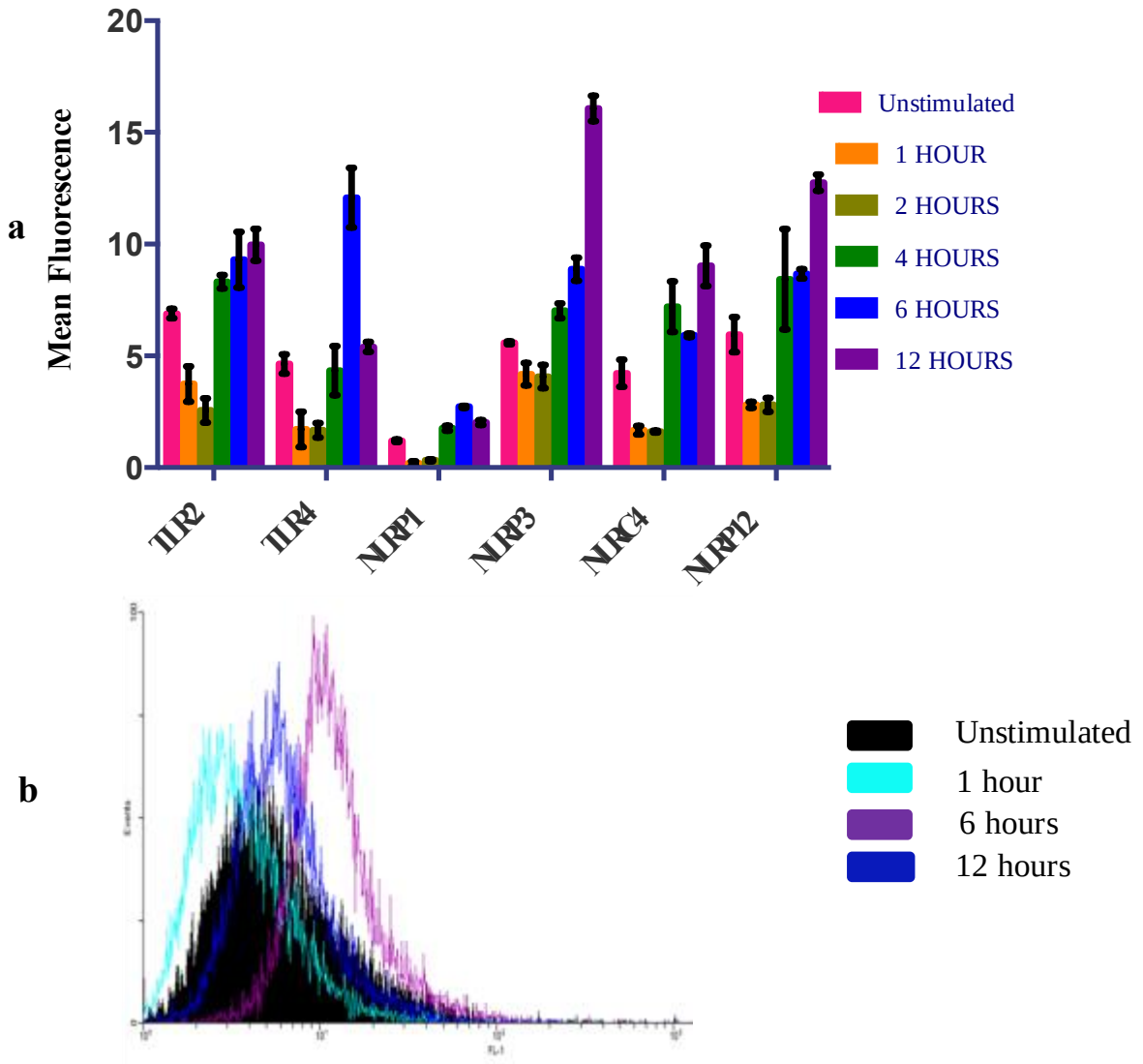


Figure 44. ***HIV-1 gp120 modulates TLR & NLR expression.*** **1x10^6 THP1 monocytes were stimulated with 500ng/ml HIV1 gp120 fixed, and probed for the depicted PRRs with the appropriate FITC-conjugated antibody. Fluorescence intensity was detected using a FACSCalibur (Becton Dickinson) counting 10,000 cells gated on THP1 settings subtracting low levels of background fluorescence. Mean background fluorescence for series = 1.42 (N=6, 95% CI=1.16-1.67, SD=0.21). Histogram shows WinMDI analysis of 500ng HIV-1 gp120 stimulation series for TLR4 expression. The data are representative of the mean of 3 independent experiments.**

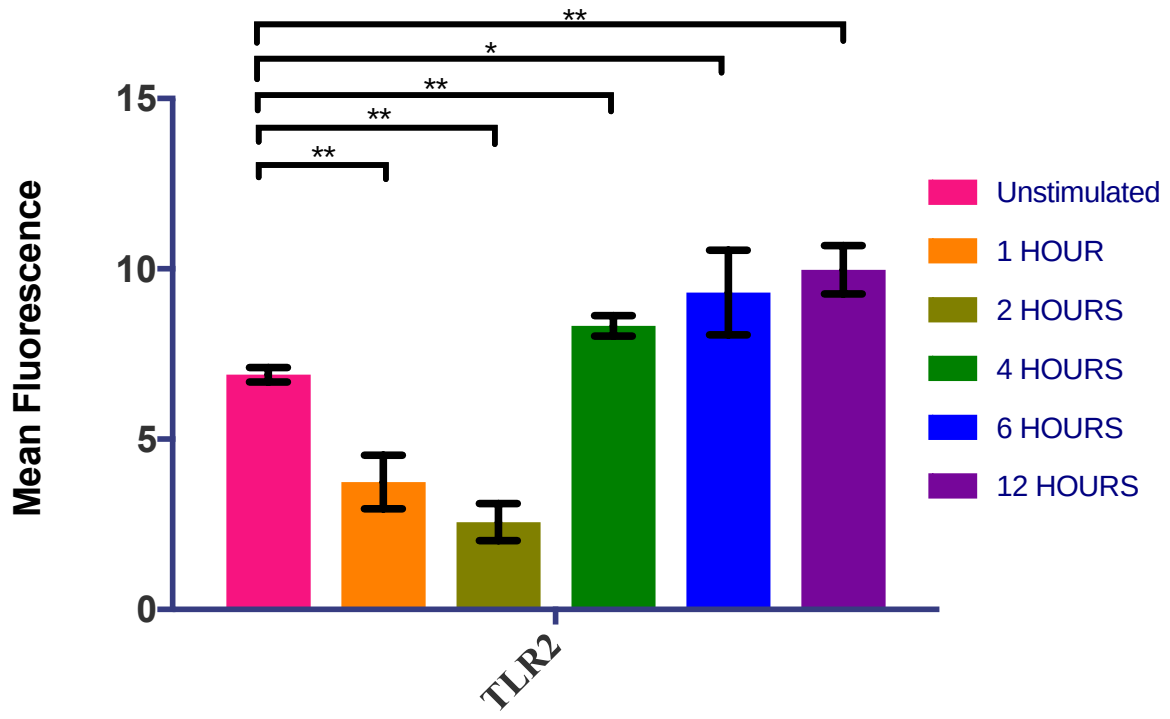


Figure 45. ***TLR2 expression in THP1 monocytes stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.30, 1.11, 0.77, 0.42, 1.76, and 0.98 respectively.**

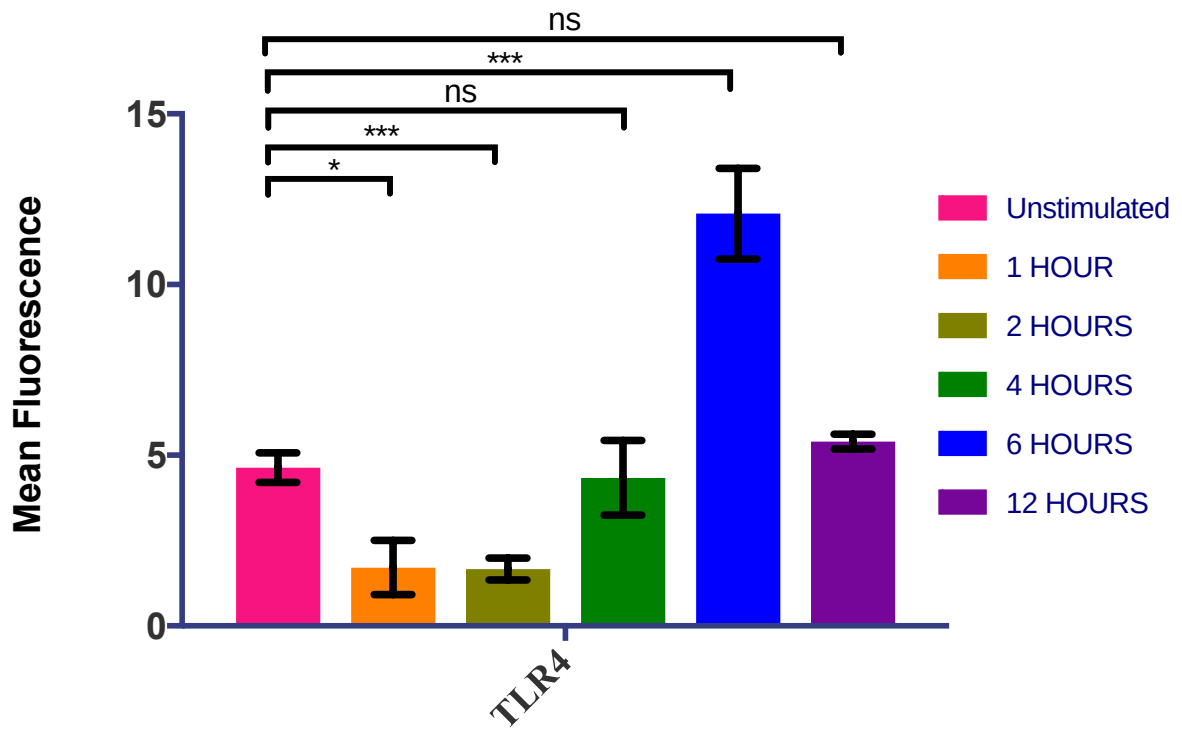


Figure 46. ***TLR4 expression in THP1 monocytes stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.62, 1.11, 0.45, 1.54, 1.88 and 0.30 respectively.**

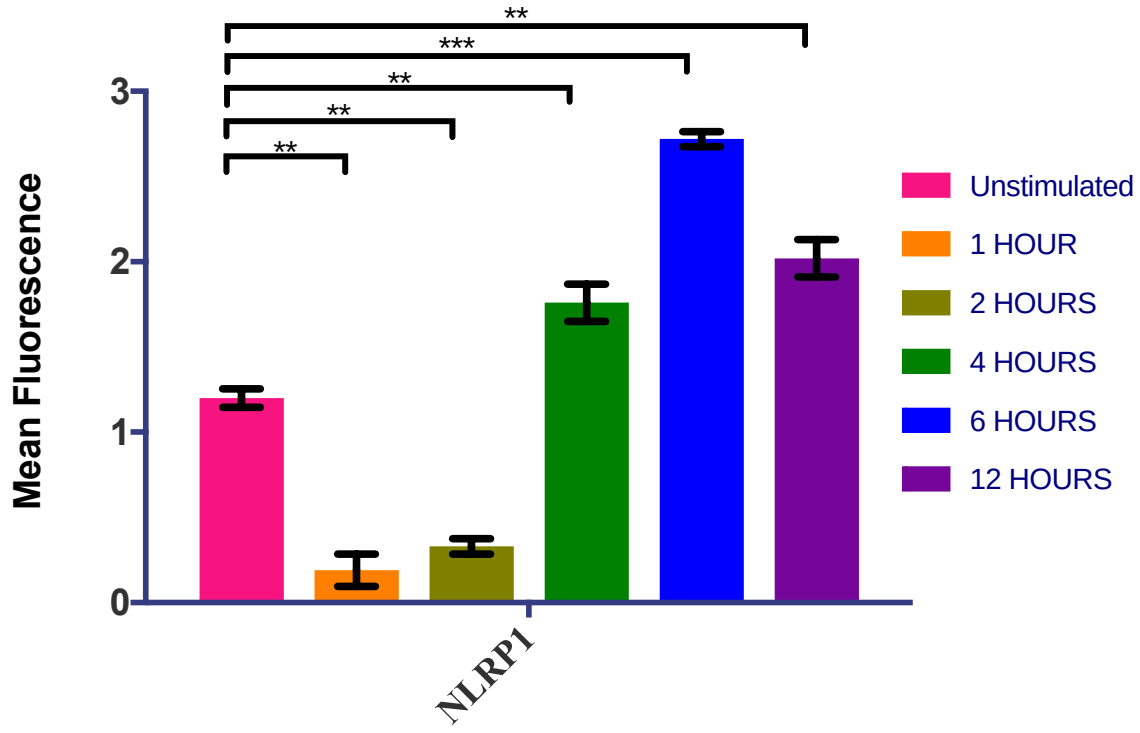


Figure 47. ***NLRP1 expression in THP1 monocytes stimulated with 500ng gp120*****. For US, 1, 2, 4, 6 and 12 hours the SD = 0.13, 0.78, 0.06, 0.16, 0.06 and 0.16 respectively.**

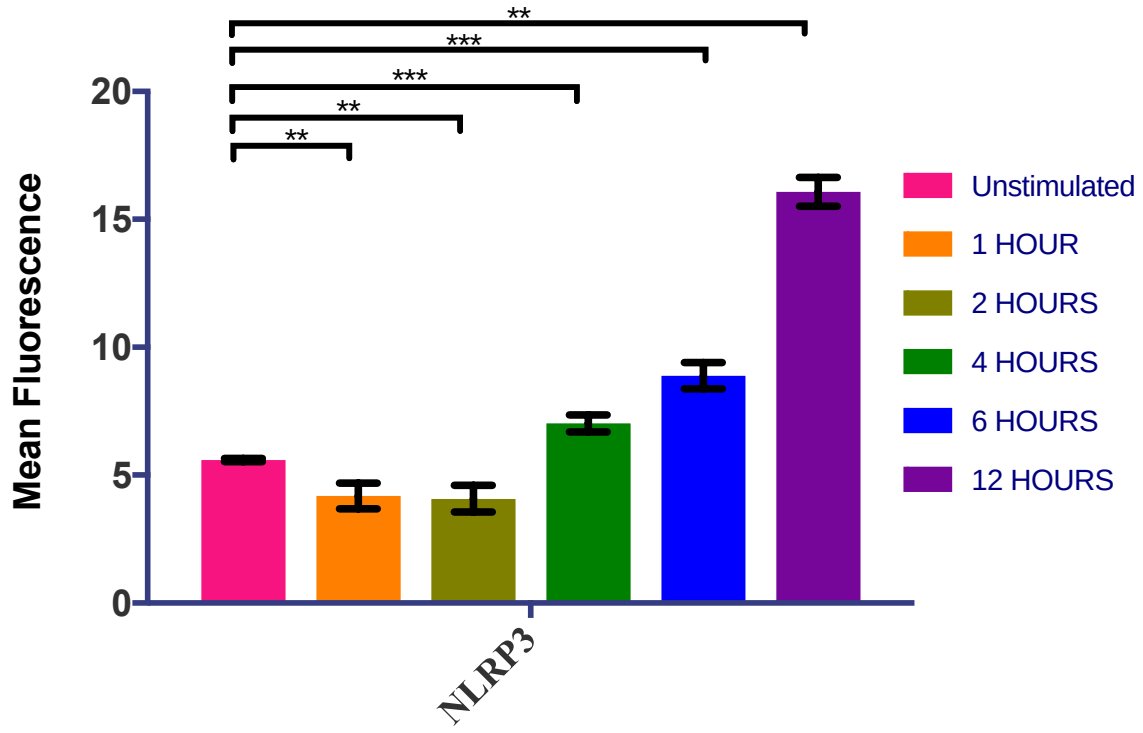


Figure 48. ***NLRP3 expression in THP1 monocytes stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.08, 0.71, 0.74, 0.72, and 0.79 respectively.**

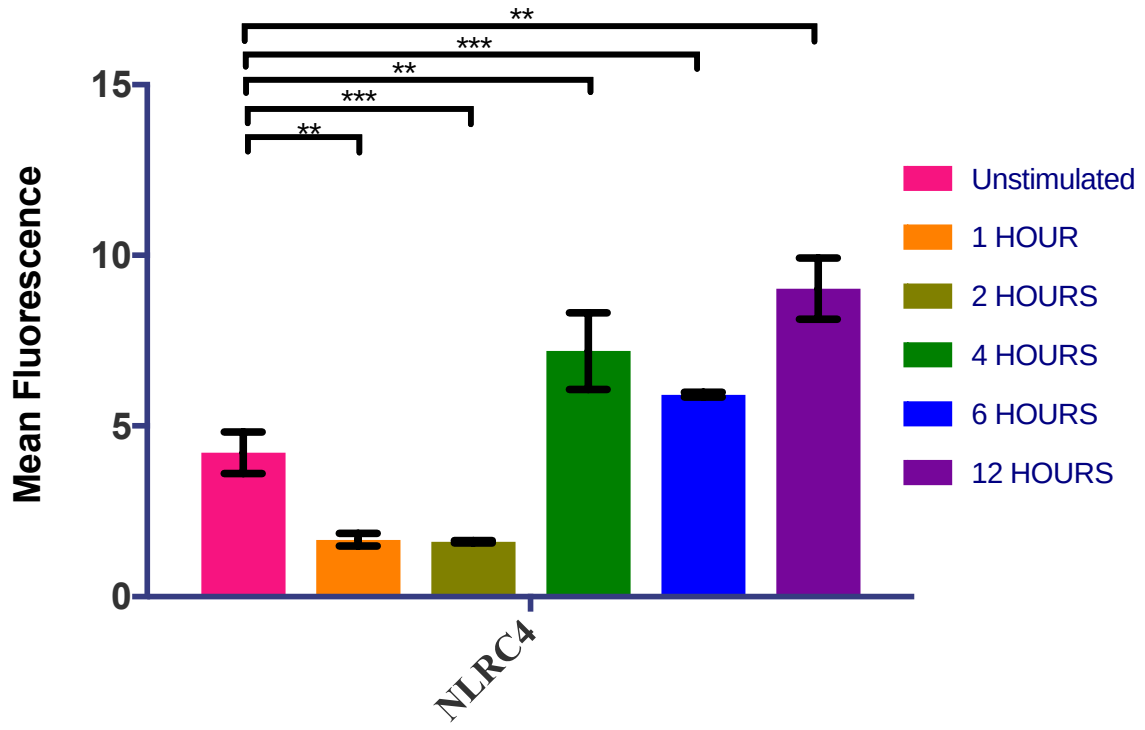


Figure 49. ***NLRC4 expression in THP1 monocytes stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.86, 0.26, 0.05, 1.59, 0.10 and 1.27 respectively.**

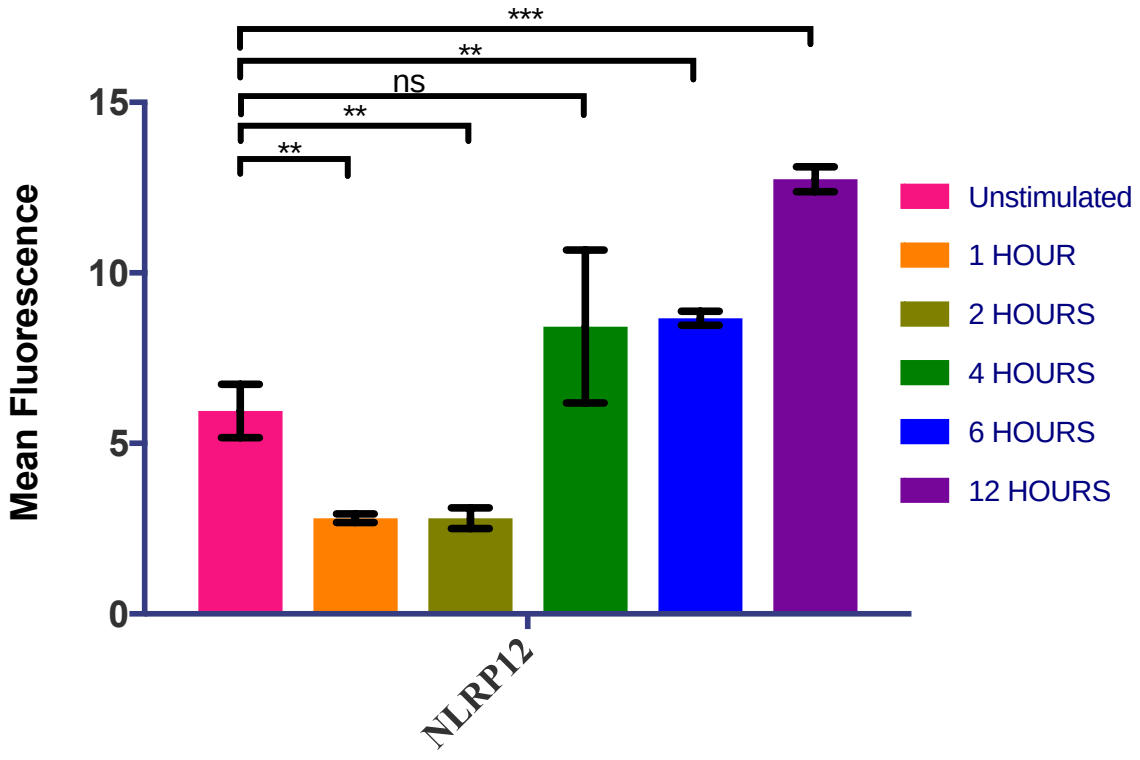


Figure 50. ***NLRP12 expression in THP1 monocytes stimulated with 500ng gp120*****. For US, 1, 2, 4, 6 and 12 hours the SD = 1.10, 0.18, 0.43, 3.17, 0.30, and 0.15 respectively.**

For THP1 monocytes stimulated with 500ng of gp120, the data showed a downregulation of TLR2 and TLR4 during the first hours of stimulation followed by recovery and upregulation (barring TLR4 at 12 hours) towards the end of the time course. A similar pattern of expression was observed for NLRP1, NLRP3 and NLRP12. The data overall appeared to show a two-phase response to 500ng gp120 stimulation of THP1 monocytes.

To determine whether TLR and NLR upregulation is functional, IL-6 which is a TLR dependent inflammatory cytokine as well as IL-1β which is an inflammasome dependent but TLR independent cytokine were measured. In addition, NFκB activation, which denotes TLR activation, was determined via phosphoIκB detection using Western blotting. Inflammasome activation was determined via caspase-1 p10 detection. Therefore, reporter cells for IL-6 and IL-1β were used to determine the presence of IL-6 and IL-1β respectively (fig. 51 overleaf).

The results in fig. 51 showed that upon 500ng gp120 stimulation there is IL-6 and IL-1β secretion as well as NFκB and inflammasome activation.

