Attenuation of Macrophage IL-10 Responses by HIV-1

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Declaration

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

Signed:

Date:

Abstract

HIV-1 infection of monocyte derived macrophages (MDM) attenuates innate immune IL-10 transcriptional responses, resulting in increased inflammation. I sought to identify the host and virus determinants of this effect to provide novel insights into HIV-associated immune dysfunction and the mechanisms that regulate IL-10 responses. I established a protocol in which THP-1 cells can be differentiated to a macrophage-like phenotype able to generate innate immune IL-10 responses and confirmed that this was attenuated by HIV-1. I found that at least one of the HIV accessory genes *vpr* or *vpu* were necessary for IL-10 attenuation in THP-1s, but not in MDMs.

I focussed my remaining experiments on MDMs in which I also introduced single cell analysis using RNA fluorescence in-situ hybridisation. In this model, neither HIV-1 accessory proteins nor productive HIV-1 infection was necessary for attenuation of IL-10. Instead I found that HIV-1 RNA was necessary and sufficient for this phenotype. TLR8-binding HIV-1 RNA motifs and a synthetic TLR8 ligand recapitulated attenuation of macrophage IL-10 responses, implicating a role for TLR8.

This interaction would be expected to lead to induction of type I interferons (IFN). Consistent with this, type I IFN attenuated IL-10 responses and its effect was reversed by blocking the type I IFN receptor. However, in the same model, HIV-1 did not induce IFN responses and HIV-1 attenuation of IL-10 was not reversed by IFN receptor blockade. In addition, transient exposure to HIV-1 achieved sustained attenuation of macrophage IL-10 responses.

My data support a model in which incoming HIV-1 RNA interacts with TLR8, leading to specific transcriptional regulation of IL-10 independently of IFN induction, most likely via epigenetic mechanisms. These data reveal a novel pathway for adaptation of innate immune responses and a potential mechanism for immune activation in HIV-1 infection due to deficient IL-10 immunoregulation.

Impact Statement

In this thesis I have investigated a novel host-pathogen interaction which has the potential to influence the immunopathogenesis of HIV-1. This uncovered that HIV-1 RNA may activate endosomal sensing via TLR8 to prime cells towards an inflammatory phenotype without triggering the secretion of type I interferons. This effect persisted in the absence of productive infection, indicating that HIV has the potential to influence innate immune responses even in cells which actively restrict virus. The resulting immune dysregulation is likely to have broader consequences for responses to coinfecting pathogens.

This could have implications for the clinical management of HIV patients. It is possible that this host-pathogen interaction contributes to chronic immune activation that in turn may contribute to progressive immunodeficiency and increased risk of cardiovascular or neoplastic disease. Likewise, the effects of HIV conditioning on macrophages may exacerbate acute immune reconstitution inflammatory syndromes in patients receiving antiretroviral treatment.

This research also has the potential to broaden our understanding of the regulation of interleukin 10. My results suggest that epigenetic modification may be responsible for generating long-lasting effects on secretion of this cytokine after exposure to HIV-1. While the mechanism is yet to be determined, the specificity of this effect to IL-10 is potentially of broader interest for our understanding of innate immune adaptation. The model of macrophage innate immune responses which has been established may also be applicable to the study of how other pathogens impact the function of these cells.

In addition to this, the RNA FISH assay and analysis pipeline which I have developed has provided the ability to analyse expression of cytokines which are not compatible with traditional immunofluorescence staining. The ability to quantify expression of multiple targets at the single cell level can also provide greater sensitivity and resolution compared to analysis of bulk cultures. Combined with high throughput imaging strategies this technology permits the study of events in rare cell populations while offering the ability to investigate features which cannot be assessed using flow cytometry, such as subcellular localisation of target proteins. The ability to detect and visualise RNA from single HIV-1 virions also has potential applications for the study of post-entry events in the viral life cycle.

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Table of Contents

Table of Figures

List of Tables

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-presenting cell
ARE	AU-rich element
ART	Antiretroviral therapy
cGAMP	Cyclic GAMP
cGAS	Cyclic GMP-AMP Synthase lic
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
EFV	Efavirenz
Env	Envelope
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HRP	Horseradish peroxidase
HS	Human serum
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFNGR	Interferon gamma receptor
IL	Interleukin
IN	Integrase
IRF	Interferon response factor
IRIS	Immune reconstitution inflammatory syndrome
ISG	Interferon-stimulated gene
IκB	Inhibitor of κB
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinase

MAVS	Mitochondrial antiviral signalling
M-CSF	Macrophage colony-stimulating factor
MDDC	Monocyte-derived dendritic cell
MDM	Monocyte-derived macrophage
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
mTOR	Mammalian target of rapamycin
Nef	Negative regulatory factor
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK Cell	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide oligomerization domain
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIC	Pre-integration complex
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
qPCR	Quantitative PCR
Rev	Regulator of Expression of Virion proteins
RLR	RIG-I-like receptor
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIV	Simian Immunodeficiency Virus
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
Tat	Transactivator of transcription

TB	Tuberculosis
TBE	Tris/borate/EDTA
Th	T helper
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumour necrosis factor
Treg	T regulatory
UTR	Untranslated region
UV	Ultraviolet
Vif	Viral infectivity factor
VLP	Virus-like particle
Vpr	Viral protein R
Vpu	Viral protein unique
Vpx	Viral protein X
VSV-G	Vesicular stomatitis Indiana virus G protein
WT	Wild type

1. Introduction

1.1. Innate and Adaptive Immunity

The immune system is responsible for generating a physiological reaction to perceived dangers, such as microbial pathogens or tissue injury. The immune response is composed of mechanisms which recognise a threat, induce antimicrobial defence mechanisms and activate tissue repair. Innate and adaptive immune responses are distinguished by their mechanisms for detecting danger [1].

Innate immunity represents responses initiated by germline-encoded host receptors. These include circulating proteins such as complement and coagulation factors, and diverse cell-associated proteins [2]. There are three groups of canonical cell-associated innate immune receptors: Cell membrane proteins, which sense the extracellular environment primarily for bacterial or fungal pathogen-associated molecular patterns (PAMPs), and exemplified by toll-like receptors (TLR) 2, 4, 5 and 6; transmembrane TLRs (7, 8 and 9) that sense nucleic acid PAMPs within endosomes; and cytosolic innate immune receptors that detect excessive, ectopic, or pathogen-associated nucleic acid motifs in the cytosol, as well as a diverse array other damage-associated molecular patterns (DAMPs) [3]. The membrane-associated innate immune receptors are primarily expressed by selected cell populations that have been identified as immune cells. In contrast, cytosolic receptors for nucleic acids are expressed ubiquitously to assist in detection of intracellular pathogens [4].

In contrast to innate immunity, adaptive immunity represents responses initiated by either the B cell or T cell receptors which generate a huge range of ligand binding specificities by genetic recombination in somatic cells [5]. Adaptive immunity evolved after innate immunity in evolutionary terms and is thought to be partly dependent on innate immunity [6]. The best example of this is the discovery that T cell responses are dependent on accessory cells, needed to ‘process’ and frame molecules in major histocompatibility complex (MHC) class 1 or class 2 proteins for recognition by CD4 and CD8 T cells respectively [7]. In addition, the innate immune activation of phagocytic antigen presenting cells (APCs) leads to upregulation of additional co-stimulatory signals which augment T cell activation [8]. Adaptive immunity allows for the generation of highly specific responses to individual pathogens to assist in control and clearance as the need arises. When activated by their adaptive immune receptors, T cells and B cells undergo cellular proliferation. A proportion of these cells form long-lived clones responsible for adaptive immune memory [9].

Both innate and adaptive immune responses are subject to a complex myriad of homeostatic regulatory mechanisms. These include the capacity for immune cells to secrete anti-inflammatory cytokines such as interleukin 4 (IL-4) and interleukin 10 (IL-10), which act to limit activation and prevent damage to healthy tissue [10]. The immune response is also controlled through the deletion or functional inactivation of autoreactive T and B cells during the development of adaptive responses, which prevents autoimmunity by ensuring tolerance of most self-antigens [11]. Immunopathology is also minimised by the production of regulatory T cell populations (Treg), which actively suppress induction and proliferation of effector T cells to prevent self-reactivity [12]. These mechanisms help to balance antimicrobial activity against immunopathology, allowing for the clearance of microbes without extensive damage to the host.

1.2. Macrophages

Macrophages are tissue resident cells that are named for their extensive capacity for phagocytosis [13]. Macrophages have diverse roles. They are involved in clearance of cellular debris following necrosis or apoptosis and tissue remodelling during organogenesis and repair. They express a diverse range of innate immune receptors and can initiate innate immune responses, primarily by secretion of pro-inflammatory molecules. In addition, macrophages have the capacity for intracellular killing of micro-organisms and function as antigen-presenting cells to initiate adaptive responses. [14]. Macrophages are also involved in the regulation and resolution of inflammatory responses [15]. In humans dysregulation of macrophage responses has been associated with the development of autoimmunity [16] and cancer [17], [18], highlighting their importance in homeostasis.

1.2.1. Macrophage Ontogeny

Macrophages, dendritic cells and monocytes all belong to the mononuclear phagocyte system. In adults, these cell types can be derived from the macrophage dendritic cell precursor (MDP) in the bone marrow [19]. Circulating monocytes produced from these precursor cells are recruited into tissues, usually under inflammatory conditions, where they can further differentiate into macrophages. However, during homeostasis monocyte-derived macrophages do not substantially contribute to tissue populations [20]. Most tissue-resident macrophages instead are of embryonic origin and exhibit the capacity for self-renewal [21]. During early foetal development macrophage populations are derived from the yolk sac (**Figure 1.1**) [22]. As development progresses these populations are gradually replaced by cells which differentiate from foetal monocytes, originating from haematopoiesis in the liver [20]. In adults, yolk sac-derived macrophages still comprise most microglia in the CNS and some Langerhans cells, while

most other tissue macrophages are derived from foetal monocytes [23], [24]. Due to their ability to self-renew these tissue-resident cells persist throughout life.

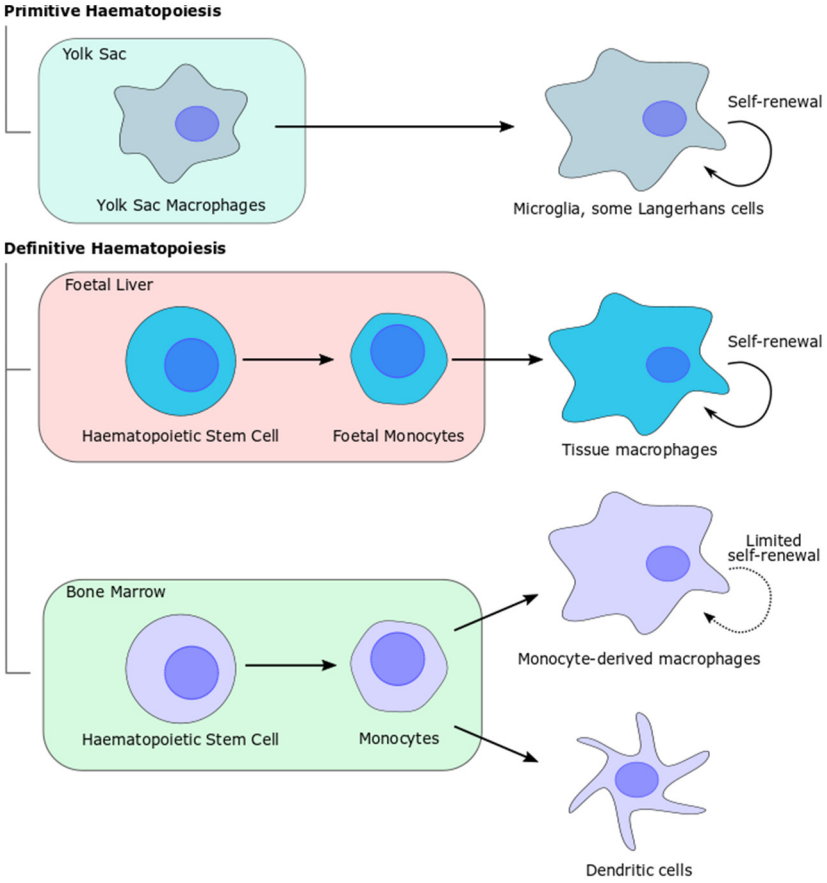

Figure 1.1: Macrophage Ontogeny

Diagram of the origins of different macrophage subsets within the body. Tissue macrophages consist of a self-renewing population of embryological origin. In the early embryo macrophages are derived from the yolk sac in a process called primitive haematopoiesis, while later in development definitive haematopoiesis is established in the liver to produce foetal monocytes. In contrast, monocytes and dendritic cells derive from mature haematopoietic precursor cells in the bone marrow and circulate in the blood. Inflammatory conditions drive recruitment of these circulating cells into tissues and differentiation into macrophages and mature DCs.

1.2.2. Macrophage Subsets

Specialised macrophage subsets have been described in most tissues around the body (**Table 1.1**) [25], [26]. Macrophage function between tissues is particularly variable, with populations such as the alveolar macrophages of the lung performing an immunological role while osteoclasts are responsible for the breakdown, repair and maintenance of bone [25]. In addition to heterogeneity driven by the cellular niche, there is also substantial functional plasticity driven by stimulation with cytokines such as IL-4 and IFN-γ, which can activate these cells with a bias towards wound healing, host defence or immune regulation [27].

Subset	Tissue	Function
Tissue Macrophage	Connective tissue	Immune surveillance
Microglia	Brain	Neuronal maintenance, immune surveillance
Langerhans Cells	Skin	Immune surveillance
Alveolar Macrophage	Lung	Clearance of surfactant, immune surveillance
Kupffer Cell	Liver	Debris clearance
Splenic Macrophage	Spleen	Immune surveillance, erythrocyte clearance
Subcapsular sinusoidal macrophages	Lymph nodes	Antigen presentation
Bone Marrow Macrophage	Bone	Regulate erythropoiesis
Osteoclast	Bone	Bone resorption
Peritoneal Macrophage	Peritoneal cavity	Immune surveillance
Adipose Tissue Macrophages	Adipose tissue	Metabolism
Monocyte-derived macrophages	Inflamed tissues	Inflammatory responses

Table 1.1: Macrophage subsets

1.2.3. Pattern recognition in macrophages

It was hypothesised as early as 1989 that germline-encoded pattern recognition receptors may be the mechanism by which pathogens are detected by the innate immune system [28]. This was proven almost a decade later when Toll-like receptors (TLRs) were described as a mechanism for T cells to discriminate self from non-self-antigens [29], [30]. Additional groups of receptors such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) have subsequently been described. These receptors are activated by the different types of PAMPs outlined in **Table 1.2** [31]–[35].

Family	PRR	Location	Example Ligand
Toll-Like Receptors	TLR2	Cell Surface	Atypical LPS
	TLR3	Endosomes	RNA
	TLR4	Cell Surface	Lipopolysaccharide (LPS)
	TLR7	Endosomes	ssRNA
	TLR8	Endosomes	ssRNA
	TLR9	Endosomes	CpG Elements
C-Type Lectin Receptors	DC-SIGN	Cell Surface	Bacterial Cell Wall
	Dectin-1	Cell Surface	Beta-Glucans
Nod-Like Receptors	NOD2	Cytoplasm	Peptidoglycan
	NLRC2	Cytoplasm	ssRNA
DNA Sensors	cGAS	Cytoplasm	DNA
	IFI16	Cytoplasm	DNA
	DDX41	Cytoplasm	DNA
RIG-I-Like Receptors	RIG-I	Cytoplasm	dsRNA
	MDA5	Cytoplasm	dsRNA

Table 1.2: Key pattern recognition receptors in macrophages

1.2.4. Toll-Like Receptors

Toll-Like Receptors (TLRs) are a class of innate immune sensors which are generally divided into cell surface and intracellular subfamilies. While cell surface TLRs generally detect bacterial and fungal proteins, ligands for endosomal TLRs are typically RNA or DNA molecules. They are expressed on innate immune cells such as macrophages and dendritic cells but are also present on non-immune cells such as epithelial cells [36]. TLRs are single subunit, membrane-spanning proteins which possess an extracellular domain with leucine-rich repeats and a cytoplasmic domain with the conserved Toll/IL-1 receptor (TIR) domain [37]. Upon ligand binding these receptors undergo a conformational change to allow them to form a homo or heterodimer. The formation of heterodimers between different members of the TLR family extends the repertoire of recognisable PAMPs which can be detected [38]. Upon dimerization cytoplasmic adaptor proteins can be recruited to the TIR domains to initiate downstream signalling (**Figure 1.2**) [39].

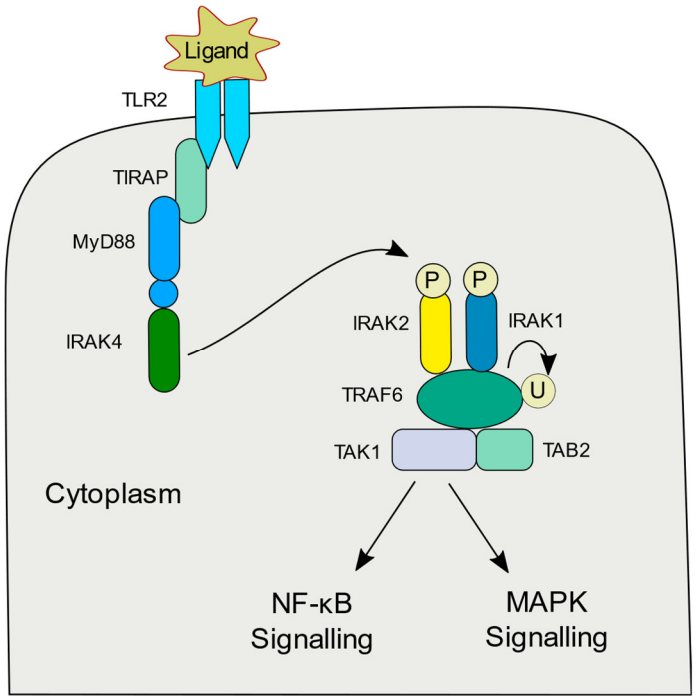

Figure 1.2: Signal transduction from toll-like receptors

Diagram of the primary signalling cascade activated upon binding of a ligand to TLR2. Receptor dimerisation triggers recruitment of the adaptor molecules TIRAP and MyD88, which activate IRAK4. This phosphorylates IRAK2 and IRAK1, which form a complex with TRAF6, TAK1 and TAB family proteins to initiate downstream signalling via the NF-κB and MAPK pathways. Some members of the TLR family are capable of binding directly to MyD88 without the need for additional adapter proteins.

The adaptor protein MyD88 is utilised by all TLRs except TLR3. MyD88 will bind directly to most activated TLRs, but the additional adaptor protein TIRAP is required for recruitment of MyD88 to TLR2 and TLR4 [40]. Once bound to a TLR MyD88 recruits and activates the protein kinase IRAK4 [41]. IRAK4 then recruits, phosphorylates and activates IRAK2 and IRAK1. IRAK1 then associates with the RING-domain E3 ubiquitin ligase TRAF6, allowing it to activate itself by self-ubiquitination. TAK1, a member of the MAPKKK family, is then recruited to TRAF6 and forms a complex along with TAB1, TAB2 and TAB3. The complex then activates TAK1, which can bind to and phosphorylate IKKβ to activate the NF-κB pathway. TAK1 activation also results in the activation of the MAPK pathway, leading to the ERK1/2 and p38 signalling cascades [36]. Due to these multiple downstream pathways the MyD88 cascade acts as an important mediator of inflammatory responses to innate immune stimuli. This role is highlighted by the fact that human patients with genetic deficiencies in this pathway are particularly susceptible to bacterial infections such as invasive pneumococcal disease [42].

MyD88-independent signalling can occur via the adaptor protein TRIF, which is recruited to TLR3 upon binding to endosomal dsDNA. TRIF can also be recruited indirectly to TLR4 through

the bridging adaptor TRAM [43]. TRIF is capable of associating with TRAF6, allowing it to activate the inflammatory signalling cascade independently of MyD88. However, TRIF also recruits TRAF3 and causes it to undergo auto-ubiquitination [44]. TRAF3 can then form a complex with TANK and NAP1, which allows for the recruitment, phosphorylation and activation of TBK1 and IKKϵ [45]. TBK1 then phosphorylates the transcription factor IRF3 to activate a type I interferon response [46]. This pathway allows for TLR3 and TLR4 to activate both NF-κB and IFN driven signalling pathways in response to pattern recognition.

1.2.5. Innate immune macrophage activation

Recognition of PAMPs in macrophages induces multiple signalling cascades which result in macrophage activation and the induction of a proinflammatory phenotype [47]. Different receptor families utilise different signalling pathways to induce responses specialised towards the type of stimulus. These responses typically lead to the activation of transcription factors such as NF-κB, AP-1 and CREB which migrate to the nucleus and induce inflammatory gene expression [36]. Other groups of PRRs utilise different adaptor proteins to activate the same signalling pathways as TLRs. C-Type lectin receptors recruit the tyrosine kinase Syk, which in turn triggers the MAP kinase cascade to activate CREB [48]. RIG-I like receptors also utilise the adaptor IPS-1 to activate the NF-κB pathway [49]. In contrast, receptors for viral PAMPs such as cGAS typically utilise the STING pathway to activate IRF3 and IRF7, which drives type I interferon responses [50].

Activation of macrophages can promote antimicrobial activity. Phagocytosis is triggered by the attachment of PAMPs to receptors on the macrophage cell surface such as scavenger receptors and complement receptors. Alternatively, opsonins such as antibodies can trigger phagocytosis by acting as a bridge between a microbe and macrophage Fc receptors [51]. Material is then engulfed using an actin-myosin contractile system to encapsulate it into an early phagosome within the cell [52]. Maturation of the phagosome acidifies this compartment, primarily through fusion with lysosomes, producing the phagolysosome [53]. Phagolysosomes efficiently degrade their contents, killing microbes and exposing cryptic ligands which can activate additional pattern recognition receptors to direct the immune response [54]. Activation by PAMP recognition also triggers macrophages to upregulate expression of MHC class II proteins alongside co-stimulatory molecules including CD80 and CD86. MHC presents peptides from degraded microbes to the rest of the immune system, which in the presence of co-stimulatory receptors can trigger activation of CD4+ T cells and promote the generation of an adaptive response [55]. These MHC class II proteins can be trafficked to endosomal compartments to acquire microbial peptides for cross-presentation and the induction of

adaptive immunity [56]. Alternative pathways such as autophagy allow macrophages to eliminate intracellular pathogens which have evaded degradation [57]. When a macrophage exhausts its capacity for phagolysosomal killing, apoptosis can also be utilised to control infection. This has been reported with pathogens such as *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* [58], [59].

Another major component of the proinflammatory responses induced by pattern recognition is the secretion of cytokines such as TNF, IL-1, IL-6, IL-8, and IL-12, which can act in a paracrine and endocrine fashion to influence other cells [60]. In immune cells inflammatory cytokine signalling can activate leukocytes and APCs, encourage microbial killing and increase cell survival [61]. In addition to this, macrophages also secrete chemokines such as CXCL10 and CCL2 to recruit additional immune cells to sites of inflammation [56]. Macrophages can also secrete type I interferons to enhance inflammation and antiviral responses (see section **1.3**). Through these functions macrophages can shape and direct the broader immune response to promote clearance of pathogens.

Macrophages also play an important role in the regulation of inflammatory responses. Inflammation can cause additional damage to host tissues during the response to invading microbes. Macrophages can exert regulatory functions in which they suppress inflammation via secretion of anti-inflammatory cytokines such as IL-10 (see section **1.4**) [27]. Macrophages can also co-ordinate tissue repair by adopting a wound-healing phenotype. In this state they secrete factors such as transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF) which induce the synthesis of collagen by fibroblasts [62]. Macrophages also secrete tissue inhibitors of metalloproteinases (TIMPs) to inhibit degradation of the extracellular matrix by matrix metalloproteinase, limiting tissue damage [25]. Together these functions promote the resolution of inflammation, the repair of tissue damage and a return to homeostasis.

In vivo monocytes and macrophages are thought to exhibit diverse phenotypes which contribute to heterogeneity observed when working with cultures of primary monocyte-derived macrophages (MDMs) [63]. Several intermediate phenotypes exhibiting both inflammatory and regulatory cytokine secretion have been described, which has prompted some to consider macrophage activation as a spectrum rather than a binary choice [60], [64].

1.3. Interferon

Interferons (IFNs) are a family of cytokines produced by cells upon detection of infection and are named for their ability to "interfere" with viral replication within host cells. IFNs are divided into three families, called type 1, 2 and 3. The principle type I IFNs are IFNα and IFNβ. Much less is known about the other variants. Type I IFNs can be produced by most nucleated cells in response to viral infection and induce an antiviral response in both infected and uninfected bystander cells [65]. In addition to this, type I IFNs modulate the immune response, with the outcome of signalling being dependent on the cellular context and nature of the microbial stimulus. Type II IFNs consist of the single protein IFNγ, which is mainly produced by T cells and NK cells. It induces activation and antigen presentation in macrophages while enhancing intracellular killing of pathogens. IFNγ also activates NK cells and promotes leukocyte migration towards sites of infection [66]. The interferon gamma receptor (IFNGR) is also present on non-immune cells, in which IFNγ signalling upregulates MHC Class I molecules and induces expression of a subset of antiviral genes [67]. Type III IFNs consist of three IFNλ proteins, which induce a similar response to the type I IFNs but in a restricted subset of cells, largely in epithelial layers [68].

Binding of these proteins to interferon receptors triggers the expression of interferon-stimulated genes (ISGs) (discussed in section **1.3.2**). These genes assist in host defence against viral infection and can also interfere with the replication of viruses in cells which are already infected [69]. In addition to this, IFNs can also help to activate immune cells such as macrophages to encourage the development of adaptive immunity [70]. IFN expression is also seen during bacterial and fungal infection, particularly in response to intracellular pathogens [65].

1.3.1. Interferon induction

Recognition of viral PAMPs is a common trigger for interferon induction. Expression of type I IFNs is mostly driven by the transcription factors NF-κB, IRF3 and IRF7 [71], [72]. The toll-like receptors TLR3, TLR7 and TLR8 detect viral dsRNA and ssRNA in the endoplasmic reticulum and endosomes [49]. While these TLRs induce general inflammation via NF-κB, IFN is simultaneously induced through signalling via the TRAF3/TBK1/IKKε axis (**Figure 1.3**) [73], [74]. Retinoic acid-inducible gene I (RIG-I) and related receptors have been identified as sensors which detect viral dsRNA in the cytosol [75]. These cytosolic RNA receptors signal via the Mitochondrial Antiviral Signalling (MAVS) adapter [76]. Cyclic GMP-AMP Synthase (cGAS) has been identified as a cytosolic sensor for viral DNA [77]. This sensor signals via the messenger molecule cGAMP, which in turn activates Stimulator of Interferon Genes (STING) [78]. Other DNA sensors such as IFI16

and DDX41 also signal via the STING axis to induce interferon [79], [80]. Both STING and MAVS induce phosphorylation of NF-κB and IRF3, which initiates inflammatory and antiviral responses [81].

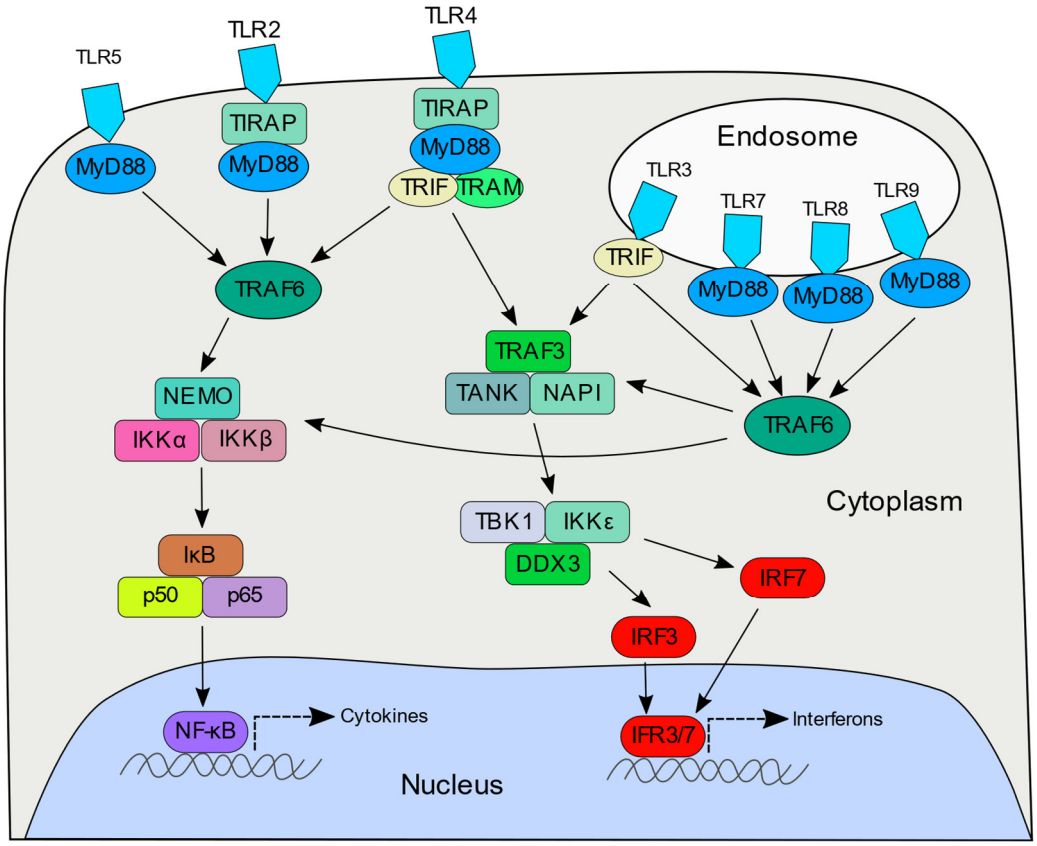

Figure 1.3: Induction of NF-κB and IFN pathways by different toll-like receptors

Pathways leading to differential NF-κB and IFN induction in response to stimulation of different toll-like receptors. Based on *Nan et al., 2014.* Signalling from toll-like receptors activates the TRAF6 complex, which leads to activation of the IKK complex in which NEMO recruits the IKKα and IKKβ subunits. The IKK complex phosphorylates IκB, which is then degraded via the ubiquitination pathway. This releases the NF-κB (p50/p65) transcription factor which can move to the nucleus to trigger inflammatory gene expression. In addition to this, TLR3 and TLR4 can recruit the adaptor protein TRIF to induce activation of the TRAF3 complex. This activates the TBK1 and IKKε kinases which phosphorylate IRF3 and IRF7 to activate these transcription factors, which trigger expression of Type I IFN.

Although interferons are commonly regarded as being antiviral in nature, these cytokines are also expressed in response to a variety of bacterial and fungal PAMPs. For example, TLR4 and TLR2 also induce type I IFNs as part of a broader inflammatory response [82], [83]. While STAT1-mediated induction of type II IFN can assist in the clearance of intracellular bacteria [66], type I IFN signalling can be beneficial or detrimental for control of extracellular bacterial

infection depending on microbial countermeasures and evasion strategies [84], [85]. The immunomodulatory genes induced by ISG signalling are thought to help to balance protective immunity against immunopathology [86]. However, some bacteria such as *Mycobacterium tuberculosis* actively secrete virulence factors which trigger interferon to modulate host immunity in their favour [87], [88]. Subversion and exploitation of the interferon system can therefore be an important feature in microbial pathogenesis.

1.3.2. Effects of interferon and the antiviral state

In response to stimulation with interferons, cells express hundreds of ISGs which have diverse effects aimed at inhibiting viral replication [89]. Different types of interferon induce unique, but partially overlapping, "ISG profiles". Many ISGs function to modify the host cell to slow down or halt viral replication. For example, IFIT proteins are reported to bind to eukaryotic initiation factor 3 and prevent translation of viral RNA [90]. Other ISGs focus on obstructing viral processes, such as TRIM5α which forms a lattice around retroviral virions to both interfere with uncoating and encourage proteasomal degradation [91], [92]. Many ISGs are PAMP sensors, which promote detection of infection within cells in this antiviral state. These include RNA and DNA sensors such as IFI16, which assists in detection of viral infection [93]. Together these factors cooperate to create a hostile environment for pathogens, with individual viruses typically being targeted by numerous ISGs [94]. This necessitates viral countermeasures, which are typically focused on counteracting the IFN-inducible effectors (e.g. HSV-1) or evading detection to prevent antiviral induction altogether (e.g. HIV-1) [95].

Aside from inducing antiviral mechanisms, binding of type I IFNs to the interferon receptor has additional effects on target cells. Stimulation of monocytes with type I IFN promotes differentiation into monocyte-derived dendritic cells, which can capture antigen and help to initiate the development of adaptive responses [96], [97]. Such signalling also promotes migration of dendritic cells into lymphatic vessels [98]. Interferon signalling may also enhance cross presentation of antigen by DCs to help activate CD8 T cells [99], [100]. These actions on dendritic cells would encourage the development of antiviral responses and assist in the clearance of viral pathogens.