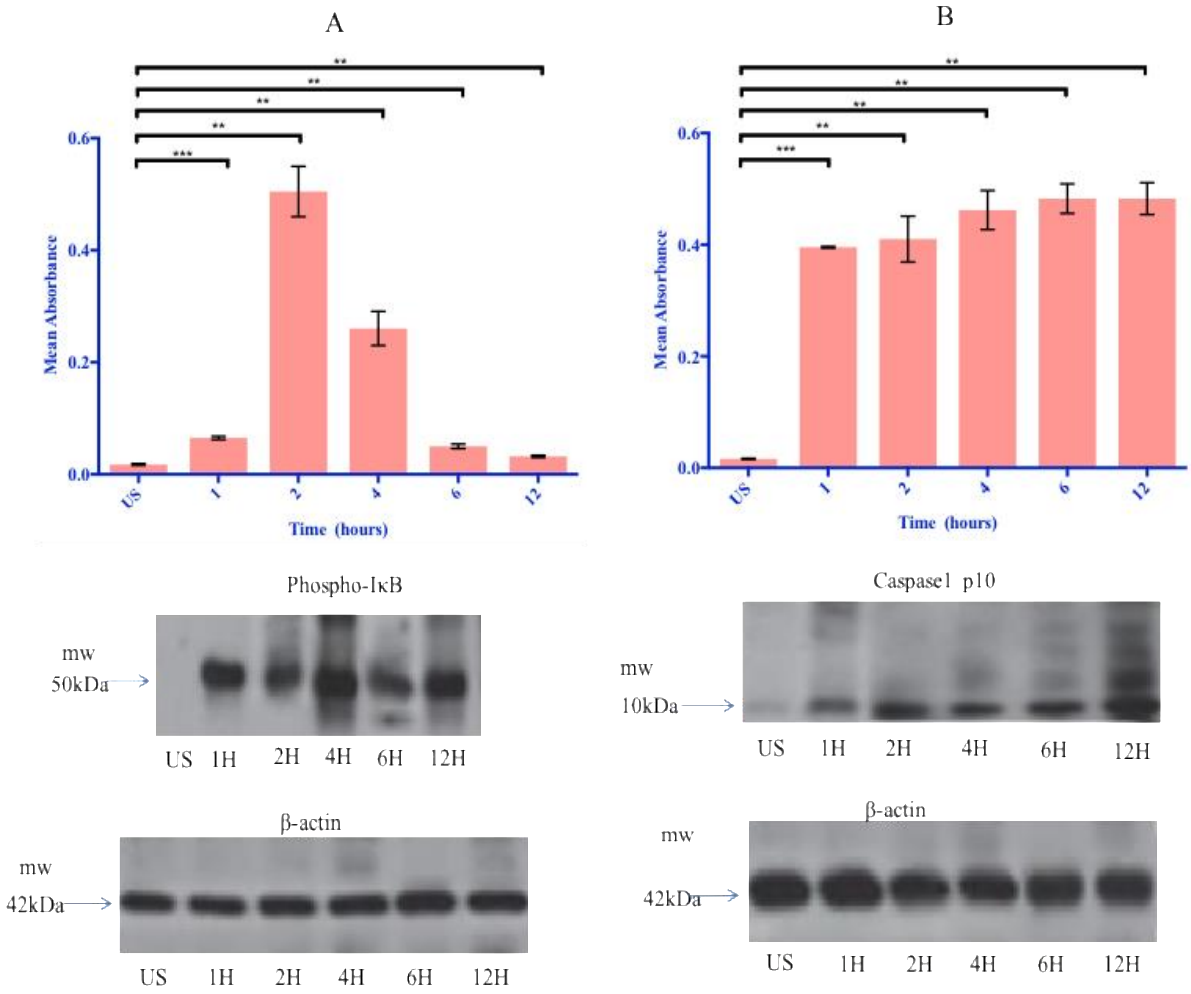


Figure 51. ***HIV-1 gp120 induces NFκB and inflammasome activation.*** **Signalling pathways underlying the IL-6 (A) and IL-1β response (B) in HIV-1 gp120 stimulations of THP1 monocytes are depicted. THP1 IL-6 reporter cells (A) or HEK 293 IL-1β (B) reporter cells were incubated with supernatants from each time-point and isotype control (US = unstimulated) for 24 hours. Supernatants were then incubated with QuantiBlue. SEAP levels were measured using a photospectrometer at 655nm. The SEAP levels correspond to the expression levels of IL-6 or IL-1β. SD for IL-6 at US, 1 hour, 2 hours, 4 hours, 6, and 12 hours = 0.000, 0.006, 0.062, 0.045, 0.008 and 0.005 respectively. SD in same order for IL-1β = 0.000, 0.001, 0.059, 0.051,0.036 and 0.039 respectively. The data represent the mean of three independent experiments. Cell samples were lysed, then proteins were separated using SDS PAGE before transfer to a nitrocellulose membrane which was probed with phospho-IκB or anti-caspase-1 p10 rabbit antibody followed by the appropriate secondary antibody conjugated to HRP. Proteins were**

visualised using via enhanced chemiluminescence. Images presented are representative of three independent experiments.

1.4 HIV-1 gp120 upregulates TLRs and NLRs in MOLT4 T cells

The binding to CD4 co-receptors by gp120 brings about an initial conformational change in the host cell membrane structure (Merk & Subramaniam 2013). Co-receptor genetic variation (Lederman MM et al. 2006) amongst individuals and cell types could influence the innate immune response. Since we saw that gp120 triggers an innate immune response in THP1 monocytes we also looked in MOLT4 CD4 T cells which are susceptible to HIV-1 infection as well to verify whether they have a similar innate response to HIV-1 gp120 or whether they trigger different PRRs (figs 52-58 below).

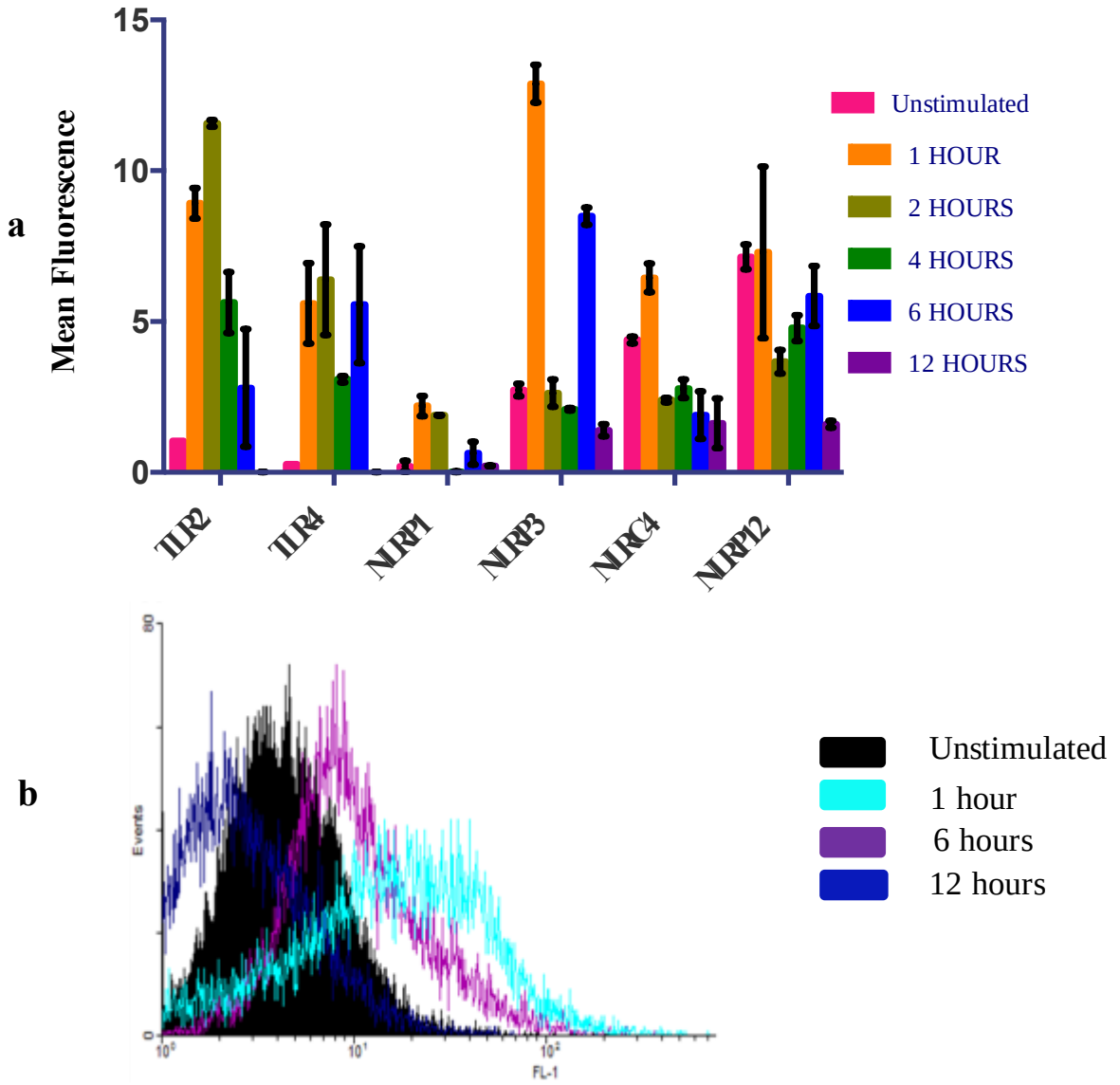


Figure 52. ***Initial study of PRR expression in CD4 T-cells stimulated with HIV-1 gp120.*** **MOLT4 CD4 T-cells were stimulated with 1µg/ml HIV1 gp120 and probed for PRRs as in figure 29. Fluorescence intensity was detected using a FACSCalibur (Becton Dickinson) counting 10,000 cells gated on THP1 settings subtracting for low background fluorescence. Mean background fluorescence = 1.42 (N=6, 95% CI=1.25-1.59, SD=0.17). Histogram (b) shows WinMDI analysis of 1µg/ml HIV-1 gp120 stimulation series for NLRP3 expression. The data are a mean of 3 independent experiments.**

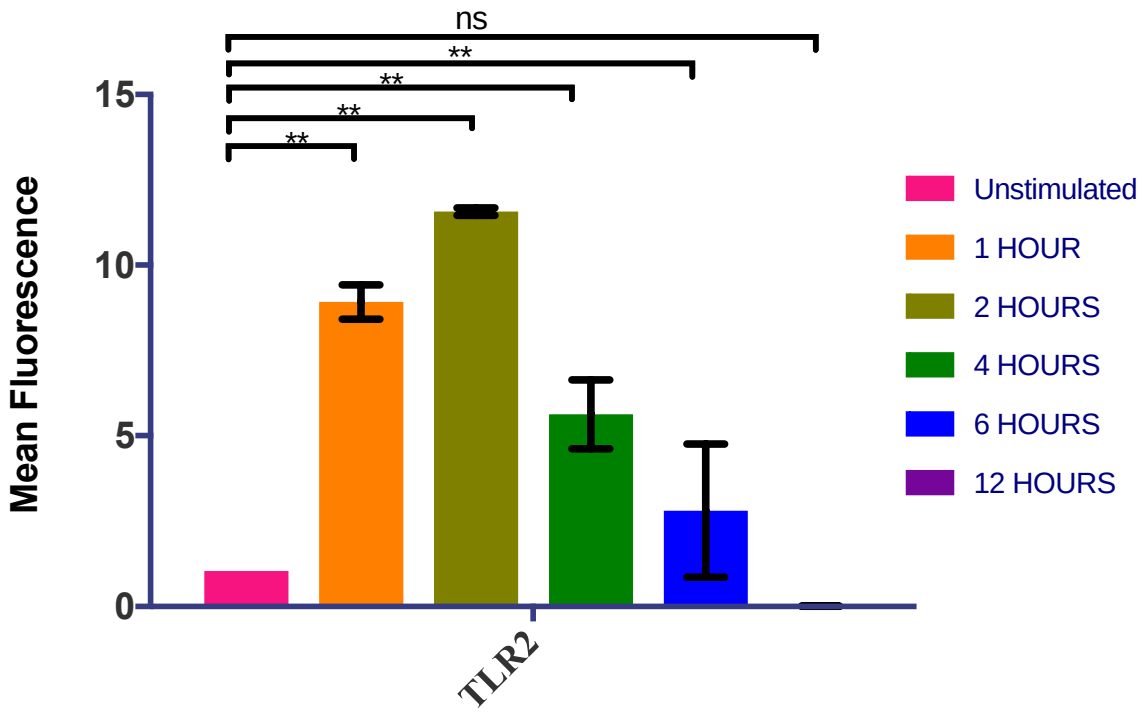


Figure 53. ***TLR2 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.00, 0.70, 0.16, 1.43, 2.77, and 0.01 respectively.**

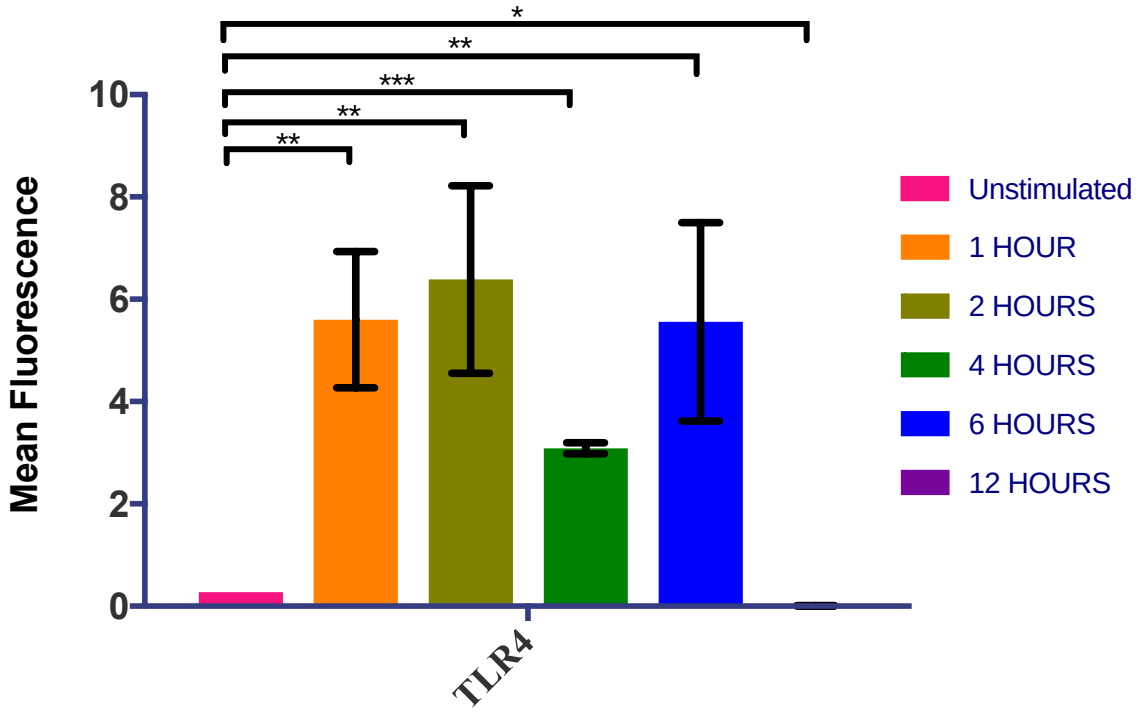


Figure 54. ***TLR4 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.00, 1.89, 2.59, 0.15, 2.74 and 0.01 respectively.**

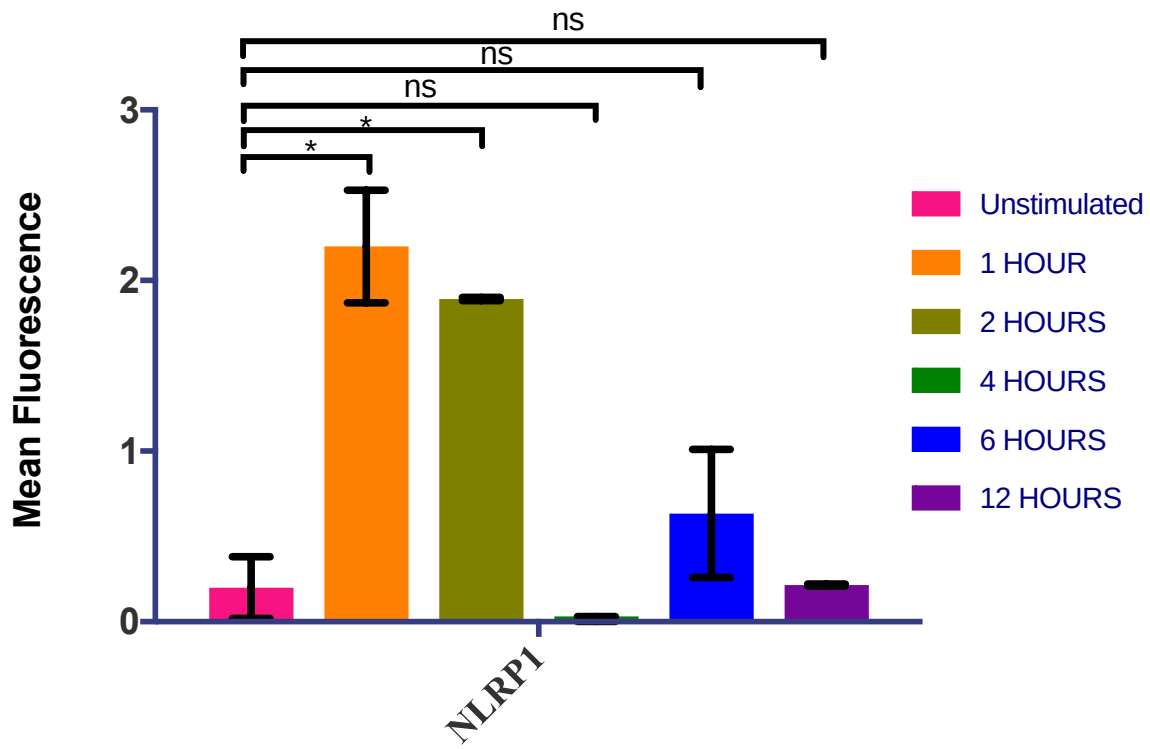


Figure 55. ***NLRP1 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.25, 0.47, 0.01, 0.02, 0.63, and 0.01 respectively.**

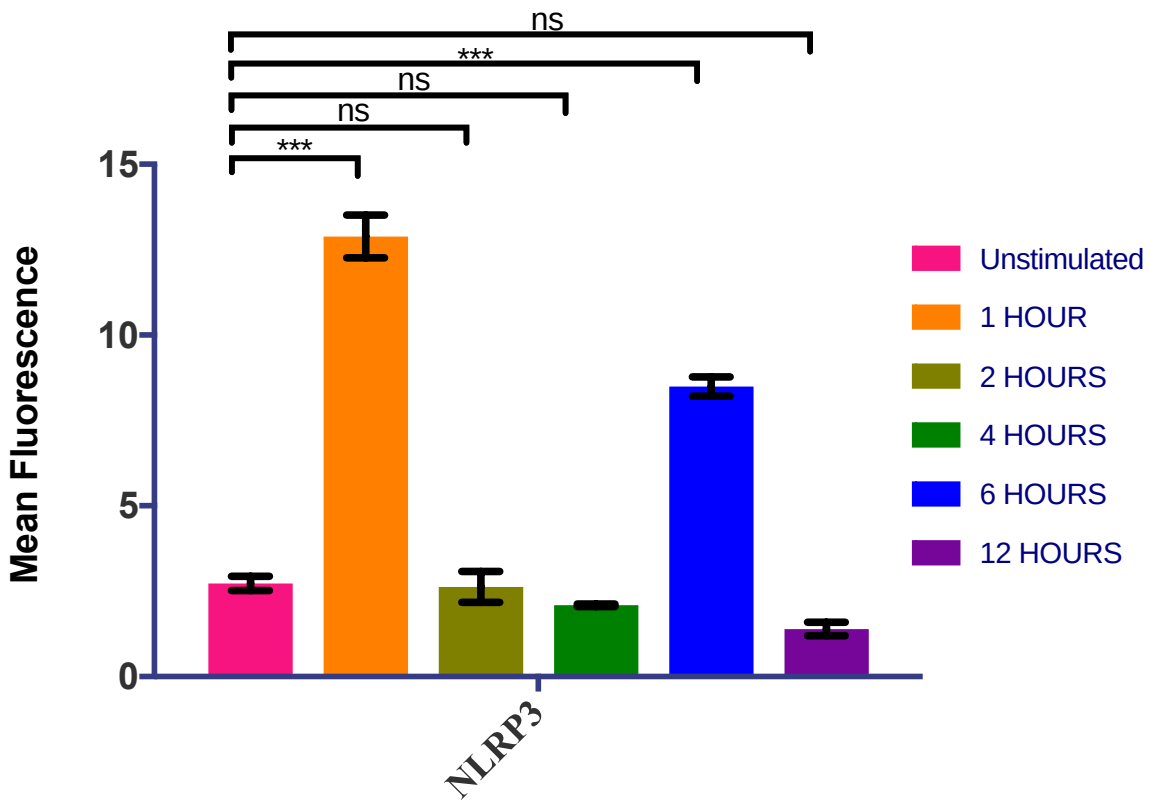


Figure 56. ***NLRP3 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.23, 0.88, 0.64, 0.06, 0.40, and 0.29 respectively.**

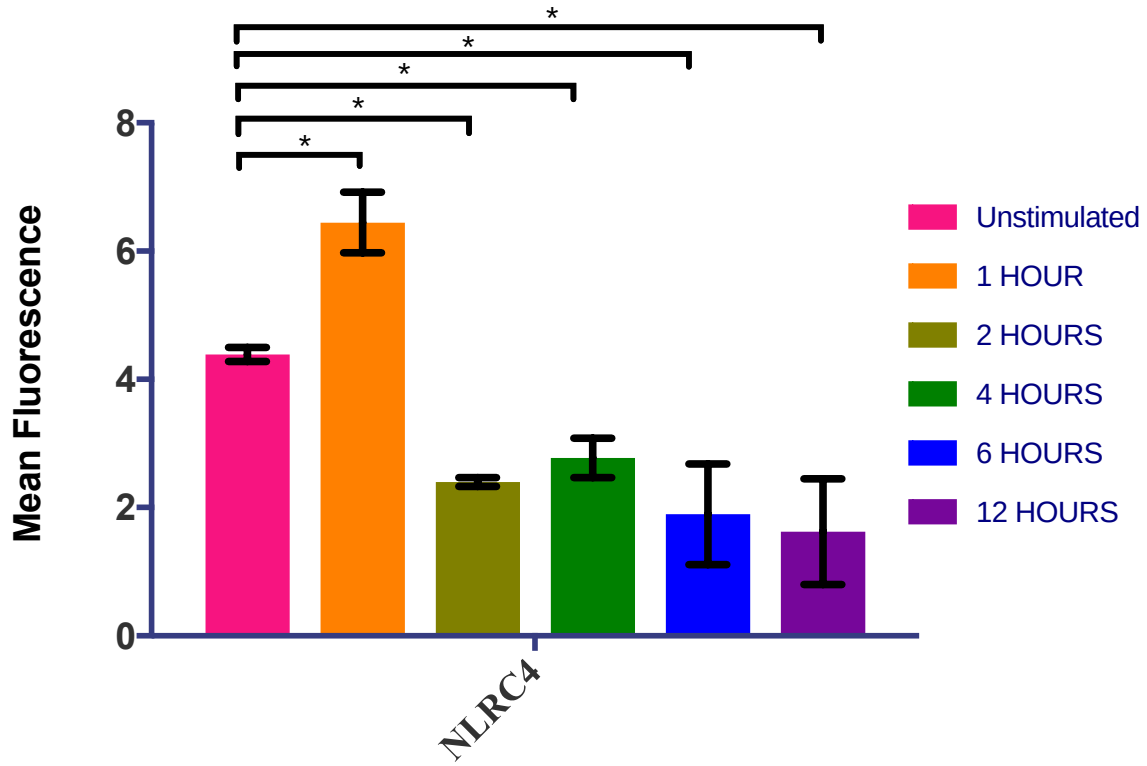


Figure 57. ***NLRC4 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.16, 0.67, 0.10, 0.43, 1.11, and 1.67 respectively.**

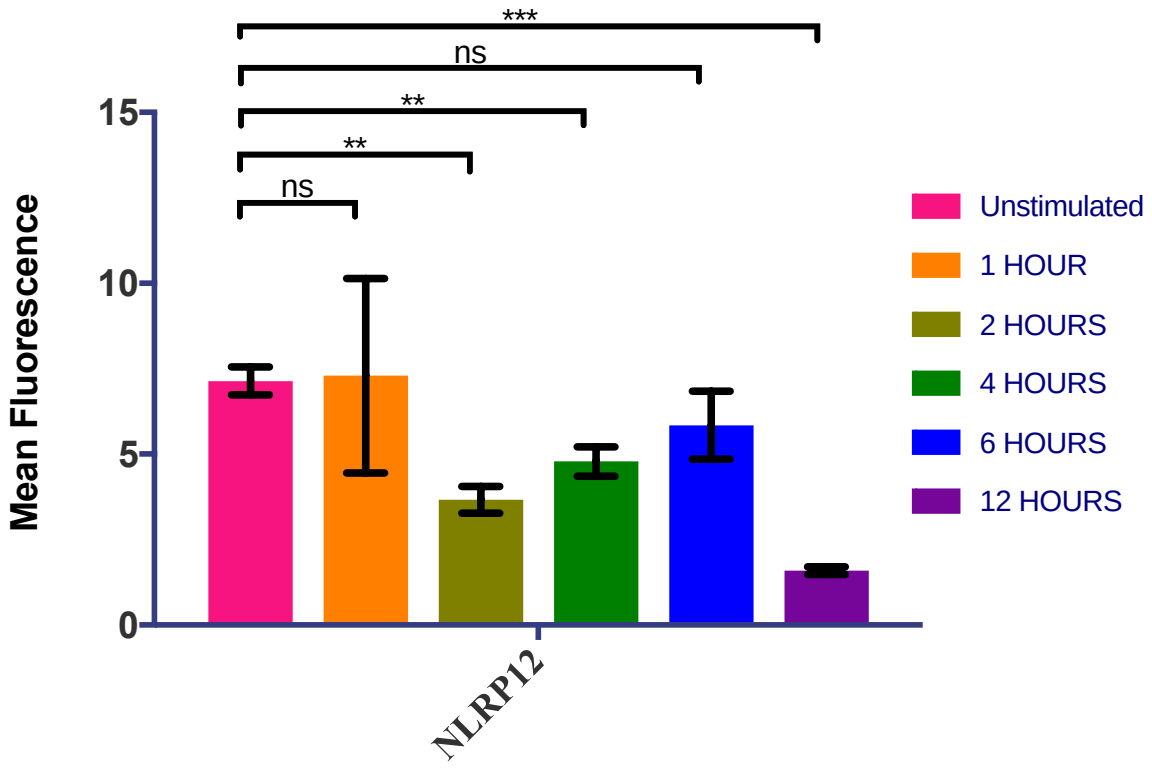


Figure 58. ***NLRP12 expression in MOLT4 cells stimulated with 1µg gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.58, 4.02, 0.55, 0.60, 1.40, and 0.16 respectively.**

The overall picture of PRR expression following MOLT4 cell stimulation with 1µg gp120 was that of marked upregulation in the early segment of the time course, then a downregulation in expression followed by a second marked spike in expression at six hours of incubation for TLR4, NLRP1 and NLRP3. Particularly for NLRP3 at 1 and 6 hours of incubation with 1µg gp120 the data showed significant differences between stimulated and unstimulated samples ($p<0.001$). There was as similarly observed for the THP1 1µg gp120 stimulations a marked level of standard deviation from the mean at specific time points: TLR2 at 2 and 6 hours; TLR4 at 1, 2 and 6 hours; NLRP1 at 6 hours as well as NLRP12 at 1 hour. Strikingly, there was little significant change in expression of NLRP1 over the course of the incubations with gp120. Thus, to control for potential PRR oversaturation by the gp120 ligand at the 1µg dose, the incubations at 1,2,4,6 and 12 hours were repeated for MOLT4 cells using 500ng gp120 along with unstimulated isotype controls (figures 59-65 below).

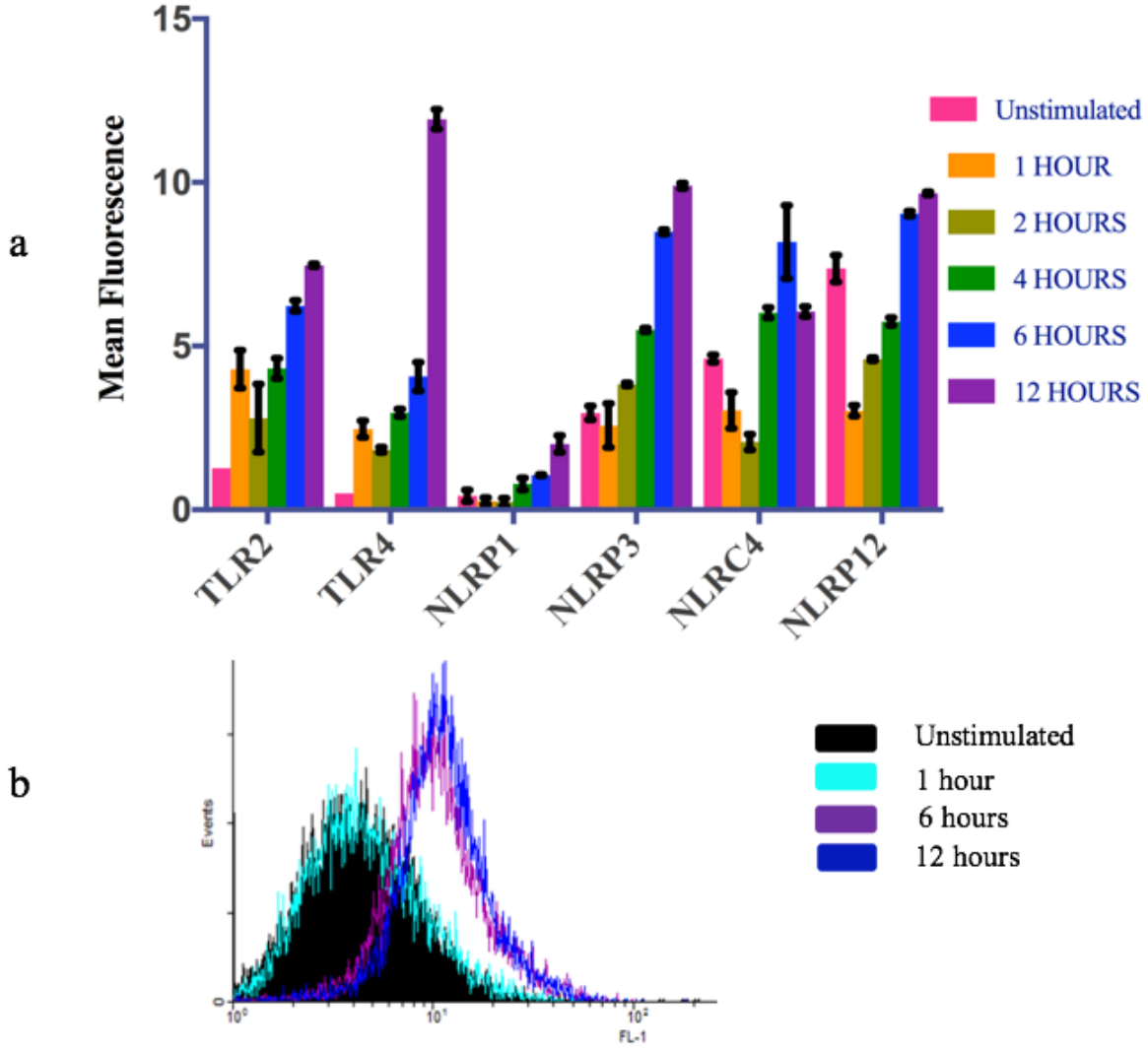


Figure 59. ***HIV-1 gp120 modulates TLR & NLR expression.*** **1x10^6 MOLT4 CD4 T-cells were stimulated with 500ng/ml HIV1 gp120, fixed in PFA, and probed for the depicted PRRs with the appropriate FITC-conjugated antibody. Mean background fluorescence for series = 1.42 (N=5, 95% CI=1.16-1.67, SD=0.21). Fluorescence intensity was detected using a FACSCalibur (Becton**

Dickinson) counting 10,000 cells. Histogram (b) shows WinMDI analysis of 500ng HIV-1 gp120 stimulation series for NLRP3 expression.

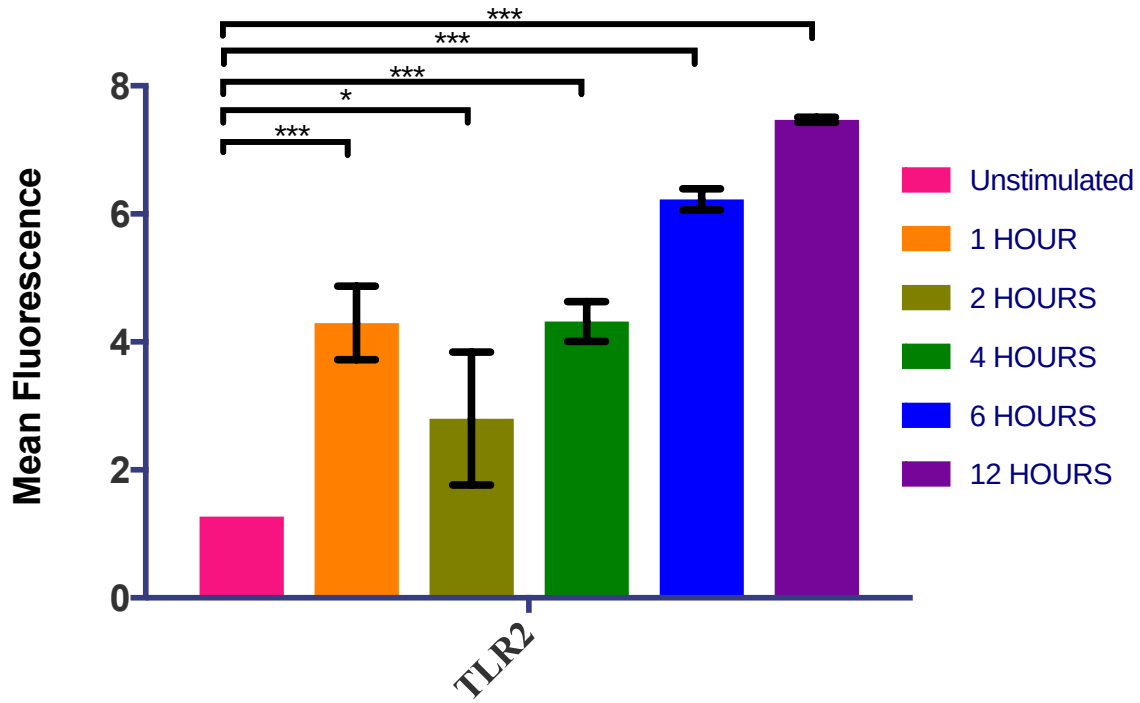


Figure 60. ***TLR2 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.00, 0.81, 1.47, 0.43, 0.23 and 0.06 respectively.**

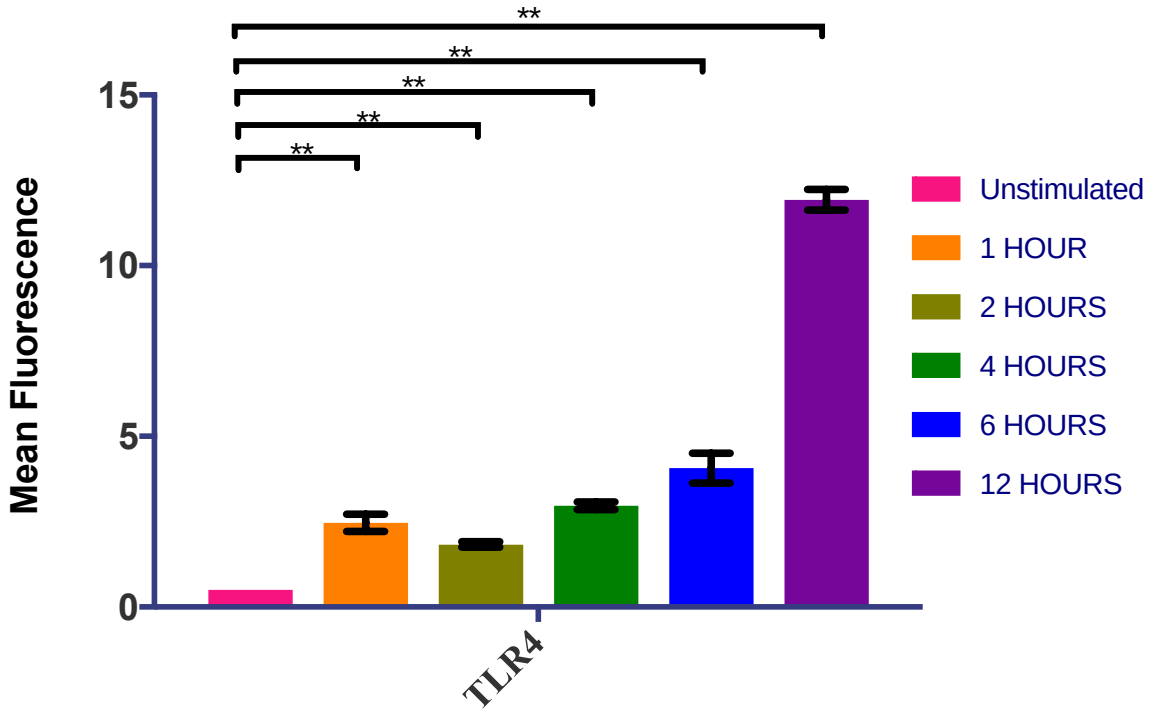


Figure 61. ***TLR4 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.00, 0.36, 0.11, 0.16, 0.62, and 0.42 respectively.**

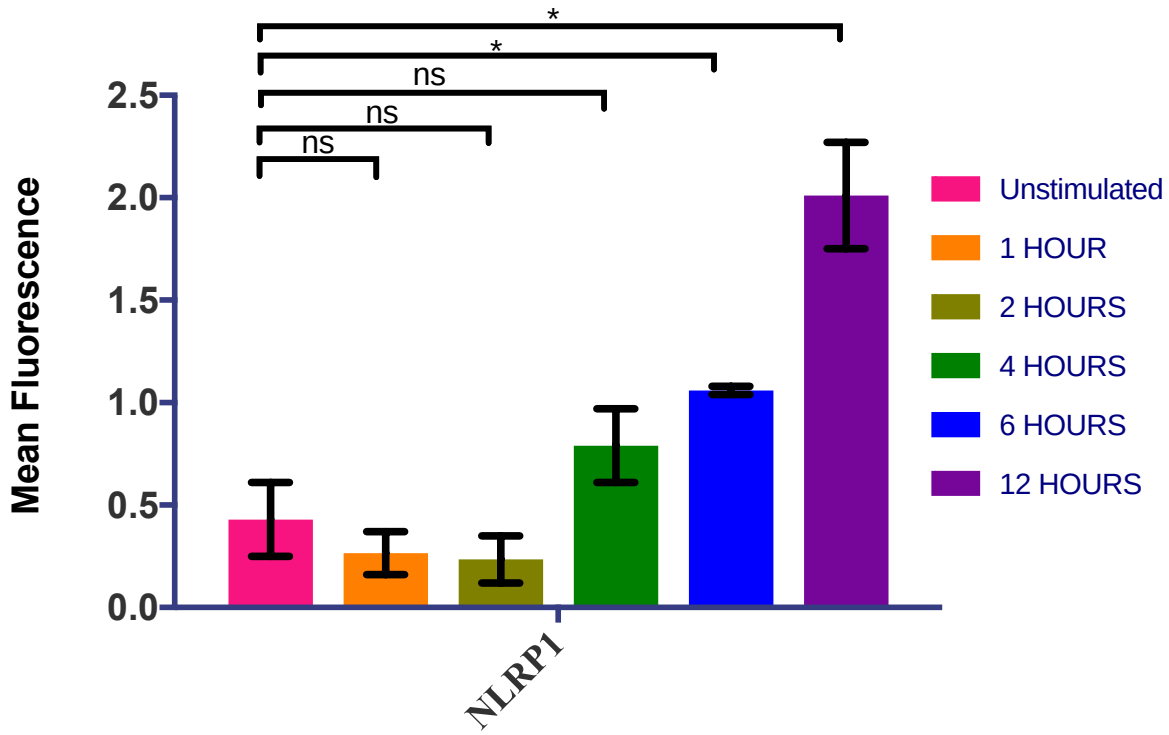


Figure 62. ***NLRP1 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.25, 0.15, 0.16, 0.25, 0.03, and 1.37 respectively.**

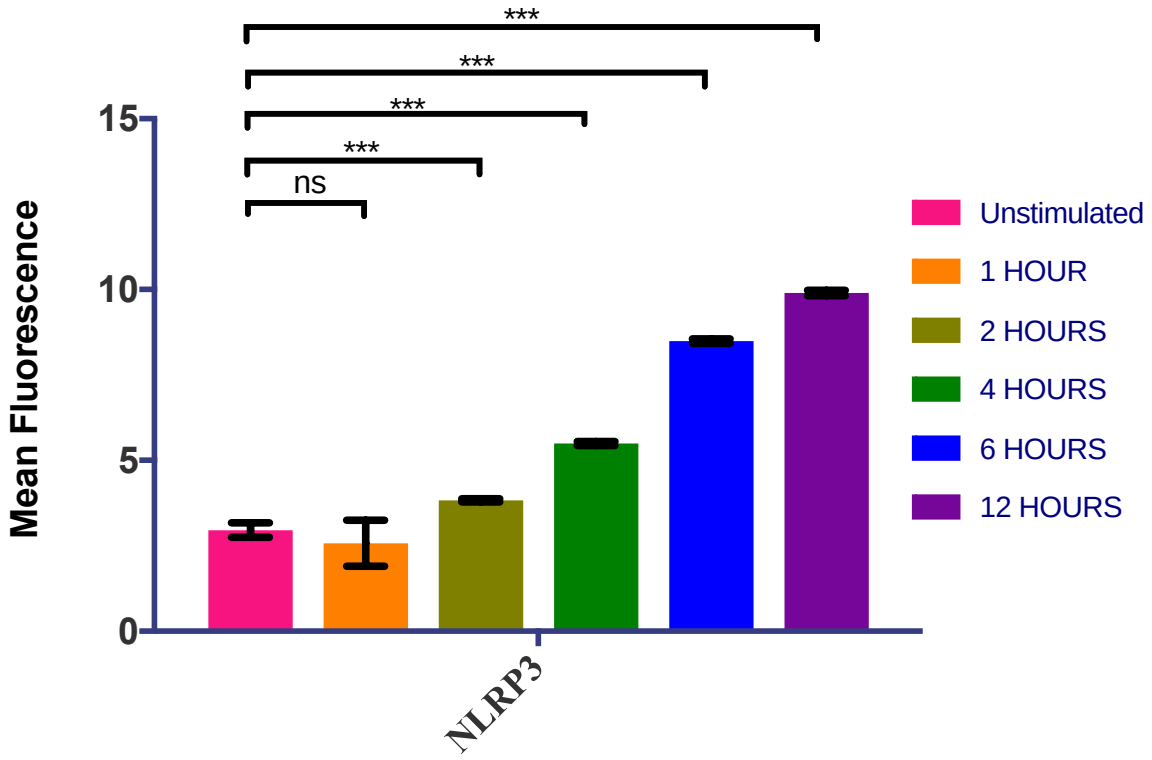


Figure 63. ***NLRP3 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.30, 0.95, 0.08, 0.09, and 0.11 respectively.**

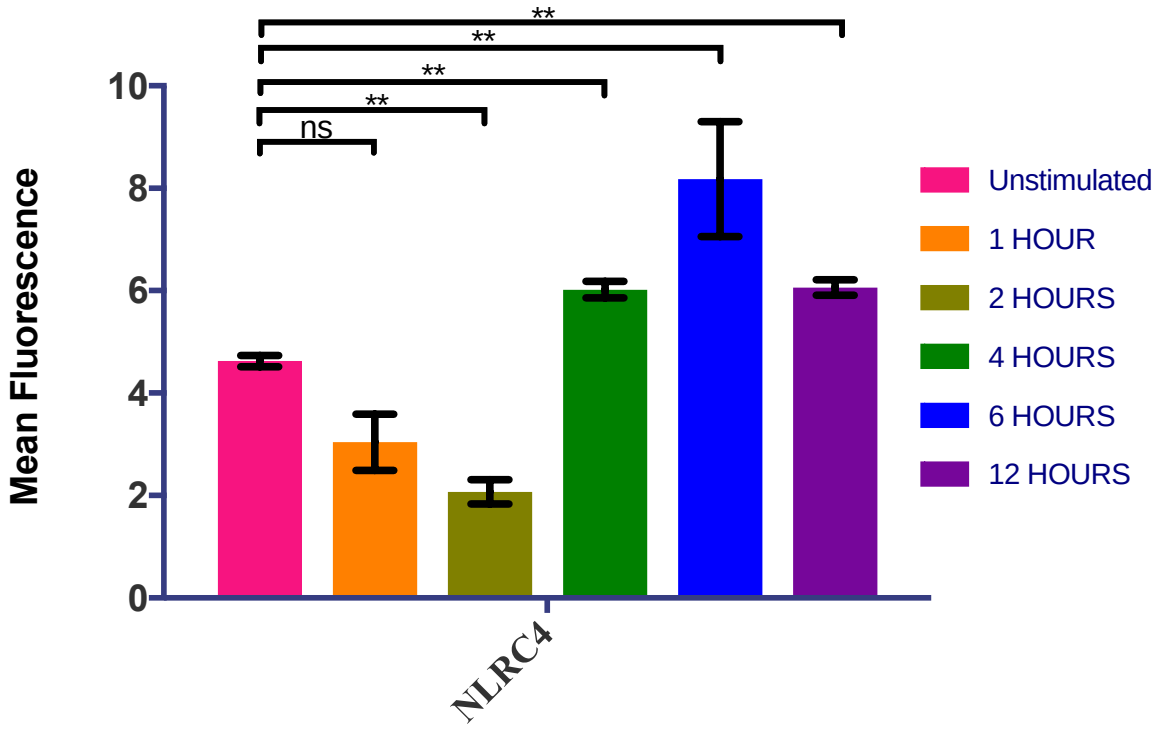


Figure 64. ***NLRC4 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.16, 0.78, 0.34, 0.23, 1.58, and 0.21 respectively.**

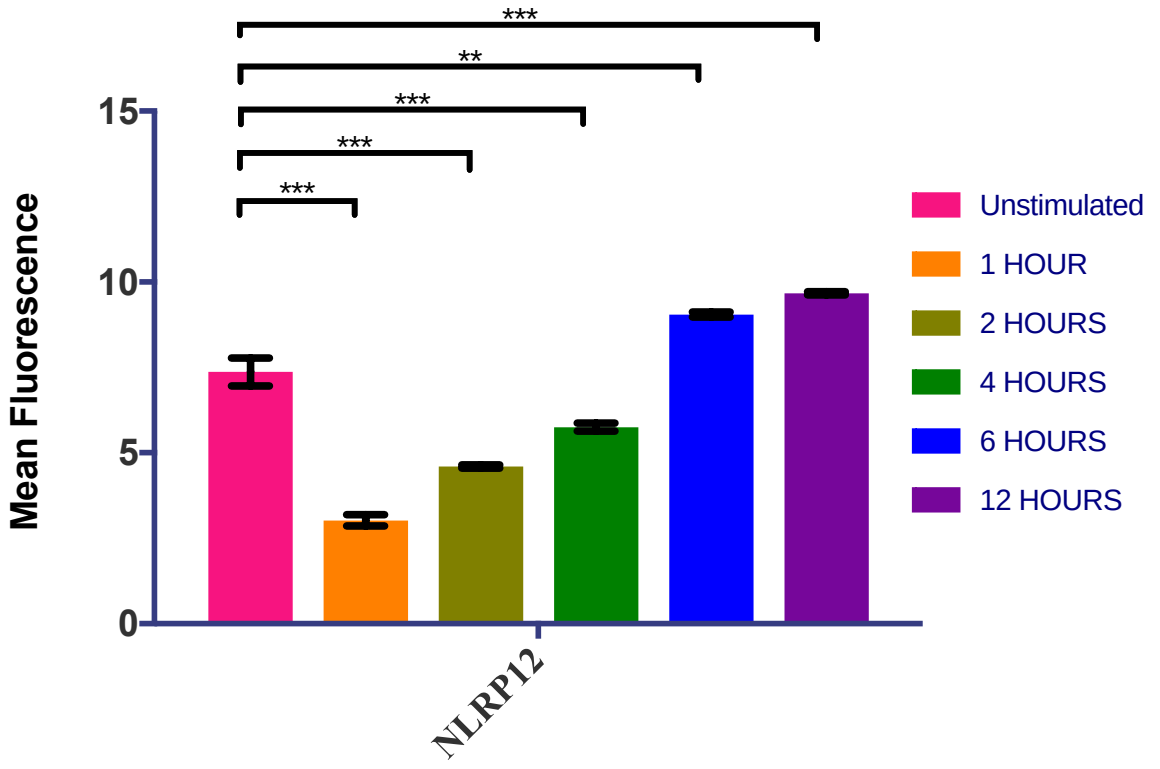


Figure 65. ***NLRP12 expression in MOLT4 cells stimulated with 500ng gp120.*** **For US, 1, 2, 4, 6 and 12 hours the SD = 0.57, 0.23, 0.07, 0.17, 0.11 and 0.06 respectively.**

The general pattern of PRR expression following stimulation of MOLT4 cells with 500ng gp120 was that of an initial downregulation from baseline levels followed by recovery and peaking in the latter stages of the experiment. Overall, the standard deviation from the mean of the different samples was much improved for the 500ng gp120 stimulation of MOLT4 cells compared to the 1µg gp120 stimulations, with the exception of TLR2 at 2 hours, NLRP1 at 12 hours, and NLRC4 at 6 hours of incubation. The 500ng gp120 experiment showed significant differences in expression levels between unstimulated isotype controls and stimulated samples for TLR2 (excluding expression at 2 hours of incubation), TLR4, NLRP3 (excluding expression at 1 hour) and NLRP12.

To determine whether TLR and NLR upregulation is functional in MOLT4 cells, IL-6 which is a TLR dependent inflammatory cytokine, as well as IL-1β which is an inflammasome dependent but TLR independent cytokine were measured. In addition, NFκB activation which denotes TLR activation was determined via phosphoIκB detection by using Western Blotting. Inflammasome activation was determined via caspase-1 p10 detection. Therefore, reporter cells for IL-6 and IL-1β were used to determine the presence of IL-6 and IL-1β respectively (figure 66 overleaf). The data showed significant upregulations compared to baseline for IL-6 and more significant upregulation of IL-1β over 12 hours. Western blotting confirmed both the activation of signal 1 by gp120 as shown from the presence of phosphoIκB, as well as the inflammasome pathway represented by the presence of the caspase-1 p10 subunit.