Type I interferons are also able to influence behaviour of T cells directly, with variable effects on proliferation, survival and function [65]. In CD4+ T cells type I IFNs promote development of Th1 cells which produce IFNγ [101]. They are also critical for sustaining clonal expansion of these cells in response to viral but not bacterial pathogens, possibly by modulating the balance of signalling between different members of the STAT family [102]. IFN signalling which activates the STAT1 pathway can inhibit T cell proliferation [103], [104]. However, in CD8+ T cells type I

IFNs can also signal through STAT3 and STAT5 to encourage cell survival [105], [106], with activated CD8+ cells expressing lower levels of STAT1 and therefore avoiding the inhibitory effects of IFN signalling [107]. IFNs may also influence the function and survival of regulatory T cell subsets, although there is currently conflicting evidence on whether they play a positive or negative role [108].

In addition to effects on T cells, IFN-mediated enhancement of B cell responses has also been documented. Type I interferon can enhance humoral immunity and promote isotype switching through activity on dendritic cells [109]. Antibody responses can also be enhanced via direct stimulation of B cells or stimulatory effects on T cells [110], [111]. However, IFNs from bone marrow macrophages may also be inhibitory towards immature B cell development [112]. Type I IFNs can also promote function and survival of NK cells which are required for efficient clearance of some pathogens [113], [114]. These cytokines may protect T cells from NK-mediated killing by upregulating expression of inhibitory NK cell receptor ligands [115], [116]. Overall this suggests a more central role for interferons in the regulation of host immunity, such that modulation of this response by viruses may have downstream consequences beyond inhibition of localised antiviral systems.

1.3.3. Importance of interferon in infection

Interferon signalling is a major protective factor against common viral pathogens such as influenza virus. Mice with interferon receptor deficiencies are highly susceptible to viral infections including vaccinia virus and vesicular stomatitis virus (VSV) [117]. Furthermore, disruption of the downstream STAT signalling pathways was found to drastically alter viral cell type tropism and allow respiratory pathogens to generate systemic disease by infecting cell types which would not normally be permissive to infection [118]. This exaggerated phenotype is thought to underpin redundancy between type I and type III interferons, with knockouts for both interferon receptor types replicating the effects of STAT deficiency [68], [119]. In humans, mutations in the shared JAK and STAT pathways which did not produce severe combined immunodeficiency (SCID) resulted in increased susceptibility to viral and mycobacterial infections [120]. Patients with impaired production of type I IFNs also exhibit susceptibility to herpes simplex encephalitis, while those deficient for type II IFN may experience severe disseminated *M. tuberculosis* infections [68], [121].

While interferons can assist in clearing infection, primarily through the antiviral ISG programme, they can also have detrimental effects. Aside from inducing excessive inflammation and tissue damage, interferons can also play a suppressive role in chronic viral infection [122]. Simian Immunodeficiency Virus (SIV) infection of most natural hosts induces minimal interferon

induction without progression to immunodeficiency, whereas some hosts such as macaques experience strong type I IFN responses and more severe disease [123], [124]. Similar observations have been made in Human Immunodeficiency Virus (HIV) patients who experience rapid progression [125]. These observations may be related to the effects of IFNs on CD8+ T cells, since excessive exposure prior to antigen stimulation can trigger apoptosis in these cells [126]. In the presence of viral infection, interferon signalling has also been reported to transiently inhibit proliferation of bystander T cells [127]. In the context of chronic viral infection high levels of systemic interferon may therefore be detrimental to effective immune responses. In agreement with this, blockade of chronic type I IFN signalling during chronic infection with lymphocytic choriomeningitis virus in mice improved control of viral load and limited organ damage [128]. Whether blockade of IFN would be beneficial to human patients with chronic viral infections has not been determined, although the application of this approach in macaques infected with HIV-1 improved immune function and reduced the viral reservoir [129].

1.4. Interleukin 10

Interleukin 10 (IL-10) is a 18kDa homodimeric cytokine which is primarily produced by lymphocytes, monocytes and macrophages in response to microbial stimulation or cytokine stimulation. It has anti-inflammatory effects on immune cells in both an autocrine and paracrine manner [130].

IL-10 binds to the IL-10 receptor, which is a multimeric complex made up of the IL-10R1 and IL-10R2 subunits. IL-10R1 contains the ligand binding domain and is expressed at low levels on haematopoietic cells, and at high levels on macrophages and dendritic cells [131]. In contrast, IL-10R2 is only involved in signalling and is constitutively expressed by most cells [132]. Binding of IL-10 to its receptor induces phosphorylation and activation of the kinases JAK1 and TYK2, which in turn triggers recruitment of the transcription factor STAT3 [133]. STAT3 is phosphorylated and forms a homodimer which translocates into the nucleus to induce gene expression [134].

1.4.1. IL-10 as a suppressive cytokine.

The response triggered by IL-10/STAT3 signalling is predominantly anti-inflammatory in nature, with a focus on limiting TNFα and NF-κB signalling. STAT3 stimulates the expression of factors which inhibit inflammatory processes, such as BCL3 which suppresses NF-κB signalling, SOCS3 which inhibits cytokine signalling and zfp36 which targets TNFα mRNA for degradation. These effects do not impact the entire inflammatory response to the same extent. A study in murine macrophages found that ~40% of the proinflammatory response to LPS was unaffected by IL-10 signalling [135]. This would suggest that IL-10 acts selectively on inflammatory signalling rather than inhibiting inflammation altogether [136].

In line with this function, treatment of cells with IL-10 has been reported to generate anti-inflammatory phenotypes. In APCs such as macrophages stimulation with IL-10 limits activation and inhibits production of inflammatory cytokines. In general IL-10 inhibits Th1-type responses, but there are also reports of this cytokine suppressing Th2-type responses in some scenarios [137]. IL-10 has also been implicated in the differentiation and function of Treg cells by promoting their expansion and expression of CTLA-4 [138]. The effects of IL-10 on B cells and NK cells remain unclear. IL-10 is also reported to have anti-apoptotic functions, although this may be an indirect result of its capacity to inhibit TNFα expression [139], [140].

As excessive inflammation in response to microbes can be harmful to the host, IL-10 is necessary to effectively balance immune responses between antimicrobial activity and immunopathology [141]. IL-10 deficiency results in immunopathology usually affecting the bowel, suggesting a defect in the regulation of immune responses to gut microbiota [142]. In keeping with this, patients with mutations in IL-10 or the IL-10 receptor often present with early-onset inflammatory bowel disease [143]. IL-10 knockout mice also develop cardiac and vascular dysfunction [144]. There is also evidence of exaggerated inflammatory responses to infectious disease in these mice, which can enhance immunopathology [145], [146], although in some instances this may assist in pathogen clearance [147]. This highlights the importance of this cytokine for the proper control of immune activation.

1.4.2. Stimuli which induce IL-10 expression

The pathways leading to IL-10 are complex and vary by cell type, with extensive regulation at both the transcriptional and post-transcriptional level. Expression of IL-10 can be triggered by the activation of pattern recognition receptors by pathogen-derived products [148], [149]. TLR2 agonists may be particularly potent inducers of IL-10 expression in antigen-presenting cells [150], [151]. TLR4 and TLR9 are also capable of inducing substantial production of this cytokine in APCs [148]. In general, macrophages produce significantly more IL-10 in response to TLR

activation than myeloid dendritic cells. Other pattern recognition receptors such as Dectin-1 are also known to be able to induce IL-10 expression [152].

CD4+ T-helper cells are reported to express IL-10 in response to antigen stimulation using a common pathway shared between multiple T cell subsets, but with additional regulation depending on subset [153], [154]. CD8+ T cells also express IL-10 following TCR activation or exposure to CD40 ligand on activated plasmacytoid dendritic cells (pDCs) [155], [156]. In contrast, Treg cells are not capable of secreting IL-10 unless they have been exposed to a cryptic signal *in vivo,* which enables IL-10 expression in Treg cells within the gut [157], [158]. B cells were observed to produce IL-10 upon stimulation with TLR4 or TLR9, or during stimulation with auto-antigens [159], [160]. TLR-activated IL-10 production in neutrophils has also been described [161], [162]. However, the molecular mechanisms leading to expression in each cell type are poorly understood and are currently the subject of intense research.

1.4.3. Pathways leading to IL-10 expression

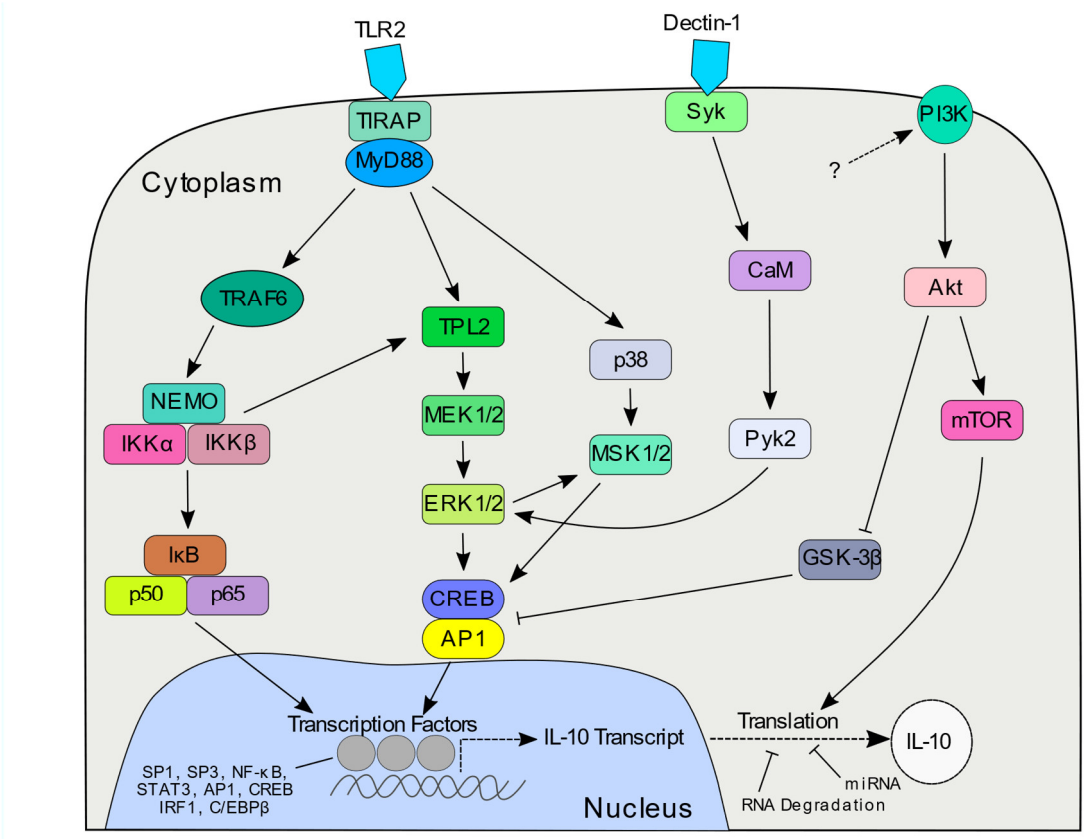

Figure 1.4: Pathways leading to IL-10 expression in macrophages

Pathways reported to influence IL-10 production in human macrophages in response to stimulation with zymosan. TLR signalling activates the IKK axis for NF-κB and the MEK/ERK axis for the transcription factor AP1, both of which are necessary for IL-10 expression. AP-1 activation can also be induced through the p38/MSK pathway. Dectin-1 activation induces the CaM pathway to activate ERK instead of signalling through MEK. Binding of a combination of different transcription factors is required, but poorly understood. Translation of IL-10 RNA is also regulated by the PI3K/Akt/mTOR axis.

Pattern recognition is thought to induce IL-10 production through several intracellular signalling pathways. Most TLRs signal through MyD88 to trigger the ERK1/2 kinase cascade, which is known to induce IL-10 expression [39], [163], [164]. Accordingly, cells from ERK-deficient mice exhibited attenuated IL-10 expression [165]. Interestingly, the strength of ERK activation in macrophages, myeloid DCs and pDCs correlates with IL-10 production, with macrophages displaying the most activation and producing more cytokine in response [166], [167]. TPL2 is an upstream activator for ERK which is degraded in the absence of NF-κB p105, depletion of either of these proteins resulted in attenuated IL-10 production compared to wild-type cells, further supporting a role for ERK in IL-10 secretion [166].

Since inhibition of ERK does not completely abolish IL-10 responses, there are thought to be ERK-independent mechanisms of regulation. MyD88 signalling can also trigger the p38 MAPK cascade to induce IL-10 expression, particularly after stimulation with LPS or CpG elements [168], [169]. A defect in MKP-1 which results in prolonged p38 activation generated increased TLR-inducible IL-10 responses in primary macrophages. [170], [171]. Chemical inhibition of p38 signalling reversed this effect, providing further evidence for the role of p38 in IL-10 signalling. Additional evidence for cooperative regulation of IL-10 comes from the finding that deficiency of MSK1 and MSK2, which are downstream of both pathways, generates an almost complete loss of IL-10 expression in LPS-stimulated macrophages [172].

Several other pathways have also been reported to regulate transcription factors which influence IL-10 expression. Dectin-1 stimulation is a particularly potent activator of IL-10 expression which utilises Syk in addition to CREB and ERK/p38, independently of TLR signalling [173], [174]. Calcium-dependent signalling via CaM kinase II and Pyk2 downstream of Syk has also been reported to induce ERK-mediated IL-10 signalling in human macrophages in response to zymosan [48]. The PI3K-Akt-mTOR axis may also be involved in the regulation of IL-10 expression through manipulation of GSK3 and CREB/AP-1 signalling [175], [176]. The manner in which these pathways interact with each other during an immune response remains poorly understood [154]. Pathways known to be involved in the regulation of IL-10 in the context of macrophage stimulation with ligands used during this thesis are depicted in **Figure 1.4**.

1.4.4. Transcriptional regulation of IL-10 expression

The expression of the IL-10 gene depends on the binding of multiple transcription factors to the IL-10 promoter. In human macrophages the transcription factors SP1, SP3, STAT3, IRF1, C/EBPβ, NF-κB and CREB are reported to be involved in the IL-10 response to different stimuli [167], [177]–[181]. The relative contribution of each factor is poorly characterised, but STAT3, NF-κB and SP1 are thought to be necessary for expression [141]. There are also distal regulatory elements for IL-10 which are located outside the IL-10 promoter. In murine macrophages the NF-κB p65 subunit was found to bind to a site 4.5kb upstream of the promoter to enhance expression during stimulation with LPS [182]. This site is conserved in humans, although it's role in human macrophage IL-10 expression has not yet been demonstrated. Overall a complex system of transcription factors regulates IL-10, which may tailor the response for specific cell types and stimuli.

IL-10 is also negatively regulated because excess secretion could prevent control or clearance of infection and enhance pathogenesis [141]. Poly (ADP) ribose polymerase 1 (PARP-1) restricts IL-10 expression in macrophages which engulf apoptotic cell debris, although this mechanism is not involved in regulating LPS-driven IL-10 production [183]. The MHC class II transactivator (CIITA) is also reported to inhibit IL-10 production in bone marrow-derived dendritic cells, with deficiency resulting in exaggerated expression [184]. STAT1 is reported to negatively regulate IL-10 expression in monocytes [185]. Murine macrophages deficient for BCL-3 were also deficient in IL-10 expression, but as the IL-10 locus lacks binding sites for this factor the effect may be indirect [186]. Alongside variation between cell types, the effectiveness of these mechanisms may be susceptible to IL-10 gene polymorphisms that reduce expression levels, which have been associated with enhanced pathology in patients [187].

Epigenetic regulation also plays an important role in initiating and sustaining transcription from the IL-10 promoter. Several studies have suggested that IL-10 is regulated by rearrangement of chromatin structure within the IL-10 locus through histone modifications [188]–[190]. This regulation is likely to be cell-type specific, with IL-10 expression in macrophages reported to be regulated by phosphorylation of histone H3 [191]. Histone H3 and H4 acetylation within the IL-10 locus has been found to influence IL-10 expression in multiple cell types [182], [192], such that disruption of histone acetylation inhibits expression [193]. Additionally, the NFκB-binding distal element is located within a DNase I hypersensitive site in macrophages and activated T cells, which is suggestive of epigenetic modification being used to render the chromatin accessible for transcription factor binding [182]. Several DNase I hypersensitive sites within the IL-10 locus which are active in most cell types have also been

reported [190]. The molecular mechanisms which initiate chromatin remodelling and epigenetic modification of the IL-10 locus have not been identified, although in macrophages TLR activation and Fc receptor binding are likely to be involved [182], [191].

Post-transcriptional control of IL-10 production provides another level of regulation for this cytokine. IL-10 mRNA is rapidly degraded after synthesis and so modulation of mRNA stability is an important mechanism by which translation is regulated. Like many cytokines, IL-10 mRNA contains multiple potentially destabilizing clusters of adenosine- and uridine-rich elements (AREs) in the 3'-untranslated region (UTR) [194]. Adenosine receptor activation reduces the repressive effect of this UTR to increase the half-life of IL-10 RNA and therefore the production of protein [195]. IL-10 is targeted by the RNA binding protein tristetraprolin (TPP), which induces rapid degradation upon binding to AREs [196]. Deletion of a long segment of the IL-10 3'UTR extended the half-life of newly synthesized transcripts from 1 h to >12 h [194]. Activity of p38-MAPK-activated 2 (MK2) is also reported to prevent TPP-mediated decay through phosphorylation, prolonging the half-life of IL-10 transcripts [197].

MicroRNAs which impact IL-10 transcript stability have also been described. mIR16 is capable of binding to AREs on RNA and cooperatively acts with TTP to silence IL-10 expression [198]. miR106a recognises a sequence in the IL-10 3'UTR in myeloid and lymphoid cells, leading to reduced mRNA stability [199]. In activated macrophages miR27a also negatively regulates IL-10 expression in response to TLR2 and TLR4 stimulation [200]. In contrast, miR466I is expressed in response to TLR stimulation but prolongs IL-10 RNA half-life by competitively inhibiting TTP binding to the transcript [201]. The cell type and stimulus-specific expression of these microRNAs may explain the variation in IL-10 production observed despite the shared pathways involved in its induction.

The balance of inflammatory and regulatory cytokine expression may also be influenced by a phenomenon known as cytokine biasing. The mTOR pathway is known to regulate the cytokine response by altering the efficiency of transcription using NF-κB (inflammatory) and STAT3 (regulatory). Inhibition of mTOR resulted in reduced IL-10 expression and enhanced inflammatory cytokine secretion [202]. It was found that PAMP recognition triggers ubiquitination of mTOR regulators such as Akt to downregulate mTOR activity and enhance inflammatory signalling [203]. This provides a mechanism of post-transcriptional regulation which can specifically influence IL-10 production as part of a broader cytokine response.

1.4.5. Interplay between IFN and IL-10

Type I interferons are reported to influence IL-10 expression, but there are conflicting reports on its effects, particularly between different cell types. In monocytes type I IFN was

inhibitory to IL-10 production while in T cells IL-10 expression was enhanced [204], [205]. In macrophages type I IFN signalling has been found to both induce [206] and inhibit IL-10 secretion [207]. It is possible that interferon induces a mixture of positive and negative effects on IL-10 regulation. In contrast, type II IFN strongly inhibits IL-10 production [176]. IFNγ-induced macrophage activation suppresses the anti-inflammatory functions of this cytokine while altering IL-10-mediated signalling to activate proinflammatory STAT1 signalling instead of STAT3 when IFNγ is also present, further limiting the regulatory effects of IL-10 [208]. This switch towards activation of STAT1 may also explain reports of proinflammatory responses to IL-10 seen *in vivo* [209].

IL-10 signalling may also influence the response to interferons. Stimulation with IL-10 suppresses IFNγ production in NK cells and T cells [210], [211]. IL-10 has also been reported to inhibit nitric oxide synthesis in response to IFNγ stimulation [212] and the expression of IFNα- and IFNγ-induced genes [213]. While the effects of IL-10 on the expression and function of type II IFN are well established, there is limited data on whether similar effects exist for type I IFNs.

1.4.6. IL-10 in infection.

IL-10 plays an important role in the response to infection. In response to highly inflammatory or extracellular bacterial infections such as *Pseudomonas aeruginosa*, IL-10 production facilitates host survival by limiting immunopathology [214], [215]. With intracellular infection or less inflammatory bacterial pathogens such as *Listeria monocytogenes*, IL-10 production may enhance bacterial dissemination and decrease survival by inhibiting the immune response [216], [217]. Some bacteria such as *Bordetella pertussis* encode virulence factors which actively induce IL-10 expression to promote their survival [218], [219]. Increased bacterial survival may partially be the result of IL-10-mediated suppression of IFNγ, since type II IFN is important for bacterial clearance [220], [221]. Control of other infections such as *Mycobacterium tuberculosis* requires a fine balance between inflammatory and regulatory signalling, with IL-10 serving protective and detrimental roles at different stages of infection [222]. In respiratory tract infections IL-10 may also serve to limit neutrophil infiltration [217]. Furthermore, high IL-10 production can interfere with vaccine efficacy when targeting intracellular pathogens such as *Leishmania major* and mycobacteria [223], [224].

In viral infections IL-10 can also play diverse roles. The induction of the antiviral state by stimulation with type I IFNs is associated with IL-10 expression in DCs and macrophages [148], [225]. Induction during early infection may limit tissue damage which would result from excessive inflammation [226]. IL-10 is produced by activated T cells during many viral infections including influenza virus and coronavirus, providing a regulatory feedback loop which limits

excessive inflammation [227], [228]. This may be mediated through IL-10 stimulating APCs to downregulate MHC expression and inhibit the production of cytokines which would stimulate trafficking of APCs to lymph nodes [229], [230]. Infected cells are generally cleared through killing by cytotoxic CD8+ T lymphocytes. IL-10 levels have also been reported to act as a regulatory trigger which initiates clearance of antiviral T cell populations upon resolution of the acute phase of infection [231]. These mechanisms serve to limit immune activation and prevent immunopathology, particularly after a viral infection has been cleared. Further evidence for the importance of IL-10 in control of viral infection comes from the fact that some viruses such as Epstein-Barr Virus and Cyprinid Herpesvirus 3 encode IL-10 homologues [232], [233]. These have the potential to modulate host immunity to favour viral replication, conferring an evolutionary advantage. Overall, IL-10 is important for directing the host response to viral pathogens, but these functions can also be exploited to favour microbial survival.

In the context of HIV infection IL-10 has been reported to have both protective and detrimental roles. Chronic infection is associated with increasing serum IL-10 levels as the disease progresses [234]. Multiple labs have found that the HIV regulatory protein Tat induces IL-10 expression in human monocytes, and several different signalling pathways have been proposed as mediators of this activity [235]–[237]. The HIV envelope gp120 subunit has also been reported to induce production of this cytokine in lymphocytes [238]. Paradoxically, IL-10 is inhibitory to HIV replication in macrophages and T cells [239], [240]. Despite this, enhancing expression may still provide a replicative advantage to the virus by inactivating effector immune responses to HIV [241]. IL-10 was also found to induce expression of the HIV coreceptor CCR5 on monocytes, facilitating their infection [242]. Studies in patients have found that IL-10 gene polymorphisms which enhance IL-10 expression are associated with slower loss of CD4+ T cell counts and reduced susceptibility to infection, but increased viral load during the acute phase of infection [243], [244]. Conversely, variant alleles resulting in reduced IL-10 production were associated with more rapid progression to AIDS [245]. This would suggest that HIV may exploit IL-10 signalling to assist in immune evasion at the expense of replication efficiency.

1.5. HIV-1

The Human Immunodeficiency Virus (HIV) is a lentivirus of the family *Retroviridae* which causes a chronic infection leading to acquired immunodeficiency syndrome (AIDS) [246]. In turn this leads to opportunistic infections and cancer, typically resulting in death around 10 years after HIV infection if left untreated [247]. HIV is largely a sexually transmitted disease, but also spreads through contaminated blood and blood products, needle sharing in intravenous drug users and by vertical transmission from mother to foetus. The virus utilises the co-stimulatory

molecule CD4 as a receptor for entry, allowing it to infect a range of immune cells if the necessary chemokine coreceptors (CCR5 or CXCR4) are also present [248]. CD4+ T helper cells are therefore thought to be the primary cell type infected by HIV [249]. As a retrovirus, HIV integrates into the genome of host cells to cause persistent infection with reservoirs of latent virus distributed around the body [250].

HIV is asymptomatic in the first few weeks after infection prior to seroconversion (**Figure 1.5**, based on [251]). After 4-8 weeks patients may experience flu-like symptoms associated with seroconversion, depletion of circulating and gut-associated CD4+ T cell counts and high plasma viral load [252]. During this time a latent reservoir of infection is also established [253]. In patients CTL escape mutants have been observed in this reservoir, suggesting that the reservoir may be constantly replenished [254]. After the initial immune response, a period of clinical latency follows in which the circulating viral load is suppressed [255]. During this time patients exhibit chronic activation of the immune system, with elevated serum levels of inflammatory cytokines and chemokines [256]. This activation may reflect HIV persistence or result from the breakdown of immunity at the gastrointestinal mucosal surface due to depleted CD4 T cell numbers, which allows microbial PAMPs such as LPS to enter the circulation and induce systemic inflammation [257]. Over time sustained activation leads to immune exhaustion, cellular senescence and a decline in regenerative capacity [258]. If left untreated, circulating CD4+ T cell counts gradually decline over the next 5-10 years and eventually control of viral load begins to break down [259], [260]. This coincides with the increased susceptibility to opportunistic infections which is characteristic of AIDS [261]. While progression to AIDS can be prevented using antiretroviral therapy, suppressed infection is still associated with other chronic health complications such as cardiovascular disease and cancer, which have been at least partly attributed to chronic immune activation [262], [263].

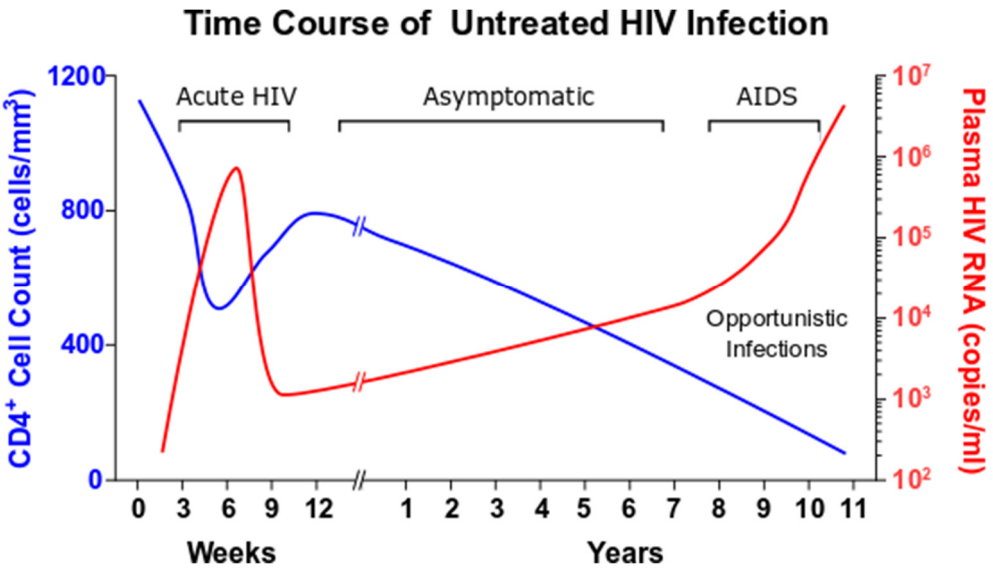

Figure 1.5: Example time course of HIV-1 infection

A time course of the acute and latent phases of HIV-1 infection. Acute viraemia is accompanied by flu-like symptoms with high viral load (red) and diminished CD4 counts (blue). A latent phase follows which can last for 5-10 years, during which patients are generally asymptomatic and $CD4^+$ T cell count gradually declines. After an extended period, viral control breaks down and AIDS symptoms begin.

1.5.1. The global HIV-1 pandemic

HIV-1 evolved from the simian immunodeficiency virus (SIV), an endemic lentivirus in non-human primates which causes persistent but non-pathogenic infection in most hosts [264]. Transmission to humans has occurred on multiple occasions to produce different groups of HIV-1 and the more distant HIV-2, but only the HIV-1 group M strains have been able to spread efficiently and become a global pandemic [265]. HIV-related disease was first reported in the 1980s and was first identified as a retrovirus in 1983 [266]. At present an estimated 36 million people are infected with HIV worldwide, with 1.8 million new cases each year [267].

Antiretroviral therapies have dramatically changed the course of HIV disease, but the high mutation rate of the virus poses a challenge for treating the disease because drug-induced escape mutations cause therapeutic failure [268]. Since the mid-1990s highly active antiretroviral therapy (HAART) has allowed for suppression of viral load in patients by using a regimen of 3 antiviral drugs, which minimises the chance of resistance developing [269], [270]. Over the following ten years this treatment changed a fatal disease into a manageable chronic condition. While availability of therapy in the developed world is widespread and has returned the life expectancy of patients to almost normal, in parts of the developing world access to treatment and adherence to the regimen is still limited [271]. Due to this, although the global

pandemic is declining, there is still a substantial need for international attention to maximise access to therapy and address the emergence of drug-resistant strains.

HAART still only results in suppression of HIV as it is unable to eradicate the integrated viral reservoir [272]. Once antiretroviral therapy is stopped most patients experience viral rebound and a return to AIDS [273]. However, a minority of patients are able to control the virus for months or years after cessation of HAART [274]. There are also a small proportion of patients who are termed “elite controllers”, these patients are able to control viral load in the absence of therapy [275]. Current research priorities in this field are focused on strategies to eliminate the viral reservoir such as “kick and kill”, which aims to reactivate latently infected cells to aid in eliminating infection [276]. This would alleviate the need for patients to take drugs for life. There has also been considerable research interest in strategies to develop a vaccine to prevent infection [277]. Both these areas require a stronger understanding of how the virus interacts with the immune system.

1.5.2. Co-infection in AIDS

The majority of the morbidity associated with AIDS occurs due to opportunistic infections which take hold as a result of the immunodeficiency generated by the virus. For example, *Cryptococcus neoformans* is a major cause of fungal meningitis in AIDS patients but is rarely pathogenic in healthy individuals [278]. Infections which are common in the general population can also spread to unusual areas of the body, such as yeast candidiasis spreading to the oesophagus [279]. Due to this, common opportunistic infections can be lethal in immunocompromised individuals. When some HIV patients begin HAART treatment the recovery of the immune system can also trigger excessive inflammation in response to opportunistic infections which couldn’t previously be controlled [280]. The resulting immune reconstitution inflammatory syndrome (IRIS) can enhance the severity of disease, particularly with infections of the central nervous system [281]. Due to these interactions the study of how HIV and AIDS impacts responses to other pathogens is important.

1.5.3. Viral structure and proteins

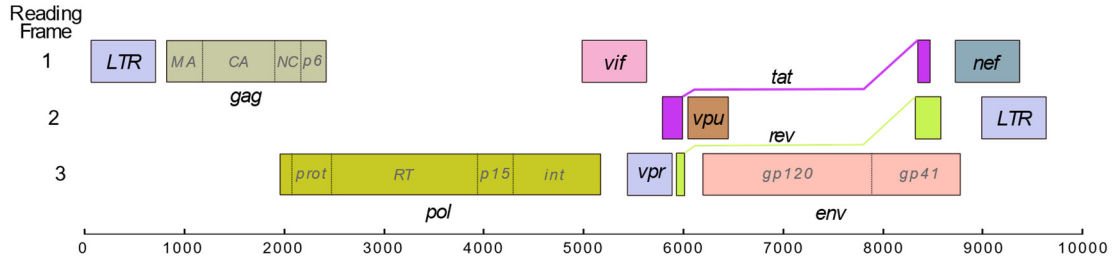

Figure 1.6: Organisation of the HIV-1 proviral genome

Arrangement of genes in the HIV-1 HXB2 isolate and derived strains when integrated into host cell's DNA. Based on the HIV sequence database, *hiv.lanl.gov.* Genes are arranged across three reading frames, with the *tat* and *rev* genes both consisting of two exons brought together by splicing. The genes *gag*, *pol* and *env* are expressed as polyproteins. The genome is bounded by long terminal repeats (LTRs).

HIV-1 is a spherical enveloped lentivirus which carries two copies of a single-stranded positive-sense RNA genome (**Figure 1.6**) [282]. The genome is approximately 9.2kB in length and encodes nine viral genes flanked by two long terminal repeat (LTR) sequences. These genes are divided into structural, regulatory and accessory proteins. New virions bud from the cell surface of infected cells in an immature state, after which viral protease-induced protein cleavage rearranges the virion to form the conical capsid of a mature infectious virus (**Figure 1.7**) [283]. Within a virion, viral RNA is packaged within a protein capsid along with the viral reverse transcriptase, integrase and the accessory protein Vpr.

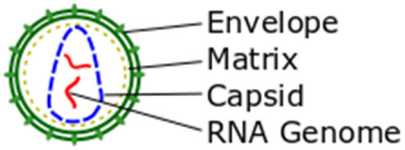

Figure 1.7: Structure of a HIV-1 virion

Diagram showing the structure of a mature HIV-1 virion. Two copies of the viral genome are packaged within a conical capsid alongside viral enzymes and the accessory protein Vpr. A membrane derived from the producing cell Is supported by the matrix protein. Env protein is carried on the envelope to permit entry into target cells.

1.5.3.1. Structural proteins

The viral Group-specific Antigen (*gag*) and Polymerase (*pol*) genes are transcribed as polyproteins which originate from the same transcript. These polyproteins undergo cleavage by viral protease into separate proteins with multiple functions [284]. Gag divides into p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC) and p6. There are also two short spacer peptides

SP1 and SP2 which separate these subunits, which are thought to be involved in maturation of the viral core [285]. The MA protein lines the inner surface of the viral envelope and is essential for targeting of nascent HIV RNA to the membrane, which is required for the initiation of particle assembly [286]. The CA capsid protein forms the viral core and encloses the viral RNA for transport from the cytoplasm to the nuclear rim [287]. NC and p6 are reported to drive the structural rearrangement of nascent RNA for packaging into the immature virion and initiate particle budding, respectively [288], [289].

The polymerase polyprotein is also cleaved into multiple subunits: protease (PR), reverse transcriptase (RT), RNase H (p15) and integrase (IN). Protease is responsible for cleaving the retroviral Gag-Pol polyprotein at nine specific cleavage sites to produce the mature viral components [284]. Reverse transcriptase transcribes the viral RNA genome into DNA prior to integration into host cell DNA. RNase H is a catalytic domain of the RT protein and degrades the RNA strand from the DNA/RNA hybrid produced by reverse transcription [290]. Finally, integrase is the enzyme which integrates viral DNA into the host cell's genome by cutting the host DNA and catalysing a strand transfer reaction [291]. As essential enzymes for HIV replication, protease, reverse transcriptase and integrase are common drug targets for antiretroviral therapy. A broad range of inhibitors have been developed for each of these proteins [292].

The HIV envelope protein is a homotrimer expressed as the polyprotein gp160 before being cleaved into two subunits (gp41 and gp120) by the host protease Furin: gp41 forms the stem of the fusion protein and assists in the later steps of fusion with a host cell [293]. This subunit is concealed by gp120, which binds to the CD4 receptor on target cells and induces a conformational change when in the presence of specific coreceptors [294]. CCR5 is reported to be the coreceptor predominantly utilised by the virus after initial infection, whereas isolates which enter using CXCR4 appear after several years of infection [295]. The conformational change induced by coreceptor binding exposes gp41 and initiates fusion [296]. The concealment of gp41 and the incorporation of uncleaved gp160 into virions assists the virus in evading immunity by limiting the exposure of domains which could be targeted by neutralising antibodies [297]. The gp120 protein is also highly genetically variable, leading to escape mutations which allow for evasion of antibody responses [298]. This variability also enables the virus to switch coreceptors, typically from CCR5 to CXCR4, which may lead to different tropism and entry routes for the virus [299].

1.5.3.2. Regulatory proteins

HIV encodes two regulatory proteins to control HIV transcription. Transactivator of Transcription (Tat) is a protein which significantly enhances viral gene expression [300]. Without

Tat HIV-1 transcripts generated by host cell machinery are predominantly short and terminate prematurely [301]. During early infection the viral genome is therefore slowly transcribed until enough Tat is present to promote efficient viral transcription. Tat binds to an RNA stem-loop called the trans-activating response element, at the 5' ends of HIV transcripts [302]. This assists with the recruitment of the transcription elongation complex to induce preferential transcription of viral RNA over host RNA. Tat also increases the processivity of RNA Polymerase II to maximise the generation of full length viral mRNAs [303]. In addition to regulating viral gene expression, Tat also modulates expression levels of cellular genes including the HIV-1 co-receptor CCR5 and MHC class I [304], [305]. It has been suggested that Tat can also be secreted from host cells in order to modulate gene expression in uninfected cells, which may render them more permissive to subsequent infection [306], [307].

The Rev protein localises to the nucleus and binds to the Rev Response Element (RRE) on incompletely spliced HIV transcripts [308]. The protein then assists in the transport of these unspliced elements out of the nucleus, which provides transcripts for viral genes and genomic RNA for packaging during late infection [309]. Rev contains a nuclear export signal which binds to and generates a dimer of the host protein CRM1, which is exploited to shuttle viral mRNA out of the nucleus [310]. Since completely spliced transcripts lack the RRE, the presence of Rev protein favours expression of HIV structural and accessory proteins over Tat, Rev and Nef [311]. This negative feedback loop may therefore enhance the efficiency of virion production.

1.5.3.3. Accessory proteins

HIV-1 encodes four accessory proteins: Vpr, Vpu, Vif and Nef. These proteins are not essential for virion structure, but instead function to manipulate the host cell to render it more permissive to viral infection and subsequent replication [312].

Viral Protein R (Vpr) is a 14kDa protein which has multiple reported roles. This protein is specifically packaged into infectious virions by binding to the Gag p6 protein [313], suggesting a role for this protein in the early stages of the virus life cycle following cellular entry [314]. Vpr recruits the Cul4-DCAF1 E3 ubiquitin ligase complex to target host proteins for proteasomal degradation [315]. The activity of Vpr results in G2/M cell cycle arrest, which may assist in viral replication as the HIV-1 LTR has been reported to be more active during the G2 phase [316]. It has been proposed that Vpr may trigger cell cycle arrest by inducing premature activation of the SLX4 complex, which is normally involved in repair of DNA damage but could interfere with active replication forks if activated inappropriately [317]. However, it has also been suggested that Vpr from some HIV-1 isolates may induce cell cycle arrest without binding to SLX4 [318]. Vpr has also been reported to participate in nuclear import of the HIV pre-integration complex

through interaction with importin-α, which promotes binding to nuclear pore proteins [319]. This may be consistent with observations made via microscopy that Vpr localises to the nuclear envelope of target cells [319]. In addition to this, Vpr has been found to promote apoptosis and/or necrosis in T cells and monocytes which may contribute to CD4 T cell depletion, although the mechanism by which this occurs remains controversial [314], [320], [321]. It is currently not clear which functions of Vpr are most important for viral replication.

Viral Protein U (Vpu) is a 16kDa membrane protein. It is not packaged into virions and is not present in HIV-2 [322]. Vpu also enhances virion release from infected cells by counteracting the host restriction factor tetherin, which anchors budding viral particles to the membrane preventing their release [323]. Vpu targets tetherin for ubiquitin-mediated degradation by interacting with the F-box protein βTrCP [324]. Tetherin exists in both a short and a long isoform originating from alternate start codons. Although both isoforms can 'tether' viral particles, the long isoform is also capable of acting as a PRR to trigger signalling cascades upon recruitment of HIV virions [325], [326]. Vpu has been found to preferentially target the long isoform for degradation, which may suggest that innate signalling from tetherin is the more important selective pressure for this accessory protein [327]. This would be supported by the observation that direct cell-cell spread of HIV is not hindered by tethering of virions [328]. Vpu also induces proteasomal degradation of CD4 in the endoplasmic reticulum through the ER-associated degradation pathway, which is likely to be important in preventing premature Env-CD4 interactions on the infected cell's surface during virion assembly and budding [329], [330]. There is also evidence to suggest that Vpu may be involved in inhibiting NF-κB activation to interfere with antiviral responses [331], [332].

The HIV Viral infectivity factor (Vif) is a 23kDa accessory protein which is packaged into virions [333]. In CD4+ T cells and some T cell lines Vif has been shown to be essential for viral replication [334], [335]. Viruses lacking Vif exhibit reduced infectivity due to the activity of the cellular restriction factor APOBEC3G [336], [337]. APOBEC3G is incorporated into virions and causes hypermutation of the viral genome, which renders the virus incapable of producing functional proteins [338]. By imitating the cellular SOCS2 protein Vif can recruit the Cul5 E3 ubiquitin ligase complex in order to induce polyubiquitination and subsequent proteasomal degradation of APOBEC3G, relieving restriction [339], [340]. It has been suggested that Vif may also bind to APOBEC3G mRNA to inhibit its translation [341]. Despite these activities, low levels of functional protein can still be detected within HIV-1 virions [342]. The mutations resulting from low levels of APOBEC3G may contribute to HIV-1 variation and therefore could aid the development of antiretroviral resistance [343], [344]. Vif has also been reported to induce G2 cell cycle arrest via interaction with the tumour suppressor p53 [345]. Recent proteomic studies

have suggested that Vif induces Cul5-depend`ent proteasomal degradation of the B56 family of serine/threonine phosphatases, although the purpose of this activity has not been established [346].

Negative Regulatory Factor (Nef) is a 27kDa myristoylated viral accessory protein (35kDa in HIV-2) which has a broad spectrum of activities in host cells [347]. While Nef primarily localises to the cytoplasm, it is also able to associate with the inner leaflet of the plasma membrane and endosomes [348]. At the plasma membrane Nef is responsible for downregulating membrane proteins from the cell surface, many of which are involved in immune recognition of infected cells. Nef is reported to impair both MHC class I expression and MHC class II antigen presentation, which could limit immune recognition by antigen-specific T cells [349], [350]. Nef-mediated downregulation of cell surface CD4 and intracellular Lck expression has also been reported, which may interfere with the development of adaptive immune responses to HIV by preventing T cell receptor signalling and subsequent activation [351]. The removal of CD4 from the cell surface may also protect infected cells from antibody-dependent cell-mediated cytotoxicity [352]. By targeting the immune modulator CTLA-4 for lysosomal degradation and lowering the threshold for T-cell activation, Nef encourages host cells to remain in an activated state which is favourable for viral replication [353]. Virions from Nef-defective mutants exhibit reduced infectivity which was recently attributed to restriction by the plasma membrane proteins SERINC3 and SERINC5 [354], [355]. The mechanism by which these proteins inhibit viral infectivity remains unknown, but Nef was observed to redirect SERINC5 into endosomal compartments rather than the plasma membrane [355], [356]. This activity prevents the incorporation of SERINC proteins into new virions, which would otherwise render virions non-infectious [354]. It has also been reported that Nef induces anti-apoptotic signalling by stimulating phosphorylation to inactivate the pro-apoptotic BAD protein, which promotes survival of the infected cell [357]. Together these functions highlight Nef's involvement in modulating the host cell to facilitate immune evasion and maximise viral replication. In keeping with this, viral strains deficient for Nef have been associated with reduced pathogenicity [358].

HIV-2 and most SIV strains carry the additional protein Vpx. Vpx has substantial sequence homology with Vpr and performs some of Vpr's functions in strains where both genes are present [359]. The most notable function of Vpx is its ability to counteract the host dNTP triphosphatase SAMHD1 by inducing ubiquitin-mediated proteasomal degradation of this protein [360]. SAMHD1 depletes free nucleotides from the cytoplasm in non-dividing cells, which restricts infection by limiting the supply of dNTPs needed for viral reverse transcription [361]. A mechanism for counteracting this restriction factor is absent in HIV-1 and other strains lacking Vpx, which may have consequences for cell type tropism [362].

1.5.4. The HIV-1 life cycle

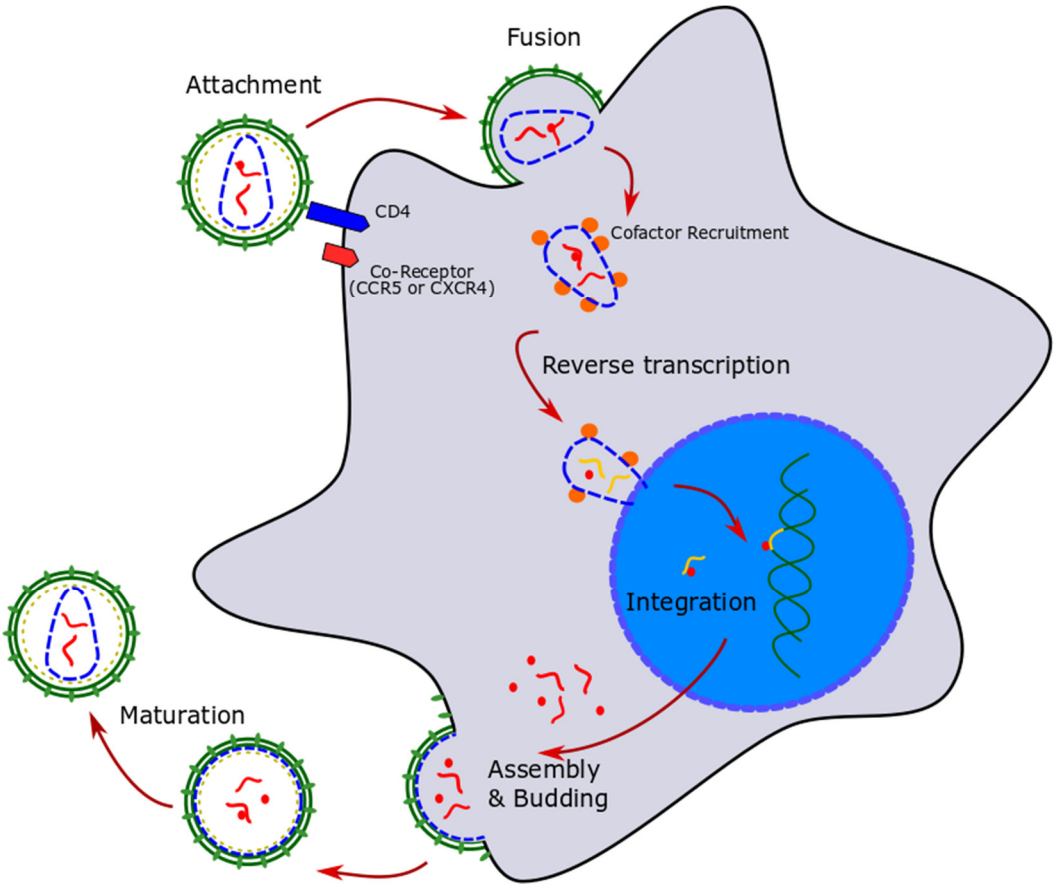

Figure 1.8: The HIV-1 life cycle.

An overview of the HIV-1 life cycle. Mature virions bind to the plasma membrane of a target cell. The viral Envelope protein then engages with the host receptor CD4 and a co-receptor (usually CCR5 or CXCR4), triggering a conformational change and fusion of the viral and host envelopes. This delivers the viral core into the cytoplasm, which then migrates to the nuclear envelope. During this time the viral RNA is reverse transcribed into DNA, which is delivered into the nucleus as part of the pre-integration complex. Viral genome is then integrated into a host chromosome. RNA for new virions is expressed from the integrated provirus to produce components for new virions, which bud from the cell surface. After budding, HIV protease activates and cleaves the viral gag protein to trigger maturation, which results in the assembly of a conical capsid.

Productive infection by HIV-1 depends on successful cellular entry, reverse transcription and integration into a host cell genome (**Figure 1.8**). Upon adhesion to a target cell, the envelope protein of a mature virion will bind to the CD4 receptor [363]. This binding triggers a conformational change in the gp120 subunit of envelope which enables engagement of a coreceptor, which can be either CCR5 or CXCR4 depending on the strain of virus [364]. Successful binding to a coreceptor triggers a further conformational change in Env which exposes the hydrophobic gp41 fusion peptide [365]. This fusion peptide then inserts into the membrane of the target cell and folds to form a six helix bundle, which brings the viral and cell membranes into close proximity to create a fusion pore [366]. The viral core is then delivered into the

cytoplasm of the target cell. There is also evidence that fusion can also take place in endosomes after virions have been taken up by phagocytosis [367].

Permissivity of a cell type to infection depends on the presence and abundance of CD4 and the coreceptors CCR5 or CXCR4 [249], meaning that HIV primarily infects CD4+ helper T cells, macrophages and dendritic cells. However, CD4+ T cells need to be activated for efficient infection with HIV [368]. Although macrophages can express both coreceptors, macrophage-tropic virions are reported to primarily utilise the CCR5 coreceptor whereas entry using CXCR4 is more limited to T-cells, [369], [370]. Entry using CXCR4 is more dependent on expression levels of CD4, and macrophages express substantially lower levels of CD4 than T cells [371]. This may partially explain the limited proportions of productively infected cells observed in HIV patients [372].

Successful fusion delivers the viral core into the cytoplasm of the target cell. The core contains the viral genomic RNA enclosed within a capsid shell [287]. Binding of cellular cofactors such as Cyclophilin A has been found to stabilise the capsid and prevent premature uncoating which would otherwise expose viral genetic material to pattern recognition receptors [373], [374]. A pore-like structure within the hexameric subunits of the capsid is capable of binding to free nucleotides and drawing them into the core, which may fuel encapsidated reverse transcription [375]. Whether reverse transcription is completed during transport of the core through the cytoplasm or initiates upon binding to the nuclear pore is an unresolved question [375]–[377].

To generate DNA from the single stranded RNA genome the host tRNA primer Lys3 binds to a primer binding site near the 5’ end of the RNA [378]. The HIV reverse transcriptase then synthesises complementary DNA at the 3’ end of the primer up to the 5’ end of RNA template, comprising the U5 and R regions [377]. During this process the RNase H activity of HIV RT simultaneously degrades the RNA template to release the ssDNA fragment, which is known as the minus strand strong stop DNA [379]. This DNA fragment then transfers to the R region at the 3’ end of the viral genome to act as a primer to initiate reverse transcription of cDNA for the rest of the genome [380]. During cDNA synthesis the RNase H activity of RT degrades the template RNA except in the PPT region. The PPT RNA is then used as a primer for synthesis of the second DNA strand [381]. RNase H then removes the PPT and tRNA primers and both strands are extended to form a complete dsDNA sequence of the viral genome. HIV integrase then binds to the dsDNA and cleaves the 3’ ends in preparation for integration into the host genome [382], [383].

Viral capsids have been observed to move to and 'dock' with the nuclear pore prior to nuclear import of the viral pre-integration complex (PIC), which consists of viral DNA associated with integrase and additional viral and host proteins [376]. The viral capsid is thought to interact with host factors such as Cyclophilin A, CPSF6 and NUP358 to assist in delivery of the PIC into the nucleus [384], [385]. Additional proteins such as NUP153 are required for import of HIV-1, although other retroviruses and mutants of HIV-1 such as N74D are capable of using other proteins such as NUP155, highlighting the potential flexibility of this mechanism [386]. Following nuclear translocation, the viral PIC binds to the host protein LEDGF/p75, which acts as a bridge to tether the PIC to the host DNA [387]. This enables a strand transfer reaction to occur during which the viral integrase catalyses the insertion of the viral genome into the host DNA [388]. This process leaves unpaired 'gaps' at the junctions between the host and viral DNA, which may be repaired by the activities of host DNA damage repair systems [389]. This integration system favours insertion into active genes, leaving a fully integrated HIV provirus which can express viral genes necessary for the formation of new virions [390].

RNA from the integrated provirus is transcribed and translated by host machinery. The viral regulatory protein Rev exports incompletely spliced viral mRNA from the nucleus to enhance expression of the structural genes *gag, pol* and *env* [391]. These genes are expressed as polyproteins which are cleaved by the viral protease enzyme. Assembly of new virions requires the viral gag polyprotein, reverse transcriptase, integrase and protease alongside two copies of the viral genome [283]. During assembly the functional subunits of Gag remain joined by flexible linker regions but perform distinct functions. The MA subunit binds the plasma membrane and may be involved in recruiting viral Env, while the CA domain mediates protein-protein interactions for assembly [392], [393]. The NC subunit recruits the viral genome through binding to the RNA packaging sequence (Ψ) [363]. The Gag p6 region also contains binding sites for the Vpr accessory protein as well as the TSG101 and ALIX proteins from the cellular ESCRT (endosomal sorting complexes required for transport) pathway [289]. This host pathway is then utilised to perform budding to produce and enveloped immature virion with a spherical core. Once separated from the host cell the retroviral protease is activated to cleave Gag into its subunits [394]. The two Gag spacer peptides SP1 and SP2 regulate the conformational changes that accompany viral maturation [283]. Maturation results in the assembly of a conical capsid within the virion which contains the viral genome and enzymes, which can then enter and infect another cell.

1.5.5. HIV-1 infection of macrophages

Although CD4+ T cells have been extensively profiled as the major cell type targeted by HIV, multiple lines of evidence suggest that HIV is also capable of infecting macrophages. The virus' ability to evade innate immune detection in these cells renders them permissive *in vitro* [395]. HIV-1-infected alveolar macrophages have been detected in patients by RNA fluorescence in-situ hybridisation (FISH), indicating that in these cells the virus is transcriptionally active [396]. Furthermore, HIV-1 strains isolated from patients with established infection exhibit tropism for MDMs *in vitro* [397]. However, HIV-1 founder viruses have been shown to have poor tropism for MDMs *in vitro* in spite of MDMs expressing the CCR5 receptor required for entry [398], [399]. While CD4+ T cells are often viewed as the primary host cell for HIV, macrophages have been proposed as a reservoir of infection due to their longevity and ability to sustain viral load in non-human primate and humanised mouse models depleted of CD4+ cells [400], [401]. Due to this, HIV infection of macrophages has the potential to influence not only responses to the virus itself but also to other co-infecting pathogens by impairing the phagocytic function of these cells [396] and disrupting the innate immune response to other pathogens [402].

1.5.6. Innate immune recognition of HIV-1

There have been numerous reports of innate immune sensors which can recognise HIV-1 RNA or DNA. GU-rich elements in genomic RNA from virions have been reported to be detected by the endosomal PRRs TLR7 and TLR8 in dendritic cells [403]. In plasmacytoid dendritic cells TLR7 activation and subsequent interferon induction was found to depend on endocytosis of virions [404]. However, there is also evidence that autophagy can allow for sensing of viral RNA from virions which have fused at the plasma membrane [405]. Furthermore, secondary structures in HIV genomic RNA can activate the cytosolic sensor RIG-I to induce a response in PBMCs [406]. In dendritic cells detection of viral RNA produces strong type I IFN responses, which may contribute to their ability to potently restrict HIV infection [407].

In contrast, viral DNA produced by reverse transcription can be sensed in the cytosol. DNA sensing via the cGAS-STING axis can trigger IFN induction if viral DNA is detected by the host cell before reaching the nucleus [408]. However, the host DNase TREX1 degrades cytoplasmic viral DNA, which may prevent infection but can also limit sensing and interferon induction [409]. In bystander cells which restrict infection during reverse transcription due to the activity of SAMHD1, partial RT products can trigger sensing via IFI16 [410].

In patients, increasing levels of type I IFNs are seen during the acute viraemic phase, which may reflect the capacity for plasmacytoid dendritic cells to secrete large quantities of IFNα upon TLR7 stimulation [411]. The array of ISGs expressed in response to IFN includes numerous factors

which can restrict HIV, such as tetherin and APOBEC proteins. Further evidence for their importance comes from the observation that transmitted/founder viruses are relatively resistant to type I IFN-mediated restriction [412], [413]. This would indicate that evasion of these systems is critical during transmission to a new host.

1.5.7. Viral evasion of innate immunity

In macrophages HIV-1 infection depends on the ability of the virus to avoid innate immune detection and subsequent production of type I interferon [414]. Endosomal RNA sensing can occur when virions are degraded by proteases during endosome maturation, although viral fusion in early endosomes may allow escape into the cytosol for productive infection [367]. The viral accessory protein Vpr has been reported to delay phagosome maturation by interfering with microtubule trafficking, which may assist evasion by allowing more time for virion fusion [415]. Furthermore, HIV infection has been observed to downregulate autophagy, which may help to prevent degradation of virions and limit sensing of genomic RNA [416]. The Vpr and Vpu accessory proteins have been found to modulate the IFN response to detection in T cells, which may minimise the impact of any sensing that does occur [331].

To avoid cytosolic DNA sensing, reverse transcription of viral RNA can occur within the viral capsid [375], [417]. By performing reverse transcription within the capsid the resulting DNA is hidden from innate sensing [374], [375]. Recent evidence has suggested that the capsid remains associated with the viral RNA/DNA until reaching the nucleus, at which it may dock at the nuclear pore to deliver the genome [376], [418]. However, a fraction of virions are reported to uncoat prematurely, which may expose viral RNA and DNA to sensors and enable a response [419], [420]. Due to this, the presence of the host DNase TREX1 may actually be beneficial for HIV infection, since it can degrade DNA from virions which have prematurely uncoated and prevent interferon induction [409].

Interestingly, HIV has been reported to exploit sensing of viral RNA via TLR8 in dendritic cells to drive the NF-κB induction necessary to initiate transcription of provirus and viral replication [421]. There have also been reports of interactions between surface TLRs and HIV-1. Binding of HIV-1 envelope protein to TLR2 is capable of inhibiting responses to other TLR2 ligands [422]. HIV Tat is also capable of binding to TLR4 to induce expression of TNFα and IL-10 [423].

1.6. Dysfunctional innate immune responses in HIV-infected macrophages.

Viral modulation of host immunity may interfere with inflammatory signalling and compromise the ability of the cell to respond to other microbes [424]. HIV is known to be capable of infecting tissue-resident macrophage populations which are important for host defence, although these populations are technically difficult to isolate from patients for study [425]. Infection of alveolar macrophages was associated with reduced phagocytic function in these cells [396]. Dysregulation of cytokine expression has also been reported in alveolar macrophages of HIV patients [426]. Even in patients treated with antiretroviral therapy, residual immune dysregulation has still been observed [427]. This suggests that infection of macrophages by HIV-1 has the potential to alter their responses to other coinfecting pathogens.

1.6.1. HIV-1 and TB coinfection.

HIV coinfection is associated with increased morbidity and mortality from diseases such as *M. tuberculosis* [428], [429]. Both HIV and *M. tuberculosis* infect macrophages, with coinfection increasing the risk of latent TB reactivation 20-fold [430]. In addition to this, TB-associated immune reconstitution inflammatory syndrome (IRIS) is a common complication when starting antiretroviral therapy in coinfected patients [431]. As a result HIV-associated TB is the leading cause of AIDS-related death, accounting for 370,000 deaths in 2016 [432]. It is therefore important to understand the interactions between HIV and other pathogens. TB infection has also been found to accelerate the progression of HIV to AIDS [433].

HIV infection has previously been reported to increase mycobacterial burden in coinfected cultures via accelerated growth of the bacteria [434]. This may be linked to the effects of HIV on autophagy, which is also required for control of intracellular mycobacterial growth [435], [436]. *M. tuberculosis* is also reported to influence HIV replication, possibly due to its ability to induce interferon signalling [437]. However, it is unclear whether mycobacterial infection promotes [438], [439] or suppresses HIV-1 replication [440]. This may depend on cell type and differentiation state [437]. These variable phenotypes may also depend on the particular *M. tuberculosis* strain which is present [441]. Samples from coinfected patients have revealed elevated viral production in the lung [442], [443].

Our lab previously utilised an *in vitro* model of human monocyte-derived macrophages to investigate the effects of coinfection with HIV-1 and *M. tuberculosis* on host-pathogen interactions [444]. Further work investigated how HIV-1 infection alters macrophage responses to *M. tuberculosis*. It was found that the virus dysregulates the innate immune response to

mycobacteria to generate exaggerated inflammatory responses [445]. Analysis of cytokine signalling at early timepoints after stimulation with *M. tuberculosis* ligands revealed that at 4 hours post-stimulation there was a specific defect in expression of IL-10. This defect in regulatory signalling was sufficient to explain the resulting enhancement of inflammatory cytokine secretion. While the mechanism was not determined, the resulting dysregulation of innate immune activation could contribute to the immunopathogenesis of HIV.

Evidence for IL-10 attenuation *in vivo* has been seen in HIV patients. Sputum and bronchoalveolar lavage samples from TB patients with HIV coinfection were found to contain lower IL-10 and higher inflammatory IL-1β content than HIV negative patients [445]. In addition to this, the transcriptome in response to challenge with the tuberculin skin test (TST) was compared between HIV patients and healthy volunteers [446]. TST-positive HIV-infected patients displayed deficient IL-10-inducible responses but normal Th1 responses to the challenge. Transcription factor binding site (TFBS) enrichment analysis was used to analyse the upstream regulators of these differences in the transcriptome. The differences between the groups primarily mapped to the STAT1, NF-κB and STAT3 transcription factors, with STAT3 being the major factor induced by IL-10 signalling. This would be consistent with the deficient IL-10 responses observed *in vitro*, providing further evidence for the clinical relevance of this phenotype.

1.6.2. Current understanding of the mechanism of IL-10 attenuation by HIV-1

To attempt to resolve the host and viral factors involved in IL-10 attenuation, a simplified model of this phenotype was developed in monocyte-derived macrophages [447]. The inducible cytokine response was measured by ELISA or qPCR after 4 hours of stimulation to investigate the early innate response (**Figure 1.9**). It was found that various bacterial and fungal stimuli could replace *M. tuberculosis* as the secondary stimulus, so the fungal cell wall derivative zymosan was selected for its capacity to induce high levels of IL-10 secretion. Furthermore, a single round HIV-1 vector produced the same phenotype as the full-length HIV-1 R9 BaL clone used previously. Use of this R9 Δenv strain with SIV VLP supplementation to provide Vpx enabled efficient infection of MDMs [448]. These changes made it possible to perform experiments at Containment Level 2 to investigate this host-pathogen interaction.

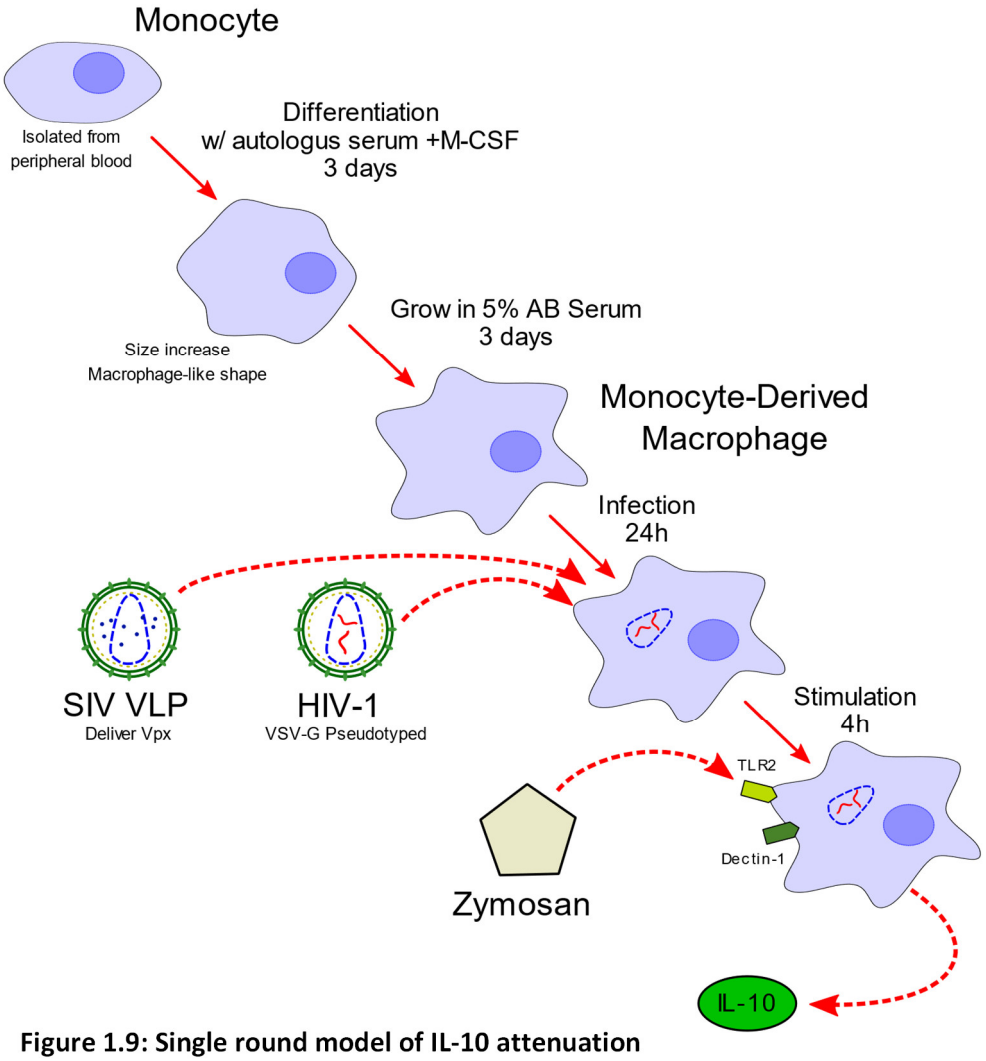

Figure 1.9: Single round model of IL-10 attenuation

Schematic of the model used in previous work to study IL-10 attenuation. Monocytes are isolated from peripheral blood by adherence before differentiation using M-CSF. After the 6-day differentiation protocol cells are infected with HIV-1 (R9 Δenv) supplemented with SIV VLPs. After 24h of infection the inducible IL-10 response to zymosan is tested. Cells are stimulated with zymosan for 4h prior to collection of supernatants for cytokine ELISA.