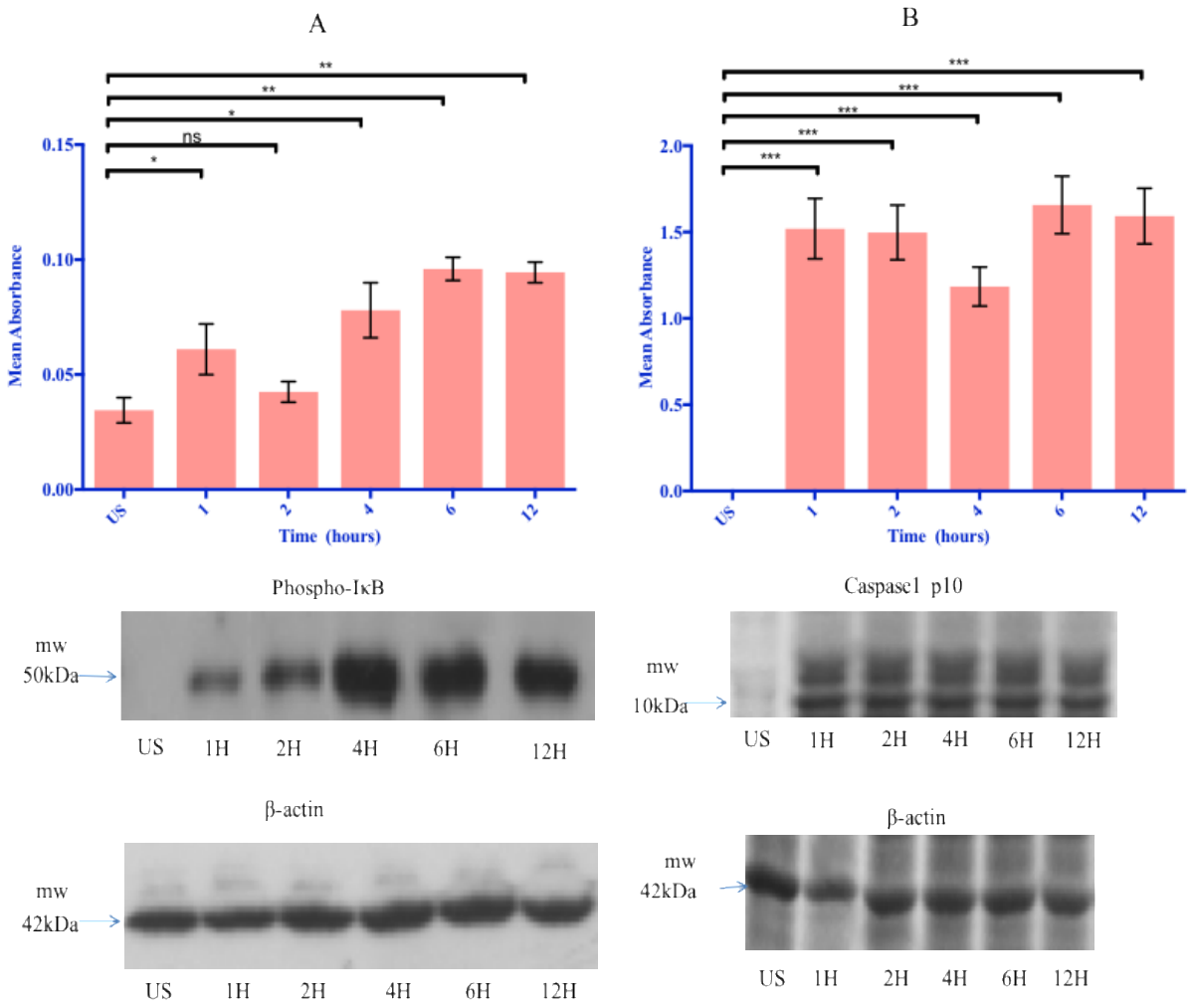


Figure 66. ***HIV-1 gp120 induces NFκB and inflammasome activation.*** **Signalling pathways underlying the IL-6 (A) and IL-1β response (B) in HIV-1 gp120 stimulations of MOLT4 CD4 T-cells are depicted. THP1 IL-6 reporter cells (A) or HEK 293 IL-1β (B) reporter cells were incubated with supernatants from each time-point and isotype control and incubated for 24 hours. Supernatants were then incubated with QuantiBlue. SEAP levels were measured using a photospectrometer at 655nm. The SEAP levels correspond to the expression levels of IL-6 or IL-1β. SD for IL-6 at US, 1 hour, 2 hours, 4 hours, 6, and 12 hours = 0.00, 0.01, 0.06, 0.05, 0.01 and 0.01 respectively. SD for IL-1β = 0.000, 0.247, 0.224, 0.160, 0.235 and 0.228 respectively. The data represent the mean of three independent experiments. Cell samples were lysed, then proteins were separated using SDS PAGE before transfer to a nitrocellulose membrane which was probed with phosphoIκB (A) or anti-caspase-1 p10 rabbit antibody (B) followed by the appropriate secondary antibody conjugated to HRP. Proteins were visualised using via enhanced chemiluminescence. Images presented are representative of three independent experiments.**

1.5 HIV-1 gp120 inflammasome activation is NLRP3 dependent

Many virus surface proteins are recognised by pattern recognition receptors. To investigate interactions between HIV-1 gp120 and germline encoded PRRs, we confirmed that the lower dose of HIV-1 gp120 could induce both signal 1 and signal 2 required for inflammasome activation. We then asked whether inflammasome activation in response to HIV-1 gp120 was NLRP3 dependent and whether activation dependent signal 1 was induced by TLR2 or TLR4 in monocytes, which are susceptible to HIV-1 infection and by virtue of their high baseline expression of all TLRs and NLRs represent a perfect experimental model to answer these questions.

Therefore we knocked down expression of TLR4, TLR2 and NLRP3 using shRNA and looked for the presence of caspase-1 p10 via Western Blotting in order to determine TLR4, TLR2 and NLRP3 significance in inflammasome activation (fig. 67 overleaf). TLR7 was also knocked down as a control since it recognizes viral ssRNA thereore it is not involved in gp120 innate recognition and the absence of TLR7 should not affect inflammasome activation (fig. 67). The results showed that TLR2 and TLR4 knockdown can severely reduce caspase-1. In addition, NLRP3 knockdown completely inhibits caspase-1 and inflammasome activation, whereas as expected TLR7 knockdown had no effect on caspase-1 p10 expression. The same pattern was observed when we looked at IL-1β secretion there was a decrease when TLR2 and TLR4 production was knocked down and there was inhibition in the absence of NLPR3.

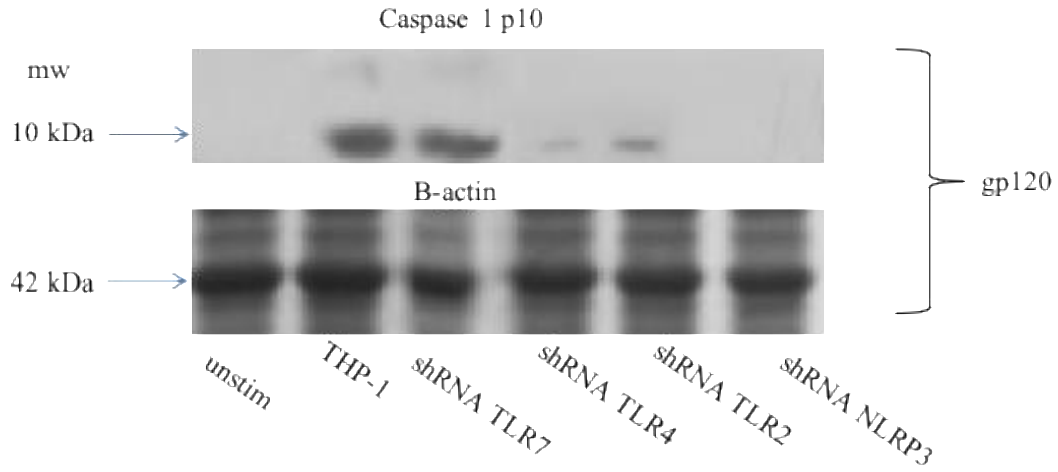


Figure 67. ***Inflammasome activation following HIV-1 gp120 stimulation is NLRP3 dependent.*** **$1x10^6$ THP1 monocytes as well as THP1 monocytes with knockdown of TLR2, TLR4, NLRP3 and TLR7 were incubated at 37°C with 500ng/ml HIV-1 gp120 for 1 hour. Lysate proteins were separated by SDS PAGE and transferred to a nitrocellulose membrane which was probed with anti-caspase-1 p10 rabbit antibody then incubated with the appropriate secondary antibody-HRP conjugate. Caspase-1 activation was visualised using enhanced chemiluminescence. β-actin controls are also displayed.**

2.0 Discussion

In this study we investigated how the host recognizes gp120 and triggers the innate immune response. We used monocytes and T cells as they are susceptible to HIV-1 infection and they express most PRRs. Initially we used a saturation concentration for HIV-1 gp120 (1μg/ml) to determine whether the viral glycoprotein could be recognised by the host. After confirming that a large dose of HIV-1 gp120 could indeed upregulate innate immune sensors, we then lowered the concentration to avoid the potential for PRR over-saturation and stimulated at the same time points with unstimulated isotype controls.

Stimulation of THP1 monocytes with 1μg/ml of HIV-1 gp120 appeared to show a marked up-regulation of PRR expression within 1 hour of stimulation, followed by a down-regulation of all PRRs investigated over a 12 hour period, suggesting that gp120 is detected early in infection, triggering innate immune responses. When 500ng/ml HIV-1 gp120 was used it resulted again in an up-regulation of PRRs although it appeared to take longer for the innate immune system to sense the presence of gp120 and trigger PRR mobilisation. To determine the effects of HIV-1 gp120 on CD4 T-cells, MOLT4 CD4 T-cells were also stimulated with different concentrations of gp120 over a 12 hour period, PRR expression as well as signalling cascade and cytokine secretion was investigated. Similar to results obtained for THP1 monocytes, MOLT4 CD4 T-cell TLRs were quickly upregulated in response to 1μg/ml HIV-1 gp120.

Interestingly, MOLT4 CD4 T-cell TLR peak expression levels were observed within 2 hours post-stimulations, whereas this occurred within 1 hour for THP1 monocytes. This suggests that innate immune system activation takes longer in CD4 T-cells compared to monocytes used in the study. In contrast, NLR expression appeared to increase within 1 hour post-stimulation and then peak at six hours. Stimulation of MOLT4 CD4 T-cells with 500ng/ml

HIV-1 gp120 appeared to up-regulate all PRRs by 6 hours post-stimulation, this occurring somewhat slower than what was observed for monocytes. This shows that monocytes and CD4 T cells could respond differently to viral pathogens. gp120 was recognized by PRRs on both cell types (THP1 monocytes and MOLT4 CD4 T-cells), and TLR4 as well as TLR2 can trigger NFκB activation and inflammatory cytokine secretion in the presence of gp120.

Furthermore, it was shown that the inflammasome is activated by gp120. This activation was NLRP3 dependent and it was abolished when NLRP3 was silenced by shRNA interference. This is consistent with the flow cytometry data. When HIV-1 gp120 was incubated in TLR2 or TLR4 pshRNA expressing THP1 monocytes, caspase-1 activation appeared to be reduced, suggesting that both TLR2 and TLR4 are involved in HIV-1 gp120 sensing in order to activate signal 1. When TLR7 was silenced as a control it had no effect since its ligand is viral ssRNA. Thus it seems that gp120 is sufficient to trigger signal 2 as well leading to caspase-1 activation and IL-1β secretion. The possible mechanism of signal 2 could be due to conformational changes that gp120 causes when it binds to the cell membrane, thus being perceived as a danger signal by the host and alerting the inflammasome into action.

Chapter 4: HIV-1 DAMPs activate the NLRP3 inflammasome

1.1 HIV-1 activates the inflammasome

HIV-1 is a single stranded RNA lentivirus responsible for the development of a plethora of opportunistic infections and cancers. Untreated HIV-1 can progress to the end stage of infection as reflected in AIDS defining illnesses and ultimately cause the death of the person living with untreated HIV-1 infection (Levy JA 2009). Various PRRs are known to recognise and respond to viral PAMPs or DAMPs depending upon their intracellular versus cell-surface localisation. TLRs 3, 7, 8, and 9 are known to sense viral nucleic acid sequences; TLRs 2 and 4 are known to sense viral envelope proteins (Loiarro et al. 2010; Kawai & Akira 2011; Takeuchi O & Akira S 2009).

Multiple studies suggest NLRP3 is activated by the HIV-1 virion (Pontillo et al. 2012; Hernandez et al. 2014). although the precise HIV-1 ligands activating the NLRP3 inflammasome are being investigated, a cell-based *in vitro* study is required to add further weight to the observation that HIV-1 activates the NLRP3 inflammasome thereby triggering caspase-1 activation and subsequent cleavage of pro-IL-1β into mature IL-1β followed by its secretion from stimulated cells.

Consequently, this study aims to clarify whether HIV-1 can activate the NLRP3 inflammasome in THP1 monocytes or primary monocytes thereby adding further evidence to an acute infection stage model of NLRP3 contribution to the cytokine dysregulation observed. This study also investigates whether inhibition of HIV-1 viroporin Vpu ion channel activity can abrogate NLRP3 activation, evaluating a candidate role of Vpu in NLRP3 inflammasome induction.

1.2 HIV-1 induced inflammasome activation

HIV-1 in the acute phase of infection and throughout the natural history of infection is known to cause significant immune dysregulation as is reflected in the observation of exaggerated cytokine profiles (McMichael et al. 2010). IL-1β secretion is known to be increased during HIV-1 infection and previous studies have implicated the NLRP3 inflammasome as being responsible for raised IL-1β levels (Pontillo et al. 2012; Hernandez et al. 2014). Prior to investigating precisely how HIV-1 proteins could be responsible for inflammasome activation, it was necessary to first confirm in the selected cell lines, in this case monocytes, that HIV-1 can activate the inflammasome in a pilot experiment. Thus caspase-1 p10 (activation) was determined via Western Blotting, which is an essential step in the pathway from NLR sensing through to IL-1β secretion (figure 68).

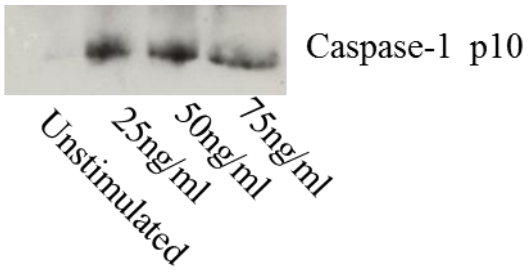


Figure 68. ***Dose ranging pilot experiment to determine optimal concentration of HIV-1 for inflammasome activation*****. HIV pNL4-3 X4 tropic virus was incubated with 1x10^6 THP1 monocytes in fresh RPMI at 37°C. Doses reflect supernatant concentration of HIV-1 in ng/ml: doses used were 25ng/ml, 50ng/ml, 75ng/ml. Cells were lysed and proteins were separated by SDS PAGE and transferred to a nitrocellulose membrane which was probed with an anti-caspase-1 p10 rabbit antibody and appropriate secondary antibody conjugated to HRP and imaged via enhanced chemiluminescence. This gel is representative of three different experiments**

1.3 Mechanisms of HIV-1 induced inflammasome activation

To discover how HIV infection can trigger inflammasome activation we looked at different mechanisms of activation. The mechanisms leading to inflammasome activation (signal 2) which have been proposed are: K^+ efflux which induces NLRP3 recruitment (Petrilli et al. 2007). release of reactive oxygen species (ROS) following lysosomal destabilisation (Hornung et al. 2008); and Ca^{2+} mobilisation (Feldmeyer et al., 2007, Murakami et al., 2012).

To test the mechanism of inflammasome activation, THP1 monocytes were incubated with HIV-1 in medium containing the appropriate concentration of different drugs (H^+ inhibitor, Ca^{2+} inhibitor or HMA which inhibits Na^+/H^+) for 60 minutes to 12 hours. Our results (figure 69 overleaf) showed that verapamil (a Ca^{2+} channel inhibitor) treatment had no effect while HMA (a Na^+/H^+ inhibitor) and rimantadine (H^+ channel inhibitor) (Jing et al. 2008) reduced caspase-1 activation. To a small extent the recruitment of ASC which is the adaptor for inflammasome assembly (figure 69) was also reduced by HMA and rimantadine. The optimal inhibition was achieved at the 6-hour time point when the inflammasome activation is at its peak. At 12 hours the effect of the inhibitors has been minimized. To exclude the possibility that the inhibitors mentioned above affected cell viability, viability of cells was determined by using 0.2% (w/v) Trypan blue and cells were examined under a light microscope.

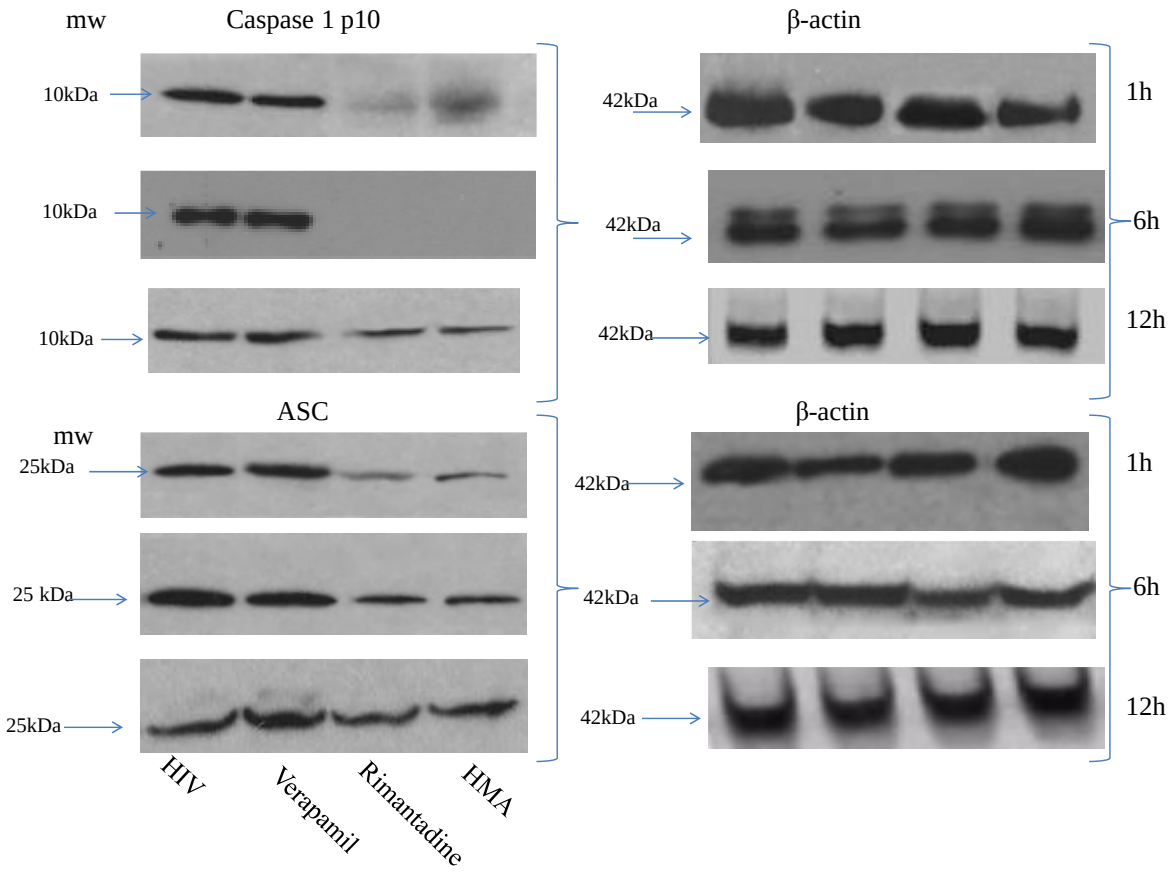


Figure 69. ***Effect of channel inhibitors on HIV-1 induced inflammasome activation.*** **THP1 monocytes were incubated at the same time with 50ng/ml HIV-1 ($1x10^6$) for 1 hour, 6 hours and 12 hours after treatment for 1 hour with verapamil (50µM), HMA (25µM) or rimantadine (6.25µM). Cell extracts were separated by SDS PAGE and analysed for the presence of caspase1 p10 and ASC by Western Blotting using an anti-caspase-1 p10 rabbit antibody and an anti ASC mouse antibody followed by the appropriate secondaries conjugated to HRP. Actin controls are displayed. The data are representative of three independent experiments**

1.4 HIV-1 Vpu activates the inflammasome

Viral infection of the human body is known to induce inflammation and inflammasome activation. In the acute phase of HIV-1 infection, this process is dramatic in terms of the range of cytokines up-regulated. IL-1β levels are no exception and they markedly increase during HIV-1 infection,(McMichael et al. 2010) likely as a consequence of NLRP3 activation.

In order to identify the main mechanism of inflammasome activation we looked at HIV viral proteins which could trigger inflammasome activation by modulating ion fluxes. HIV-1 encodes Vpu, an ion channel protein which has been well-studied as a viroporin in biophysical models (Mehnert et al. 2008; Li et al. 2013). There are several studies showing that viroporins encoded by other viruses, such as the IAVA M2 protein channel, activate the inflammasome. For example, the SH viroporin of RSV and the 2B viroporin of HRV can activate the NLRP3 sensor, via cognate ion channel activity, resulting in the recruitment of caspase-1 and secretion of IL-1β (Ichinohe et al. 2010; Ito et al. 2012; Triantafilou, Kar, van Kuppeveld, et al. 2013; Triantafilou, Kar, Vakakis, et al. 2013). Of particular interest is the M2 viroporin of IAVA which shares striking homology to Vpu as encoded by HIV-1 (Pinto et al., 1992, Nieva et al., 2012, Triantafilou and Triantafilou, 2014).

Thus we proceeded to determine whether Vpu was sufficient to stimulate inflammasome activation. THP1 monocytes were transfected with a plasmid expressing HIV-1 Vpu (pcDNA-Vphu) (figure 70) and inflammasome activation was determined by detecting the

presence of caspase-1 via Western Blotting (figure 71). Our results showed that Vpu expression was sufficient to stimulate inflammasome activation.

1.5 Vpu expression in monocytes

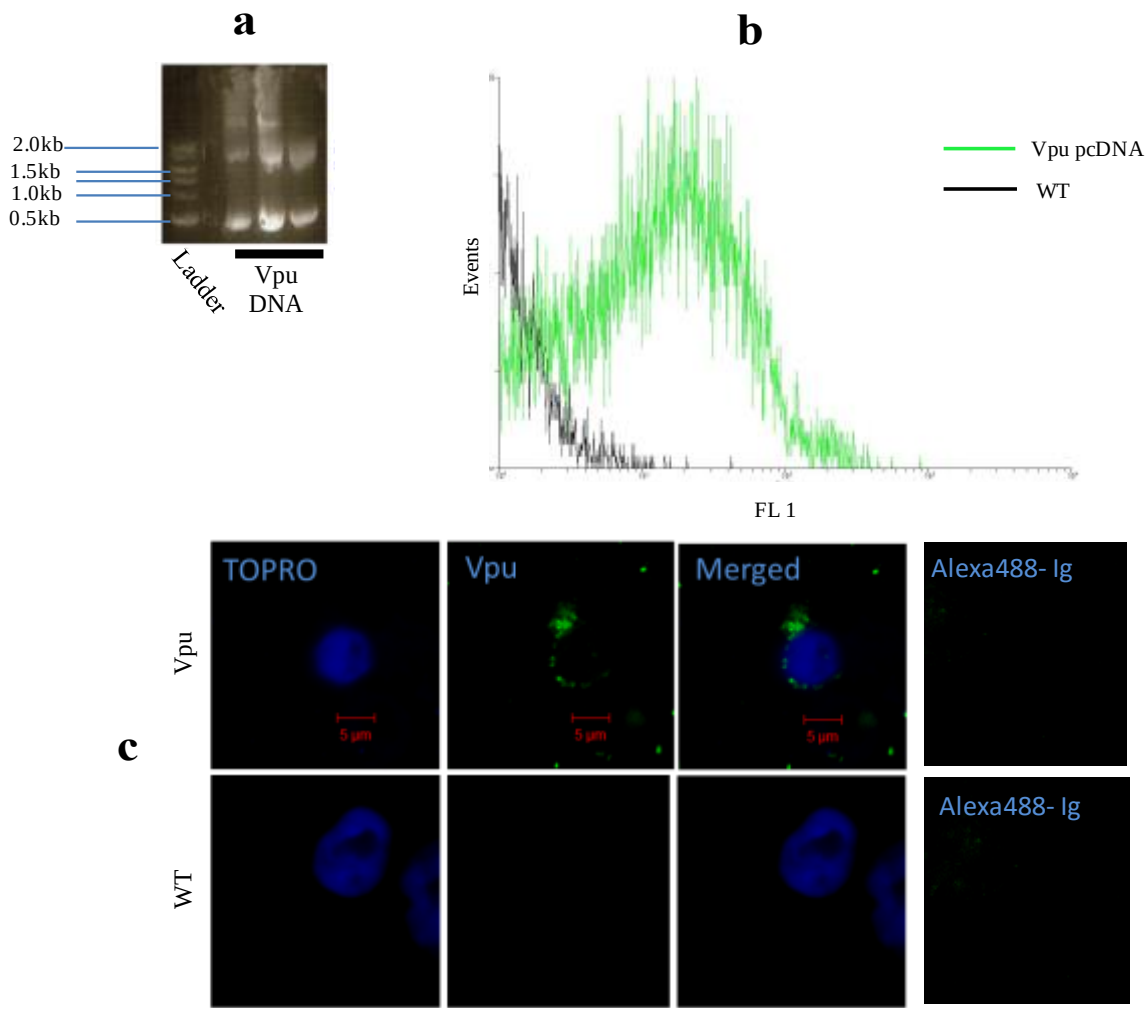


Figure 70. ***Confirmation of HIV-1 Vpu transfection of THP1 monocytes*****. pcDNA-Vphu plasmid was extracted and purity was confirmed by agarose gel electrophoresis loading 100ng in three wells adjacent to the 1kb DNA ladder (BioRad) and visualised by UV transillumination (A). Efficient transfection of pcDNA-Vphu (5µg) and expression of Vpu was confirmed comparing Vphu-transfected THP1 monocytes (referred to as Vpu) to THP1 monocytes (WT) via indirect immunofluorescence and flow cytometry using a BD FACSCalibur (B), as well as confocal microscopy using a Carl Zeiss 510 confocal C). Anti-rabbit Vpu antibody followed by Alexa 488-goat anti rabbit Ig was used to label Vpu HIV-1 protein whereas TOPRO-3 was used as a nuclear stain (blue). Alexa 488 donkey anti rabbit antibody used as a control without the**

addition of primary anti Vpu antibody is also shown. The images were obtained sequentially (not a simultaneous scan) thus there was instrumental separation of the fluorophore emission to prevent any bleedthrough between channels. In each experimental run 20-25 cells were imaged to confirm results. The data are representative of three independent experiments.