Treatment of infected cells with the protease inhibitor indinavir did not prevent IL-10 attenuation, despite blocking the spread of infection. This indicated that production of infectious particles is not required, and the HIV protease enzyme is not involved in the phenotype. This would also mean that the single round infection model and the spreading infection model are functionally equivalent and comparable. The timing of IL-10 attenuation after infection was also studied. Defective IL-10 production could be observed after 24h of infection with HIV-1, but not after 4h. This suggested that that a time-dependent process was required. The cell type specificity of the phenotype was assessed, with no effect of HIV-1 infection seen in monocyte-derived dendritic cells or MDMs differentiated with GM-CSF rather than M-CSF. However, this may be the result of the limited IL-10 production in general seen in these cell types.

To investigate the mechanism, previous work aimed to determine which stage of IL-10 production was influenced by HIV-1 infection. Since IL-10 can be regulated post-transcriptionally via effects on mRNA stability [194], actinomycin D was used to inhibit transcription and the decay of IL-10 RNA was traced over time. In HIV-infected MDMs the IL-10 transcripts' half-lives were longer, but the overall level of RNA was reduced. This indicates that infection primarily affects IL-10 at the transcriptional level, eliminating the post-transcriptional regulatory mechanisms as a hypothesis.

Small molecule inhibitors were used to investigate which pathways involved in regulating IL-10 may be affected by HIV-1 infection. Inhibition of p38, ERK and Pyk2 all significantly attenuated the IL-10 response to zymosan, but this was not specific and also inhibited IL-6 expression (unlike HIV). In contrast, inhibitors of the PI3K-Akt-mTOR axis specifically effected IL-10 without off-target effects, phenocopying the effects of the virus. However, no impact on the phosphorylation of Akt at one important site (Ser473) was seen by Western blotting. These data would suggest that this pathway may be involved in IL-10 attenuation by HIV-1, although care must be taken when interpreting these results due to the multitude of other cellular functions regulated by pathways such as MAPK. Total inhibition of signalling pathways may not be representative of any effects of HIV-1.

Overall, previous work from the lab suggested that HIV infection inhibited IL-10 responses at the transcriptional level, potentially via inhibition of signalling through the PI3K axis without affecting the pro-inflammatory cytokine response. However, the precise host and viral mechanisms remained unclear.

These findings were of interest for three reasons. First, it reflects a previously unknown host-virus interaction. Second, it offers a potentially novel mechanism that may contribute to chronic immune activation in progressive HIV disease, and third, it provides a model by which to explore the molecular pathways that control differential regulation of cytokine production in the innate immune response.

1.7. Research Objectives

In this thesis I sought to address the gaps in our understanding of IL-10 attenuation in macrophages. My overall research objectives for this thesis are as follows. These will be expanded upon within each chapter.

1) Identify the components of HIV-1 necessary for specific attenuation of macrophage IL-10 responses
2) Identify the components of HIV-1 sufficient for specific attenuation of macrophage IL-10 responses
3) Investigate the host factors that are affected by HIV-1 for specific attenuation of macrophage IL-10 responses

2. Methods

2.1. Reagents, solutions and media.

2.1.1. Buffers and Solutions

Solution	Composition
Phosphate-buffered saline (PBS)	950mg/L Phosphate (as sodium phosphates) 201mg/L Potassium Chloride (KCl) 8120mg/L Sodium Chloride (NaCl) 1 tablet (Gibco) in 500ml of dH_2O For sterile cell culture, pre-formulated PBS (Gibco) with and without Ca and Mg was used.
PBS-Tween	PBS (as above) with 0.05% Tween-20 (Fisher Scientific)
Lysis Buffer	50mM Tris pH 8.0, 150mM NaCl, 1mM EDTA, 10% glycerol, 1% Triton X-100, and 0.05% NP-40 supplemented with protease inhibitors (Roche). Phosphatase inhibitor cocktail tablets (Roche) were added when blotting phospho-proteins.
MES Running Buffer	Proprietary (Life Technologies, Novex). 50ml in 1L dH_2O
Transfer Buffer	**10x** – 500ml dH_2O, 15g Tris, 72g Glycine **1x** – 350ml dH2O, 100ml Methanol (Sigma), 50ml 10x transfer buffer
Loading Buffer	Proprietary NuPAGE LDS Sample Buffer (4x) (Life Technologies)
Tris/borate/EDTA TBE	**10x:** 108g Tris, 55g boric acid, 7.5 g EDTA disodium salt, made up to 1L with dH_2O **1x:** 10x diluted 1 in 10 with dH_2O
20% Sucrose	20% sucrose (Sigma) in sterile PBS, 0.22µM filtered
TFB1	30mM Kac, 100mM RbCl, 10mM $CaCl_2$, 50mM $MnCl_2$, 15% Glycerol in dH_20.
TFB2	10mM PIPES pH 6.5, 10mM RbCl, 75mM $CaCl_2$, 15% Glycerol in dH_20.
Milk Blocking Buffer	10% dehydrated milk powder (Sainsbury's) in PBS
BSA Blocking Buffer	10% weight/volume Bovine Serum Albumin (Sigma) in PBS
NGS Blocking Buffer	10% volume normal goat serum (Life Technologies) in PBS
Cell Freezing Buffer	10% DMSO (Sigma) in heat-inactivated FBS (Biosera)
FACS Buffer	1% (Biosera) FCS in PBS

Table 2.1: Contents of buffers and solutions

2.1.2. Cell Types, growth conditions

Cell Type	Medium	Culture Conditions
NP-2 Cells	DMEM (Sigma) supplemented with 10% FCS (Biosera S1800), puromycin (1µg/ml; Sigma) and G418 (100µg/ml, Sigma)	37°C, 5% CO2
HEK293T Cells	DMEM (Sigma) supplemented with 10% FCS (Biosera)	37°C, 10% CO2
THP-1 Cells	Standard Media: RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biosera) Selection Media: RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biosera) and 100µg/ml hygromycin (Invitrogen)	37°C, 5% CO2
X4R5 GHOST Cells	RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biosera), 500µg/ml G148 (Sigma), 100µg/ml hygromycin (Invitrogen) and 1µg/ml puromycin (Calbiochem)	37°C, 5% CO2
Primary Human MDMs	Differentiation Media: RPMI-1640 supplemented with 10% heat-inactivated autologous serum and M-CSF (20ng/ml, R&D Systems)	37°C, 5% CO2
	Maintenance Media: RPMI-1640 supplemented with 5% heat-inactivated pooled human AB serum (Sigma)	
	FCS Maintenance Media: RPMI-1640 (Invitrogen) supplemented with 10% FCS (Biosera)	

Table 2.2: Culture conditions for mammalian cells

Adherent cell lines were split by detaching with Trypsin (Invitrogen) according to the manufacturer's instructions.

2.2. Culture of primary cells.

Written informed consent was obtained from all blood donors. The joint University College London/University College London Hospitals National Health Service Trust Human Research Ethics Committee approved this study.

2.2.1. Isolation of PBMCs

Up to 120ml of blood was obtained from volunteers and collected into heparinised syringes. This was mixed in a 2:1 ratio with DPBS (with Ca and Mg, Gibco) and layered over Ficoll Paque PLUS density gradient media (GE Healthcare) or separated using pre-filled Leucosep tubes (Greiner Bio One). The blood was centrifuged for 20 minutes at 800g with the lowest brake to separate the PBMC fraction. To transfer the PBMCs into a fresh 50ml falcon, a pasteur pipette was used to collect cells from Ficoll tubes while the layer from Leucosep tubes was collected by removing excess serum and pouring off the desired fraction. The PBMC-containing tubes were then topped up to 50ml with DPBS and centrifuged at 800g for 10 minutes. Following this the supernatant was poured off and the cells were resuspended in 50ml DPBS before spinning for 5 minutes at 400g. This process was repeated for a total of 3x 5-minute washes. PBMCs were then counted and resuspended at 1_{x10}^{7}/ml in maintenance media before seeding according to **Table 2.3**.

When autologous serum was needed, up to 20ml of blood was collected into unheparinised syringes and spun at 1000g for 10 minutes in serum separation tubes (Starstedt). The serum fraction was collected into a fresh 15ml falcon using a pipette and then heat inactivated for at least 30 minutes in a 56^{o}C water bath.

Dish	Culture surface (cm^2)	Seeding Volume	Culture Volume (ml) /well	Estimated MDMs	Stimulation Volume (µl)
96-well	0.32	100µl	200µl	5 x10^4	50µl
48-well	1.1	200µl	500µl	1 x10^5	100µl
24-well	1.9	400µl	1ml	2 x10^5	200µl
12-well	3.5	800µl	2ml	5 x10^5	400µl
6-well	9.6	2.0ml	3ml	1 x10^6	1ml
10 cm Dish	56.7	10ml	12ml	5 x10^6	5ml

Table 2.3: Seeding densities for MDMs

2.2.2. Selection of monocytes by adhesion and differentiation into MDMs.

PBMCs were seeded onto plates as described above. After 1 hour of incubation at 37^{o}C cells were washed 3 times with DPBS (+Ca, +Mg) to remove non-adherent cells. The adherent monocytes were then cultured for 3 days in autologous differentiation media containing M-CSF, described in **Table 2.2**. At day 3 the media was then replaced with fresh maintenance media

with human AB or foetal bovine serum depending on the experiment. At day 6 differentiated macrophages were ready to be used. Cells can be maintained for several weeks by providing fresh maintenance media every 3-4 days. Typical yields are described in **Table 2.3**, with less than 5% lymphocyte contamination [444].

2.3. Culture of cell lines

2.3.1. THP-1 culture

Non-adherent THP-1 cells were maintained between 1_{x10}^5 and 1_{x10}^6 per ml by splitting the culture every 2-3 days. Cells were kept in media and conditions described in **Table 2.2**.

2.3.2. THP differentiation.

THP-1 cells were seeded onto plates at the densities described in **Table 2.4**. Cells were 'differentiated' by treatment with 200nM phorbol 12-myristate 13-acetate (PMA) for 48 hours. After this the differentiation media was changed with fresh culture media and cells were 'rested' for 5 days as described by the Dockrell lab [449]. Any non-adherent cells after the resting period were removed before stimulation or infection.

	Seeding Density	Growth Medium (ml)
6-well	1.2 x 10^6	3 – 5
12-well	0.4 x 10^6	1- 2
24-well	0.2 x 10^6	0.5 - 1.0

Table 2.4: Seeding densities for THP-1 cells

2.3.3. HEK-293T culture

Human Embryonic Kidney 293T cells were cultured in RPMI + 10% FCS according to **Table 2.2** and split 1:4 three times each week. Every 20 passages fresh stocks were thawed from liquid nitrogen. There cells were primarily used for virus production.

2.3.4. NP2 culture

This astrocytoma cell line was stably transduced with HIV-1 co-receptors CD4 and CXCR4 for use in titrating HIV-1 vectors. Cells were cultured in DMEM-based selection media as described in **Table 2.2**. Cells were passaged 1:10 each week.

2.3.5. X4R5 GHOST culture

This human osteosarcoma (OST)-derived cell line was stably transduced with HIV-1 receptors CD4, CXCR4 and CCR5 to produce a permissive cell line for vector titration. An additional GFP reporter driven by HIV-1 Tat was also stably transduced to simplify detection of

infection. These cells were passaged 1:8 twice a week and maintained in an **RPMI**-based selection media as described in **Table 2.2**.

2.4. HIV-1 vectors

All *env*-deleted strains were pseudotyped with VSV-G. As single-round vectors these were used in Category 2 laboratories under appropriate safety precautions.

2.4.1. Production

Vectors were produced by transfecting T150 flasks of HEK293T cells grown to 70% confluency. Media was changed 4 hours prior to transfection with 15ml of fresh growth media per flask. Plasmid DNA was prepared in 500µl of Optimem (Life Technologies) and 30µl of Fugene 6 (Promega) per flask, with plasmid concentrations as described in **Table 2.5**.

Type	DNA per T150 flask
Virus-like particles (VLPs)	8µg packaging plasmid (e.g. SIV3+) 2µg pMDG (VSV-G)
Two plasmid vectors	8µg vector genome (e.g. R9 delta env) 2µg pMDG (VSV-G)
Three plasmid vectors	8µg vector genome (e.g. CSGW) 8µg packaging plasmid (e.g. 8.91) 2µg pMDG (VSV-G)

Table 2.5: Plasmid mixes for vector production

The DNA mix was left at room temperature for 20 minutes to mix, after which it was added drop-wise to the cultures. The following day the culture media was replaced with 24ml of fresh growth media per flask. Every 24h for up to 3 days, virus-containing supernatant was collected into 50ml falcons and filtered through 0.45µm syringe filters (Millipore) to remove contaminating cells. This could then be stored at -80^{o}C until further purification.

2.4.2. Purification

Virus-containing supernatant was thawed at 37^{o}C before being added to ultracentrifuge tubes (36ml, Beckman-Coulter). 25ml of supernatant was layered in each tube over a 5ml cushion of filter-sterilised 20% sucrose solution. The tubes were then spun in an ultracentrifuge for 2 hours at 23,000 RPM at 4^{o}C using a Sorvall Sure-Spin 630 rotor. Pelleted virus was resuspended in serum-free RPMI-1640 medium, aliquoted and stored at -80^{o}C.

2.5. Titration

NP2 cells were initially used for titration of viruses encoding HIV Gag. During this study the GHOST cell line containing a Tat-inducible GFP reporter became available, which was adopted as the preferred method for titrating Tat-expressing vectors. Vectors with GFP-expressing genomes such as CSGW, LAI and SFXUC were also titrated using the GHOST line by detecting expression of the genome-encoded marker. For Gag and Tat-deficient vectors the Roche RT ELISA assay was initially used to analyse reverse transcriptase activity. This was later superseded by the SG-PERT qPCR assay and the genome copy qPCR assay developed by the Towers lab (UCL), both of which are more sensitive than the ELISA.

2.5.1. GHOST Titration

GHOST cells were seeded onto 6-well plates 24 hours prior to titration. Cells were then inoculated with virus serially diluted 1-in-3. After 48 hours of incubation cells were detached with trypsin and fixed with paraformaldehyde. The fixed cells were analysed using a BD Accuri C6 flow cytometer, which was used to quantify the percentage expressing GFP (indicating infection). Titration results were determined by calculating the infectious units per ml of input virus.

2.5.2. RT ELISA

For non-integrative vectors, reverse transcriptase (RT) activity was measured using a colorimetric kit as per the manufacturer's instructions (Roche). Each nanogram of RT activity corresponded to approximately $1_{x10}{}^{6}$ infectious units as detected in other assays. It should be noted that RT activity alone does not necessarily indicate infectious virus.

2.5.3. SG-PERT

The SG-PERT is an RT-qPCR assay used to measure activity of viral reverse transcriptase [450]. Virus was lysed, then the reaction was set up as per the published protocol using the primers in **Table 2.6**. Recombinant HIV RT (Applied Biosystems) was used to create standards for quantitation. The reaction was run on an ABI 7500 FAST system using the program in **Table 2.7**. This assay quantifies the amount of active reverse transcriptase in a sample of virus, but it should be noted that this does not necessarily correspond to infectious vector. By using an equivalent dosage of RT during infection it was possible to utilise virus-like particles and other non-integrative vectors which weren't compatible with other titration methods.

Component	Sequence
Forward primer	TCCTGCTCAACTTCCTGTCGAG
GAPDH reverse primer	CACAGGTCAAACCTCCTAGGAATG

Table 2.6: Primers for qPCR

	Step	Time	Temperature (°c)
1 cycle	**Reverse Transcription**	20 min	42
	Taq initial heat activation	15 min	95
40 cycles	**Denaturation**	10 sec	95
	Annealing	30 sec	60
	Extension	15 sec	72

Table 2.7: qPCR cycling conditions for SG-PERT

2.5.4. Genome Copy qPCR

This assay was used to detect copies of the HIV-1 RNA genome in vectors deficient for reverse transcriptase activity. RNA was extracted and purified as with the SG-PERT assays, but reverse transcription was performed using SuperScript III (Thermo) rather than viral enzyme in the reaction mix recommended by the manufacturer's protocol. Cycling conditions for the RT step are indicated in **Table 2.8**, below.

	Step	Time	Temperature (°c)
1 cycle	**Denaturation**	5 min	25
	Reverse Transcription	60 min	50
	Heat Inactivation	15 min	70

Table 2.8: Cycling conditions for genome copy qPCR

Following RT, qPCR for viral DNA was performed with the same primers and cycling conditions as the SG-PERT assay. Unlike the SG-PERT, results represent the total amounts of viral RNA in a sample as opposed to viral RT activity.

2.6. Plasmid amplification and cloning

Molecular cloning was used to produce novel deletion mutants.

2.6.1. Preparation of competent bacteria

The *E.coli* strain HB101 was used for production and amplification of retroviral vectors due to its lack of *RecA*. Bacteria were grown on a shaker at 37°C in a small volume of LB broth (Sigma) overnight to produce a starter culture. This was then added to 200ml of fresh broth and

incubated in a 30°C shaking incubator until the culture reached an OD550 of 0.45-0.55. The culture was then chilled on ice for 10 minutes before centrifugation at 3000RPM for 20 minutes. The pellet was resuspended in 20ml of buffer TFB1 (**Table 2.1**) before incubation for 5 minutes on ice. The centrifugation was repeated before the pellet was resuspended in 2mls of cold buffer TFB2 (**Table 2.1**). After 10 minutes on ice the bacteria were aliquoted and stored at -80°C.

2.6.2. Transformation

Up to 1µg of plasmid DNA was added to 30µl of competent bacteria stock. The bacteria were incubated for 20 minutes on ice and then heat-shocked at 42°C for 45 seconds. After further incubation on ice for 10 minutes the bacteria were spread onto LB Agar plates (Sigma, with relevant antibiotic as required). Plates were incubated overnight at 37°C to allow colonies to grow.

2.6.3. Plasmid amplification

Plasmids were amplified by picking colonies from transformed plates and inoculating into LB broth. Bacteria were then grown overnight at 37°C before purification using Qiagen Mini/Midi/Maxiprep kits according to the manufacturer's instructions. Extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher) and stored at -20°C.

2.6.4. Restriction digestion

Restriction digestions were performed on 1µg of plasmid DNA using the necessary restriction enzymes (Promega/New England Biolabs) according to the manufacturer's instructions. For example, cloning of accessory gene mutations was usually achieved by using a BamH1-Sal1 digest at 37°C for 1 hour. Digested DNA was then run on 1% agarose gels (+ ethidium bromide) in TBE buffer at 200v for 30 minutes before the desired bands were visualised using UV light and cut out of the gel. Fragments were then extracted using QiaQuick Gel Extraction Kits (Qiagen) according to the manufacturer's instructions.

2.6.5. Ligation

Ligations were performed using T4 Ligase (New England Biolabs) at 15°C for up to 16 hours. Ratios of fragments were calculated using the NEB ligation calculator at a 3:1 ratio and used in a reaction volume of 20µl.

2.7. Lentivector strains

A variety of VSV-G pseudotyped vectors were utilised in this study. These are described in **Table 2.9**.

Vector	Description
R9 Δenv	This clone was created from the full length R9 BaL clone [451] by Dr Elspeth Potton (UCL). A 445bp deletion in the *env* gene was used to create a single round vector.
R9 Δenv Δnef	Provided by Dr Elspeth Potton (UCL). The fragment with a premature stop codon in the *nef* gene was cloned from a full length NL4.3 clone [452] into the R9 Δenv backbone.
R9 Δenv Δvif	A fragment from the virus VH17 [453], which has nonsense mutations in Vif was cloned into the R9 Δenv backbone by Dr Lucy Bell (UCL).
R9 Δenv Δvpr	Dr Jane Rasaiyaah (UCL) provided a full length NL4.3 molecular clone with a mutation in the start codon of Vpr. R9 was based on an NL4.3/HXB2 hybrid sequence but has subsequently had most HXB2 sequence removed, so the R9 Δenv BamHI-SalI fragment containing the Env deletion was cloned into this backbone by Dr Lucy Bell (UCL) to produce this construct.
R9 Δenv Δvpu	An env-deleted NL4.3 clone with an early frameshift mutation in the vpu gene leading to a premature stop codon was obtained from Prof Stuart Neil (KCL). The BamHI-SalI fragment containing the vpu and env mutations was then cloned into the R9 Δenv backbone.
R9 Δenv Δvpr Δvpu	The *vpu* deletion from R9 Δenv Δvpu was cloned into R9 Δenv Δvpr to produce this double mutant.
R9 Δenv ΔRT	A segment of the RT sequence was deleted from R9 Δenv in the middle of the gene, resulting in a frameshift and premature termination.
LAI Δenv GFP	This virus was obtained from Dr Becky Sumner (UCL) and contains a GFP gene in place of *nef* to enable easy detection of infection.
LAI Δenv GagLuc GFP	This virus was obtained from the Towers Lab. A luciferase gene has been inserted into the coding sequence for HIV capsid within Gag.
CSGW	Initially described by Bainbridge *et al.* (2001) [454]. CSGW is a self-inactivating HIV-1 vector modified from HR' [455]. Viral genes have been inactivated and replaced with an SFFV MLV LTR driving GFP expression.
SFXUC/G	This HIV-1 vector was derived from CSGW by David Escors. A transgene is inserted under the SFFV promoter, while an additional ubiquitin promoter drives expression of either GFP (SFXUG) or mCherry (SFXUG).

Table 2.9: Viral strains used in this study

2.7.1. R9

R9 BaL is related to the laboratory strain R9. This vector is predominantly based on the Group M strain NL4.3, with a *nef* sequence from HXB2 and the *env* from BaL. A 500bp deletion in the *env* gene was generated by Elspeth Potton (UCL) to create a single round vector. This has been termed “R9 delta env” in this study.

2.7.2. LAI GFP

HIV LAI is another laboratory strain from Group M. The vector's *nef* gene had been replaced with GFP to enable straightforward visualisation of infection.

2.7.3. Empty genomes

To test whether HIV-1 genes are necessary, vectors typically used in gene therapy were tested. CSGW is a simplified viral genome encoding only GFP under a CMV promoter, bounded by viral LTRs. SFXUC/G is a similar construct, but with two promoters: SFFV for a desired transgene and an ubiquitin promoter to drive a GFP or mCherry reporter.

2.7.4. Packaging plasmids

Viruses defective for essential proteins, such as those with empty genomes, were packaged by supplying viral genes on a separate packaging plasmid during transfection. These plasmids lack LTR and packaging signals and so are not readily incorporated into virions but can provide the necessary proteins for virus formation. Two such plasmids were used in this study: 8.2 which encodes all viral genes except for *env*, and 8.91 which also lacks the accessory proteins *vif, vpr, vpu* and *nef*.

2.7.5. Virus-like particles

Virus-like particles were produced by transfecting packaging and envelope plasmids in the absence of viral genome plasmid. This generates mature virions which lack viral genomes and so can be used to deliver viral proteins without genetic material. It is currently unknown to what extent host cell RNAs may be incorporated into these structures in place of viral RNA.

2.8. Infection of cultured cells.

Cells were infected with vectors for 24 hours. Frozen virus stocks were thawed and added to warm media before being added to cells. An MOI of 10IU_{GHOST}/cell was used to transduce approximately 20-40% of MDMs. When supplemented with SIV VLPs (for Vpx-mediated degradation of the restriction factor SAMHD1) up to 100% of the cells could be infected.

2.9. Innate immune stimulation.

Innate immune stimuli were added to media at the concentrations listed in **Table 2.10**. During secondary stimulation cells were stimulated for 4 hours prior to harvest of supernatant, RNA or fixed cells for staining.

Innate immune stimuli	Concentration	Manufacturer
Zymosan from *Saccharomyces cerevisiae* cell wall	0.4mg/ml	Invivogen
Zymosan from *Saccharomyces cerevisiae* cell wall, Alexa Fluor 488 Conjugate	0.4mg/ml	Life Technologies
Pam3-Cys-Ser-Lys4 (Pam3CSK4)	100ng/ml	Axis-Shield
Curdlan (β-1,3-glucan from *Alcaligenes faecalis*)	0.1mg/ml	Wako Chemicals
LPS	100ng/ml	Invivogen
Poly(I:C)	10µg/ml	Invivogen
IFNγ	10ng/ml	Peprotech
IFNα (clinical grade)	200IU/ml	Teva
IFNβ (clinical grade)	200IU/ml	Merck Serono
ssRNA40	5µg/ml	Invivogen
ssRNA41	5µg/ml	Invivogen
CL075	100ng/ml	Invivogen

Table 2.10: Innate immune stimuli used in this thesis

2.10. ELISA for cytokine secretion

Cytokine production was quantified by testing culture supernatants with eBioscience Ready-Set-Go! ELISA Kits (**Table 2.11**). Kits were used according to the manufacturer's protocols with supernatants diluted 1:6 for all kits except for TNF, which was diluted 1:100 due to high production. Standards were generated using a 1:2 serial dilution with 4000pg/ml as the top concentration. All samples were tested in duplicate wells.

Kit Target	Product Code	Analytical Sensitivity (pg/ml)
Human IL-10	88-7106-88	12
Human IL-6	88-7066-88	12
Human IL-8	88-8086-88	12
Human TNF-α	88-7346-88	400

Table 2.11: ELISA kits (Thermo Fisher) used in this thesis

2.11. qPCR for RNA expression

RNA from stimulated cells in 24 well plates was lysed with 350µl of buffer RLT (Qiagen) from the RNeasy Mini Kit. This was stored at -80^{o}C until extraction.

2.11.1. RNA Extraction

RNA samples in buffer RLT were thawed and then extracted using RNeasy Mini Spin Columns according to the manufacturer's instructions. RNA was eluted in 30µl of molecular grade water (Sigma) before being quantified using a Nanodrop 2000 spectrometer.

2.11.2. DNase treatment

To remove DNA contamination from extracted RNA, samples were treated with a Turbo DNA-Free kit (Thermo) according to the manufacturer's instructions.

2.11.3. Reverse transcription

RNA was reverse transcribed into cDNA using a qScript cDNA Synthesis Kit (Quanta Bio) according to the manufacturer's instructions. 5µl of the extracted RNA was added to the reaction mix and incubated in a thermal cycler for 30 minutes at 42°C, followed by 5 minutes at 85°C in a reaction volume of 20µl. The resulting cDNA was then stored at -80°C.

2.11.4. qPCR

qPCR was performed using an Applied Biosystems 7500 FAST qPCR machine, using the Taqman reaction system (Applied Biosystems). Inventoried assays are detailed in **Table 2.12**. Reactions were made up using 5µl of reaction mix, 1µl of primer and 1µl of cDNA for the inventoried assays (made up to 10µl with dH_2O). Quantification was performed using comparative CT relative to GAPDH, which used a custom primer-probe set detailed below (**Table 2.13**). 2.4µl of GAPDH primer and 0.4µl of probe were used in each 10µl reaction.

Gene Target	Applied Biosystems Assay ID
IL10	Hs00961622_m1
IL6	Hs00985639_m1
TNFA	Hs00174128_m1
CXCL10	Hs00171042_m1
IFI16	Hs00986757_m1
HIV-1 LTR	Pa03453409_s1

Table 2.12: TaqMan assays used in this thesis

Component	Sequence
GAPDH forward primer	GGC TGA GAA CGG GAA GCT T
GAPDH reverse primer	AGG GAT CTC GCT CCT GGA A
GAPDH probe	TCA TCA ATG GAA ATC CCA TCA CCA

Table 2.13: Control primers used with TaqMan assays

2.12. Detection of proteins

Antibodies were used to stain proteins and cytokines for for immunofluorescence or Western blotting. Antibodies used in this study and their relative staining concentrations are detailed in **Table 2.14**. Cells were left in PBS for imaging and stored at 4°C.

Antibody Target	Species	Product Code	Dilution	Supplier
Primary Antibodies				
HIV-1 p24	Mouse	E365/366	1:1000	NIBSC
MCM2	Mouse	610700	1:300	BD
β-actin	Mouse	4697	1:10000	Cell Signal
Tubulin	Mouse	Ab7291	1:10000	Abcam
VCP	Rabbit	sc-20799	1:1000	Santa Cruz
GSK3	Rabbit	5676	1:1000	Cell Signal
Phospho GSK3 (Ser21/9)	Rabbit	8506	1:1000	Cell Signal
PI3K	Rabbit	4257	1:1000	Cell Signal
Akt	Rabbit	4691	1:1000	Cell Signal
Phospho Akt (Ser473)	Rabbit	4058	1:1000	Cell Signal
Phospho Akt (Thr308)	Rabbit	13038	1:1000	Cell Signal
Erk1/2	Rabbit	9102	1:1000	Cell Signal
Phospho Erk1/2 (Thr202/Tyr204)	Rabbit	4377	1:1000	Cell Signal
P38	Rabbit	9212	1:1000	Cell Signal
Phospho P38 (Thr180)	Rabbit	9211	1:1000	Cell Signal
NFKB P65	Rabbit	sc-372	1:1000	Santa Cruz
IRF3	Rabbit	11904	1:1000	Cell Signal
Secondary Antibodies				
Anti-mouse AF488 Conjugate	Goat	A11009	1:1000	Invitrogen
Anti-mouse AF555 Conjugate	Goat	A21422	1:1000	Invitrogen
Anti-mouse AF647 Conjugate	Goat	A21235	1:1000	Invitrogen
Anti-rabbit AF488 Conjugate	Goat	A11008	1:1000	Invitrogen
Anti-rabbit AF546 Conjugate	Goat	A11010	1:1000	Invitrogen
Anti-rabbit AF647 Conjugate	Goat	A21244	1:1000	Invitrogen
Anti-mouse BGAL Conjugate	Goat	1010-16	1:400	Southern Biotech
Anti-mouse IRDye 800CW Conjugate	Goat	925-32210	1:10000	Li-Cor
Anti-rabbit IRDye 800CW Conjugate	Goat	925-32211	1:10000	Li-Cor

Table 2.14: Antibodies used in this thesis

2.12.1. Inhibition of cytokine secretion

Treatment with the golgi transport inhibitor Brefeldin A (Biolegend) at 5µg/ml for up to 4 hours was used to prevent secretion of cytokines and therefore enable fluorescent staining.

2.12.2. Fixation

Cells were fixed and permeabilised prior to staining. Chilled 4% paraformaldehyde (Sigma) in PBS was added to cells for 30 minutes at room temperature, after which cells were washed

with PBS (Gibco). 0.1% Triton-X100 (Sigma) was added for 20 minutes to permeabilise cells fixed via the PFA method, followed by another PBS wash. Cells in PBS were stored at 4°C prior to staining.

Alternatively, cells were fixed with -20°C 1:1 methanol:acetone solution applied for 10 minutes before PBS washing and storage of fixed cells. Since methanol is destructive to many fluorophores this method was not compatible with cells expressing markers such as GFP, but it does eliminate the need for a permeabilization step.

2.12.3. Protein staining

Fixed and permeabilised cells were blocked with 10% normal goat serum (Life Technologies) in PBS for 30 minutes. Primary antibody in blocking buffer was then added and incubated at room temperature for 1 hour. The cells were then washed 3 times with PBS before applying the secondary antibody in blocking buffer for 1 hour at room temperature. Finally, cells were washed again as above to remove unbound antibody.

2.12.4. Nuclear staining

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) is a fluorescent dye which binds dsDNA. DAPI (Sigma) was applied at a concentration of 1µg/ml in PBS for 5 minutes before cells were washed 3 times with PBS to remove residual dye. DAPI enters live cells much more slowly than fixed or dead cells, and so on live cells Hoechst 33342 (Life Technologies) was used instead at a concentration of 1µg/ml, following the same protocol.

2.13. FISH

Some proteins, such as IL-10, were not amenable to antibody staining. Therefore, I utilised the ViewRNA (eBioscience/Thermo) single-molecule RNA FISH system to stain for RNA instead, allowing for single-cell detection of RNA expression.

2.13.1. Principal

To achieve sufficient sensitivity and specificity, permeabilised cells are incubated with sets of 20-40 oligomers specific to the target of choice. These ~20bp oligomers each include an additional site which can bind a preamplifier molecule in locations where two oligomers have annealed to RNA in close proximity to each other. Multiple amplifier molecules are then

hybridised to the preamplifiers, which can then have fluorescent probes bound to them to allow visualisation of the RNA. A schematic is depicted in **Figure 2.1**.

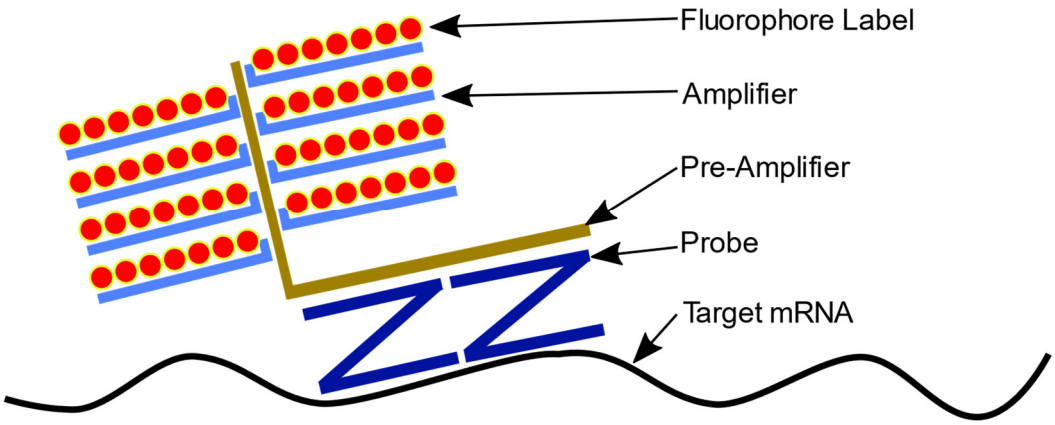

Figure 2.1: RNA FISH probe hybridisation structure

Schematic of RNA FISH probes bound to an RNA molecule. Sequential binding of RNA probes and amplifiers provides sensitivity and specificity. Two probes must bind in close proximity for subsequent amplification.

2.13.2. Fluorophores and microscope

The ViewRNA system can be multiplexed by using oligos which bind amplifiers specific for different fluorophores. The probes used and their relative fluorophores are detailed in **Table 2.15**.

Target	Fluorophore (Type)	Probe Set ID
IL10	Alexa Fluor 546 (Type 1)	VA1-10840
IL6	Alexa Fluor 488 (Type 4)	VA4-15969
CXCL10	Alexa Fluor 488 (Type 4)	VA4-19075
IFIT1	Alexa Fluor 488 (Type 4)	VA4-18833
TNFA	Alexa Fluor 647 (Type 6)	VA6-11200
HIV-1 gagpol	Alexa Fluor 647 (Type 6)	VA6-17396

Table 2.15: RNA FISH probe sets used for microscopy

2.13.3. Hermes FISH

Imaging of RNA FISH was carried out on a Hermes Wiscan (IDEA Bio-Medical) wide field, high-content microscope using a 20x or 40x objective. Staining in captured images was quantified using the Metamorph 7 software suite, followed by data analysis using R.

2.14. Image analysis

Multiple image analysis techniques were used during this study to quantify staining and other features within cells of interest. At least 10,000 cells were analysed in each experiment.

2.14.1. Metamorph

Metamorph 7 was used to process images from the Hermes and EVOS FL Auto II systems in an automated fashion. The inbuilt journals system was used to create scripts which loop through images, identify different channels and perform analysis. For detection of infection, the Multi Wavelength Cell Scoring package was used to evaluate staining in multiple colours for each cell. To analyse nuclear translocation, the Translocation-Enhanced package was used to compare staining inside and outside of nuclei.

During FISH assays many experiments lacked unused fluorescent wavelengths which could be used for a cell mask, which was necessary for compatibility with the Cell Scoring packages. To alleviate this, I used the Translocation-Enhanced algorithm to analyse an area immediately adjacent to the nuclei for RNA staining. This was sufficient for detection of cells expressing a target RNA as part of an immune response, which typically produced large quantities covering the whole cytoplasm.

2.14.2. Athena

During this study, the Athena platform (IDEA Bio-Medical) was released for the Hermes system. This software enables common analysis tasks to be performed with minimal user input. This was utilised for infection assays and cell counts to produce summary statistics for each condition.

2.14.3. ImageJ

ImageJ (NIH) was used for creation of image overlays and measurements of cellular statistics.

2.14.4. R

The R programming language was used to further analyse data from the image analysis scripts and generate relevant plots and figures as required. Graphs were generated using the ggplot2 and ggridges packages.

2.15. SDS-PAGE and Western Blotting

2.15.1. Sample preparation

Cells were lysed in 100µl of the lysis buffer detailed in **Table 2.1**. The buffer was pipetted up and down rigorously to lyse the cells before being transferred into Eppendorf tubes. NuPage Sample Buffer (Thermo) was added to the lysate, after which the samples were boiled at 100^{o}C for 5 minutes to denature the proteins.

2.15.2. Gel Loading

Up to 25µl of sample was loaded into each well of pre-cast 4-12% NuPage Gels (Thermo), which were loaded in MES buffer (Thermo) within SureLock tanks as per the manufacturer's instructions. 5µl of PageRuler Prestained Plus ladder (Thermo) was used to evaluate size of bands.

2.15.3. Gel Running and Transfer

Gels were run for 40 minutes at 200V under reducing conditions. Gels were then removed from their casts and left to equilibrate in transfer buffer (See **Table 2.1**) for 10 minutes. A stack was made consisting of transfer buffer-soaked filter paper, the membrane, the gel and then another filter paper. Protein bands were then transferred onto the nitrocellulose membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). This was run at 20V for 35 minutes.

2.15.4. Blocking

The nitrocellulose membranes were cut and then transferred into 50ml falcon tubes and loaded onto a roller. Membranes were blocked for 30 minutes using 5% Milk in PBS-Tween solution.

2.15.5. Antibody staining

Primary antibodies were diluted in milk/PBS-tween solution according to **Table 2.14**. 5ml of primary antibody solution was added into the falcon tubes with the membrane and incubated on the roller overnight at 4^{o}C.

Membranes were then washed 3 times for 5 minutes with PBS-Tween at room temperature. 5ml of secondary antibodies conjugated with infrared fluorophores (Li-Cor Biosciences) were added at 100ng/ml and incubated for 2 hours in the dark. Following this, membranes were washed 3 times with PBS-Tween as before and then rinsed in PBS for 5 minutes before being imaged.

2.15.6. Detection with Li-Cor

Completed blots were scanned using a Li-Cor Odyssey infrared imager. This system was capable of multiplexing stains using a red (700nm) and green (800nm) channel. Acquired scans were quantified using the Image Studio Lite (Li-Cor Biosciences) software package.

2.16. Statistical analysis of data

Statistical analysis of results was performed using the GraphPad Prism V6 software suite. Tests used are indicated in the relevant figure legends.

P values are represented as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

3. Results 1 – Which viral genes are necessary for IL-10 attenuation?

3.1. Introduction

The HIV-1 accessory proteins Vif, Vpr, Vpu and Nef have been described to modulate a diverse range of host cell functions (see section **1.5.3.3**), with each viral protein evidently able to interact with multiple host factors [456]. Therefore, I hypothesised that attenuation of IL-10 responses by HIV-1 infection of macrophages may be the result of a novel interaction between one or more viral accessory proteins and the host cell.

It has previously been shown that HIV-1 attenuation of innate immune IL-10 responses in monocyte-derived macrophages is evident in the *env*-deleted, single round vector R9 Δenv, which enabled this phenotype to be studied using a simplified model in Containment Level 2 [447]. Attenuated IL-10 responses were seen in response to a variety of innate immune stimuli after 24 hours of HIV-1 infection. The TLR2/Dectin-1 ligand zymosan, a glucan derived from the cell wall of *Saccharomyces cerevisiae* (yeast), was selected for modelling this phenotype due to the particularly high IL-10 production induced by this compound in macrophages [174].

The use of a single-round viral infection also enabled the investigation of the role of individual viral accessory genes through the use of knockout mutants, without any confounding effect these mutants may have on viral propagation. In order to identify the role of host molecular pathways that interact with the virus, I reasoned that it may be advantageous to use immortalised cell lines amenable to genetic manipulation by knockdown or gene editing approaches. Theoretically, such a model may also eliminate some of the donor to donor variability evident in primary cells and overcome the need for volunteer blood donors. However, innate immune responses are not always preserved in immortalised lines compared to primary cells [457].

In this chapter I sought to test the hypothesis that HIV-1 attenuation of IL-10 responses in monocyte-derived macrophages could be modelled by non-replicative HIV-1 virus infection of THP-1 cells, and to test the hypothesis that one or more HIV-1 accessory proteins were necessary for this phenotype.

The objectives of this chapter are as follows:

1) Determine whether THP-1 cells will produce IL-10 upon stimulation.
2) Investigate whether attenuation of IL-10 responses by single-round HIV-1 infection can be recapitulated in these cells.
3) Utilise HIV-1 deletion mutants to determine if any of the HIV-1 accessory genes were necessary for this phenotype.

3.2. Results

3.2.1. A resting step after differentiation enables THP-1 cells to secrete IL-10 in response to zymosan.

The THP-1 cell line was derived from an acute monocytic leukaemia patient in the early 1980s [458]. These monocyte-like cells can be "differentiated" via treatment with phorbol 12-myristate 13-acetate (PMA) to produce macrophage-like cells which do not divide [459]. This process causes the cells to become adherent, increase in size and express selected macrophage markers [460]. However, it was not known if THP-1 cells are capable of producing IL-10 in response to innate immune stimulation.

My first aim was therefore to test whether THP-1 cells produced IL-10 in response to zymosan stimulation. A previous report suggested that their macrophage-like features such as increased cytoplasmic to nuclear ratio and high phagocytic capacity could be optimised by 'resting' the cells for five days after PMA stimulation [449]. I measured IL-10 by ELISA in cell culture supernatants four hours after zymosan stimulation in monocytic THP-1 cells and PMA treated THP-1 cells, with and without a five-day resting period after treatment. Monocytic THPs did not secrete detectable IL-10 upon stimulation with zymosan. Small quantities were seen after PMA treatment (**Figure 3.1**), and substantially higher levels after resting. I therefore selected the resting protocol for subsequent experiments. Interestingly, detectable levels of IL-10 production were also evident in rested THP-1 cultures prior to stimulation.

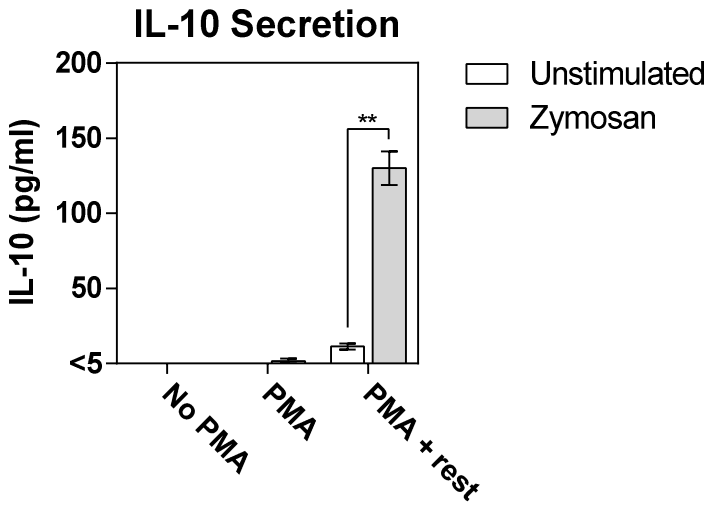

Figure 3.1: THP-1s secrete IL-10 in response to zymosan after PMA treatment

PMA-treated and untreated THP-1 cells, with or without a 5-day resting step after treatment, were stimulated for 4 hours with zymosan (0.4mg/ml). IL-10 content in supernatants was quantified by ELISA. Graph shows means ± SEM. Representative of 3 experiments. Significance was determined by paired t-test.

3.2.2. HIV-1 infection of THP-1 cells

Next, I tested the permissively of THP-1 cells to HIV-1 infection when differentiated with PMA and rested for five days. In the monocyte-derived macrophage model, HIV-1 infection can be enhanced through the addition of virus-like particles (VLPs) derived from Simian Immunodeficiency Virus (SIV). The SIV accessory protein Vpx, contained within these VLPs, counteracts HIV restriction by degrading the host factor SAMHD1 that otherwise depletes the cell of dNTPs required for viral reverse transcription [448] [461]. It was not known whether a similar system of Vpx complementation would enhance infection in differentiated THP-1 cells, so I performed a titration of virus with and without supplementation with SIV VLPs. Vpx complementation did not significantly enhance infection in the THP-1 clone used in this thesis (**Figure 3.2a**), allowing the SIV VLPs to be removed from the THP model in subsequent experiments. I then performed an expanded dose titration of virus to determine the optimal amount of virus for use with these cells (**Figure 3.2b**). An MOI of 5 $IU_{(NP2)}$/cell was selected as a dose which delivers virus to all cells without inducing noticeable cell death.

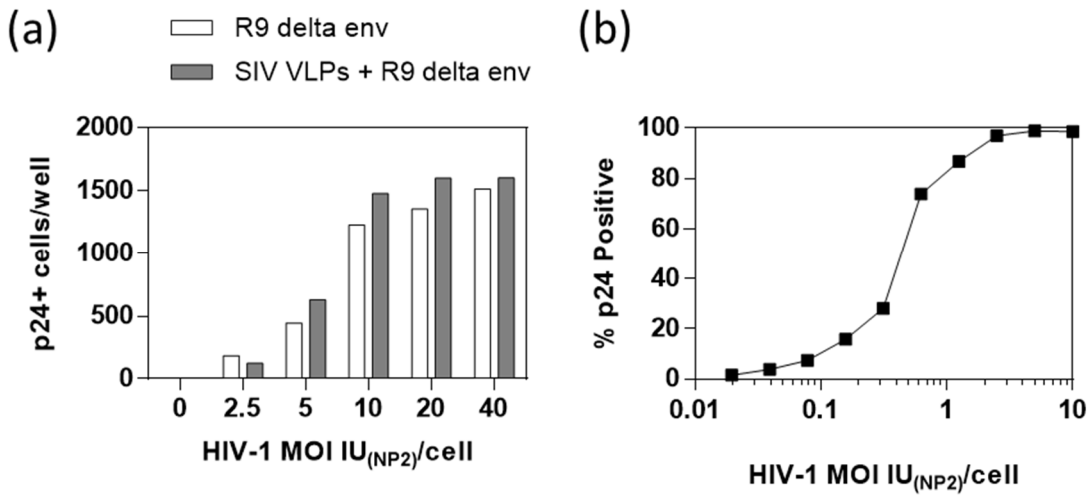

Figure 3.2: Infection of THP-1 cells with HIV-1.

THP-1 cells were infected for 48 hours with HIV-1 in a dose titration experiment. **(a)** Comparison of infection with and without complementation with SIV VLPs as determined by staining for p24+ cells using X-Gal. **(b)** An expanded dose titration in the absence of SIV VLPs, with infection determined by fluorescent staining of HIV p24 against DAPI staining of nuclei, allowing for quantitation of percentage infection. No cell death was observed up to 10 IU/cell. Representative of three experiments.

3.2.3. HIV-1 attenuates IL-10 responses in THP-1 cells

I then tested the effect of HIV-1 infection of PMA-differentiated and rested THP-1 cells on IL-10 production in response to zymosan stimulation. IL-10 in the cell culture supernatant was measured by ELISA after 4 hours of stimulation. In previous work within our group, attenuation of cytokine responses to zymosan was specific to IL-10. In the present experiment, I therefore also measured TNF and IL-6 production by THP-1 cells in order to test the specificity of any effect on IL-10. HIV-1 consistently attenuated IL-10 production in this model, while the inflammatory cytokines IL-6 and TNF were unchanged (**Figure 3.3**). These results mirrored previous observations in the primary monocyte-derived macrophage model, which demonstrated that attenuation of IL-10 responses by HIV-1 could be successfully replicated in this THP-1 cell line. The finalised model can be seen in **Figure 3.4**.

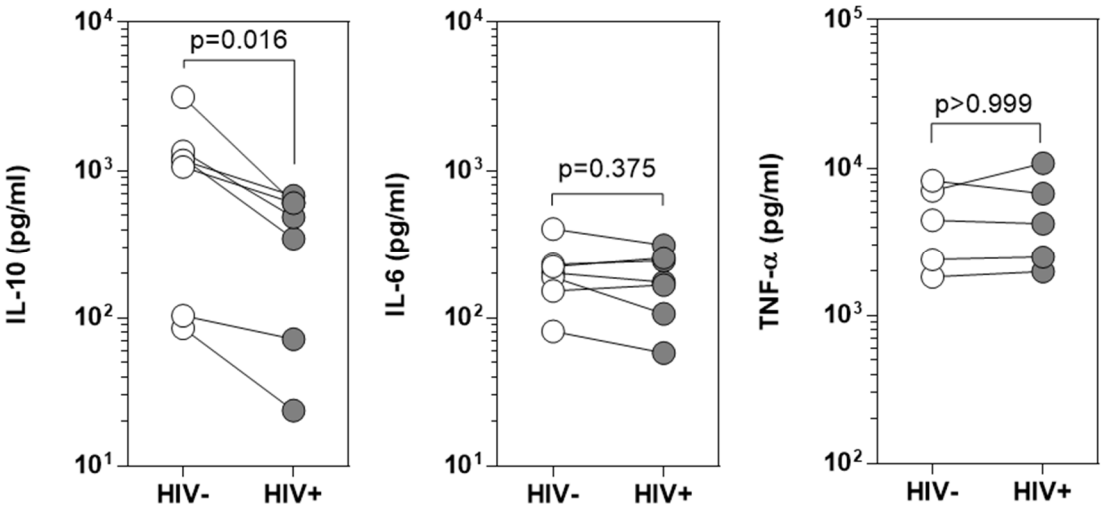

Figure 3.3: IL-10 responses are attenuated in HIV-infected THPs.

Supernatants were collected from HIV-infected and uninfected THP-1 cells stimulated with zymosan for 4 hours. Secreted IL-10, IL-6 and TNF-α was quantified by ELISA. Statistical significance was evaluated using the Wilcoxon signed-rank test. Results from 6 experiments.

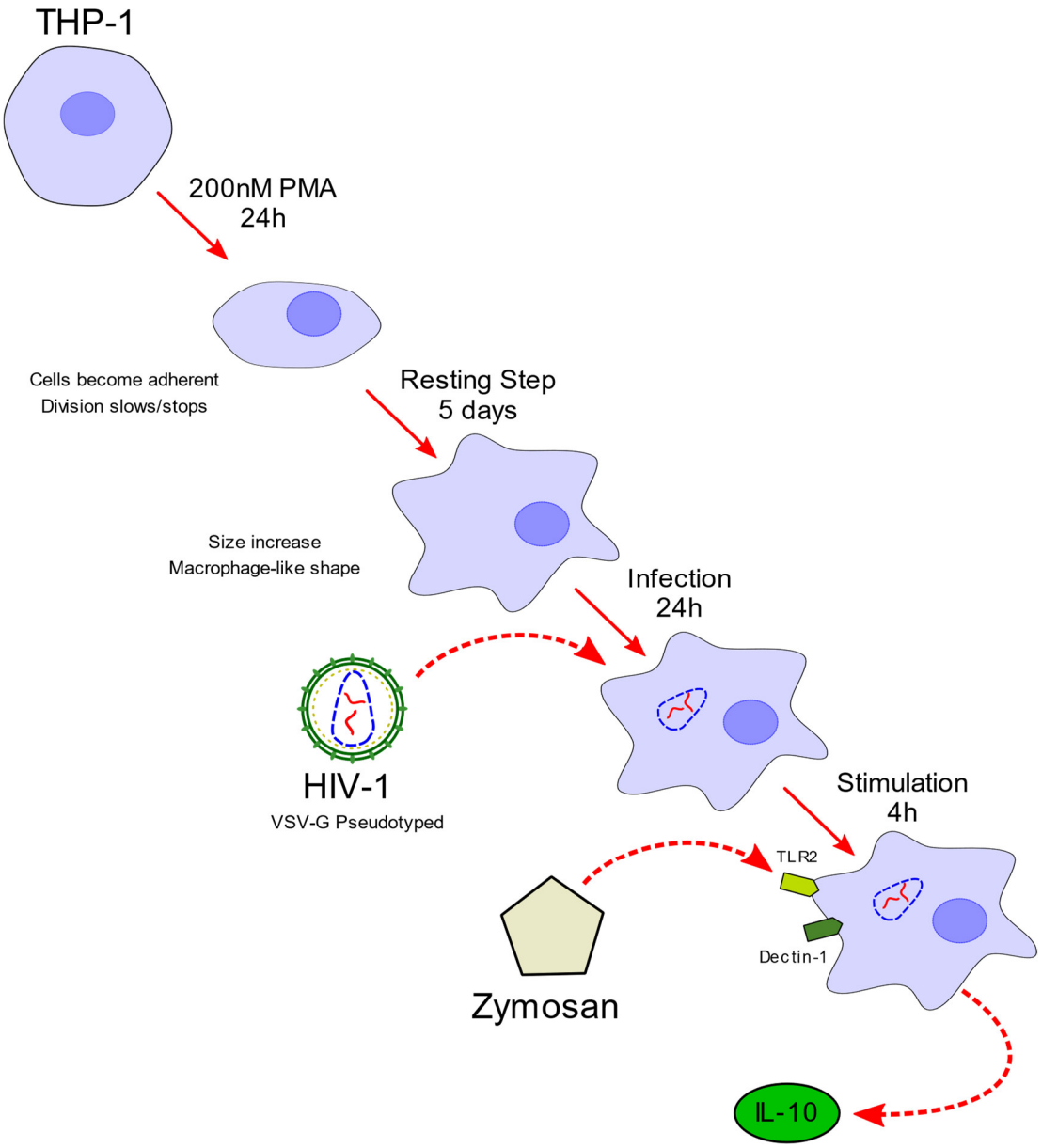

Figure 3.4: The THP-1 model of IL-10 attenuation.

Overview of the PMA treatment protocol, infection and subsequent stimulation used to model IL-10 attenuation in THP-1 cells. Cells in log phase are seeded onto plates in the presence of 200nM PMA and incubated for 24h. The media is then replaced with growth medium without PMA and the cells are 'rested' for 5 days. Cells are then infected with HIV-1 for 24h prior to stimulation with zymosan for 4h. Culture supernatants were assayed for cytokine content.

3.2.4. Attenuation of IL-10 responses in HIV-1 accessory protein deletion mutants in THP-1 cells.

The HIV-1 accessory proteins Vif, Vpr, Vpu and Nef have been reported to have immunomodulatory functions, which primarily serve to evade host restriction factors. I aimed to test whether one of these genes was responsible for IL-10 response attenuation. I obtained or generated knockout mutants (**Table 2.9**) for each of the accessory genes and tested them in the THP-1 model. No significant difference in infection was observed between the different mutants (**Figure 3.5a**). All HIV-1 mutants displayed some capacity to attenuate IL-10 responses, although the Vpr and Vpu mutants produced inconsistent results (**Figure 3.5b**). IL-6 expression was also altered by some mutants, which may indicate a role for some accessory proteins in regulating inflammatory cytokine secretion. In these experiments most vectors only produced a 2-fold inhibition of IL-10 expression, which is weaker than had previously been observed in primary cells.

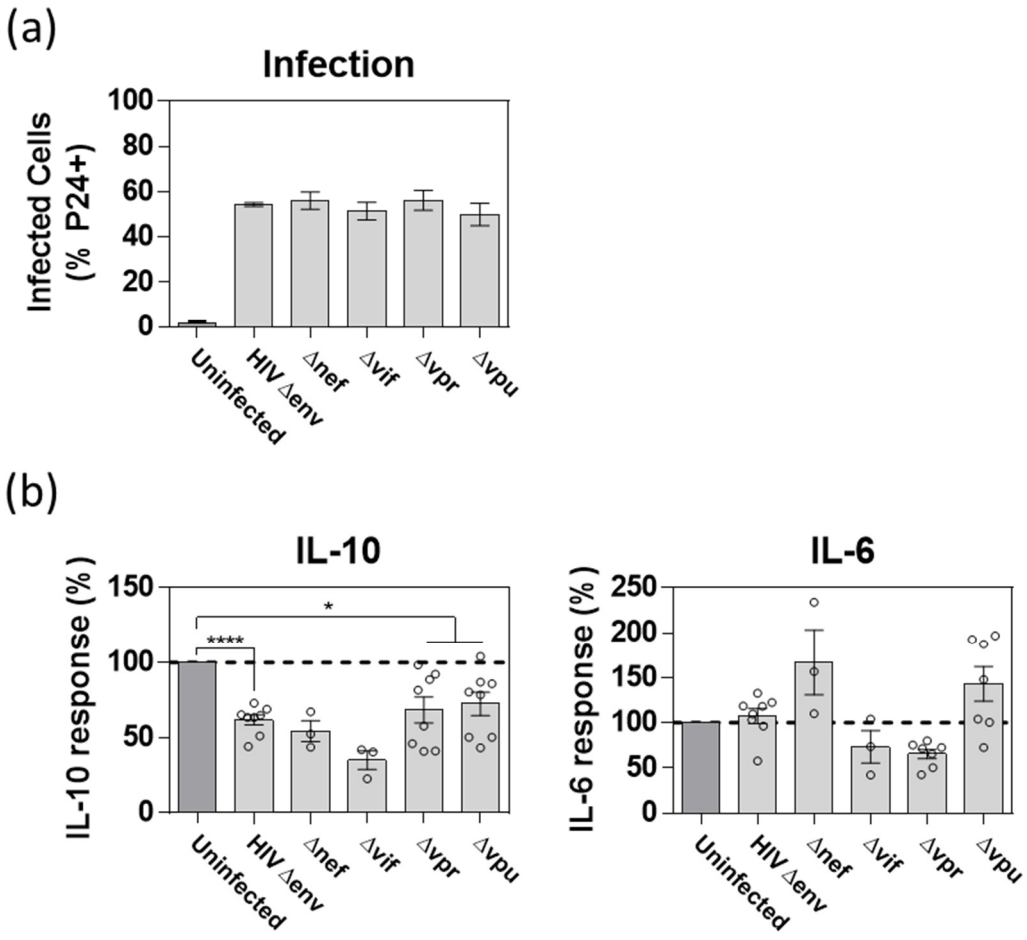

Figure 3.5: Accessory protein deletion mutants in THP-1 cells.

IL-10 responses when infected with HIV-1 or accessory protein deletion mutants. **(a)** Secreted IL-10 and IL-6 in the supernatant after 4 hours of stimulation with zymosan. **(b)** Percentage of infected cells as determined by fluorescent p24 staining. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from at least 3 donors per condition.

3.2.5. A double knockout of Vpr and Vpu fails to attenuate IL-10 in THP-1 cells

The IL-10 attenuation phenotype was weakest following infection with either the *vpu* or *vpr* mutant vectors. Therefore, I reasoned that each of these accessory proteins may have a partial effect and sought to test the hypothesis that a double mutant may completely abrogate HIV attenuation of IL-10 responses. I therefore created a double knockout of Vpr and Vpu and tested this in the present model. Interestingly, the double deletion mutant did not induce significant IL-10 response attenuation (**Figure 3.6a**). Even with two accessory genes knocked out there was no impact on infectivity in this cell line (**Figure 3.6b**). These genes may be more important during a replicative infection, but it is also possible that the THP-1 cell line lacks some of the host defence mechanisms which are present in primary cells.

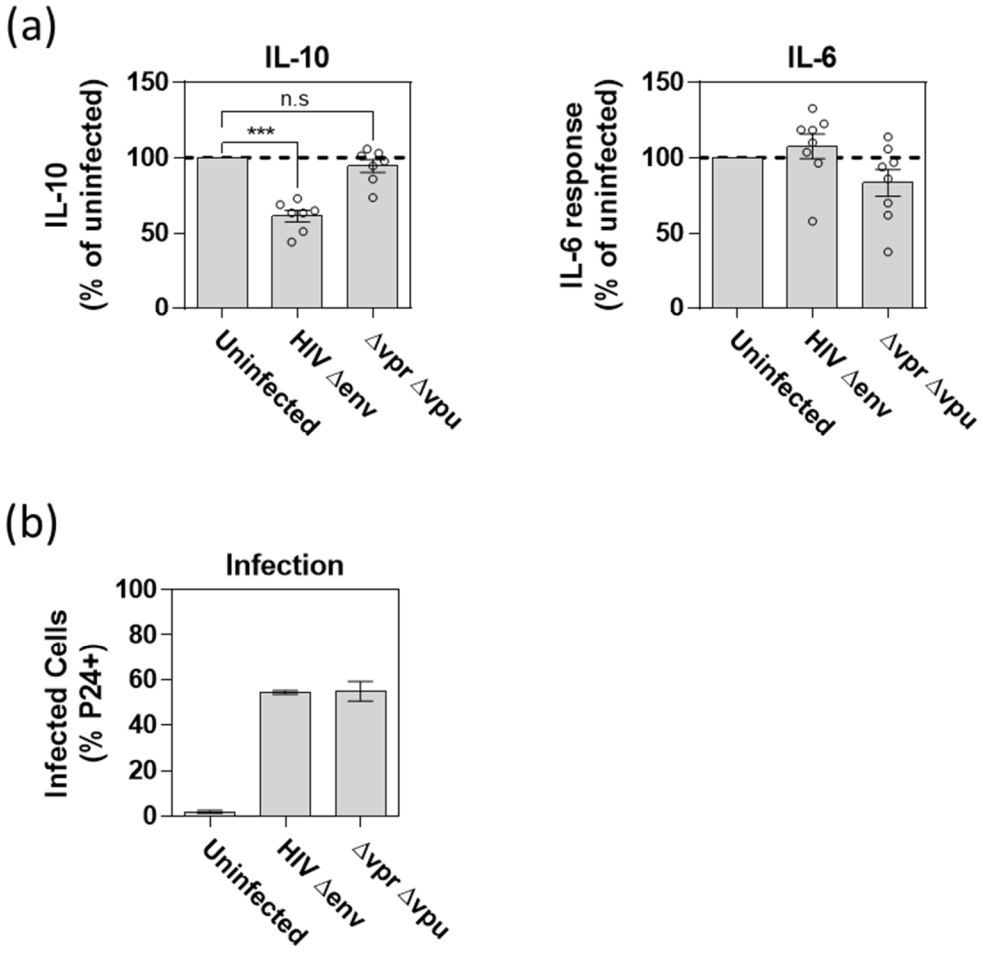

Figure 3.6: A double deletion mutant of Vpr and Vpu in THP-1 cells.

IL-10 responses when infected with HIV-1 or a mutant lacking the accessory proteins Vpr and Vpu. **(a)** Secreted IL-10 and IL-6 in the supernatant after 4 hours of stimulation with zymosan. **(b)** Percentage of infected cells as determined by fluorescent p24 staining. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 7 donors.

3.2.6. *De novo* viral gene expression is required to attenuate IL-10 in THP-1 cells

It has been reported that some HIV-1 accessory proteins are packaged into virions in order to deliver them into target cells [283]. Because of this I aimed to determine whether delivery of viral accessory proteins packaged in virions would be sufficient to attenuate IL-10 responses. To investigate this, I generated HIV-1-derived vectors which were packaged with the gene therapy vector CSGW, encoding only GFP within a HIV-1 LTR sequence (termed "empty vectors") [454]. The components needed for viral assembly were delivered on a separate transfected packaging plasmid. Two packaging plasmids were tested: 8.2 which encodes all HIV-1 genes (except *env*) and the variant 8.91 which is deficient in all the accessory proteins. I reasoned that the resulting 8.2 empty vectors should therefore contain packaged accessory proteins while those vectors made with 8.91 should not. Both types of empty vector were unable to induce IL-10 attenuation, indicating that packaged viral proteins are not sufficient for this phenotype (**Figure 3.7a**). Slightly greater levels of infection were seen with the empty vectors, which may be a result of their shorter genomes reverse transcribing more efficiently in target cells (**Figure 3.7b**).

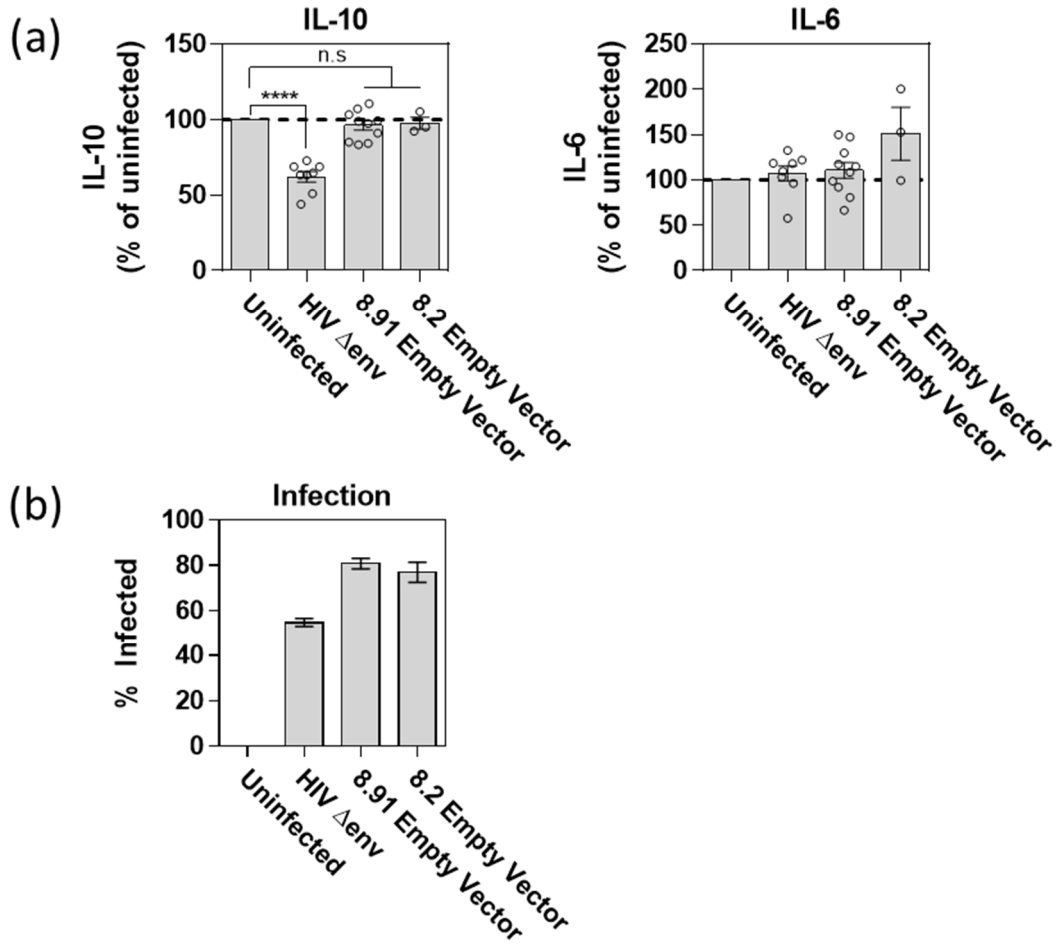

Figure 3.7: "Empty" vectors fail to attenuate IL-10 in THP-1 cells.