1.6 HIV-1 Vpu activates caspase-1 causing IL-1β secretion

Caspase-1 activation is the crucial step in the pathway of NLR signalling. Successful activation of caspase-1 infers upstream NLR stimulation by a ligand representing a sign of cell damage (DAMP) (Martinon et al. 2002). The presence of caspase-1 was analysed using Western Blotting and laser scanning confocal microscopy. It was observed from Western Blotting and HEK IL-1β reporter cell assays that THP1 monocytes transfected with HIV-1 Vpu exhibited caspase-1 activation and IL-1β secretion compared to wild-type monocytes which did not exhibit caspase-1 activation nor IL-1β secretion (figure 71 overleaf). The Invitrogen pcDNA3.1 empty plasmid which was used as a cloning vector for Vpu was also used as a negative control and exhibited no inflammasome activation as expected. The presence of caspase-1 p10 was also confirmed by confocal microscopy (figure 72).

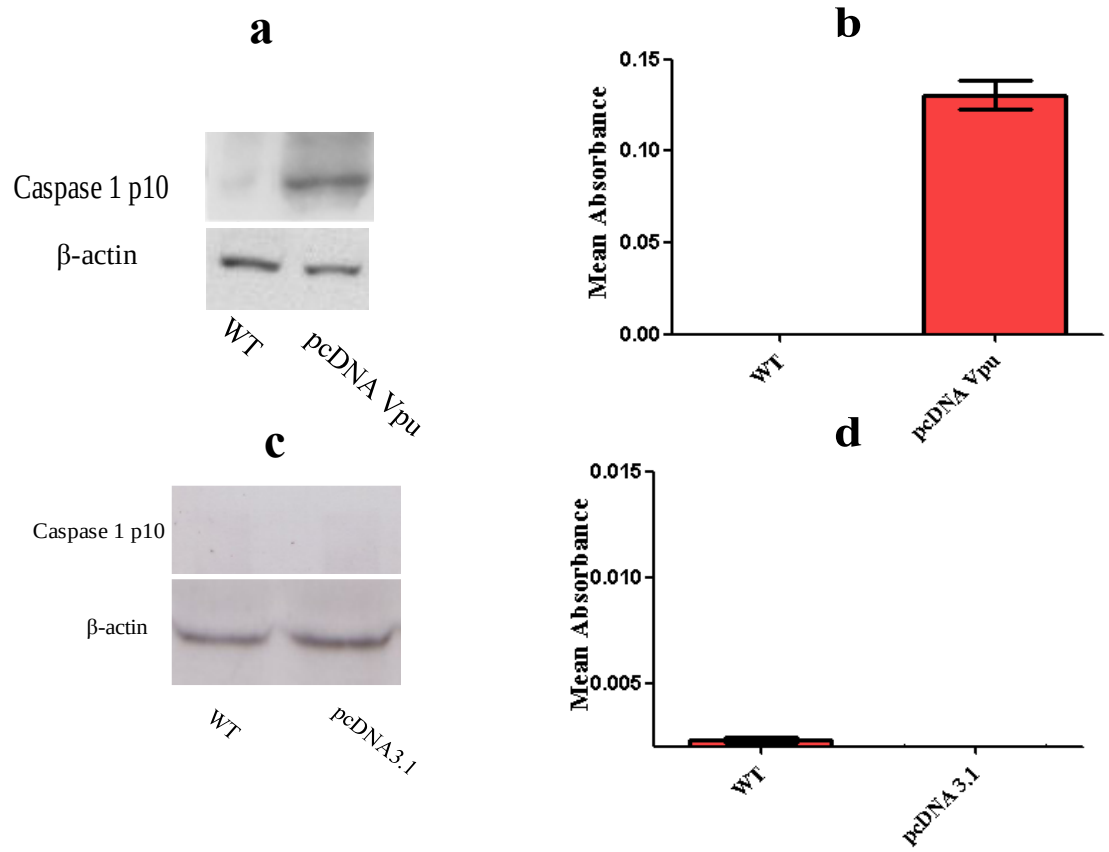


Figure 71. ***HIV Vpu activates the inflammasome*****. THP1 monocytes were untransfected or transfected with either 5µg pcDNA-Vphu plasmid (A & B) or transfected with 5µg Invitrogen pcDNA3.1 empty plasmid as a negative control (C & D). Cell extracts from THP1 monocytes (WT), pcDNA-Vphu transfected and 'mock' empty vector-transfected (pcDNA3.1) were analysed for the presence of caspase-1 p10 by Western Blotting. Supernatants were collected and tested for IL-1β secretion using HEK Blue IL-1beta reporter cells where SD for pcDNA Vpu = 0.02. The data are representative of three independent experiments.**

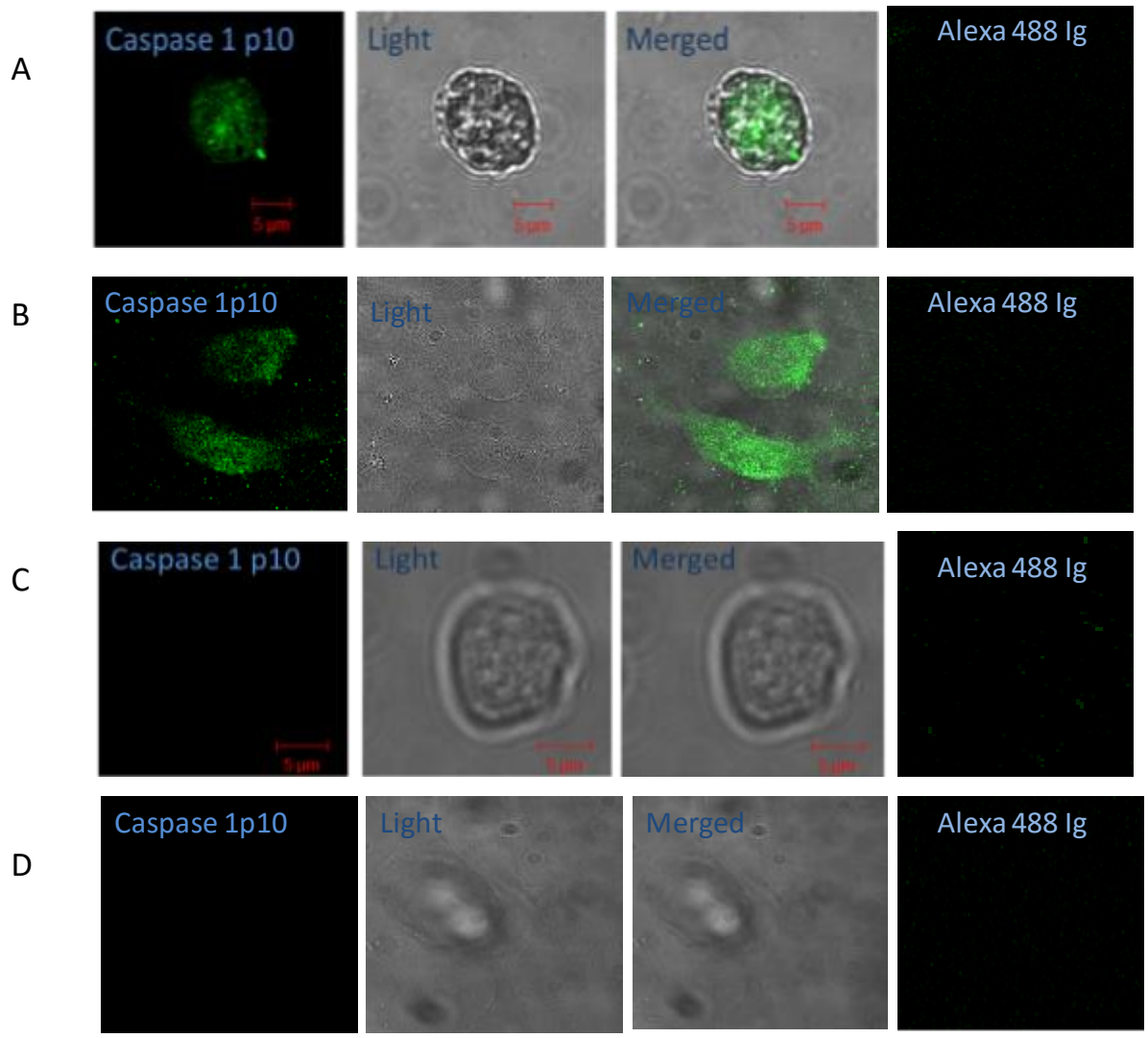


Figure 72. ***Caspase-1 detection in THP1 monocytes.*** **The presence of caspase-1 p10 was determined by confocal microscopy using a Carl Zeiss 510 confocal microscope in THP1 monocytes expressing Vpu (Vpu) (A,B) as well as THP1 monocytes that do not express Vpu (WT) (C, D). Caspase-1 p10 was labelled by using an anti-caspase-1 p10 rabbit antibody followed by a secondary antibody conjugated to Alexa 488. Alexa 488 donkey anti rabbit antibody used as a control without the addition of primary antibody is also shown. The images were obtained sequentially (not a simultaneous scan) thus there was instrumental separation of the fluorophore emission to prevent any bleed through between channels. In each experimental run 20-25 cells were imaged to confirm results. The image is representative of three independent experiments.**

1.7 NLRP3 detects HIV-1 Vpu

To determine which NLR is responsible for Vpu detection and subsequent triggering of IL-1β secretion. NLRP3, NLRP1, NLRC4, or NLRP12 were knocked down using shRNA in THP1 monocytes then the cells were transfected with pcDNA-Vphu. pshRNA with a scrambled sequence was also used as a control. When IL-1β secretion as well as caspase-1 expression was examined the data showed a significant reduction in IL-1β secretion as well as a reduction in caspase-1 expression when NLRP3 was knocked down (figure 73 A and C). There was no significant change in either IL-1β or caspase-1 when NLRP12, NLRP1 or NLRC4 were knocked down. To confirm NLRP3 specificity, NLRP3 was knocked down by pshRNA interference and THP1 monocytes were infected with HIV-1. The results show an inhibition in terms of the presence of caspase-1 p10 as well as IL-1β (figure 73 B and D).

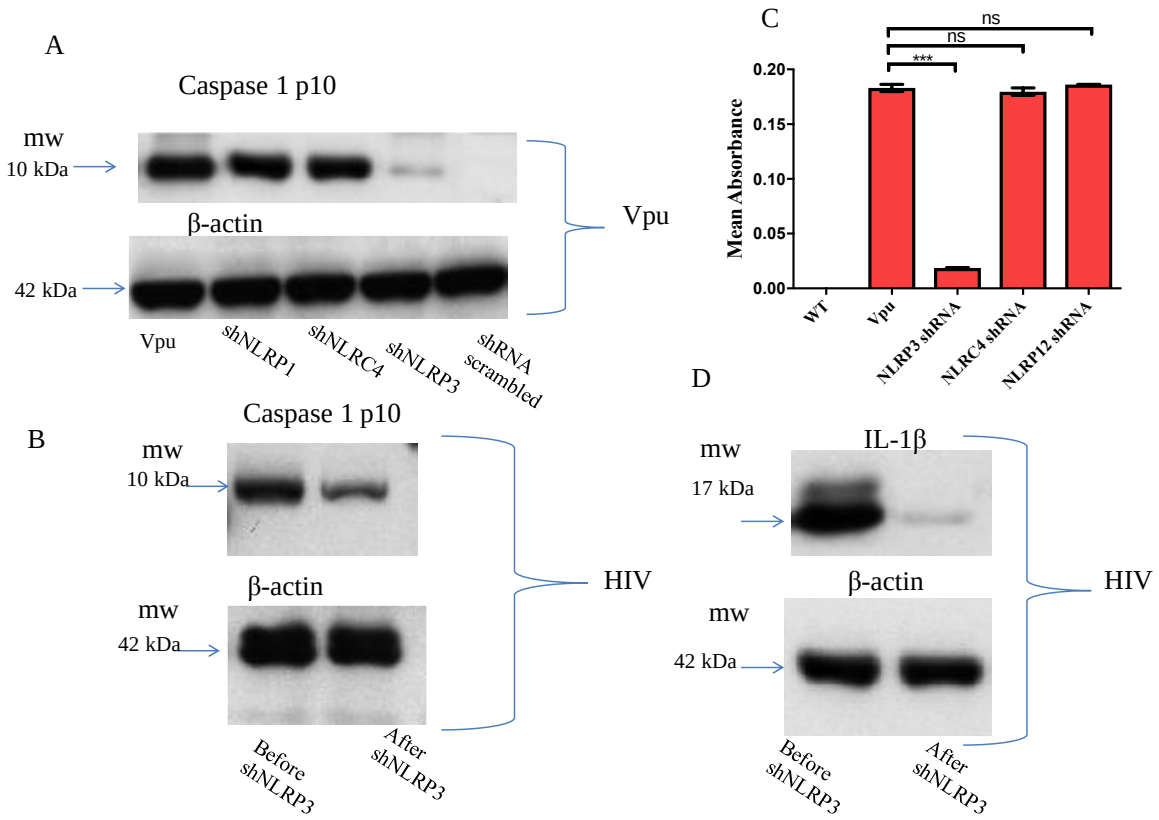


Figure 73. ***NLRP3 plays a role in HIV induced inflammasome activation*****. THP1 monocytes silenced for NLRP3, NLRP1, NLRC4, NLRP12 or NLRP3 and NLRC4 by shRNA (pshRNA with scrambled sequence was used as a control) were transfected with pcDNA-Vphu (5µg). Cells extracts were analysed for the presence of caspase1 p10 by western blotting (A). Supernatant was collected and analysed for IL-1β (C) using HEK-IL-1β reporter cells. SD for Vpu = 0.05, NLRP3 shRNA <0.01, NLRC4 shRNA = 0.1 and NLRP12 shRNA < 0.01. In addition, NLPR3 expression in THP1 monocytes was knocked down by shRNA and the cells were infected with HIV-1. Cells extracts 6 hours post infection were analysed for the presence of caspase-1 p10 (B) and IL-1β (D) by Western blotting. The data represents the mean of three independent experiments.**

1.8 Inhibition of Vpu ion channel activity reduces caspase-1 activation and IL-1β secretion

To investigate whether we could inhibit viroporin induced inflammasome activation, we used a permeant Ca^{2+} chelator, BAPTA-AM; verapamil, which blocks Ca^{2+} channels;(Stuart & Brown 2006); amantadine or rimantadine which block the IAVA M2 channel (Ichinohe et al. 2010) as well as HMA which blocks Na^{+}/H^{+} ion channels (Lemaitre et al. 2004) and N-acetyl cysteine (NAC) which is a ROS inhibitor. THP1 monocytes transfected with Vpu were treated with different concentrations of each drug, viability of cells was determined by using 0.2% (w/v) Trypan blue and examining cells under a light microscope. Cell-free supernatant was collected at 1hr, 6hr and 12hr post-incubation and analysed for IL-1β (figure 74) HMA was successful in inhibiting IL-1β secretion and additionally to a lesser extent rimantadine and amantadine had an inhibitory effect. The presence of caspase-1 p10 was also determined by Western blotting (figure 75).

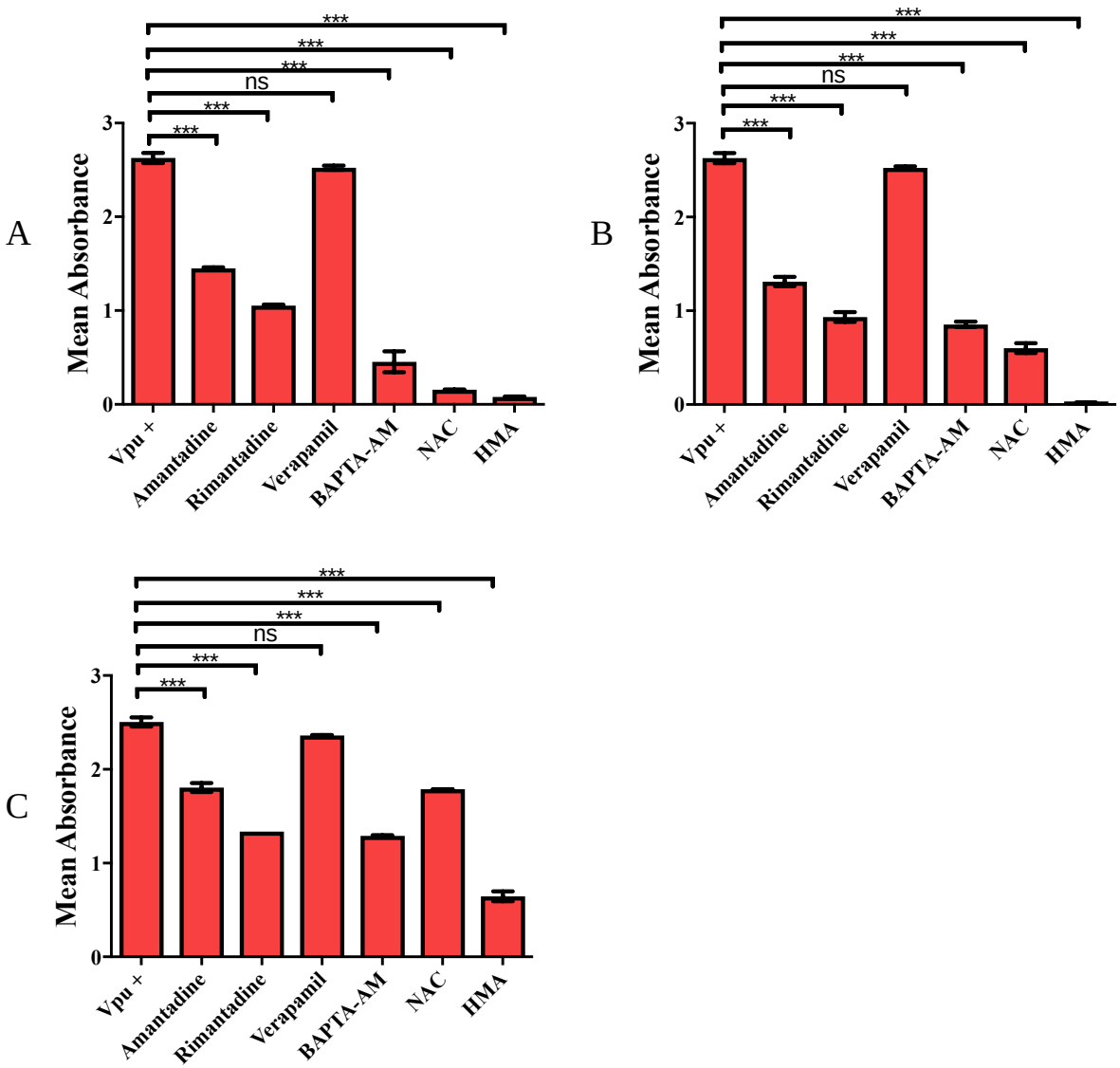


Figure 74. ***Effects of inhibitors on Vpu-induced inflammasome activation*****. THP1 monocytes (1x10^6) expressing Vpu were incubated over 12 hours with various inhibitors or no inhibitors ('Vpu'). Supernatants were collected at 1 (A), 6 (B) and 12 (C) hours and tested for IL-1β secretion using HEK blue IL-1β reporter cells. Inhibitor concentrations: Amantadine, 10µl/ml; rimantadine, 6.25µM; verapamil, 50µl/ml; BAPTA-AM, 10µl/ml; NAC, 20mM and HMA, 25mM). A: SD for Vpu = 0.05, amantadine = 0.01, rimantadine = 0.01, verapamil = 0.02, BAPTA-AM = 0.11, NAC = 0.00 and HMA = 0.00. B: SD for Vpu = 0.08, amantadine = 0.07, rimantadine = 0.07, verapamil = 0.03, BAPTA-AM = 0.04, NAC = 0.07, and HMA = 0.01. C: SD for Vpu = 0.05, amantadine = 0.05, rimantadine < 0.00, verapamil = 0.01, BAPTA-AM = 0.01, NAC < 0.00 and HMA = 0.05. The data represent the mean of three independent experiments.**

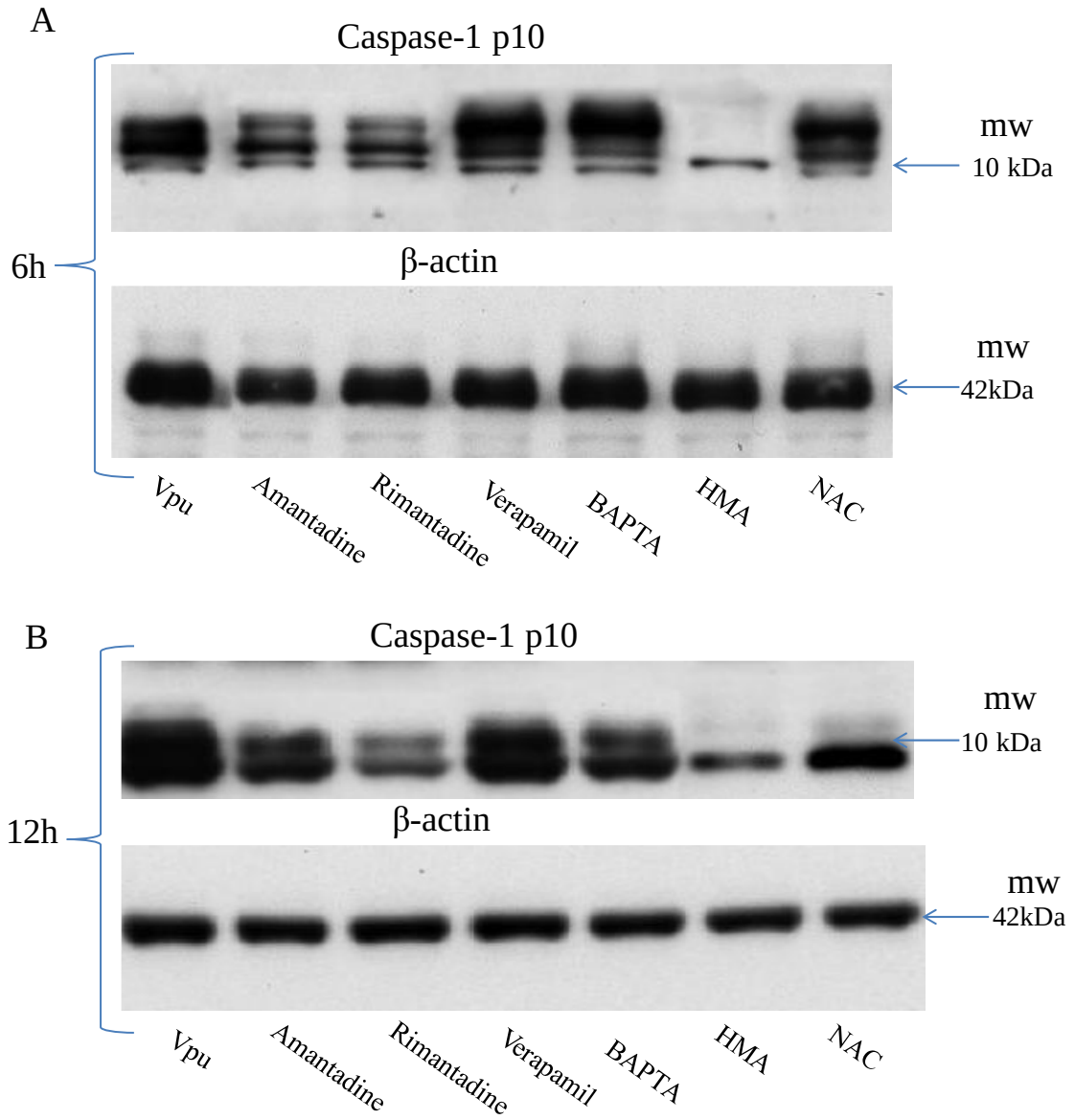
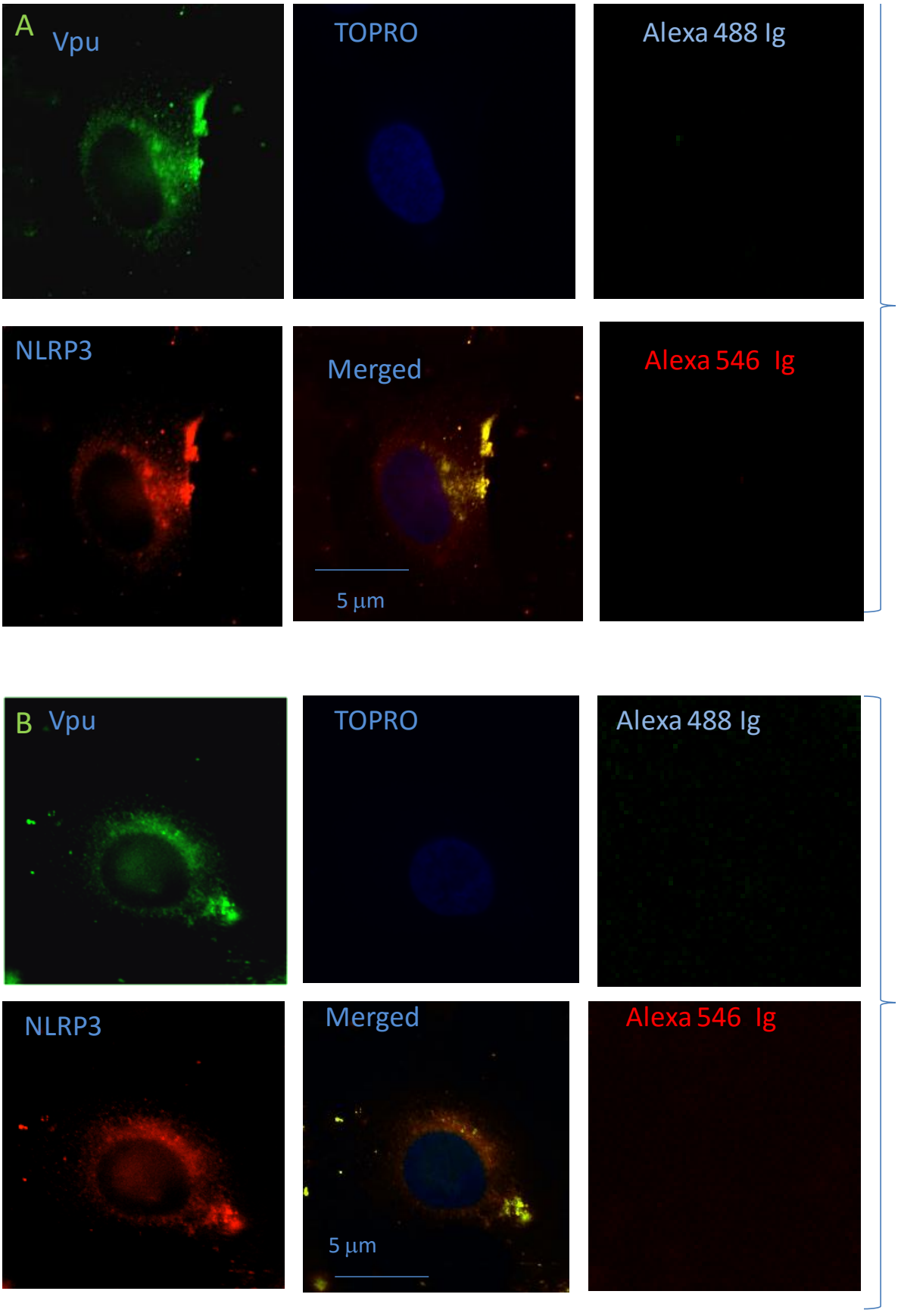