PMA-treated THP-1 cells were infected with virus encoding only GFP, packaged with (8.2) or without accessory genes (8.91). **(a)** Cytokine ELISAs were performed on supernatants from cells stimulated with zymosan for 4h. **(b)** Infection was quantified by intracellular p24 staining and flow cytometry for GFP expression. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Each virus was tested in at least 3 blood donors.

3.2.7. A double mutant of Vpr and Vpu attenuates IL-10 in primary monocyte-derived macrophages.

In order to confirm that the THP-1 cells reflected the biology of primary monocyte-derived macrophages, I sought to replicate my findings that HIV-1 *vpr* and *vpu* genes were both able to attenuate IL-10 responses in primary cells. In this case, I infected monocyte-derived macrophages with the HIV-1 deletion mutant lacking Vpr and Vpu while also delivering SIV VLPs to enhance infection (see **Figure 1.9**). SIV VLPs did not significantly attenuate IL-10 responses to zymosan and as expected HIV-1 infection did do so. Surprisingly however, the double Vpr and Vpu knockout mutant also significantly attenuated IL-10 in this model (**Figure 3.8**), in contrast to my findings in THP-1 cells.

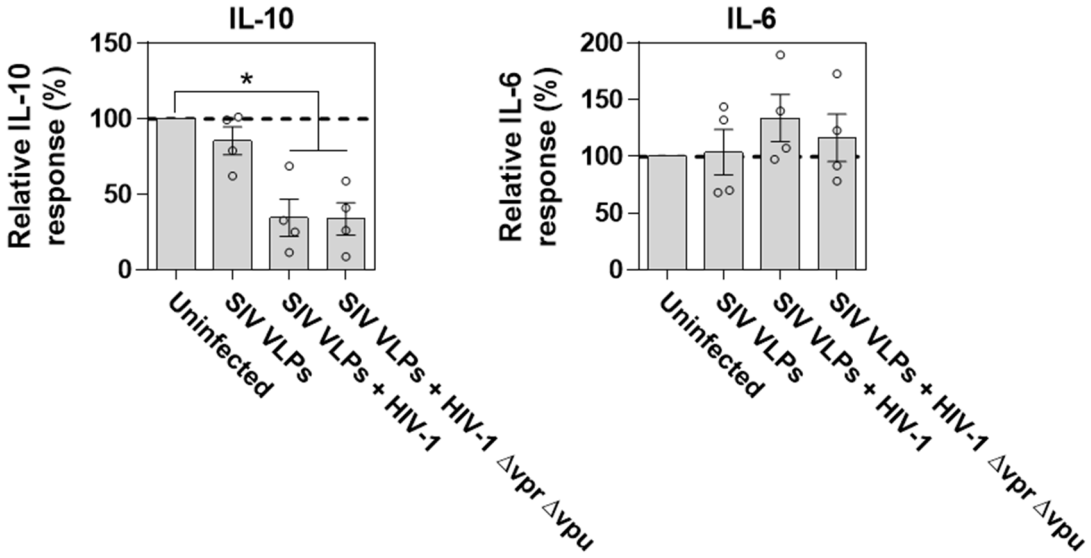

Figure 3.8: A double deletion mutant of Vpr and Vpu attenuates IL-10 in human MDMs.

Primary monocyte-derived macrophages were infected for 24 hours with Vpx supplementation before being stimulated for 4 hours with zymosan. Supernatants were assayed for IL-10 secretion by ELISA. Significance was determined by one-way ANOVA. Graphs show means ± SEM. Results from 4 donors.

3.2.8. Culturing MDMs in Foetal Calf Serum induces a G1-like state

The need to use SIV VLPs may have confounded the results in my macrophage experiments, for example through the action of SIV accessory proteins packaged in the VLPs, even though SIV VLPs were insufficient to attenuate IL-10 responses by themselves. To eliminate this potential confounding factor, I aimed to remove the VLPs from the MDM model.

It was recently reported that culturing of monocyte-derived macrophages in foetal calf serum (FCS) instead of pooled human serum (HS) causes some of the cells to enter a state similar to the G1 phase of the cell cycle. In this state SAMHD1 becomes inactivated via phosphorylation, which renders macrophages more permissive to HIV infection [462]. I therefore used FCS within my monocyte-derived macrophage model to eliminate the need for supplementation with SIV virus-like particles.

In order to confirm that culturing monocyte-derived macrophages in FCS would induce a permissive state, cells grown for 3 days in media containing FCS or human serum were fixed and stained for expression of MCM2, a cell cycle marker [462]. In agreement with the literature, FCS induced increased expression of MCM2 in the nuclei of the macrophages (**Figure 3.9a**). While less than 5% of cells cultured in human serum were in this cycling-like state, FCS appears to induce this state in 30-60% of macrophages depending on donor (**Figure 3.9b**). In these cells SAMHD1 is expected to be inactivated by phosphorylation, which would render them more permissive to HIV-1 infection.

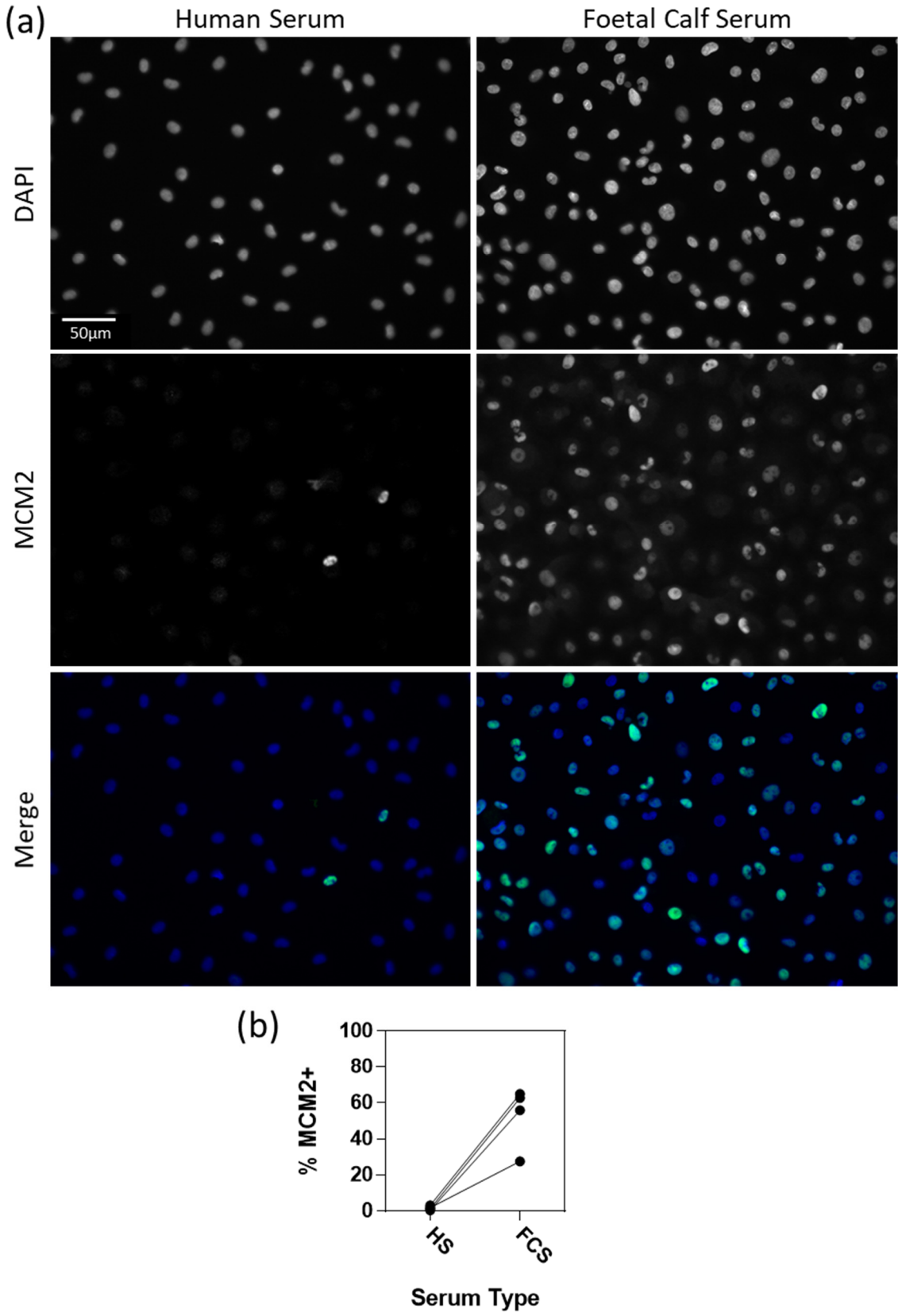

Figure 3.9: MDMs cultured in FCS exhibit MCM2 positivity.

(a) Macrophages cultured in media containing either FCS or human serum (HS) were fixed and stained for MCM2 by immunofluorescence. DAPI was used to counter-stain nuclei. **(b)** Quantitation of MCM2 expression performed using Metamorph 7 to detect MCM2 expression in the nuclei. Results from 3 donors.

3.2.9. FCS MDMs are permissive to HIV-1 infection without Vpx complementation

I next tested whether MDMs treated with FCS are permissive to HIV-1 infection without Vpx complementation. To do this, macrophages in both serum conditions were infected with a VSV-pseudotyped, single-round HIV-1 vector, with or without SIV VLPs. After 48 hours cells were fixed and stained for intracellular p24 expression, indicative of productive infection. As expected macrophages in FCS were significantly more permissive to HIV, to a level that was comparable to macrophages in human serum also exposed to SIV VLPs (**Figure 3.10**). Therefore, I adopted the FCS-modified macrophage model without SIV VLPs in order to eliminate the potentially confounding effects of SIV proteins on my experiments.

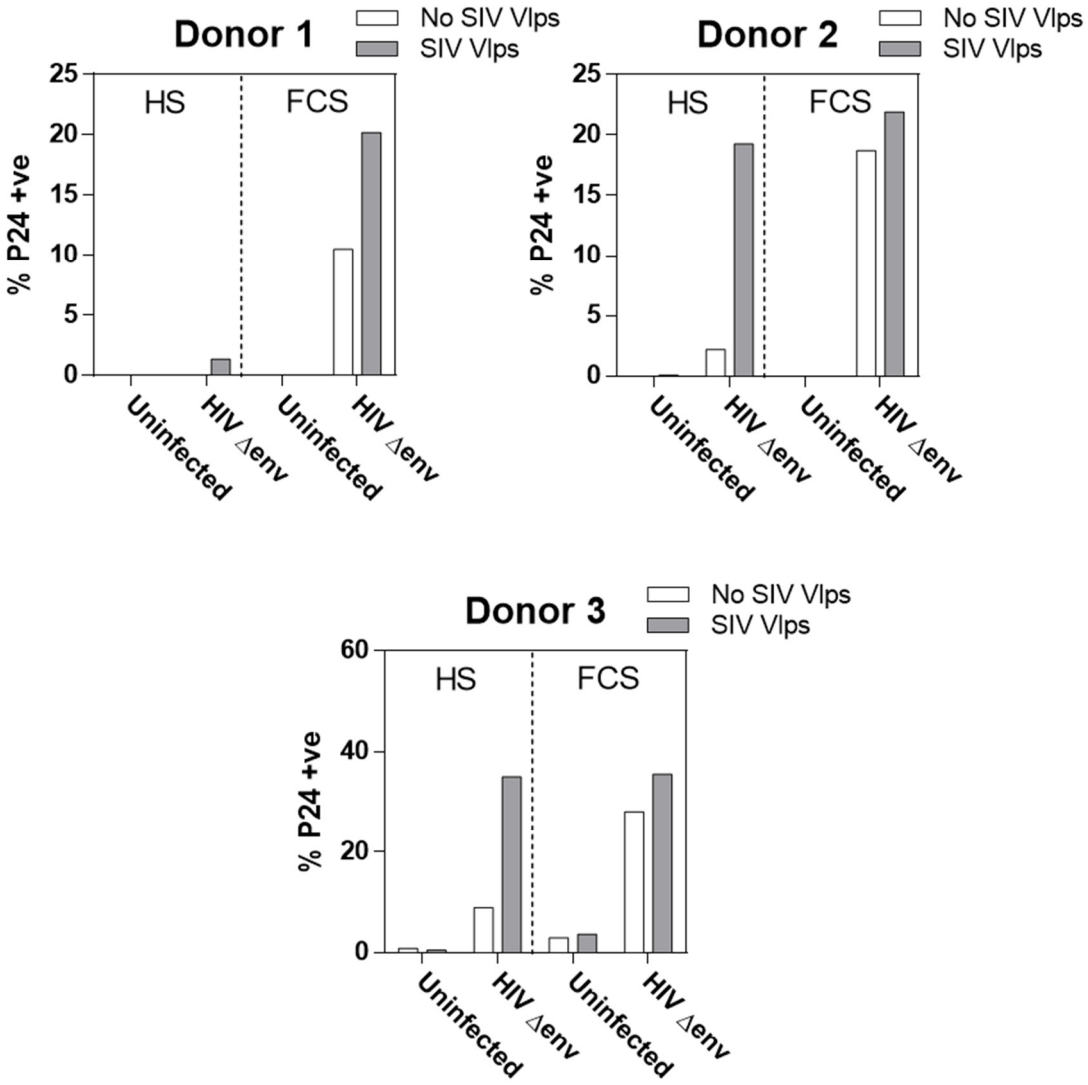

Figure 3.10: Infection of MDMs in different serum conditions.

MDMs cultured for 3 days in media containing foetal calf serum (FCS) or human serum (HS) were infected with HIV-1 in the presence of absence of SIV VLPs. After 48 hours cells were fixed and stained for p24 expression by immunofluorescence. Productive infection was quantified using Metamorph 7 to detect cells expressing high levels of p24. Data from 3 donors, with >10,000 cells per condition.

3.2.10. Cytokine responses in FCS and HS-cultured macrophages

Another important question that arose from this work was how the change in serum treatment would affect immune responses of monocyte-derived macrophages. While *in vitro* differentiation of primary monocytes into monocyte-derived macrophages has been extensively profiled [463], a direct comparison between the two serum conditions has not been reported. Despite this, culture in both types of serum is common in the literature, with various protocols described for generating different macrophage phenotypes [464].

To investigate whether the change in serum type would affect cytokine responses to innate immune stimulation, cells grown in each condition were stimulated with zymosan and the resulting cytokine production was measured by ELISA (**Figure 3.11**). While IL-6 expression was unchanged between serum conditions, IL-10 secretion was significantly enhanced in the cultures grown in foetal calf serum.

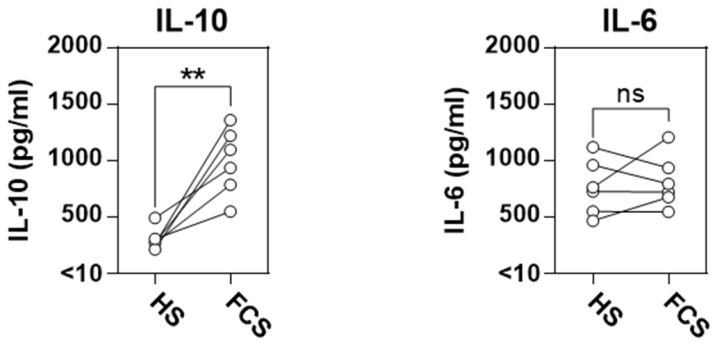

Figure 3.11: Cytokine responses in macrophages cultured in FCS or human serum.

Cytokine ELISA on supernatants from MDMs stimulated with zymosan for 4 hours after 3 days of culture in foetal calf serum (FCS) or human serum (HS). Statistical significance was evaluated using the Wilcoxon signed-rank test. Results from 6 donors.

3.2.11. Vpx complementation is not necessary to model IL-10 attenuation in FCS MDMs

To investigate whether the IL-10 attenuation phenotype could also be seen in the FCS-treated MDM model, cells cultured in each type of media were infected with HIV-1 with and without Vpx complementation before being stimulated with zymosan. IL-10 production was normalised relative to the uninfected, FCS-treated cells due to the variability in overall IL-10 production between different donors. As described in **Figure 3.11**, MDMs in human serum consistently secreted less IL-10 than their FCS counterparts (**Figure 3.12**). In the human serum-treated cells, HIV-1-induced IL-10 attenuation only approached significance when cells were supplemented with SIV VLPs. In contrast, FCS MDMs exhibited significant attenuation with or without the complementation with Vpx. This may be due to the loss of SAMHD1-mediated restriction in FCS-treated cells which have taken on the 'cycling', MCM2-positive phenotype which renders them permissive to infection. However, another possibility is that the enhanced IL-10 secretion induced by FCS may simply make attenuation easier to detect, as the cytokine levels produced by human serum-treated cells at these early time points were approaching the detection limits of our assay (~10pg/ml).

Based on these results, FCS treated MDMs were used without SIV VLPs in subsequent experiments in order to simplify the model.

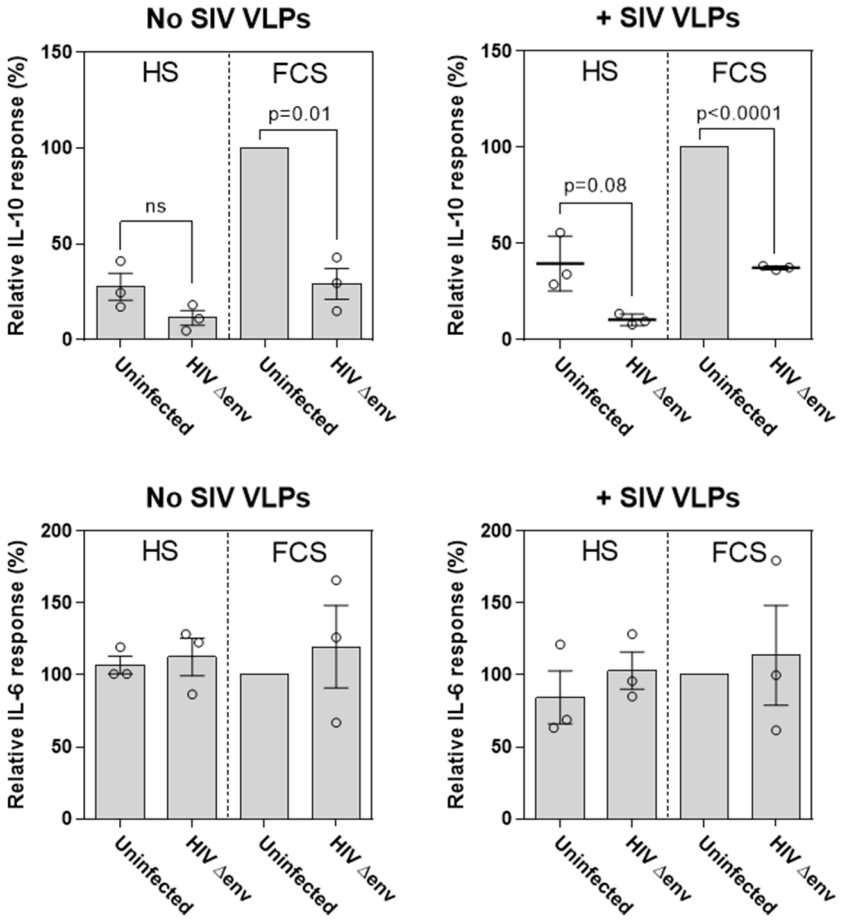

Figure 3.12: SIV VLPs are not necessary to detect IL-10 attenuation in *MDMs*.

MDMs grown in FCS or human serum were infected with HIV-1 for 24 hours, with or without Vpx complementation via SIV VLPs. Cells were then stimulated with zymosan for 4 hours and supernatants were assayed for cytokine expression. Graphs show means ± SEM. Significance was determined by paired t-test. Results from 3 donors.

3.2.12. HIV-1 accessory protein deletion mutants attenuate IL-10 in primary MDMs cultured in FCS.

I then replicated the deletion mutant experiments performed in the THP-1 model in the FCS-treated MDM model. Cells were infected with each viral vector for 24h before being stimulated with zymosan for 4h, and the resulting supernatant subjected to ELISAs for IL-10 and IL-6. Infection was evaluated by fixing cells and staining for intracellular p24. All deletion mutants, including the double knockout of Vpr and Vpu, were still capable of attenuating IL-10 responses in primary cells (**Figure 3.13**). Productive infection was seen in ~30% of cells across all mutants, indicating that at least single round infection efficiency is not impacted by these knockouts.

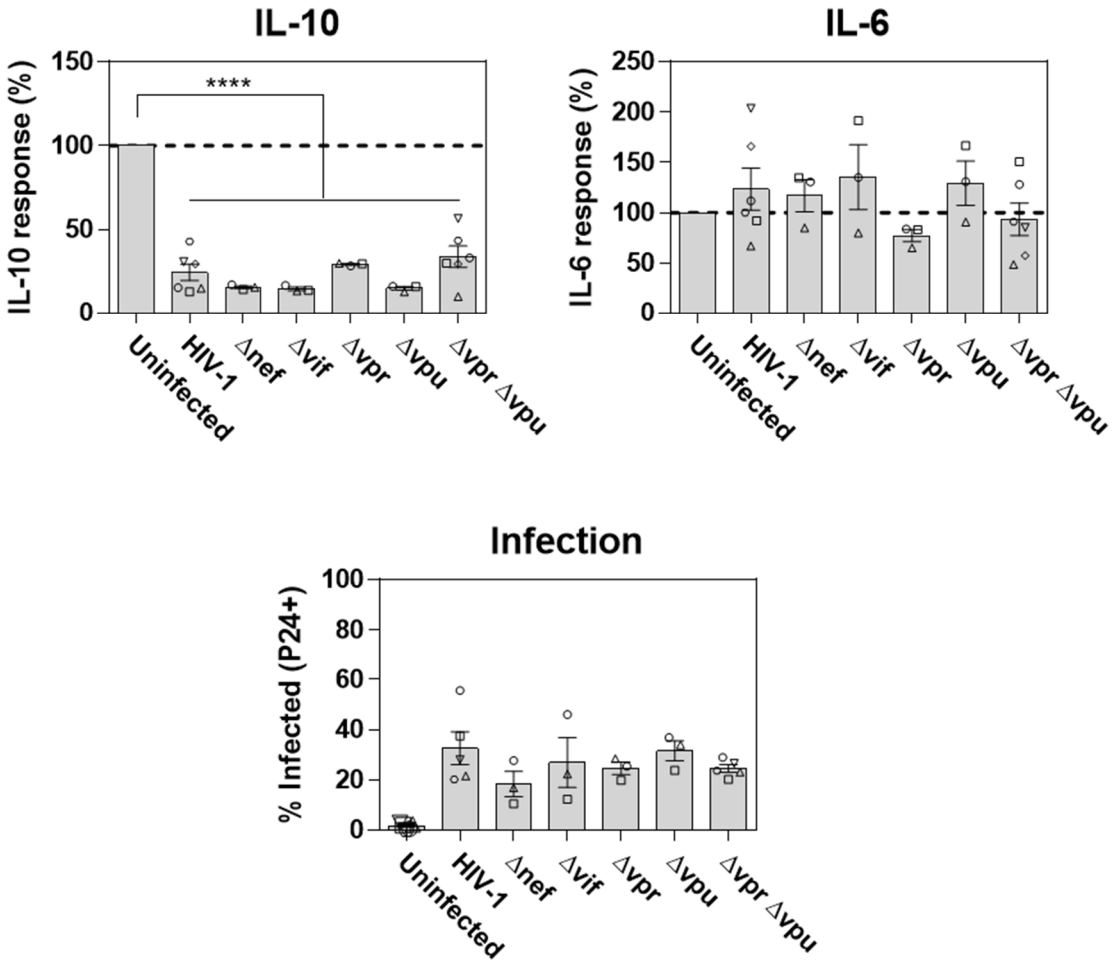

Figure 3.13: IL-10 responses are attenuated by infection with HIV-1 accessory protein deletion mutants in primary MDMs.

FCS MDMs were infected for 24 with HIV-1 deletion mutants. Supernatants were collected after stimulation with zymosan for 4 hours and tested for cytokine expression. Infection was detected using fluorescent p24 staining. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 3 donors for single deletion mutants and 6 donors for HIV-1 and ΔvprΔvpu.

3.2.13. *De novo* viral gene expression is not required in primary macrophages

Since the deletion mutants behaved differently in primary cells, I also tested the "empty" vectors expressing only GFP on their genome in this model. MDMs were infected with vectors packaged using 8.91 (therefore lacking accessory proteins) with a CSGW genome. 24h after infection they were stimulated with zymosan as previously described and the resulting cytokine secretion was quantified from the supernatant. In this model the CSGW "empty" vectors were capable of attenuating IL-10, providing further confirmation that HIV-1 accessory proteins are not necessary for this phenotype (**Figure 3.14**). Together, these findings suggest that a factor delivered with the incoming vector is responsible for deficient IL-10 responses in macrophages.

One possible explanation for the disparity between results from the THP-1 and MDM models was that a higher dosage of the CSGW vector may be required to attenuate IL-10 compared to the full HIV-1 vector. To test this, I titrated both vectors in the MDM model to compare their effects on IL-10. Attenuation of IL-10 expression was dose-dependent, with increased dosage of virus producing a greater defect in both the full length (*env*-deleted) and the CSGW vectors (**Figure 3.15**) across multiple donors. However, there was no significant difference in infectiousness or IL-10 inhibition between the CSGW and HIV-1 vectors in this model.

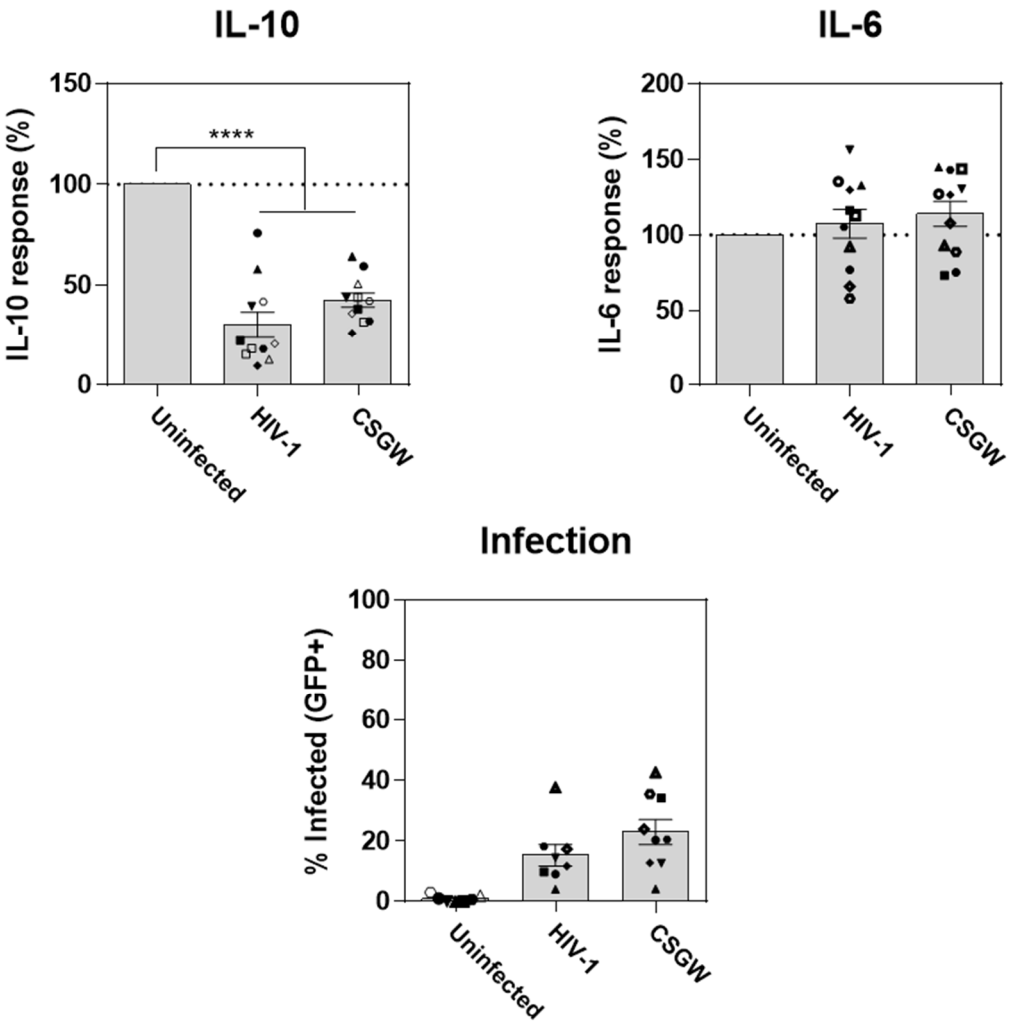

Figure 3.14: "Empty" vectors are still able to attenuate IL-10 in primary MDMs.

FCS MDMs were infected with HIV-1 or a vector packaged with the minimal packaging plasmid 8.91 and the CSGW genome which lacks HIV-1 gene expression. Cells were stimulated with zymosan for 4h before supernatants were assayed for cytokine expression. Infection was quantified by imaging for GFP expression. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 11 donors.

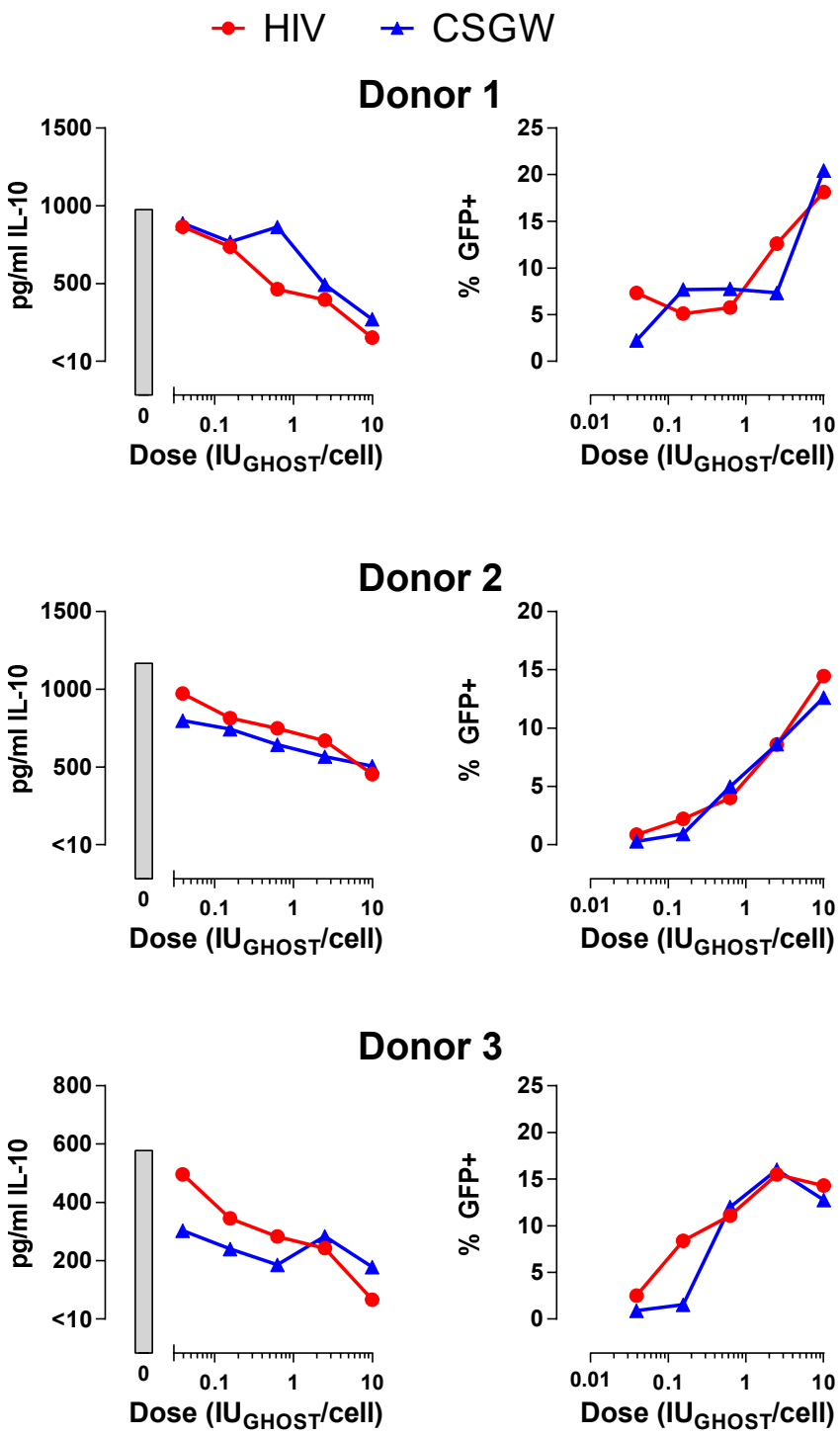

Figure 3.15: IL-10 attenuation is dose dependent.

FCS MDMs were infected with a dose titration of HIV-1 or a vector packaged with the minimal packaging plasmid 8.91 and the CSGW genome which lacks HIV-1 gene expression. Cells were stimulated with zymosan for 4h before supernatants were assayed for cytokine expression. Infection was quantified by imaging for GFP expression.

3.3. Chapter Discussion

In this chapter, I found that THP-1 cells could be made to secrete IL-10 in response to zymosan using a PMA differentiation protocol with a post-treatment resting step. I was able to infect these cells with HIV-1 and recapitulate the IL-10 attenuation phenotype previously observed in monocyte-derived macrophages. Treatment with deletion mutants suggested that Vpr and Vpu may both exert an effect on IL-10 expression, with a double mutant failing to attenuate IL-10 responses in the THP model. Delivery of these proteins within vectors was not sufficient to affect IL-10, suggesting that *de novo* expression of these viral genes in the target cells may be necessary for this phenotype in cell lines.

The THP-1 model provides the opportunity to investigate host factors via the creation of knockout mutant lines via techniques such as CRISPR [465]. This would expand the options for further experiments into the pathways involved in IL-10 expression, since such techniques are generally not compatible with primary cells. However, the IL-10 attenuation phenotype in this model is weaker than had previously been seen in primary monocyte-derived macrophages. THP-1 cells displayed only a 2-fold decrease in expression when infected. This may be partially due to the complex regulation of IL-10 expression being different in THP-1 cells compared to primary macrophages, perhaps with cells expressing less IL-10 to begin with. It should be noted that this weaker phenotype could make it difficult to achieve significance and confidence in results, since it may be difficult to distinguish a defective virus from a genuine rescue of IL-10 expression.

Overall, while IL-10 attenuation could be recapitulated in the THP-1 cell line, the prolonged differentiation protocol, variable IL-10 production and weaker attenuation phenotype place limitations on the usefulness of this model. To confirm that this THP-1 model accurately represented the phenotype as seen in primary cells I opted to try to replicate these findings in the monocyte-derived macrophage system. In doing this I found that the double knockout mutant still attenuated IL-10 in the original primary cell model.

Due to the potential for SIV VLPs to deliver SIV variants of HIV accessory proteins and interfere with the assay, I refined the model to eliminate the need for Vpx complementation. Culturing macrophages in foetal calf serum caused them to enter a cycling-like state in which SAMHD1 is inactivated by phosphorylation, rendering cells permissive to HIV-1 without the need for Vpx-mediated degradation of this restriction factor. Interestingly, while this treatment had no effect on IL-6 expression, IL-10 production was enhanced in FCS-treated cells. This could reflect a generalised increase in expression or the FCS could be promoting development of a subset of cells which potently express this cytokine. This enhancement of IL-10 production may

be beneficial to the model due to the ability to detect cytokine secretion that would have otherwise been below the assay detection limit. In the FCS model IL-10 attenuation could be detected without complementation with Vpx-containing VLPs, allowing them to be removed from the model.

When using the refined model, in contrast to results with the THP-1 model, HIV-1 accessory protein mutants were all capable of inhibiting IL-10 expression. I found that vectors with genome encoding only GFP were also capable of inducing this phenotype, meaning that viral accessory proteins or indeed *de novo* expression of any viral gene is not required. It is possible that the mechanism of IL-10 attenuation may be entirely different in cell lines, especially considering the multiple levels of regulation that have been described for IL-10. Since the magnitude of the effect is weaker in THP-1 cells, it is possible that the lack of Vpr and Vpu results in only a partial rescue of IL-10 expression due to reduced viral fitness, which the THP assay is not sensitive enough to distinguish from a complete loss of phenotype.

4. Results 2 – Which viral factors are sufficient for IL-10 attenuation?

4.1. Introduction

In my first results chapter, I established not only that the HIV-1 accessory proteins were not necessary for attenuation of innate immune IL-10 responses in HIV-infected macrophages, but also that HIV-encoded gene expression was not necessary. These observations indicate that this phenotype is mediated by host interactions with the incoming virus. We already know that the native HIV-1 envelope is not necessary because attenuation of macrophage IL-10 responses is also induced by VSV pseudotyped virus, suggesting a role for post-entry interactions. Host interactions with viral capsid are well established [287], [466]. Of particular interest in macrophages is that these may serve to inhibit innate immune sensing of nascent HIV DNA [374]. Notwithstanding these observations, reports of innate immune IFN responses to HIV DNA in the absence of a host cell endonuclease, TREX1, indicate that some viral DNA can be detected by the macrophage [467]. TLR recognition of viral RNA has also been reported albeit not in monocyte-derived macrophages [403]. In general, these interactions are thought to lead to IFN responses, which were previously found to be absent in our experimental model [444]. In the present chapter I sought to investigate whether any of these interactions may be responsible for attenuation of macrophage IL-10 responses.

Of note, in my experimental model, HIV-1 infection of macrophages revealed by intracellular p24 staining indicates only 20-40% of cells become productively infected. I hypothesised that this is likely to underestimate the proportion of cells that are exposed to incoming virus due to factors such as host restriction and defective virions which prevent productive infection. Therefore, attenuated IL-10 responses may be mediated by effects in cells beyond the population represented by p24 staining. In order to test this hypothesis, I needed to detect both HIV-1 and cytokine responses at the single cell level. Although intracellular cytokine immunostaining is widely used for selected cytokines, it has not generally been reported for IL-10. I undertook exhaustive trials of all the commercially available antibodies for human IL-10 and was unable to find a suitable reagent for fluorescent intracellular cytokine staining in macrophages. In view of the fact that the production of many cytokines is regulated at the transcriptional level, an emerging alternative strategy to intracellular immunostaining for cytokines is RNA fluorescence in-situ hybridisation (FISH). This approach offers the versatility to design specific RNA probes to detect transcriptional upregulation of selected cytokines and HIV-1 RNA, using different fluorophores in order to quantify multiple targets in the same cell.

Importantly, if IL-10 expression within macrophage cultures is heterogenous, single cell analysis may also reveal a greater effect size by overcoming the averaging effect of measuring protein in culture supernatants.

In this chapter, I combined development of RNA FISH staining for cytokines and HIV-1 with experiments to investigate incoming components of the HIV-1 virus that may mediate attenuation of macrophage IL-10 responses.

The objectives of this chapter are as follows:

1) Determine whether viral genomic material is necessary or sufficient to attenuate IL-10.
2) Identify the nature of the virion component responsible for inducing IL-10 attenuation.
3) Quantify IL-10 innate immune responses and productive HIV-1 infection at single cell level.
4) Test the hypothesis that attenuation of IL-10 responses is restricted to HIV-1-infected macrophages.

4.2. Results

4.2.1. Intact virions are necessary for IL-10 attenuation

Based on results from the previous chapter I hypothesised that viral material delivered within the virion may be responsible for the effects of HIV on IL-10. I next sought to test the hypothesis that either a protein component of the virion or genomic material may be necessary for this effect. Both of these may be denatured by boiling the virus preparation. In addition, the viral RNA may be damaged by UV irradiation. Both treatments reversed HIV attenuation of macrophage IL-10 production in my experimental model (**Figure 4.1**). On the basis that UV-inactivation is not expected to denature proteins, I interpreted these data to indicate a role for viral genomes.

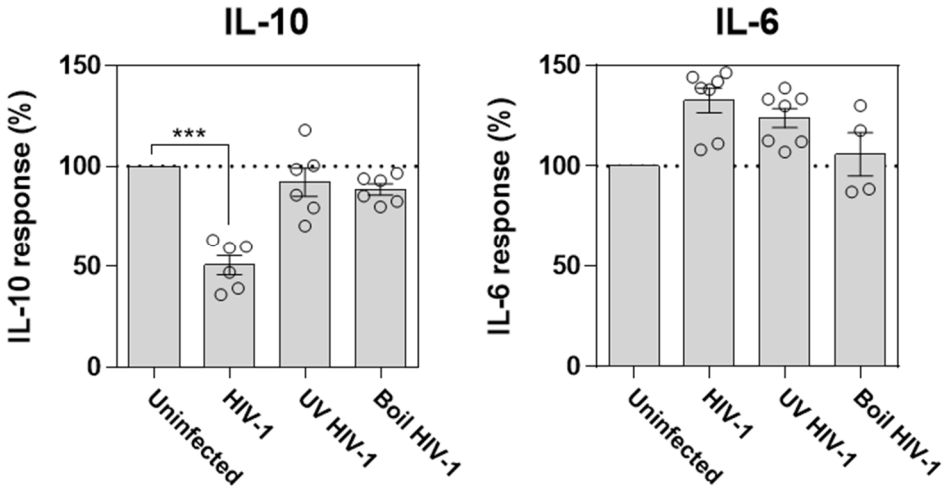

Figure 4.1: Inactivated HIV-1 virions fail to attenuate IL-10 expression.

HIV-1 virions were boiled for 5 minutes or UVC-irradiated for 10 minutes before transducing MDMs for 24 hours. 4-hour supernatants from zymosan stimulation were assayed for cytokine secretion. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 6 donors.

4.2.2. Viral genomic material is required for IL-10 attenuation

The role for viral genomes in my model was also supported by previous data showing that incoming virions free of any accessory proteins were sufficient to attenuate IL-10 responses. In order to build on these findings further, I also tested the effect of virus-like particles (VLPs) which were packaged without a viral genome using the 8.91 packaging plasmid. Unlike VLPs with the CSGW genome, which I have previously showed to attenuate macrophage IL-10 responses (**Figure 3.14**), the genome-free VLPs will not contain the viral LTR sequence, perform reverse transcription or undergo integration. The HIV-1 packaging process remains poorly understood [468], [469]. Packaging signals are required for efficient incorporation of viral RNA into the virion, so these have been removed from the 8.91 packaging plasmid. However, evidence suggests that viral capsid may form around an RNA molecule during assembly to assist stabilisation [470], [471]. In the absence of genomic RNA mature virions are produced, but it is not clear whether capsid assembles without RNA or if random transcripts are incorporated from the host cell. Despite this, such vectors would be expected to contain significantly less viral genomic material than normal virions.

Consistent with the previous results indicating a role for viral genomes, genome-free virions were not capable of inducing IL-10 attenuation (**Figure 4.2**).

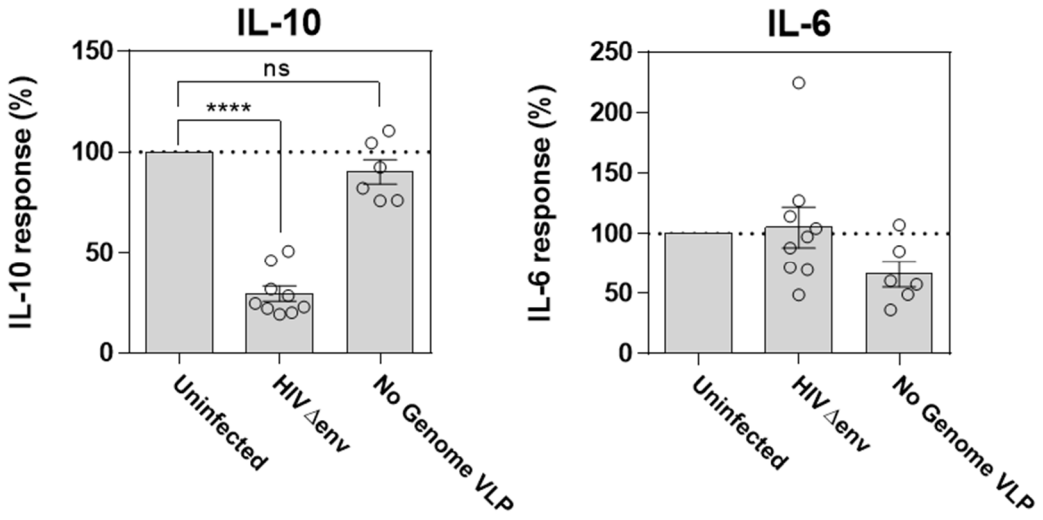

Figure 4.2: Viral genomic material is required for IL-10 attenuation

Genome-free VLPs were packaged with the p8.91 packaging plasmid and used in the FCS MDM model. Cytokine content in the supernatant was quantified by ELISA after 4 hours of stimulation with zymosan. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 7 donors.

4.2.3. A subset of macrophages express high levels of IL-10

Macrophages have previously been reported to exhibit substantial heterogeneity in their cytokine responses. Multiple different activation profiles have been described [27]. At the single cell level substantial variability has been observed [472]. Single cell measurements of IL-10 transcripts by RNA FISH would allow me to assess for the first time whether macrophages exhibit heterogeneity in IL-10 responses.

I found minimal detectable IL-10 RNA in unstimulated macrophages. After stimulation with zymosan there was clear upregulation of IL-10 RNA levels evident in 20-50% of cells, albeit with some donor-to donor variability (**Figure 4.3a-b**).

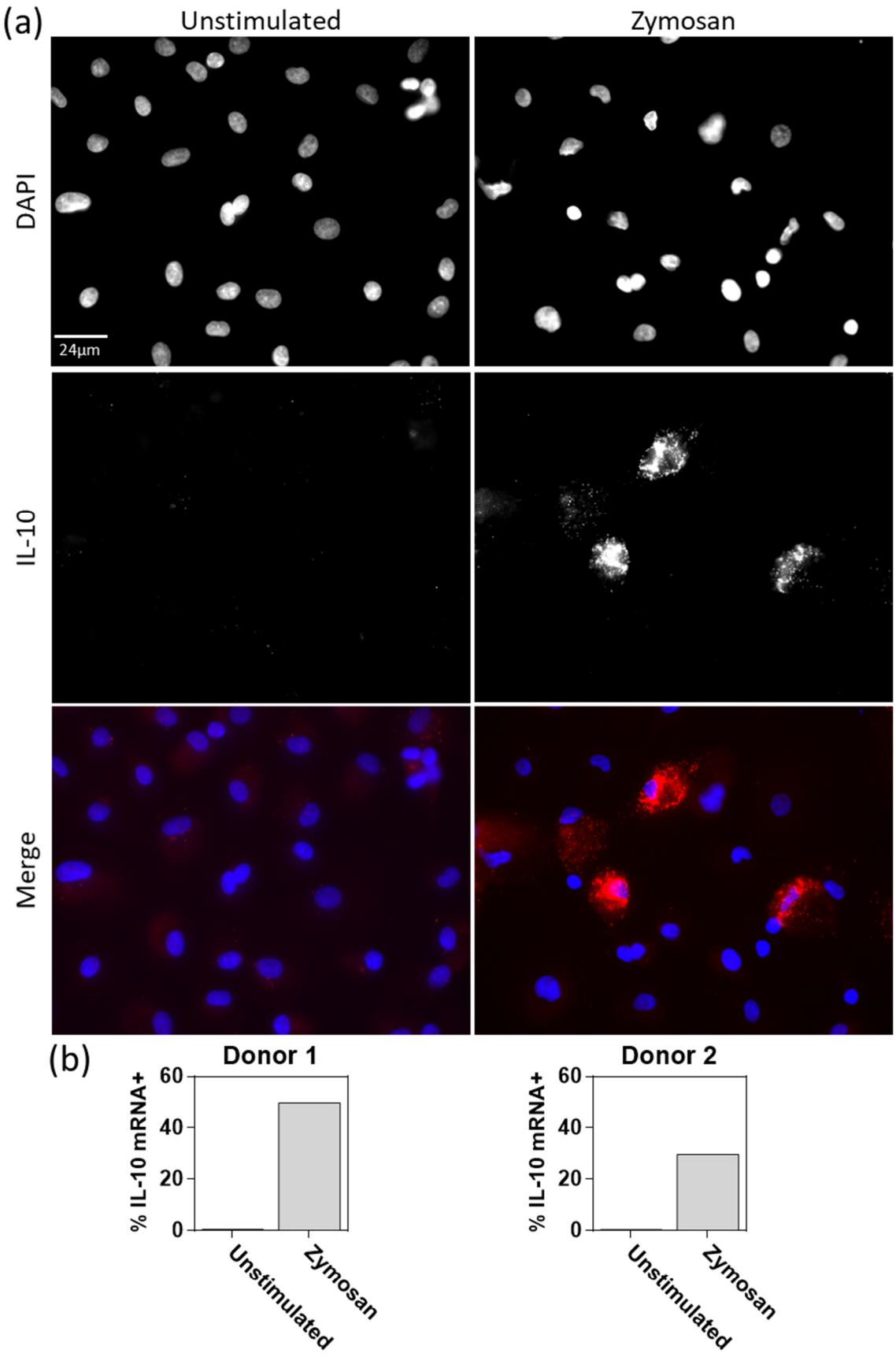

Figure 4.3: Single molecule RNA FISH for IL-10

(a) Monocyte-derived macrophages were stimulated for 4 hours with zymosan prior to fixation and staining for IL-10 RNA (red) using ViewRNA FISH probes (Thermo). **(b)** Proportions of cells expressing IL-10 were quantified using Metamorph 7. Images represent a typical donor.

4.2.4. Analysis strategy for RNA FISH

Due to the substantial variability in IL-10 expression between different cells, I reasoned that using a simple threshold to distinguish between IL-10 positive and negative cells in binary fashion may limit the ability to resolve small changes in expression levels. I therefore investigated alternative analysis strategies for the single-cell quantitation of RNA expression by immunofluorescence. In the RNA FISH staining method I used, a single molecule of RNA appears as a single spot, theoretically allowing the enumeration of the total number of transcripts [473], [474]. Software packages such as FISH-quant are available for the analysis of FISH data [475], but these rely on the segmentation of spots representing individual RNA molecules. These were not suitable for use with this assay due to the high expression of some targets, which rendered single spots impossible to resolve using the wide-field hardware available to us. Instead, I generated an analysis strategy based on determining the average intensity of cytoplasmic staining in each wavelength within each individual cell. Cells were identified based on DAPI staining of nuclei, but a limited number of fluorescence channels were available on the microscope used, which prevented counter-staining with a cell mask to help define the cytoplasm. Instead, an analysis script was used which estimates cytoplasmic staining by evaluating a region in a defined radius (ring mask) around each nucleus (**Figure 4.4**). This strategy allowed for quantitation by sampling fluorescence intensity in the perinuclear zone without having to define the outer cytoplasmic margins.

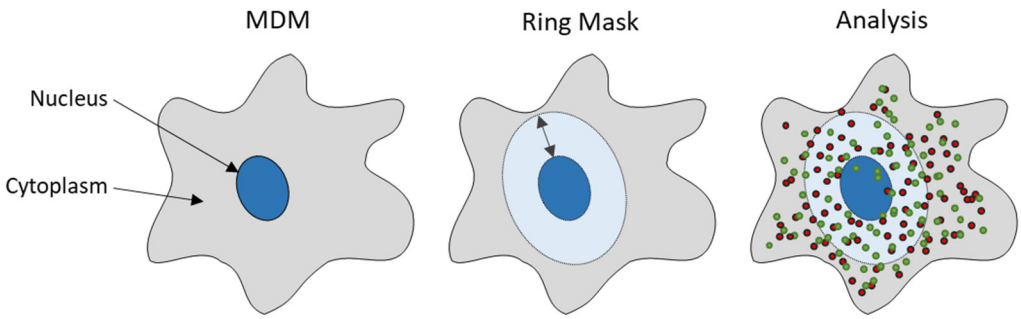

Figure 4.4: Analysis strategy for RNA FISH experiments

RNA FISH data was analysed using a ring mask strategy to evaluate staining within a defined radius of each nucleus, generating an approximation of RNA staining in each cell. DAPI-stained nuclei are detected as objects and used to generate a dilated ring to estimate cytoplasmic staining in each wavelength.

4.2.5. Attenuated IL-10 responses can be detected using RNA FISH.

To validate whether the RNA staining could replicate results seen by ELISA, MDMs stimulated with zymosan for 4h were stained by RNA FISH, with or without HIV infection. IL-10 attenuation in HIV-1 infected macrophage cultures was clearly evident (**Figure 4.5a**). IL-10 expression was not induced by HIV infection of macrophages alone. Quantitation of staining represented by a frequency distribution of intensity across all cells revealed an overall decrease in IL-10, visualised as a histogram shift to the left (**Figure 4.5b**). Small increases in average intensity within a cell can reflect a substantial increase in IL-10 transcripts as additional spots occupy more of the analysis region. Cells were therefore classified into IL-10 'high' and 'low' populations using an intensity threshold. This revealed a 2-fold decrease in cells expressing high levels of IL-10. These results were consistent with our observations of IL-10 attenuation at the protein level.

Next, I sought to confirm that the effect of HIV-1 was specific to IL-10 (as suggested by previous protein and qPCR assessments of bulk cell cultures) by using RNA FISH for IL-6 as a control (**Figure 4.6a**). While not all cells expressed IL-6, low levels of staining were observed in most cells rather than a small population expressing high numbers of transcripts as seen with IL-10 (**Figure 4.6b**).

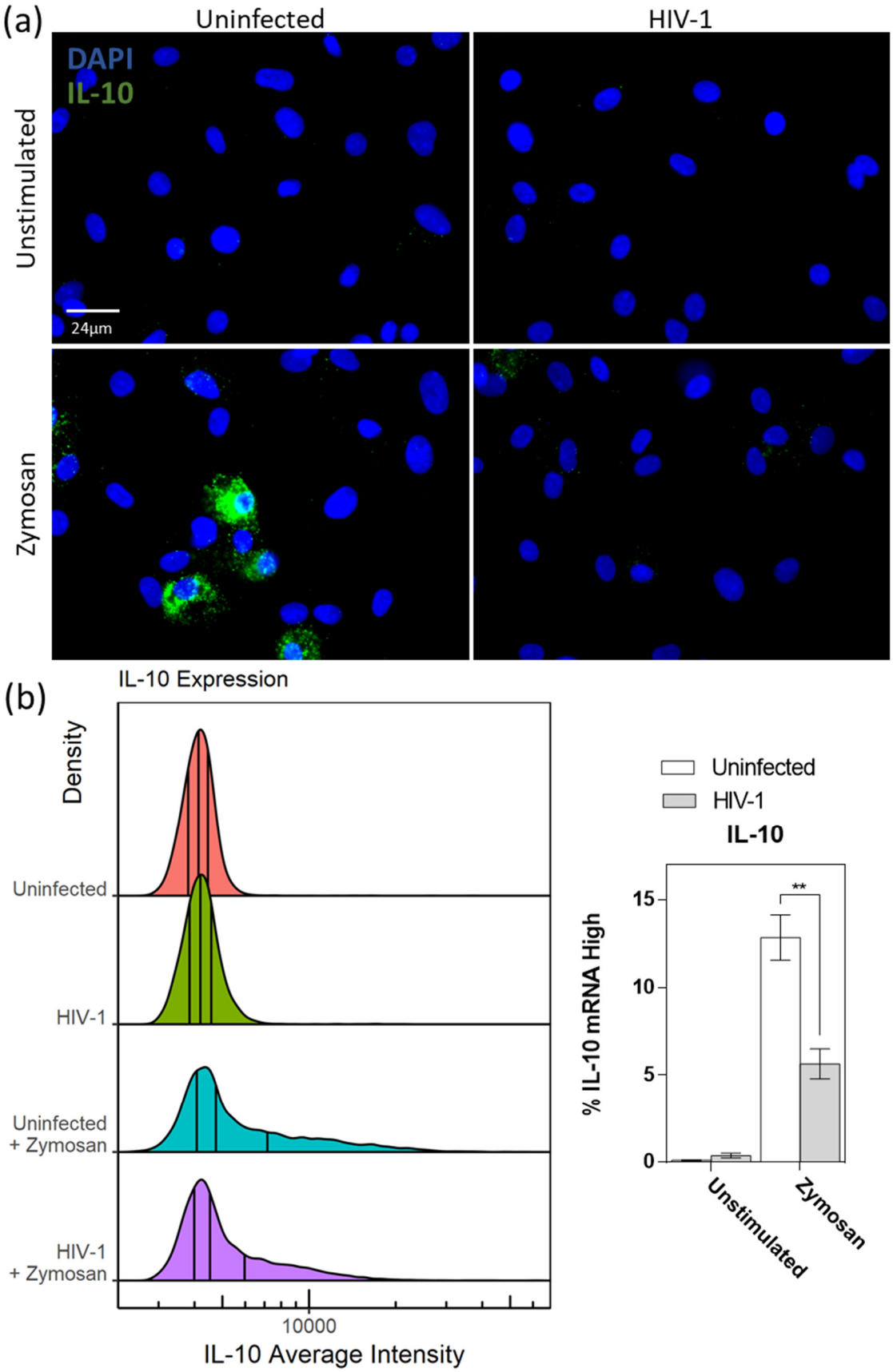

Figure 4.5: Attenuation of IL-10 responses by HIV-1.

(a) Infected and uninfected MDMs were stimulated with zymosan for 4 hours before fixation and staining for IL-10 by RNA FISH. **(b)** Staining was quantified using Metamorph 7, using an intensity threshold of 10,000. Graphs show means ± SEM across 3 replicate wells. Significance was determined by paired t-test.

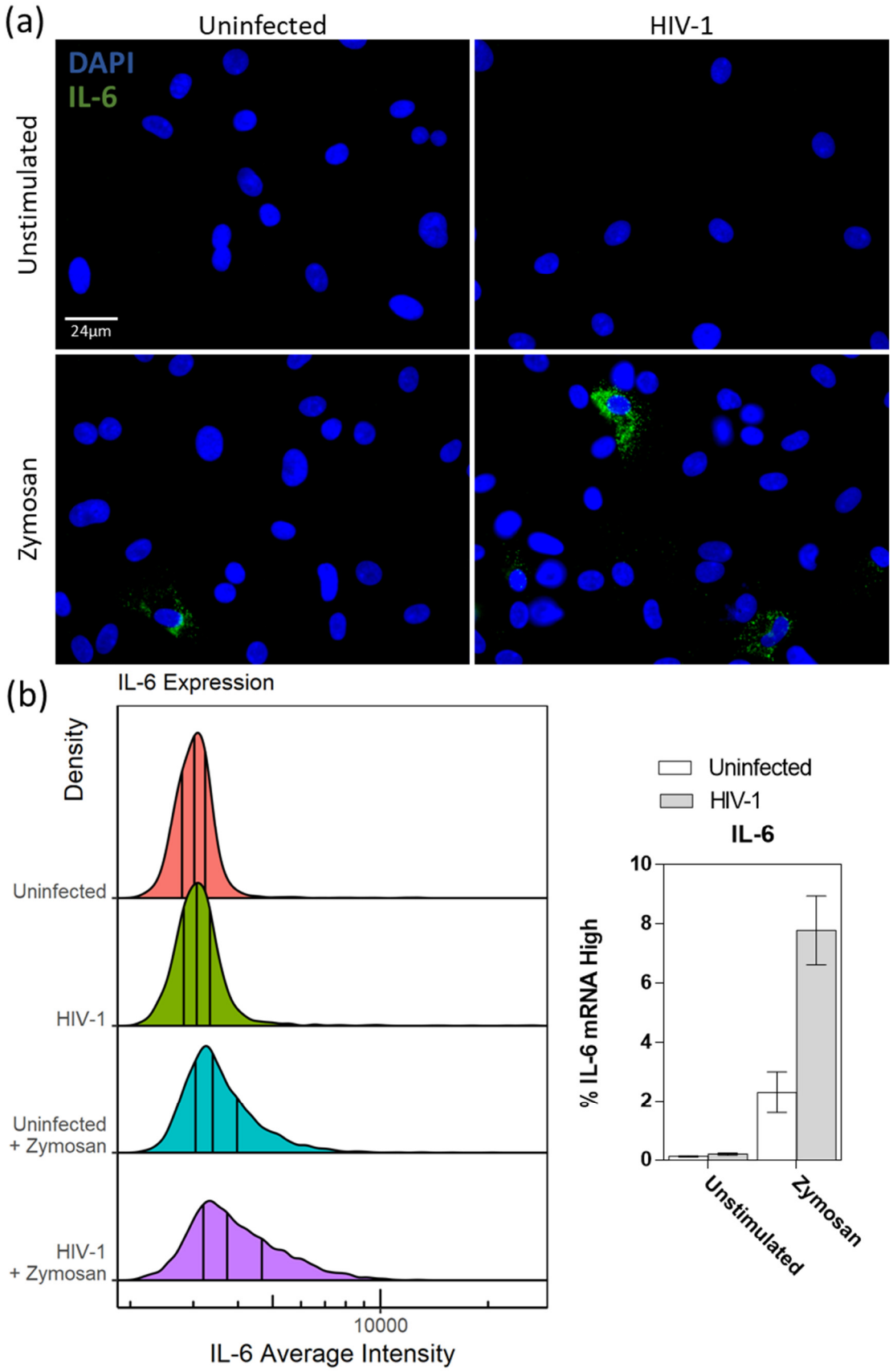

Figure 4.6: IL-6 responses are enhanced by HIV-1.

(a) Infected and uninfected MDMs were stimulated with zymosan for 4 hours before fixation and staining for IL-6 by RNA FISH. **(b)** Staining was quantified using Metamorph 7, using an intensity threshold of 7,500. Graphs show means ± SEM across 3 replicate wells.

4.2.6. RNA FISH can resolve heterogeneity in response to different stimuli.

To further validate the RNA FISH staining and to investigate the single cell variability seen in the response to zymosan, I compared the IL-10 and TNF response to stimulation with zymosan, LPS and Poly(I:C). At the doses used both LPS and zymosan induced strong expression of IL-10 in only a subset of cells, while Poly(I:C) did not induce a significant IL-10 response at this time point (**Figure 4.7a**). Staining for TNF expression revealed that zymosan induced expression of this cytokine in almost all cells in a culture, whereas LPS stimulated much less and Poly(I:C) induced minimal expression (**Figure 4.7b**). Expression of IL-10 and TNF in individual cells was independent, meaning that expression of one cytokine did not influence the expression of the other (**Figure 4.7c**). Cytokine mRNA expression was highly variable between individual cells for all the stimuli tested here, suggesting that this is a general feature of the cell culture model rather than being specific to particular stimuli.