Figure 75. ***Effects of inhibitors on Vpu-induced inflammasome activation.*** **THP1 monocytes ($1x10^6$) expressing Vpu were incubated over 12 hours with various inhibitors or no inhibitors ('Vpu'). Inhibitor concentrations: Amantadine, 10µl/ml; rimantadine, 6.25µM; verapamil, 50µl/ml; BAPTA-AM, 10µl/ml; NAC, 20mM and HMA, 25mM). Cells extracts were analysed for the presence of caspase-1 p10 by Western blotting at 6 hours (A) and 12 hours (B) together with β-actin controls from the same sample.**

To elucidate the intracellular interactions of NLRP3 and Vpu we looked at Vpu trafficking in the cell using confocal imaging. Vpu colocalisation with NLRP3 (figure 76 split over two pages, the legend follows last image) was observed since both proteins display the same distribution and there is colocalisation (panels A) in the merged image (yellow), which could explain the subsequent inflammatory activation (de Jong et al., 2008). To confirm that their interaction was specific, NLRP1 and Vpu distribution was also examined. The NLRP1 seems to be more perinuclear and does not overlap with Vpu (figure 76 – second page). Thus, there is no colocalisation between NLRP1 and Vpu.

To further confirm that NLRP3 was sensing Vpu upstream of caspase-1 activation, we probed Vpu transfected THP1 monocytes for both NLRP3 as well as ASC, an important adaptor protein for inflammasome activation mediated by this NLR sensor (Agostini et al., 2004). The results show NLRP3 association with ASC during Vpu mediated inflammasome activation (figure 77). Control images of the secondary antibodies used are also shown to confirm the absence of non-specific binding.


A
Vpu
TOPRO
Alexa 488 Ig
NLRP3
Merged
Alexa 546 Ig
5 µm
B
Vpu
TOPRO
Alexa 488 Ig
NLRP3
Merged
Alexa 546 Ig
5 µm

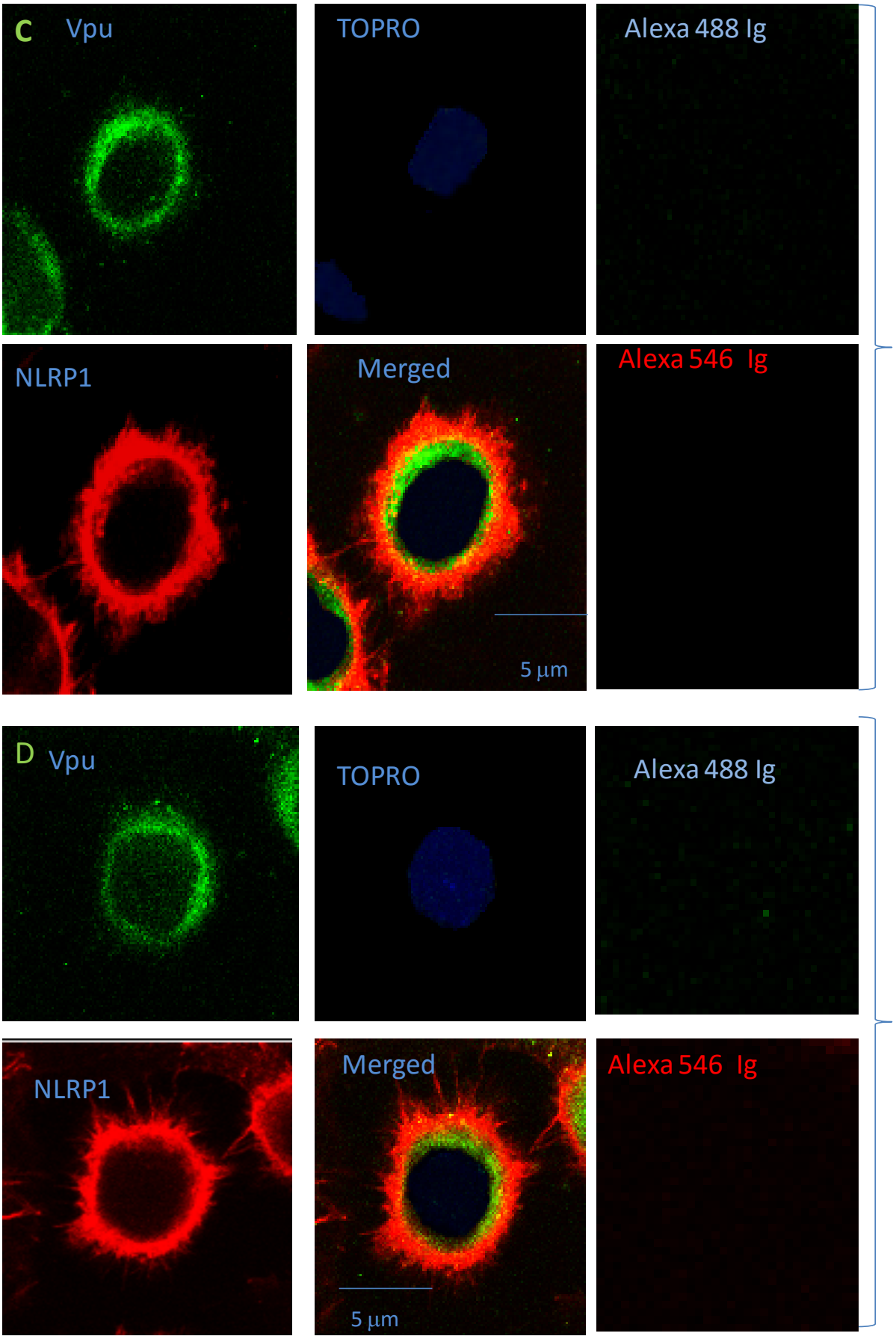

C
Vpu
TOPRO
Alexa 488 Ig
NLRP1
Merged
Alexa 546 Ig
5 µm
D
Vpu
TOPRO
Alexa 488 Ig
NLRP1
Merged
Alexa 546 Ig
5 µm

Figure 76. ***Visualising NLRP3 and Vpu in Vpu-expressing THP1 monocytes.*** **Vpu was stained using an anti-Vpu rabbit antibody followed by a secondary donkey anti-rabbit Ig conjugated to Alexa 488. NLRP3 was labelled using a goat anti NLRP3 antibody followed by donkey anti-goat Ig conjugated to Alexa 546. TOPRO nuclear stain was also used (A, B). NLRP1 was stained using a goat anti NLRP1 antibody and a donkey anti goat Ig conjugated to Alexa 546 (C, D). Images of donkey anti-rabbit Ig -Alexa 488 as well as donkey anti goat Ig-Alexa546 are also displayed. These images are representative images of three independent experiments. In each experimental run 20-25 cells were imaged to confirm results. The images were obtained sequentially (not a simultaneous scan) thus there was instrumental separation of the fluorophore emission to prevent any bleedthrough between channels. Cells were imaged using a Zeiss 510 confocal microscope.**

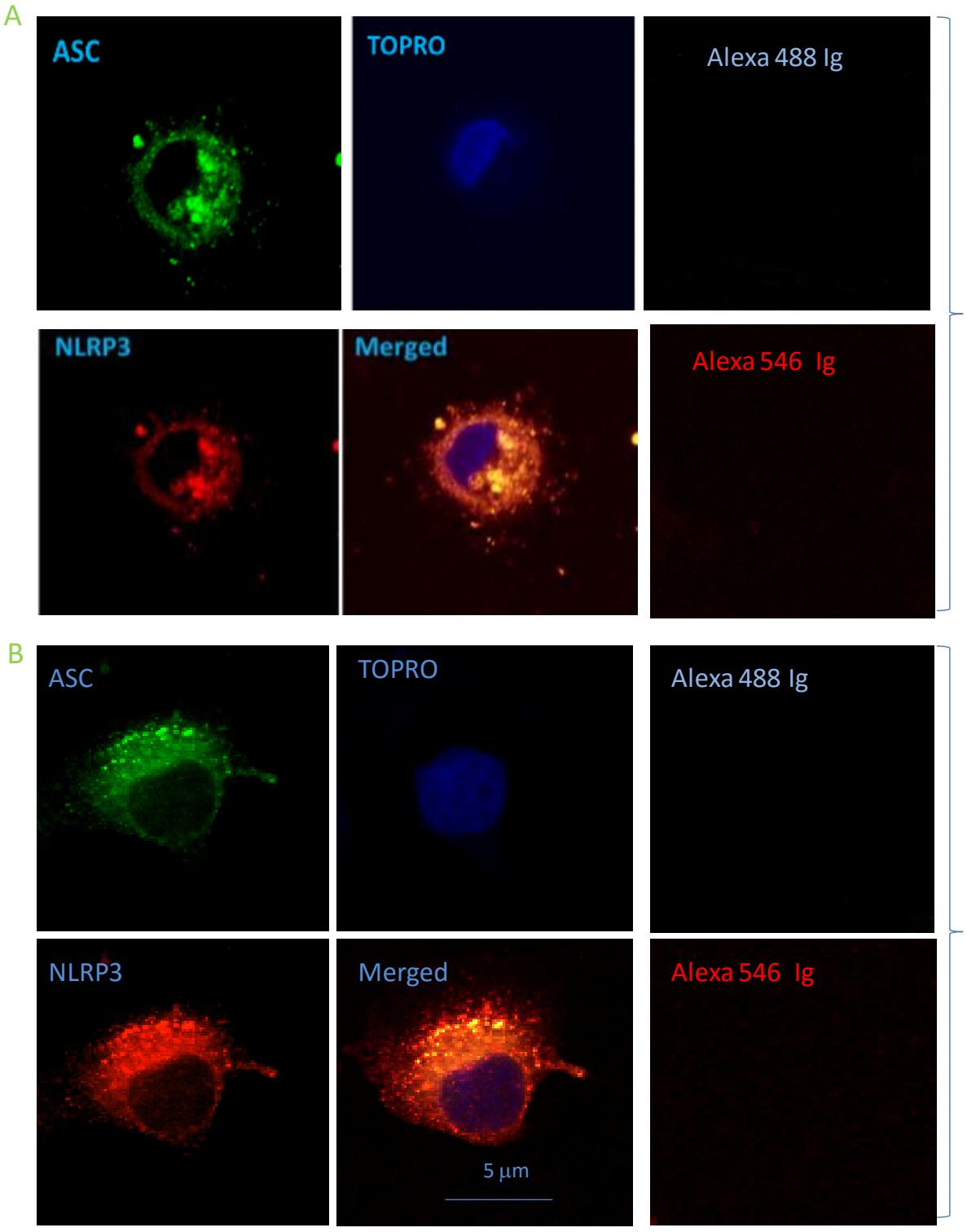


Figure 77. ***Visualising NLRP3 and ASC interactions.*** **Vpu-transfected THP1 monocytes were fixed and probed for ASC and NLRP3 (A, B). ASC was stained using a goat anti ASC antibody followed by a secondary donkey anti-goat conjugated to Alexa 488. NLRP3 was labelled using a rabbit anti NLRP3 antibody followed by donkey anti-rabbit secondary conjugated to Alexa 488. Images of donkey anti-rabbit Ig -Alexa 488as well as donkey anti goat Ig-Alexa546 are also displayed. TOPRO nuclear stain was also used. These images are representative images of**

three independent experiments. In each experimental run, 20-25 cells were imaged to confirm results. The images were obtained sequentially (not a simultaneous scan) thus there was instrumental separation of the fluorophore emission to prevent any bleed through between channels. Cells were imaged using a Zeiss 510 confocal microscope.

2.0 Discussion

We aimed to confirm whether HIV-1 virion activates the inflammasome in monocytes and whether inhibiting putative pathways of inflammasome activation – cation flux, ROS generation and viroporin activity – could reduce or remove caspase-1 activation and IL-1β secretion. This component of the study also aimed to clarify HIV-1 interaction with the NLRP3 inflammasome and asked whether Vpu, like viroporins from other viruses, was able to similarly activate the NLRP3 inflammasome.

Our ability to analyse supernatants from live-virus experiments was limited by the absence of a photospectrometer located in the onsite class III biosafety facility and the related well-justified biosafety regulations preventing the removal of sample supernatants likely containing live-virus from such facilities for analysis in a class II biosafety facility. There exists the potential for limited contribution towards IL-1β production from cognate non-canonical inflammasome activation, a process which was not investigated in this study (Kayagaki et al. 2011; Lupfer et al. 2014) .

HIV-1 induced inflammasome activation was reduced by inhibiting Vpu channel activity with HMA a proton (Na^+/H^+) inhibitor and an additional role for ROS inhibition was identified. The ability of Vpu to independently activate the NLRP3 inflammasome and subsequent IL-1β secretion was also shown, with this process similarly reduced by treatment with HMA. As Vpu is known to exhibit bidirectional monovalent cation transduction (Mehnert et al. 2008; Wang et al. 2011): the study seems to show that NLRP3-mediated caspase-1 recruitment and activation is highly sensitive (Triantafilou and Triantafilou, 2014) to small fluctuations in cation concentration caused by HIV-1 Vpu cation conduction.

Chapter 5: Mechanisms underlying HIV-1 induced IL-18 secretion

1.1 Introduction

The innate immune system, is the first line of defence against pathogens, it responds rapidly to invading microbes through pattern recognition receptors (PRRs) which can distinguish self from non-self, and mount an inflammatory response. Interleukin-1β (IL-1β) is one of the major cytokines that modulate the outcome of viral infection. Its protective role for the host against intracellular pathogens, including viruses, has been well documented (Martinon et al. 2002). IL-18 is triggered by the hosts innate immune response in order to increase the cytolytic potential of NK cells and regulate the innate and adaptive immune responses to pathogens (Nakanishi et al. 2001; Okamura et al. 1998). Induction of IL-1β and IL-18 production seems to be an integral part of the host's innate response to viral pathogens their secretion is tightly controlled by a diverse class of cytosolic complexes known as the inflammasome (Martinon et al. 2002; Martinon & Tschopp 2007).

HIV-1 infection results in a strong activation of innate immunity. Among the innate immune responses triggered by HIV-1 is the inflammasome. There has been observed elevated IL-18 in the serum of adult and paediatric HIV-1 patients (Torre et al. 2000). Evidence has shown that HIV-1 infection engages the NLRP3 inflammasome complex (Pontillo, Silva, et al. 2012; Guo et al. 2014; Hernandez et al. 2014). The mechanisms that regulate IL-18 in acute HIV-1 infection have not been fully elucidated and may be more complicated than previously thought. In this study we have shown that NLRP3 and NLRC4 co-operate and become activated mainly by Cl^- ion disturbances and to a lesser degree by K^+ ion disturbances caused by HIV-1 gp41 which localises in GM-1 enriched lipid raft membranes and triggers inflammasome activation and IL-18 production.

1.2 HIV infection triggers IL-18 secretion

Studies have shown that IL-18 and IL-1β are elevated in the serum of HIV patients, (Iannello A et al. 2010; Ahmad R et al. 2002) since these cytokines are released upon inflammasome activation we proceeded to determine if HIV is detected by the inflammasome, thus we investigated inflammasome activation by measuring IL-1β and IL-18 secretion from primary monocytes incubated with HIV-1. We saw that the HIV-1 incubation resulted in IL-1β and IL-18 secretion (figure 78).

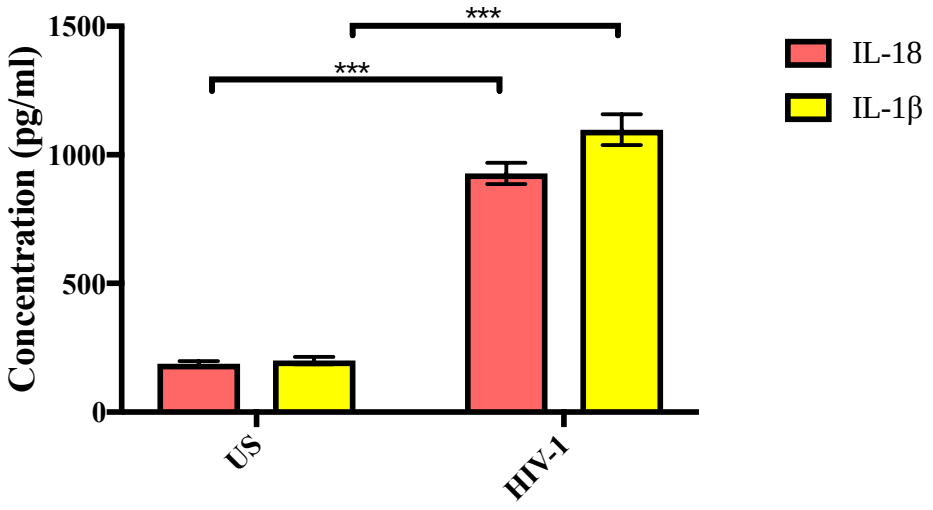
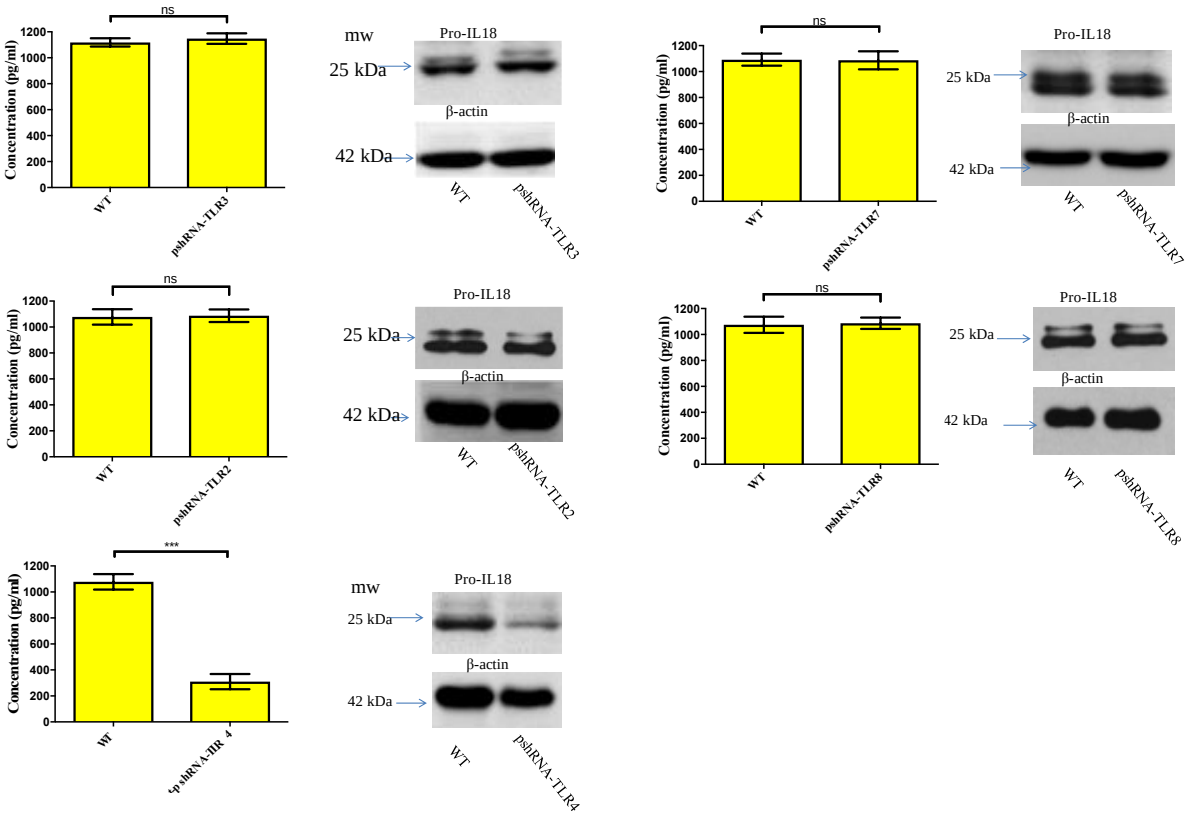


Figure 78. ***HIV activates the inflammasome.*** **Primary monocytes were incubated with HIV-1 or left untreated for 6 hours. Supernatant was collected following incubation and analysed for IL-1β and IL-18 secretion using the human IL-18 and human IL-1β ELISA kits (eBiosciences). SD (IL-18 series): US = 7.93, HIV-1 = 41.63. SD (IL-1β series): US = 10.00, HIV-1 = 59.65.**

Next, we sought to determine the pathway responsible for caspase-1 activation and subsequent IL-18 release. To determine the role TLRs play in inflammasome activation we individually knocked down TLR3, TLR2, TLR4, TLR7 or TLR8 and subsequently incubated them with HIV-1 (figure 79). Knockdown of TLR3, TLR2, TLR8 or TLR7 had no effect in IL-18 production. TLR4 knockdown however resulted in a reduction in pro-IL-18 and IL-18 secretion (figure 79).

To verify if inflammasome activation was NLR specific, NLRP3 expression as well as NLRP1, NLRC4 and NLRC5 expression was reduced using shRNA knockdown (figure 80). Primary monocytes were incubated with HIV-1 (shRNA controls with scrambled sequence were also used). NLRP3 and NLRC4 knockdown resulted in a significant reduction in IL-18 secretion, while NLRP1 and NLRC5 expression did not appear to affect IL-18 production. NLRP3 and NLRC4 knockdown showed a major significant inhibition in IL-18 production (figure 80).



	WT	pshRNA vs. PRR
TLR3	31.93	40.11
TLR2	59.61	48.60
TLR4	59.66	58.60
TLR7	46.39	69.10
TLR8	108.97	76.07

Figure 79. ***HIV-1 incubation with primary monocytes leads to TLR4 dependent pro-IL-1β expression.*** **TLR2, TLR4, TLR7, TLR8 or TLR3 expression by primary monocytes was 'knocked down' by shRNA interference (5μg appropriate plasmid) and the cells were incubated with HIV-1 for 6 hours. Supernatant was collected and analysed for IL-18 using the human IL-18 ELISA kit (eBiosciences). Cells extracts were analysed for the presence of pro-IL-18 by Western Blotting. The data represent the mean of three independent experiments. Standard deviations for each silencing experiment are depicted in the table above.**

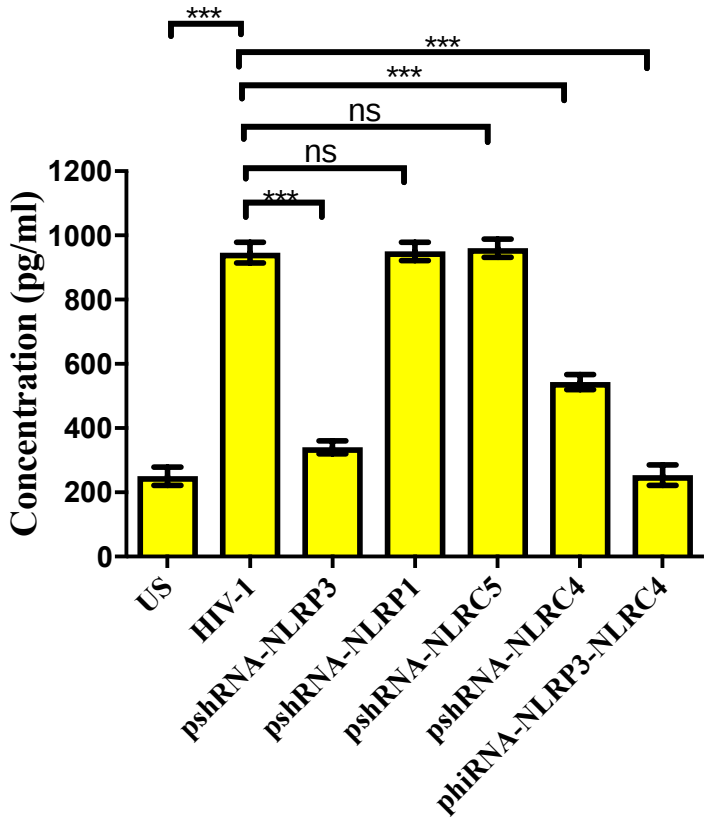


Figure 80. ***NLRP3 and NLRC4 are involved in IL-18 secretion.*** **NLRP3, NLRP1, NLRC4, or NLRC5 expression on primary monocytes was knocked down by pshRNA (5μg of the appropriate plasmid) and the cells were incubated with HIV-1 and examined for IL-18 secretion. SD: US = 50.00, HIV-1 = 55.08, pshRNA-NLRP3 = 36.06, pshRNA-NLRP1 = 49.20, pshRNA-NLRC5 = 50.00, pshRNA-NLRC4 = 40.12, psh-NLRP3-NLRC4 = 55.08. The data represent the mean of three independent experiments.**

In order to identify the main mechanism of IL-1β and IL-18 secretion we looked at HIV viral proteins which enhance membrane permeability since it has been shown that viroporins trigger inflammasome activation by modulating ion fluxes (Triantafilou & Triantafilou 2014). The IAVA M2 ion channel induces inflammasome activation, by modulating the intracellular K^+ concentration (Ichinohe et al. 2010) while human RSV viroporin SH and HRV 2B protein can activate inflammasomes by changing intracellular ion homeostasis (Triantafilou, Kar, Vakakis, et al. 2013; Triantafilou, Kar, van Kuppeveld, et al. 2013).

HIV-1 Vpu which is an oligomeric, type I transmembrane phosphoprotein is a viroporin. Vpu forms ion channels in the cell membranes and we have seen that they trigger inflammasome

activation. HIV-1 gp41, which contains the transmembrane anchor domain for anchoring the envelope protein of the virion into membranes, as well as the fusion domain which is responsible for entry into cells has also been classified as a viroporin (Costin et al. 2007).

Therefore, primary monocytes were transfected with a plasmid expressing HIV-1 Vpu (pcDNA-Vphu) or expression plasmids for HIV-1 gp41 (pgp41-myc-DKK) and inflammasome activation was determined by using HEK blue IL-1β and HEK blue IL-18 reporter cells as well as detecting the presence of caspase-1 p10 via Western Blotting (figure 81). Our results showed that Vpu expression was sufficient to stimulate inflammasome activation and IL-1β secretion. IL-18 secretion seemed to be dependent on the presence of gp41.

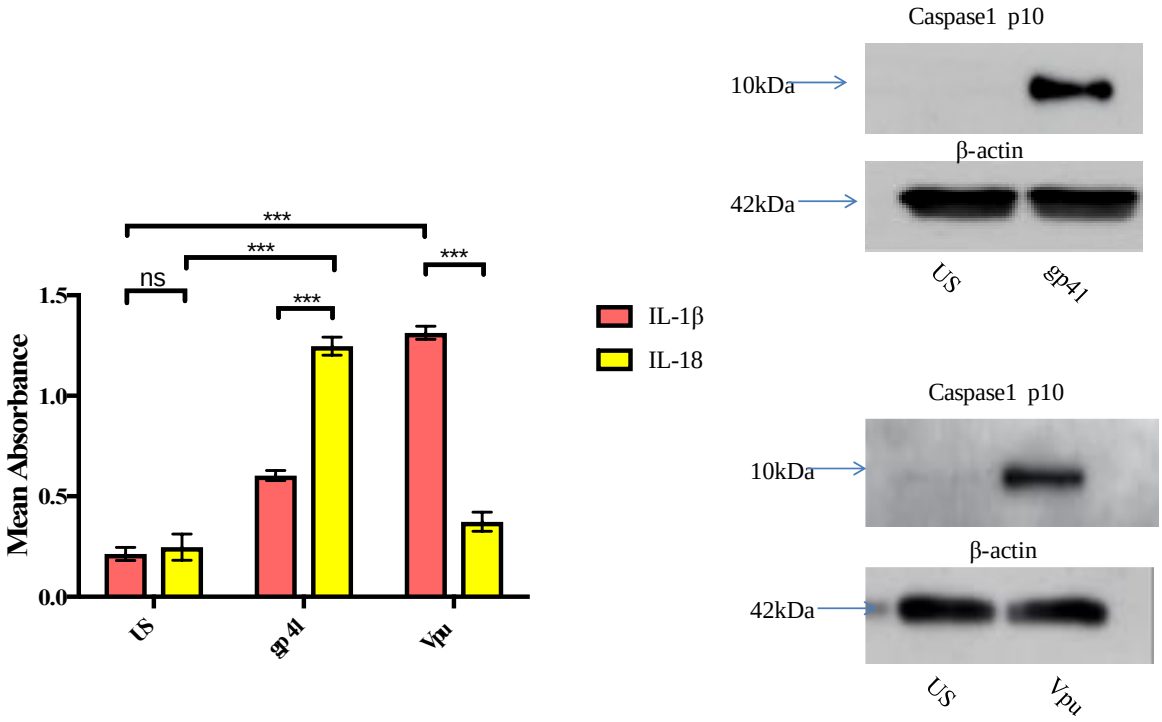
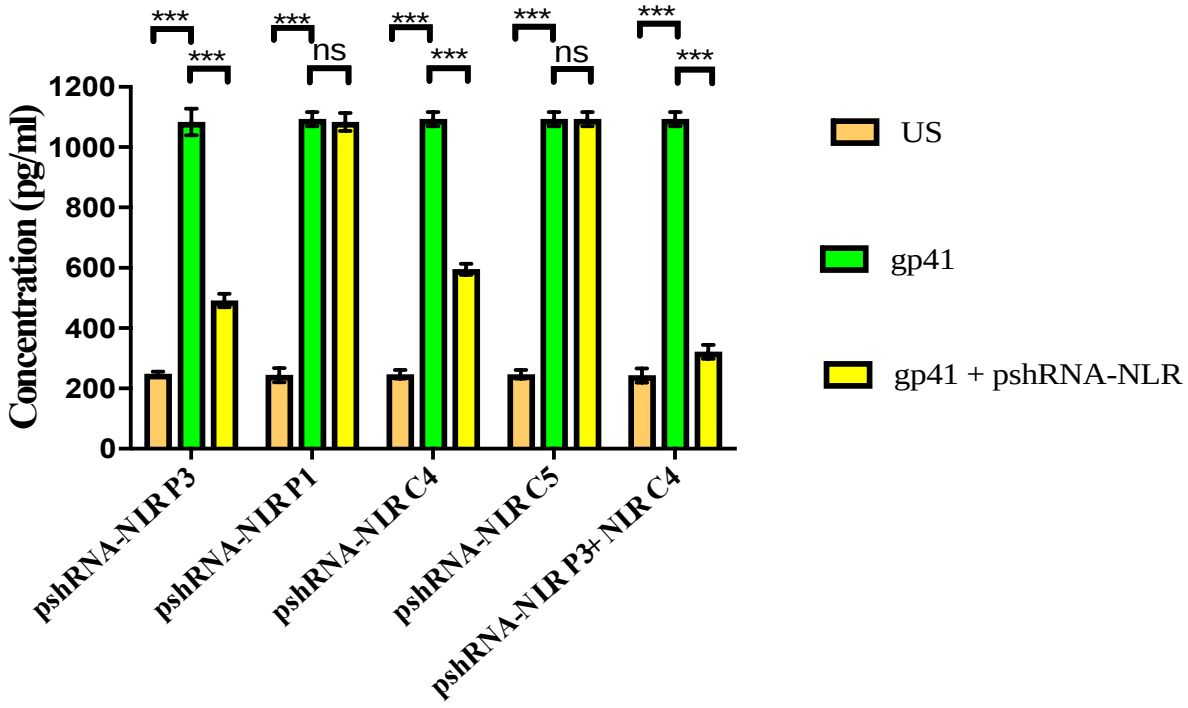


Figure 81. HIV viroporins trigger inflammasome activation. Primary monocytes (1x10^6) were either left untreated or transfected with 5µg of pcDNA-Vphu or 5µg pgp41-myc-DKK plasmid expressing gp41. Supernatants were collected and tested for IL-1β and IL-18 secretion using HEK blue IL-1β or HEK blue IL-18 reporter cells. Cells extracts from mock ('US') or transfected cells were analysed for the presence of caspase-1 p10 by Western Blotting. SD (IL-1β series): US = 0.03, gp41 = 0.03, Vpu = 0.04. SD (IL-18 series) US = 0.07, gp41 = 0.05, Vpu = 0.05. The data represent the mean of three independent experiments.

To verify the significance of gp41 in IL-18 production, knockdown of NLRP3, NLRC4, NLRP1, NLRC5 was achieved using shRNA interference. Primary monocytes were transfected with 5µg pgp41-myc-DKK or the control vector pg-myc-DKK (also 5µg). When IL-18 secretion was analysed the data showed a significant reduction in IL-18 secretion when NLRP3 was reduced by shRNA knockdown in cells expressing gp41 (figure 82). There was no significant change in when NLRC5, or NLRP1 were knocked down by shRNA interference though shRNA knockdown of NLRP3 and NLRC4 showed significant reduction



pshRNA-NLR	US	gp41	gp41 + pshRNA-NLR
NLRP3	12.58	76.38	38.19
NLRP1	41.33	40.42	52.04
NLRC4	25.17	40.43	32.79
NLRC5	25.17	40.16	40.12
NLRP3 + NLRC4	40.42	40.42	40.10

Figure 82. ***NLRP3 and NLRC4 involvement in gp41 recognition.*** **Primary monocytes were transfected with 5µg pgp41-myc-DKK in addition monocytes silenced for NLRP3, NLRP1, NLRC4, NLRC5 or NLRP3 and NLRC4 by shRNA were transfected with pgp41-myc-DKK. Supernatant was collected and analysed for IL-18 using the human IL-18 ELISA kit (eBiosciences). Standard deviations are given in the table.**

of IL-18 in the presence of gp41 (figure 82 above).

1.3 HIV gp41 is transported in lipid rafts

Recent studies have suggested that gp41 assembly occurs within lipid-raft structures on the cell surface and more specifically at the edge of the raft (Yang et al. 2015). It was therefore interesting to determine whether gp41 cellular location plays a role in inflammasome activation. Primary monocytes were incubated with HIV-1 and then stained for HIV gp41, NLRP3, ASC which is the inflammasome adaptor molecule. TLR4 as well as the Golgi apparatus was also stained and we used confocal microscopy to determine where inflammasome assembly takes place. The images showed a localized distribution to the Golgi that was rich in TLR4 and NLRs as well as ASC. The presence of gp41 was also detected (figure 83).

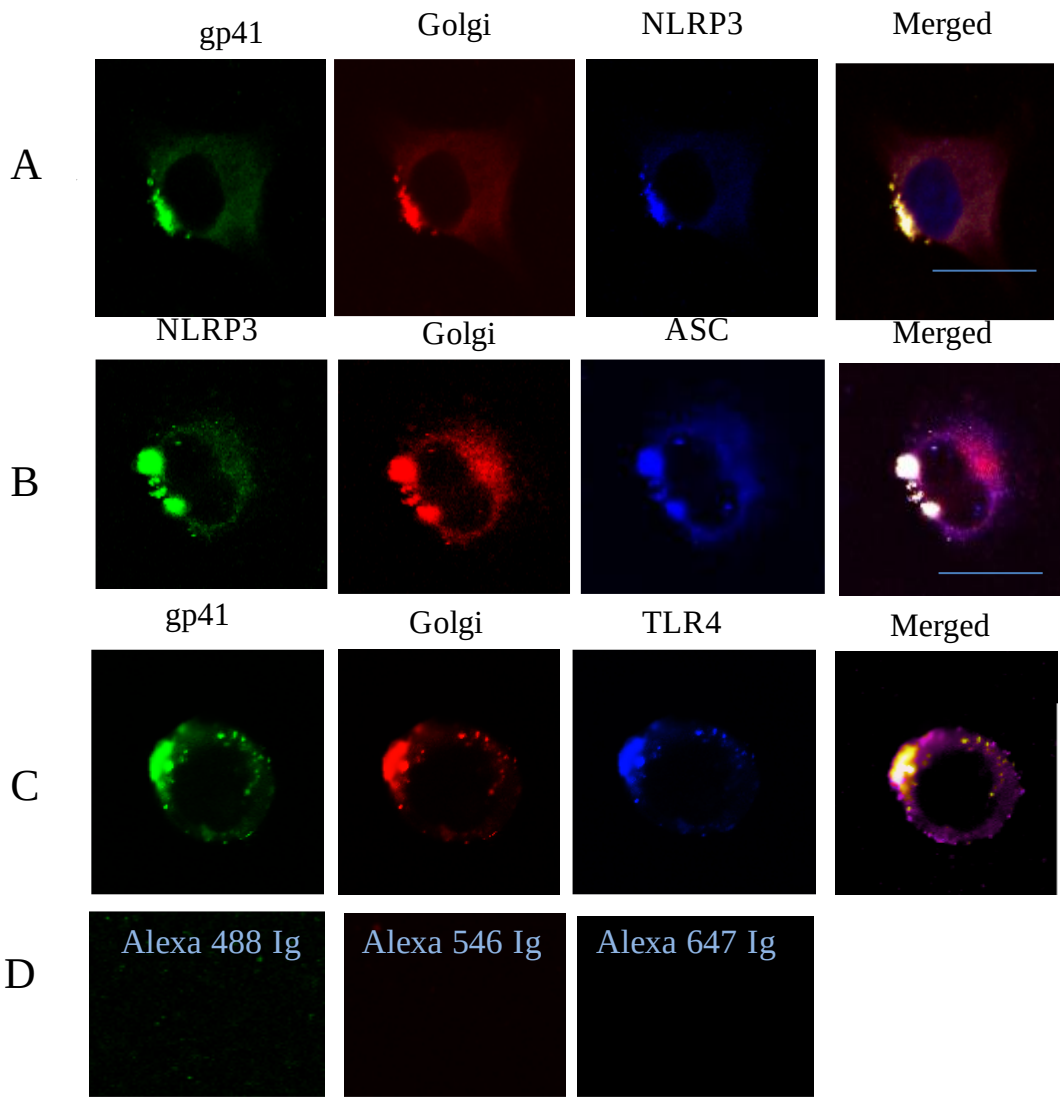


Figure 83. ***NLRP3 assembly in the Golgi.*** **NLRP3 assembly in the Golgi. Primary monocytes incubated with HIV-1 were stained for HIV gp41 (A) an anti-rabbit gp41 antibody conjugated to Alexa 488 was used to detect gp41. Golgi was stained with GM130 mAb followed by Alexa 546-Fab mouse specific Ig to label the Golgi system. Goat anti NLRP3 was used to label NLRP3 followed by either donkey anti goat Alexa 647 or donkey anti goat Alexa 488. ASC (B) was labelled with a goat anti ASC antibody and TLR4 (C) with a goat anti TLR4 followed by donkey anti goat Alexa 633. Control images with only Alexa 633 Ig. Alexa 488 Ig and Alexa 546 Ig are also depicted (D) Cells were imaged using a Zeiss 710 confocal microscope. Bars shown are 5μm. In each experimental run, 20-25 cells were imaged to confirm results. The images were obtained sequentially (not a simultaneous scan) thus there was instrumental separation of the fluorophore emission to prevent any bleedthrough between channels. The data presented representative of at least 20 cells over four independent experiments.**

To support the confocal data we also used Fluorescence resonance energy transfer (FRET). FRET can occur over 1-10 nm distances, and effectively increases the resolution of light microscopy to the molecular level. Recent studies have suggested that gp41 assembly occurs within lipid-raft structures on the cell membranes and more specifically at the edge of the raft. It was therefore interesting to determine whether gp41 concentrates in lipid rafts. Primary monocytes were incubated with HIV-1 and we proceeded to measure FRET between gp41, the lipid rafts and the PRRs of interest. A rabbit gp41 antibody conjugated to FITC was used to label gp41 (FITC-anti gp41) and GM-1 ganglioside, a raft-associated lipid, using Rhodamine-conjugated cholera toxin (CTX-B-TRITC) (from List Laboratories California, USA) on human monocytes (figure 84).

A positive control was used using probes for two different epitopes on the same molecule, in this case TLR4 molecule was used as a positive control. The probes were HTA-125 FITC and C18 TRITC for two different epitopes on TLR4. The maximum FRET signal for the TLR4 molecule was 30±1.3% thus this was our maximum signal. A negative control was also used choosing two molecules that are known not to associate. We have chosen MHC-class-I and TLR4 as a negative control since they do not associate. The W6/32-TRITC probe was used to target MHC-class-I and HTA-125 FITC was used for TLR4. The signal between these molecules was 4±0.7% (figure 84).

gp41 and GM-1 ganglioside were imaged. There was large dequenching observed once the rhodamine was photobleached giving FRET signal of 26±0.9%, suggesting that gp41 concentrates in lipid rafts which contain predominantly GM-1. Since TLR4 seems to play a role in pro-IL-18 secretion we also investigated whether TLR4 was in the raft. Similar results were obtained when FITC-anti TLR4 was used (E=24±0.8%) showing that TLR4 is in the raft containing GM-1 and associated with gp41 (E=17±1.0%). In contrast, TLR4, was not

found to be associated with lipid rafts prior to HIV-1 infection, giving FRET values of 8±0.7 (figure 84).

Figure 84. ***FRET data reflecting HIV-1 induced associations between PRRs and lipid rafts.***

Donor	Acceptor	E%
Anti TLR4 (HTA 125-FITC)	Anti TLR4 (C18-TRITC)	30±1.3% (positive control)!
Anti TLR4 (HTA 125-FITC)	Anti- MHC class I W6/32-TRITC	4±0.7% (negative control)
Unstimulated		
Anti TLR4-FITC	CTX-B-TRITC	8±0.7%!
Anti NLRP3-FITC	CTX-B-TRITC!	6 ±0.5%!
Anti NLRC4-FITC!	CTX-B-TRITC!	7.3 ±0.5%!
Anti ASC-FITC!	CTX-B-TRITC!	5 ±1.2%!
HIV-1 incubation		
Anti TLR4-FITC	CTX-B-TRITC	24±0.8%!
Anti gp41-FITC	CTX-B-TRITC	26±0.9%!
Anti NLRP3-FITC	CTX-B-TRITC!	18 ±1.1%!
Anti NLRC4-FITC!	CTX-B-TRITC!	17 ±0.7%!
Anti ASC-FITC!	CTX-B-TRITC!	20 ±1.2%!
Anti gp41-FITC	Anti TLR4-TRITC	17 ±1.0%!

It has been shown that lipid rafts, which are generally supposed to be a feature of plasma membranes are present in the Golgi membranes and exocytic vesicles(Resh 2004; Surma et al. 2012). Therefore, we also analysed FRET in the Golgi region (figure 84) using both positive (anti TLR4) and negative (anti MHC class 1) controls. Our results showed associations between lipid rafts and NLRP3 (E=18±1.1%) or ASC (E=20± 1.2%) as well as the presence of NLRC4 in lipid rafts (E=17±0.7%) after HIV incubation. FRET levels also showed gp41 and TLR4 in lipid rafts after HIV-1 infection. The data shows that TLR4, NLRP3 and ASC do not reside in lipid rafts but are recruited there after activation.

1.4 Functional significance of lipid raft integrity

In order to investigate the functional significance of lipid raft integrity we evaluated the ability of HIV to trigger inflammasome activation when lipid rafts are disrupted by nystatin, a fungal metabolite that binds membrane cholesterol and disrupts raft integrity; was used. It was shown that IL-18 secretion was inhibited by nystatin (figure 85). Therefore, it seems that gp41 concentrates in lipid rafts forming microclusters and triggers inflammasome assembly.

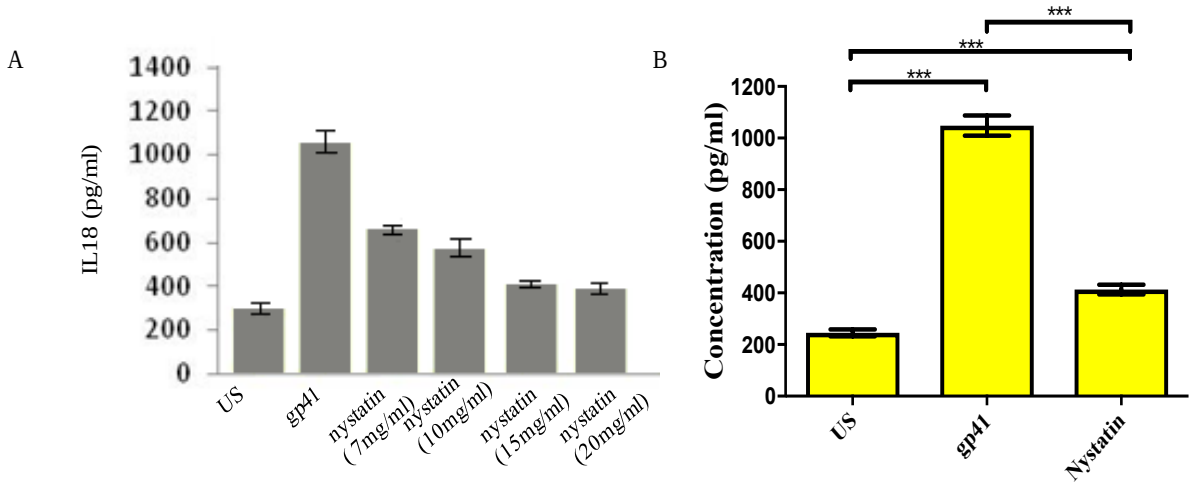


Figure 85. ***Nystatin disrupts IL-18 secretion.*** **A nystatin dose response was measured via IL-18 secretion in primary monocytes expressing gp41. Viability of the cells was determined microscopically using 0.2% Trypan blue (concentrations up to 20µg had no effect on cell viability). Supernatants were collected and tested for IL-18 secretion using ELISA (eBiosciences) from unstimulated or from cells treated with nystatin (7-20µg/ml) (A). A concentration of 15µg/ml of nystatin was used as an optimum concentration (B). SD: US = 22.91, gp41 = 67.52, Nystatin = 32.15 (B). These experiments were repeated three times.**

1.5 Mechanisms of gp41 induced IL-18 secretion

After identifying that gp41 could trigger inflammasome assembly and IL-18 secretion we wanted to identify the mechanism of activation. To test how gp41 triggers inflammasome activation, primary monocytes expressing gp41 were treated with medium containing the appropriate concentration of different drugs (figure 86). Diphenyliodonium (DPI) as well as N-acetylcysteine (NAC) are inhibitors of ROS production. Ion channel inhibitors 5-(*N*-Ethyl-*N*-isopropyl) amiloride (EIPA), which block Na^+/H^+ ion channels, tetraethylammonium (TEA), a broadly acting blocker of potassium (K^+) channels, and verapamil, which blocks Ca^{2+} channels were also used. The results showed little inhibition in IL-18 production with ROS or calcium channel inhibitors, however there was some inhibition when TEA was used showing that K^+ channels had an effect (figure 86).

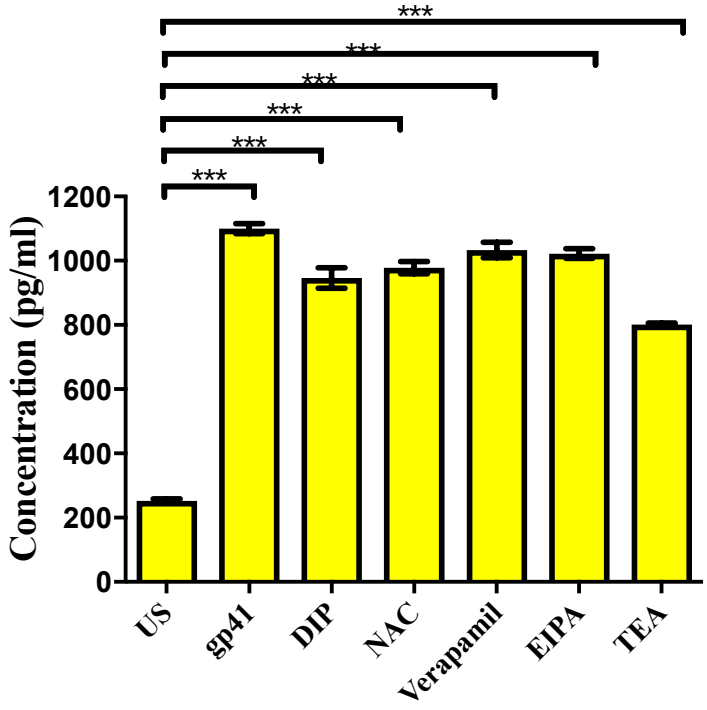


Figure 86. ***Use of ion channel inhibitors in gp41 induced inflammasome activation.*** **Primary monocytes expressing gp41 were treated with and cultured in the presence or absence of EIPA (25µM), verapamil (50µM), TEA (10mM) DPI (20mM), NAC (20mM) for 12 hrs. Supernatants were collected and tested for IL-18 (B) secretion using ELISA. SD: US = 10.79, gp41 = 26.46, DIP = 55.1, NAC = 33.30, Verapamil = 41.64, EIPA = 26.11, TEA = 7.64. The data are the mean of three independent experiments.**

1.6 Cl^- channel inhibition is important for gp41 induced IL-18 secretion

To determine how gp41 triggers IL-18 secretion we looked at Cl^- channels since they have been implicated in the infectious cycle of other viruses (Igloi et al. 2015). To verify the importance of at Cl^- channels, we used well-characterized Cl^- channel blockers, such as 5-nitro-2-3-phenylpropylamino benzoic acid (NPPB) and indyanyloxyacetic acid 94 (IAA-94).The results showed significant inhibition of IL-18 by NPPB and IAA-94 (at these inhibitory concentrations, NPPB and IAA-94 did not affect cell viability) (figure 87). The data showed that gp41 induced IL-18 secretion may be due to enhanced Cl^- influx.

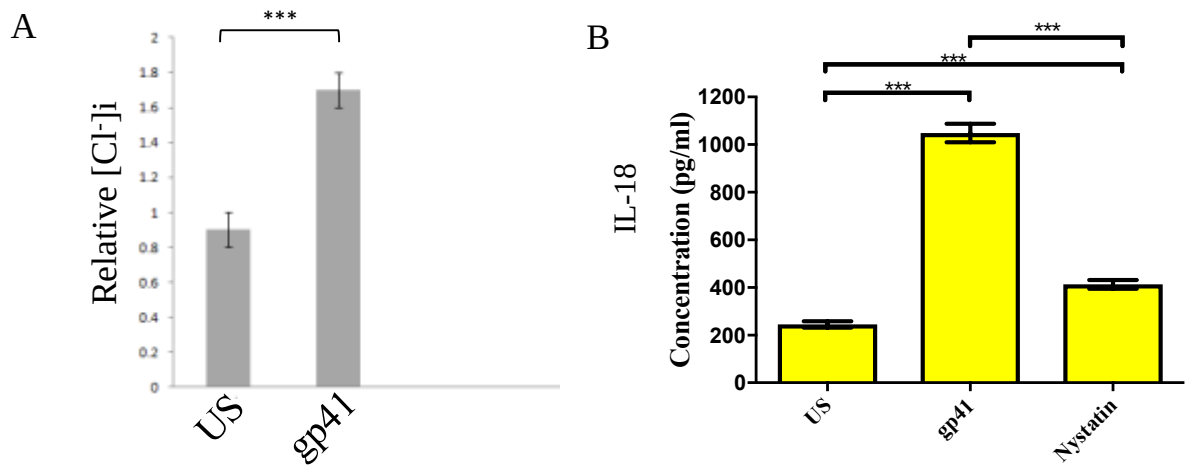


Figure 87. Supernatants were also collected and tested for IL-18 secretion from untransfected (US = WT) primary monocytes or from primary monocytes transfected with 5µg gp41 plasmid, they were loaded with 5mM MQAE in Dulbecco modified Eagle medium (DMEM) for 1 h at 37°C. Following incubation, cells were washed three times with DMEM and Mean fluorescence per cell was determined by flow cytometry using a BD FACSCalibur from a minimum of three independent experiments performed in triplicate (A). Relative [Cl^-]i SD: US = 0.20, gp41 = 0.96. Primary monocytes either untreated or transfected with gp41 plasmid were treated with Cl^- channel blockers NPPB (10µM MNPPB) and IAA-94 (100µM MNPPB). Supernatants were collected and tested for IL-18 (B) secretion using ELISA. (B) SD for IL-18: US = 10.79, gp41 = 5.13, NPPB = 15.28, IAA94 = 15.18. The data are the mean of three independent experiments.

2.0 Discussion

HIV infection causes significant morbidity and mortality worldwide. 35 million people were living with HIV globally at the end of 2013. Although the risk of AIDS has been reduced with antiretroviral therapy a growing body of evidence implicates chronic HIV-1 inflammation and immune activation in the development of non-AIDS conditions such as cardiovascular, respiratory and neurologic diseases which lead to increased mortality in HIV-1 positive patients (Appay & Sauce 2008; Miedema et al. 2013).

The innate immune response of the host to this virus activates several important inflammatory pathways which include inflammasome activation. Inflammasomes are cytosolic multimeric protein complexes that act as surveillance systems which when triggered fight infection and eliminate pathogens (Martinon et al. 2002; Martinon & Tschopp 2007). In this study we investigated the mechanisms of inflammasome activation in HIV-1 infection. Our data show that IL-18 is triggered by the gp41 viral protein. Our data also shows that TLR4 provides the first priming signal for IL-18 production, while HIV-1 gp41 viroporin accumulates in lipid rafts and provides the second signal by influencing Cl^- channel activity in lipid rafts.

Chapter 6: Discussion

In healthy humans, the innate and adaptive immune systems work to deliver a formidable defence against infection. When an effective, specific immune response against various invading pathogens is required the effector cells of what is classically defined as the adaptive immune system must receive confirmatory cytokine signals from CD4 T-helper cells. HIV-1 infection causes disease primarily by infecting and destroying CD4 T-helper cells as well as other critical immune cells such as macrophages. Chronic CD4 T-helper cell depletion gives rise to an overwhelmed immune system unable to control pathogens, even those it would have normally kept in check at acceptable commensal populations. The progression of HIV-1 infection and consequent risk of developing opportunistic infections can be impeded by total adherence to HAART. Such a combination of antiretroviral drugs suspends different specific stages of the HIV-1 lifecycle in cells where HIV-1 is actively replicating. As a consequence of the latency of HIV-1 of infection, HAART cannot as yet completely remove the HIV-1 genome from every single infected cell in the human body nor is it designed to overcome the immune dysregulation seen even in treated HIV-1 infection.

IL-1β and IL-18 are critical pro-inflammatory cytokines generated by the caspase-1 inflammasome following sensing of PAMPs or DAMPs by germline encoded PRRs such as NLRP3 or NLRC4.(Vanaja et al. 2015) IL-1β and IL-18 secretion in addition to inflammasome activation was shown to be dysregulated in HIV-1 infection in numerous studies (Yamato K et al. 1990; Arditi et al. 1991; Corasaniti MT et al. 2001; Pontillo et al. 2012). We therefore aimed to clarify mechanisms of HIV-1 induced immune dysregulation by investigating the interactions between HIV-1 and the innate immune system as the first responder to any invading pathogen, focusing on the mechanisms underlying HIV-1 induced IL-1β and IL-18 secretion.

The first stage of the HIV-1 lifecycle involves fusion of the virion with the host cell membrane which is mediated by HIV-1 *Env* proteins gp120 and gp41. HIV-1 gp120 interacts with target cell surface proteins CD4 as well as CCR5 or CXCR4, effectively hijacking receptors otherwise used beneficially by the host. These actions trigger a conformational change in the *Env* fusion apparatus exposing gp41 to interact directly with the membrane releasing the HIV-1 genome and accessory proteins into the cytosol. Previous work shows that HIV-1 gp120 is recognised by the innate immune system leading to the release of IL-1β (Corasaniti MT et al. 2001) though the role of gp120 and gp41 in inflammasome activation during HIV-1 infection has not as yet been characterised.

Innate immunity to virus infection involves recognition of pathogen-associated molecular patterns (PAMPs), or danger associated molecular patterns (DAMPs), by pattern recognition receptors (PRRs). During virus infection, viral DNA and RNA are detected by a myriad of PRRs whose activation elicits antiviral responses and inflammation (Takeuchi O & Akira S 2009). To mediate the innate immune response, binding of RNA or DNA to PRRs results in activation of NFκB, IRFs, AP-1 and other transcription factors followed by pro-inflammatory gene expression. These include type I and type II interferons, interleukin (IL)-6, and TNF-α that immediately affect an antiviral state and initiate inflammation.

In addition, pro-IL-1β and pro-IL-18 are also induced by PRR activation (‘signal 1’) but are inactive. Pro-IL-1β and pro-IL-18 are processed into their functional forms and released from cells through multi-protein complexes known as inflammasomes (Latz E 2010; Franchi et al. 2012). Inflammasomes come in many forms and are mainly activated by nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs). Following recognition of PAMPs or DAMPs the active NLR oligomerises and initiates formation of the inflammasome complex that usually consists of the adaptor apoptosis-associated speck-like protein

containing a CARD (ASC) and the CARD domain containing pro-caspase-1. Inflammasome complex assembly results in caspase-1 activation and cleavage of pro-IL-1β and pro-IL-18, leading to their activation and release from cells (Martinon et al. 2002).

In this study, we investigated the mechanisms of inflammasome activation in HIV-1 infection. We investigated whether HIV-1 induces an inflammatory cytokine secretion and tried to identify the host mechanism that triggers this response. Using monocytic (THP1) and CD4 T cell lines (MOLT4) we stimulated cells with the envelope glycoprotein gp120 to determine whether it is recognized by PRRs. Our results showed that gp120 was a PAMP for the host's PRRs and could trigger IL-6 as well as IL-1β secretion. The data showed a downregulation of TLR2 and TLR4 during the first hours of stimulation followed by recovery and upregulation towards the end of the time course. A similar pattern of expression was observed for NLRP1, NLRP3 and NLRP12. The data overall appeared to show a two-phase response to gp120 stimulation of THP1 monocytes.

In the MOLT4 cells there was a marked upregulation in the early segment of the time course, then a downregulation in expression followed by a second marked spike in expression at six hours of incubation for TLR4, NLRP1 and NLRP3 showing a biphasic response over time. Overall this could mean that gp120 induces a biphasic response in T cells and monocytes. Thus, an early and late phase of PRR activation was detected. This biphasic innate immune response triggers an initial inflammatory response by the host's PRR but then allow tissues to remain quiescent under low viral burdens while responding specifically and strongly after time to damage-inducing stimuli such as ROS production or ion channel disturbances induced by the viral proteins.

Concerning inflammasome activation in response to gp120 our data showed that TLR2 and TLR4 sensing of gp120 is required for inflammasome activation, which is consistent with recent work investigating female reproductive tract innate sensing of gp120 where it was found that both TLRs sense this PAMP and trigger an immune response (Nazli et al. 2013). In agreement with the gp120 experiments, it was found that HIV-1 virion sensing by TLR4 is required to provide signal 1, though in contrast TLR2 was not required. The disparity could be explained by the notion that large amounts of gp120 quickly engage and saturate TLR4, whereas TLR2 is contributing to a synergistic effect by assisting in gp120 recognition and contributing to a full IL-1β response.

To confirm TLR4 and TLR2 involvement in inflammasome activation, we knocked down expression of TLR4, TLR2 and NLRP3 using shRNA and looked for the presence of caspase-1 p10 and IL-1β secretion in order to determine TLR4, TLR2 and NLRP3 significance in inflammasome activation TLR7 was also knocked down as a control since it recognizes viral ssRNA thereore it is not involved in gp120 innate recognition and the absence of TLR7 should not affect inflammasome activation. The results showed that TLR2 and TLR4 knockdown reduced the presence of caspase-1. In addition, NLRP3 knockdown completely inhibits caspase-1 and inflammasome activation, thus confirming that downstream of the TLRs, NLRP3 is the main NLR that contributes to inflammasome assembly and IL-1β release, possibly by detecting the conformational changes of gp120 when it binds to the cell membrane as a danger signal and assembling the inflammasome into action.

The involvement of other HIV-1 proteins in the host's innate immune response was also investigated. Our data showed that HIV-1 could trigger IL-1β and IL18 secretion and that gp120 was recognized as a PAMP by TLR4, TLR2 and NLRP3, however we wanted to investigate whether other HIV-1 proteins contribute to the innate immune recognition. Studies have shown that viroporins from RNA viruses can trigger inflammatory responses (Ichinohe et al. 2009; Triantafilou et al. 2013) .

Viroporins are small hydrophobic viral proteins that oligomerise to form aqueous pores on cellular membranes. HIV-1 Vpu which is an oligomeric, type I transmembrane phosphoprotein is a viroporin. Vpu forms ion channels in cell membranes (Schwartz et al. 1990; Schubert U et al. 1996). HIV-1 gp41, has also been classified as a viroporin which contains the transmembrane anchor domain for anchoring the envelope protein of the virion into membranes, as well as the fusion domain which is responsible for entry into cells has also been classified as a viroporin (Costin et al. 2007). Therefore we used these proteins for further studies to determine whether viroporins are involved in inflammasome activation.

Our data showed that IL-18 and IL-1β are activated by different viral components. HIV-1 gp41 could trigger IL-18 release. TLR4 was providing the first priming signal for IL-18 production as HIV-1 gp41 viroporin accumulated in lipid rafts and provided the second signal by influencing Cl^- channel activity in lipid rafts. By silencing different NLRs it was found that HIV-1 gp41 could activate both NLRP3 and NLRC4 which lead to caspase-1 activation and IL-18 production. To discover the mechanism of inflammasome activation by gp41 we looked at ion inhibitors which block viral ion channels as well as different ion channel inhibitors. The results showed that gp41 is selective for Cl^- ions.

Yang and colleagues (Yang et al. 2010) have suggested that gp41 assembly occurs within lipid-raft structures. Thus we decided to use FRET in order to determine whether gp41 proteins localises within lipid rafts. Our FRET studies confirmed that gp41 was indeed localised intracellularly in lipid rafts in the Golgi compartment membranes. Interestingly, NLRP3 and NLRC4 was also shown to associate with the lipid raft marker GM1, upon HIV-1 infection.

Lipid rafts are specialized membrane microdomains that are rich in sphingolipids and cholesterol. These rafts provide a favourable environment for intra-molecular cross talk but also aid signal transduction from pattern recognition receptors (Triantafilou & Triantafilou 2002; Triantafilou & Triantafilou 2003). In this study, lipid raft disrupting drugs resulted in re-distribution of NLRP3 and NLRC4 and complete abrogation of caspase-1 and IL-18 secretion. These intracellular raft structures seem to be crucial for the accumulation of ion channels and their modulators. Sensing of cellular stress imposed by imbalances in ionic concentrations in intracellular vesicles could be a general viral recognition pathway that can be utilised by the infected host cell to signal the activation of NLR inflammasomes.

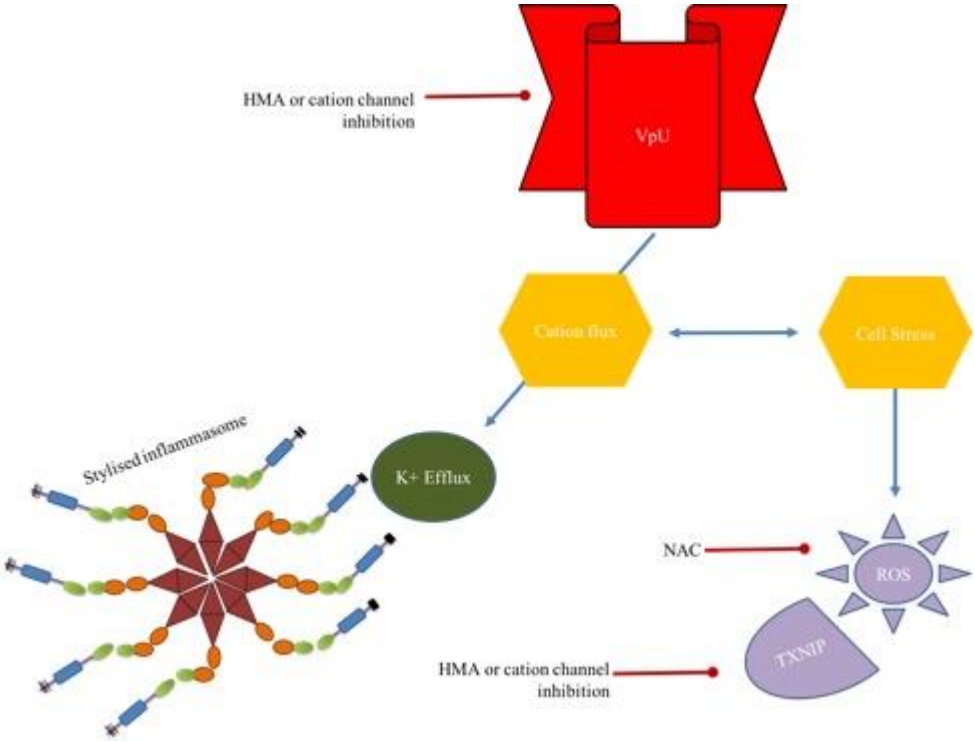


Figure 88. ***Inter-related sources of signal 2 in the mechanism of HIV-1 Vpu mediated inflammasome activation*****. It is currently unknown whether Vpu interacts with K^+ channels directly to provoke inflammasome activation by initiating K^+ efflux or whether the viroporin effects other pathways leading to the generation of ROS or other DAMPs to activate the inflammasome.**

On the other hand, IL-1β secretion seems to be triggered via NLRP3 activation by recognizing HIV gp120 and HIV-1 Vpu. With the use of ion channel inhibitors such as HMA (H^+/Na^+) we concluded that Vpu causes ion efflux possibly via membrane depolarization (figure 54). This Na^+/K^+ flux likely facilitates the maturation and release of IL-1β. It remains to be determined whether Vpu interacts with the channel protein directly causing the channel to open or whether the effect on K^+ activity is indirect, through the generation of other DAMPs such as ROS mediating TXNIP-NLRP3 binding (Zhou et al. 2010) .

The ability of RNA viruses such as influenza virus, rhinovirus, HIV-1 to utilise lipid rafts for assembly, budding and replication (Veit & Thaa 2011; Takeda et al. 2003; Nayak & Barman 2002) has important implications for host defence. Thus, the lipid bilayer is not an inert solvent for membrane proteins as was previously thought. This understanding is now superseded by the idea that membrane lipids play an integral part in the regulation of ion channel function and HIV-1 seems to take advantage of lipid raft structures.

The host has evolved cytosolic danger sensing signalling platforms that can detect perturbations in immune homeostasis and damage associated molecular patterns (DAMPs) caused by viral infection which trigger release of IL-1β and IL-18 and the initiation of pyroptosis in the infected cell. Viroporin activity of viruses can disrupt the balance of intracellular ion concentration. This ion imbalance activates the NLRP3 inflammasome. The use of ion channel protein blockers can affect this activation process. Hence, viroporins represent a novel group of molecules that activate the NLRP3 inflammasome. Specific viroporin blockers or ion chelators could be used as effective antiviral drugs.

Conclusions and future directions

Inflammasome activation is an essential part of the immune response to virus infection and holds the promise of many potential therapeutic drug- or vaccine-based interventions for either the control of virus replication or resolution of excessive inflammation and immunopathology. In the clinic, inhibition of NLRP3 or IL-1β is already used for several or autoimmune diseases, but there has been little clinical research directed at using such treatments during viral infection. The range of viruses detected by NLRP3 has grown immensely in the last few years.

Many therapeutic advances have been achieved in the treatment of HIV-1, the mechanisms underlying the immune system-dysregulating inflammatory response observed during the course of infection however remain understudied. This study contributes to the general understanding of HIV-1 recognition by the human innate immune system through its various intrinsic PAMPs, and generated DAMPs acting upon the NLRP3 and NLRC4 inflammasomes. Our study also illuminates how HIV-1 causes striking dysregulation in cytokine profiles, as reflected in IL-1β and IL-18 levels in people living with HIV-1 at various stages of infection: we found this to occur largely by causing ion flux which is sensed by NLRs, as is summarised in figure 89 overleaf.

It is becoming clear that all viroporins could universally activate the NLR-inflammasome, though further work is particularly indicated to clarify exactly how gp120 interacts with TLR4 (as signal 1) and how this leads, via signal 2, at the molecular level with respect to NLRP3 to bring about inflammasome activation. To overcome the limitations of the *in vitro* and *ex vivo* experiments which we performed in a baseline HIV-1 naïve model, this study therefore represents a sign-post for large *ex vivo* studies in HIV-1 patients perhaps in both antiretroviral naïve and antiretroviral adherent patients to further elucidate the mechanisms

underlying HIV-1 induced innate immune dysregulation accounted for in part, by inflammasome activity.

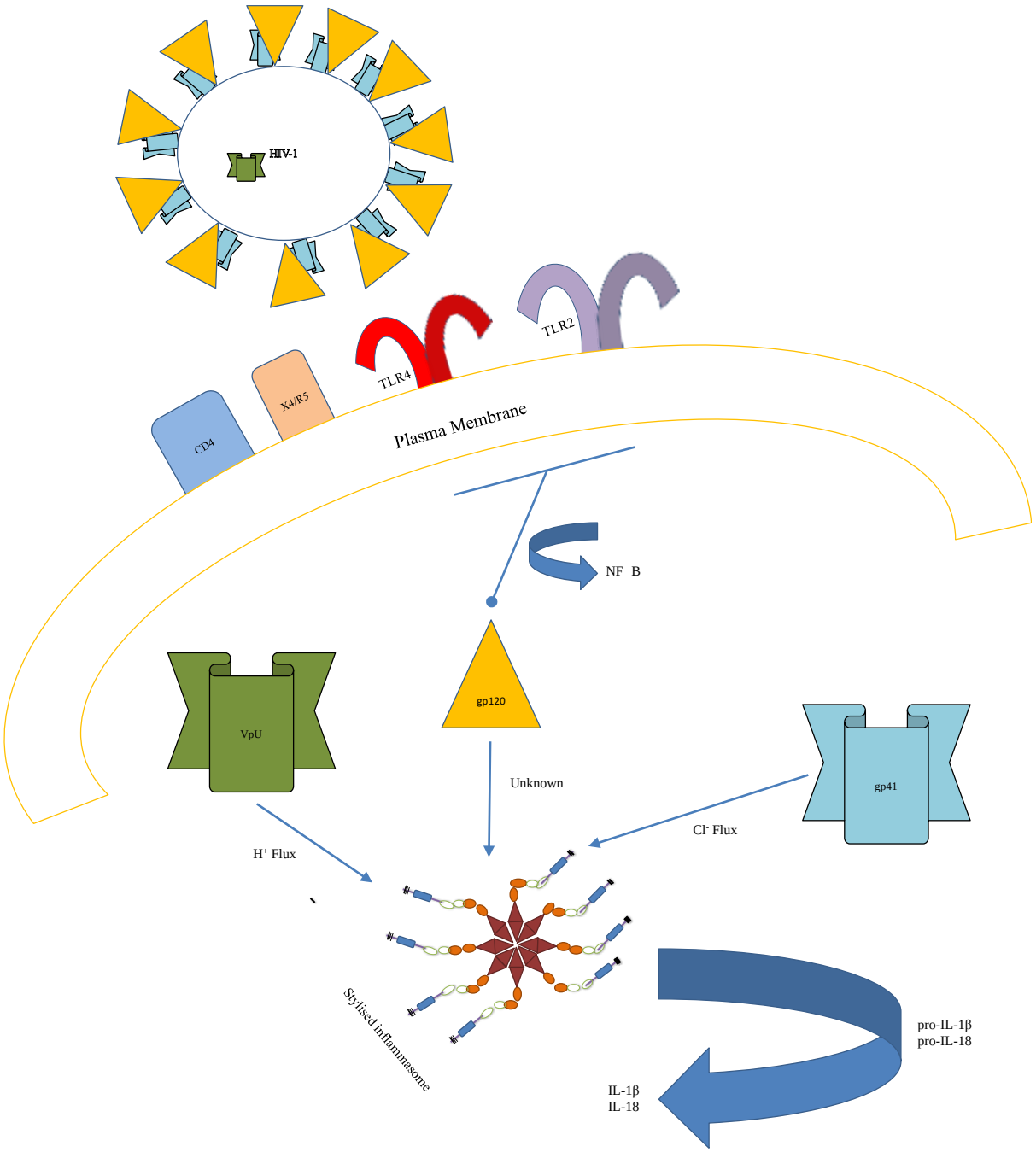


Figure 89. ***Summary of the mechanisms found to be underlying HIV-1 induced inflammasome activation.*** **TLR2 and TLR4 interactions with gp120 appeared to account for the first signal required for inflammasome activation ('signal 1'). Precisely how gp120 triggers the second signal ('signal 2') for inflammasome activation remains unknown and a potential focus for future study. It was however found that Vpu and gp41 triggers signal 2 via ion flux activating either NLRP3 alone (Vpu) is in keeping with conventional understanding of inflammasome activation (Munoz-Planillo et al. 2013); or both NLRP3 and NLRC4 in the case of gp41.**

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