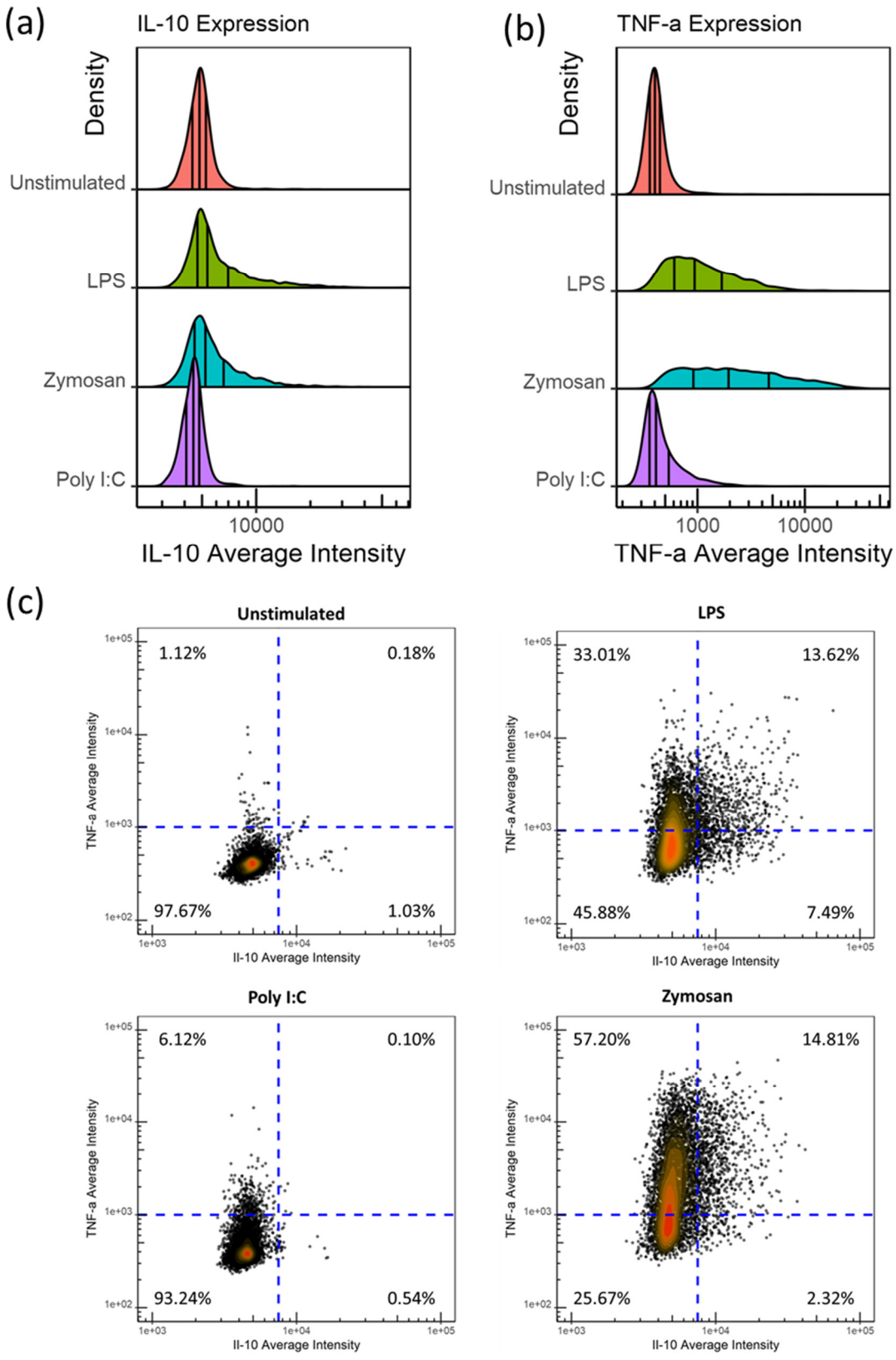

Figure 4.7: Cytokine expression heterogeneity in macrophages

MDMs were stimulated for 4 hours with LPS (100ng/ml), zymosan (0.4mg/ml) or Poly(I:C) (10µg/ml) before fixation and staining for IL-10 and TNFα RNA. **(a-b)** Quantitation was performed using Metamorph. **(c)** R was used to quantify staining above/below indicated thresholds. Stimulation and staining was performed by Matthew Solomons (UCL).

4.2.7. IL-10 expression in macrophages is independent of MCM2 status

It is possible to combine the RNA FISH staining with immunofluorescent staining for MCM2. This was of interest because I had observed that macrophages grown in FCS produced greater quantities of IL-10 (**Figure 3.11**). Since HIV induces up to a 6-fold attenuation of IL-10 expression when only 20% of cells are infected, I hypothesised that HIV may preferentially infect a subset of cells which express IL-10 to induce such a large effect. As the 'cycling' MCM2 positive cells are more permissive to HIV-1 infection I aimed to test whether these cells express high levels of IL-10. To test this experimentally, cells from both serum conditions were stimulated with zymosan for 4 hours before fixation and staining for IL-10 RNA and MCM2 protein (**Figure 4.8a**). As expected, macrophages cultured in FCS exhibited substantially greater MCM2 staining compared to macrophages cultured in HS. However, the proportion of IL-10 expressing cells was elevated in both MCM2 positive and negative cells treated with FCS (**Figure 4.8b**). Expression of IL-10 was therefore increased in the FCS-treated MDMs independently of cycling status, meaning that cells which express IL-10 are not preferentially infected by HIV-1.

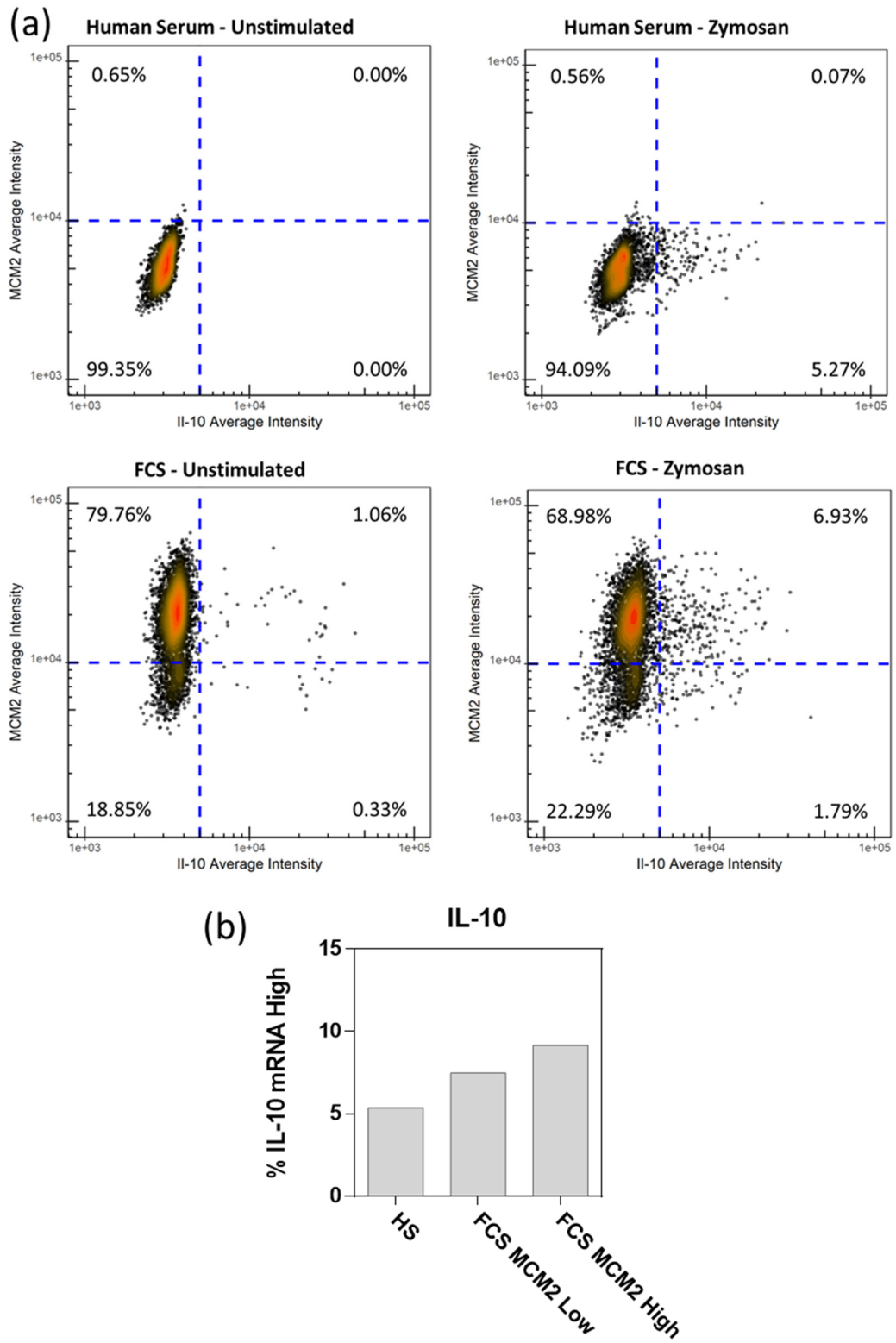

Figure 4.8: IL-10 expression and MCM2 status are independent.

MDMs were cultured in either FCS or human serum prior to simulation with zymosan for 4 hours. Cells were stained for IL-10 and MCM2 expression. **(a)** Staining was quantified using Metamorph 7, staining above/below marked thresholds was determined using R. **(b)** Quantitation was summarised using an MFI threshold of 5,000.

4.2.8. HIV-1 infection can be quantified using RNA FISH

A key objective in this chapter was to test the hypothesis that IL-10 responses are only attenuated in productively infected cells, but this required the ability to co-stain IL-10 and HIV-1. GFP-expressing viruses were unsuitable for these experiments because the RNA FISH staining process is destructive to most fluorophores. I evaluated RNA FISH staining of HIV-1 in macrophages with a view to multiplexing this with cytokine staining in subsequent experiments. I used RNA FISH probes targeting the HIV *gagpol* gene, which have the potential to stain both incoming virus and nascent viral RNA expression. These probes revealed different profiles of staining in macrophage cultures infected with HIV-1 for 48 hours (**Figure 4.9a**).

I hypothesised that low levels of HIV RNA staining represented incoming viral RNA, whilst high levels of HIV RNA staining represented nascent viral RNA expression. To test this, I assessed RNA FISH staining for HIV at 2h, 6h and 48h post-infection in the presence of absence of the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (**Figure 4.9b**). At the two and six-hour time points, only low levels of RNA were evident and unaffected by the RT inhibitor, confirming that this level of staining represented incoming HIV RNA pre-RT. In contrast, high level HIV RNA staining was only evident at 48 hours and was sensitive to the RT inhibitor, confirming that this level of RNA staining represented nascent HIV gene expression post-RT. This conclusion was further supported by showing that the proportion of macrophages containing high levels of HIV RNA was consistent with the proportion of cells expressing GFP in duplicate wells, which was also sensitive to the RT inhibitor (**Figure 4.9c**).

Pre-RT or 'incoming' virus could still be seen at the later time point, which may reflect the fact that infected cells would still be expected to produce new virions lacking envelope. While non-infectious, these virions could still be taken up by other cells by phagocytosis. Alternatively, this population may represent persistent RNA from non-productive infection in which reverse transcription failed, perhaps in cells which restricted infection. Despite this the clear difference in fluorescence intensity between this population and the productively infected cells allowed for the use of this assay as a means of detecting infection during RNA FISH experiments.

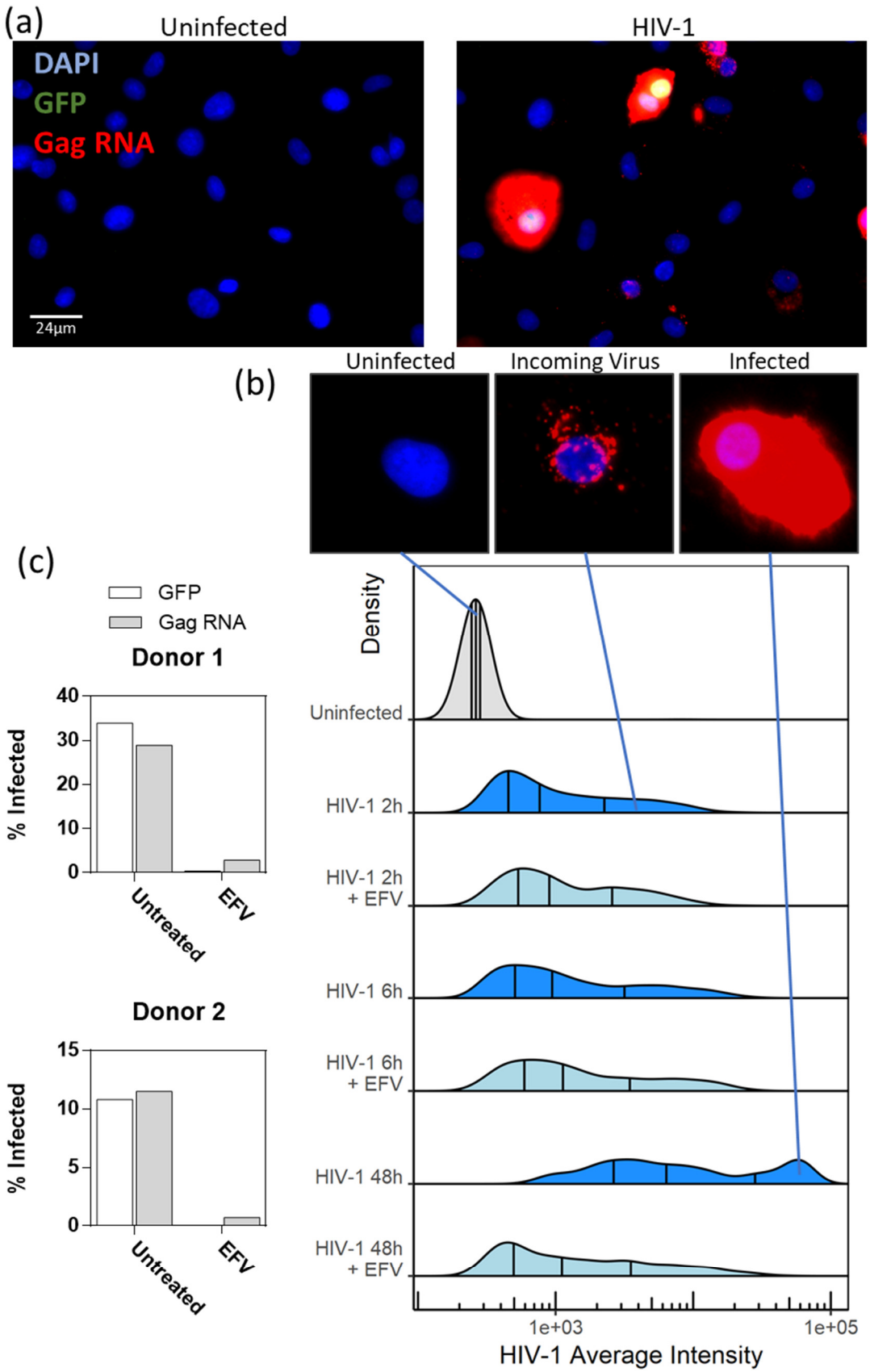

Figure 4.9: Detection of HIV-1 infection with RNA FISH

(a) Cells infected with HIV LAI-GFP for 48h were stained for HIV-1 RNA to visualise productively infected cells. **(b)** A time course of infection in the presence and absence of the reverse transcriptase inhibitor efavirenz (EFV), as detected using Metamorph 7. **(c)** Comparison of quantitation of infection by GFP expression and HIV-1 RNA detection as determined by thresholding in R.

4.2.9. IL-10 attenuation is not stimulus specific

During development of the RNA FISH assay I found that zymosan exhibited some green autofluorescence, which interfered with my ability to detect some of the weaker probe sets. In order to overcome this limitation, I sought to replace zymosan with an alternative secondary stimulus. Previous work had established that IL-10 attenuation was present in response to a variety of innate immune stimuli [447]. Curdlan, a β-1,3-glucan from *Alcaligenes faecalis*, was therefore selected as an alternative to zymosan in this model. Consistent with previous data, I confirmed that HIV-1 infection of macrophage cultures attenuated IL-10 responses in response to this stimulus (**Figure 4.10**).

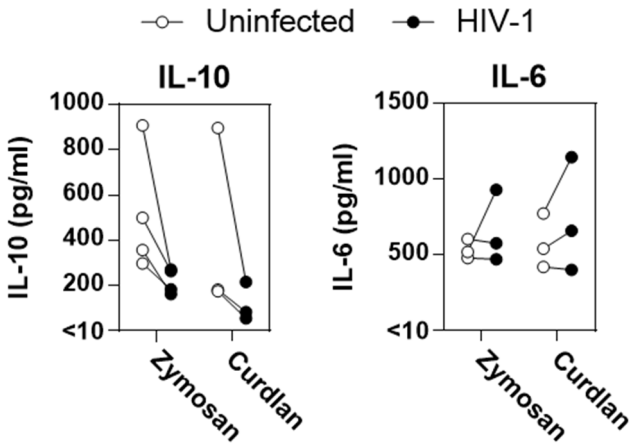

Figure 4.10: Attenuation of IL-10 responses is not specific to zymosan

MDMs infected with HIV were stimulated with zymosan (0.4mg/ml) or curdlan (0.1mg/ml) for 4 hours, after which supernatants were collected and assayed for cytokine expression. Results from 3 donors.

4.2.10. IL-10 attenuation is not restricted to infected cells

Next, I tested the hypothesis that IL-10 attenuation is restricted to productively infected macrophages. As before, HIV-1 infection of macrophage cultures attenuated IL-10 responses compared to uninfected cultures (**Figure 4.11a**). I then separated IL-10 staining data from cells within HIV-1-infected cultures, with and without high levels of HIV RNA staining indicative of viral gene expression. Both the productively infected ("Infected") and uninfected (but "Exposed") groups of cells revealed significantly attenuated IL-10 responses to curdlan compared to cells from wells without HIV (**Figure 4.11b**). Using a threshold to classify cells as expressing high or low levels of IL-10 demonstrated a 2- to 4-fold loss of the high IL-10 expressing population in three separate donors, regardless of infection status (**Figure 4.11c**). I therefore concluded that productive infection is not required for IL-10 attenuation.

These data also confirmed my prior conclusion that HIV-1 attenuation of macrophage IL-10 responses did not require HIV-1 gene expression.

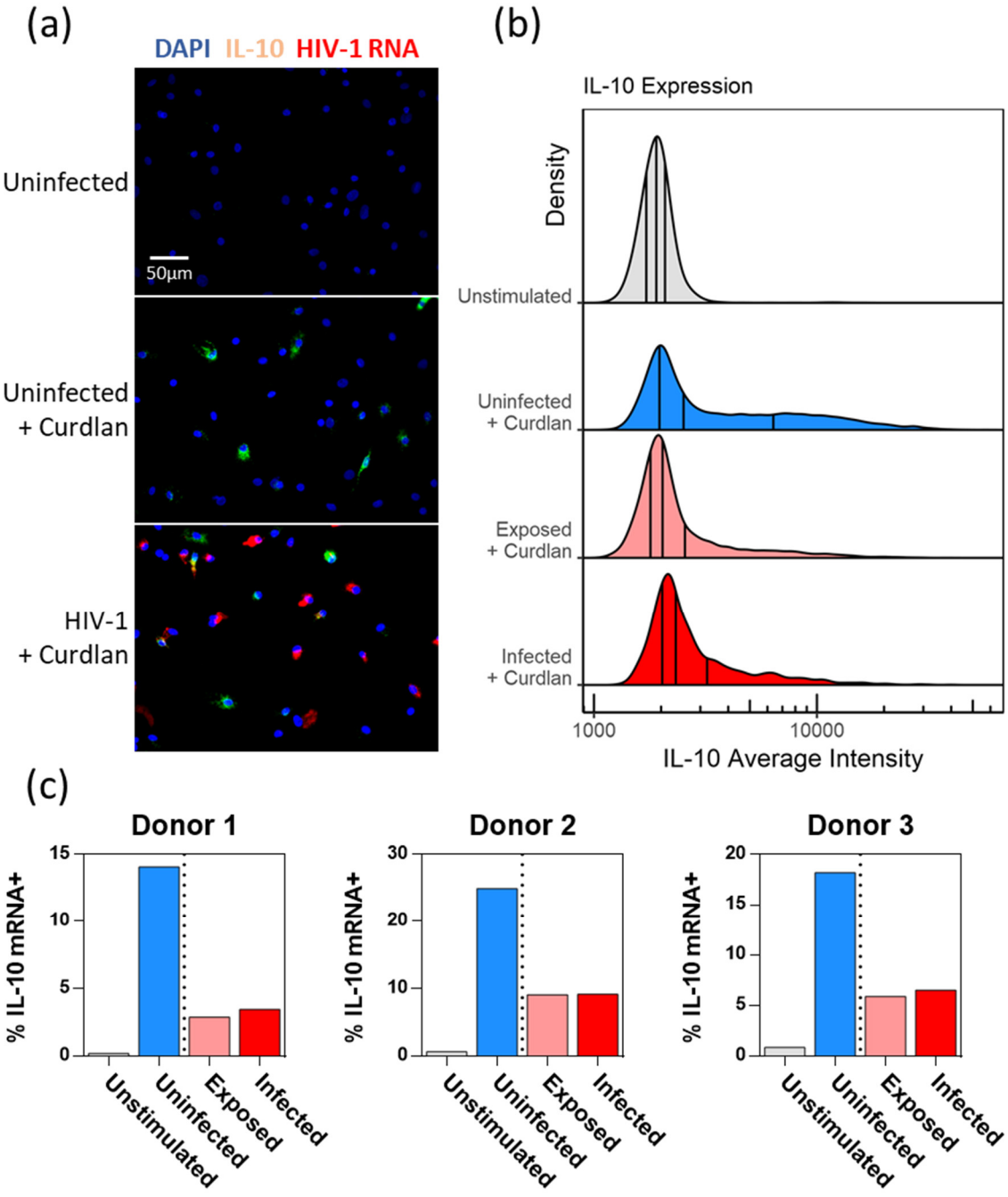

Figure 4.11: Single cell analysis of IL-10 attenuation and infection.

MDMs were infected with HIV-1 for 24h prior to stimulation for 4h with curdlan. Cells were then fixed and stained for HIV-1 RNA and IL-10 RNA **(a)**. **(b)** Expression was quantified using Metamorph 7 and a ringmask-based analysis strategy to generate MFI for each cell. An infected well was divided into productively infected cells ("**Infected**") and uninfected cells ("**Exposed**") using HIV-1 RNA staining to look for *de novo* viral RNA expression. Representative of 3 donors. **(c)** Quantitation was summarised for 3 donors using a threshold for "high expressing" cells at a MFI of 7,500.

4.2.11. Most cells are exposed to HIV-1 virions but restrict infection.

I then sought to test the hypothesis that MDMs which do not become productively infected have been exposed to virions which are restricted by the target cell or fail to integrate into the genome. To achieve this, I fixed cells at 4h post-infection and stained using RNA FISH for *gagpol* RNA to try to visualise virus which had been taken up into cells. Uptake between individual cells varied substantially, with some cells taking up large quantities of virus (**Figure 4.12a**), but this still did not approach the intensity of signal previously seen after productive infection (**Figure 4.9**). Quantitation revealed that at the dosages selected for these experiments (10IU/cell) most cells in a culture had taken up virus (**Figure 4.12b**).

The presence of intracellular viral RNA and possibly DNA could provide a potential agonist for innate immune sensors such as cGAS and RIG-I [476], [477]. Given that most cells do not become infected but do take up virus, restricted virions may provide the necessary stimulus to induce IL-10 attenuation in this population.

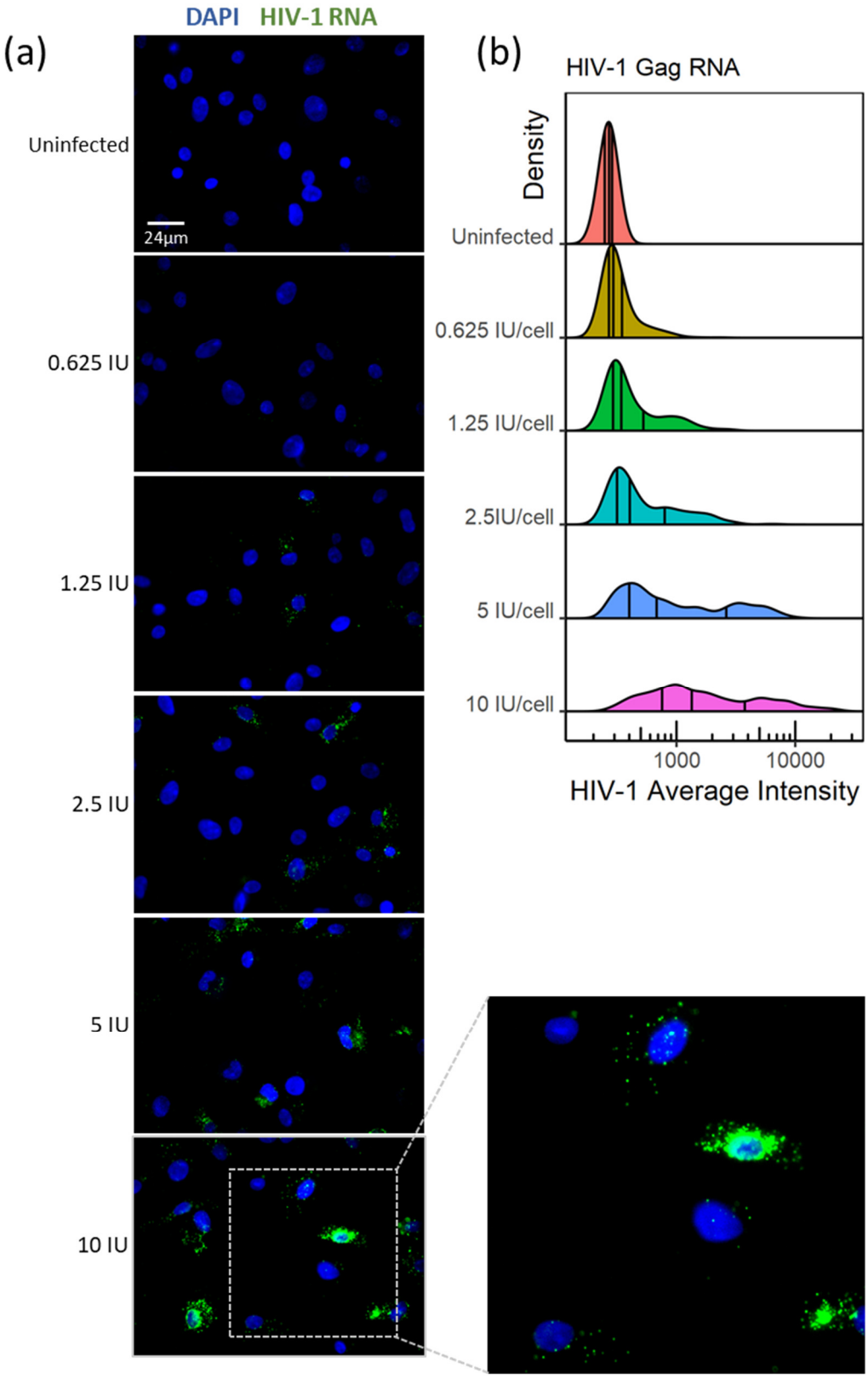

Figure 4.12: Imaging of HIV-1 RNA uptake in a dose titration of infection.

MDMs were infected for 4 hours with a dose titration of HIV-1. **(a)** Cells were fixed and stained for HIV-1 gagpol RNA. **(b)** Viral content of cells was quantified using MetaMorph 7. Data representative of 3 donors.

4.2.12. Viral reverse transcription is not required for IL-10 attenuation

My findings so far suggested that incoming viral genomes were responsible for attenuation of macrophage IL-10 responses, and that almost all cells in my experimental model are exposed to these genomes. Host cells have diverse mechanisms for detecting microbial genomes. These mechanisms are typically grouped as RNA or DNA sensors. HIV RNA has been reported to interact with TLR7 and TLR8 [403], although there are no prior reports of this in monocyte-derived macrophages. In addition, the fact that TREX1 deficiency leads to IFN responses to HIV in macrophages indicates that these cells also harbour DNA sensors [409]. Therefore, I sought to test whether viral RNA or DNA was responsible for attenuation of macrophage IL-10 responses.

To do this, I infected cells with HIV-1 in the presence of efavirenz. At 48h post-infection EFV completely inhibited productive infection (**Figure 4.13a**) as measured by both GFP expression and high viral RNA content detected by RNA FISH, indicative of viral gene expression. In the presence of EFV some viral RNA remained in the cytoplasm of cells, most likely from the incoming virus, while GFP expression was absent (**Figure 4.13b**). Even in the presence of the inhibitor, zymosan-inducible IL-10 responses were still attenuated (**Figure 4.13c**), suggesting that viral reverse transcription was not required for this phenotype.

Even in the presence of reverse transcriptase inhibitors there is the potential for small quantities of viral DNA to be transcribed before the action of an inhibitor, although this is primarily an issue with competitive nucleoside reverse transcriptase inhibitors (NRTIs) [478]. To build further confidence in my results, I also utilised an RT knockout mutant of my HIV-1 vector and tested this in the model. As expected, this virus could not be titrated using GHOST cells and produced negligible RT activity readings by SG-PERT, so genome copies were quantified by qPCR and used to normalise dosage compared to the wild type strain for infection. Quantitation of intracellular viral RNA at 48h post-infection revealed an absence of the high levels of RNA expected from *de novo* expression from integrated virions. This confirmed that this mutant was unable to productively infect MDMs (**Figure 4.14a-b**), incoming RNA having been mostly degraded by this time point. Consistent with results from the RT inhibitor (**Figure 4.13**), the RT mutant also attenuated IL-10 responses to subsequent innate immune stimuli (**Figure 4.14c**).

These results demonstrated that reverse transcription was not necessary for this phenotype. Combined with my previous findings which showed that viral genomic material is required (**Figure 4.2**), this strongly suggests that RNA sensing is sufficient to attenuate macrophage IL-10 responses.

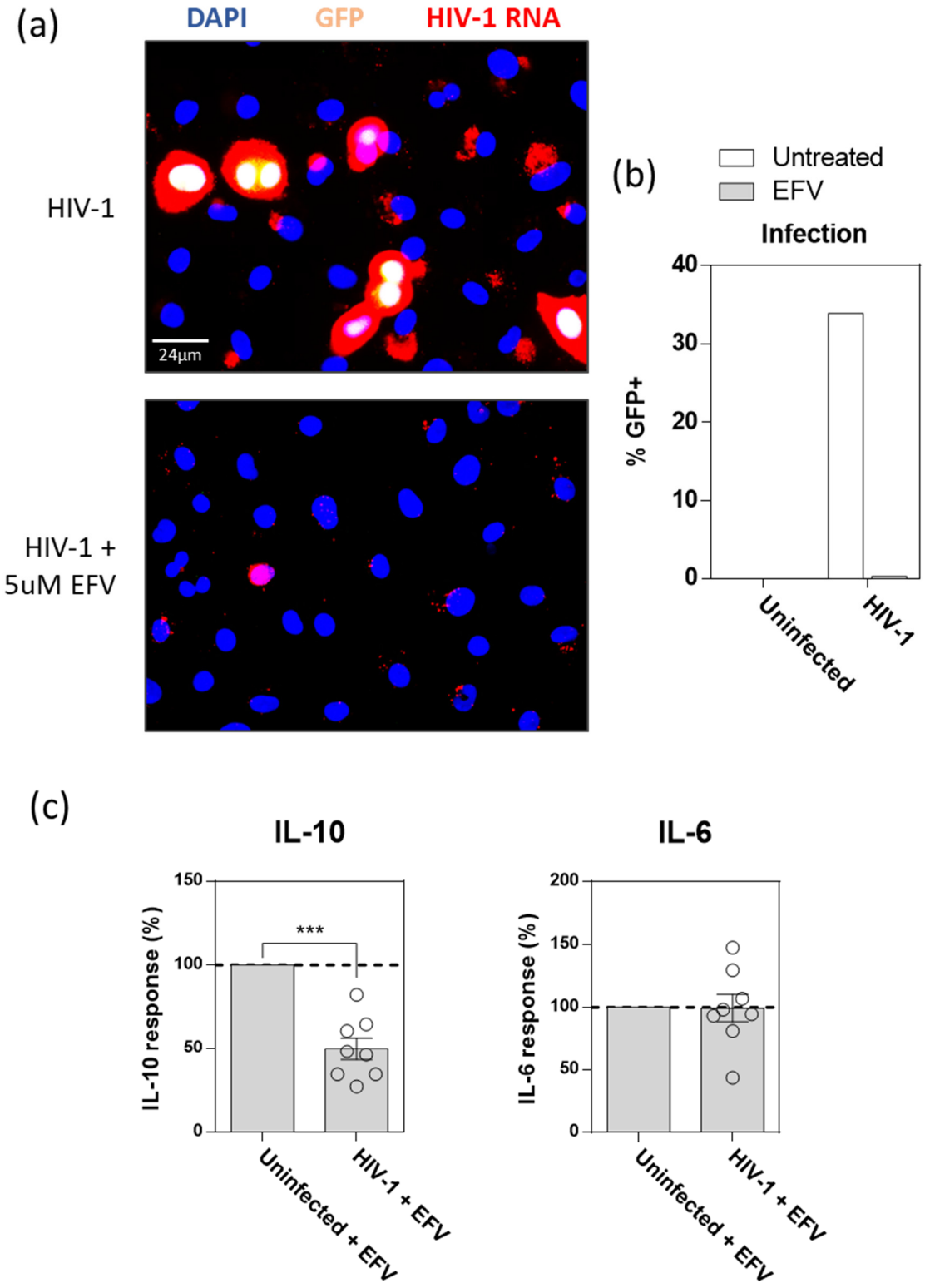

Figure 4.13: A reverse transcriptase inhibitor does not prevent IL-10 attenuation

MDMs were treated with 5µM EFV for 2 hours prior to infection with HIV-1. **(a)** After 48 hours infection was quantified by looking for GFP+ nuclei and staining for HIV-1 RNA. **(b)** Quantification of infection by counting of GFP expressing cells. Representative of 3 donors. **(c)** Cytokine ELISA on 4-hour supernatants in EFV-treated wells. Graphs show means ± SEM. Significance was determined by paired t-test. Results from 8 donors.

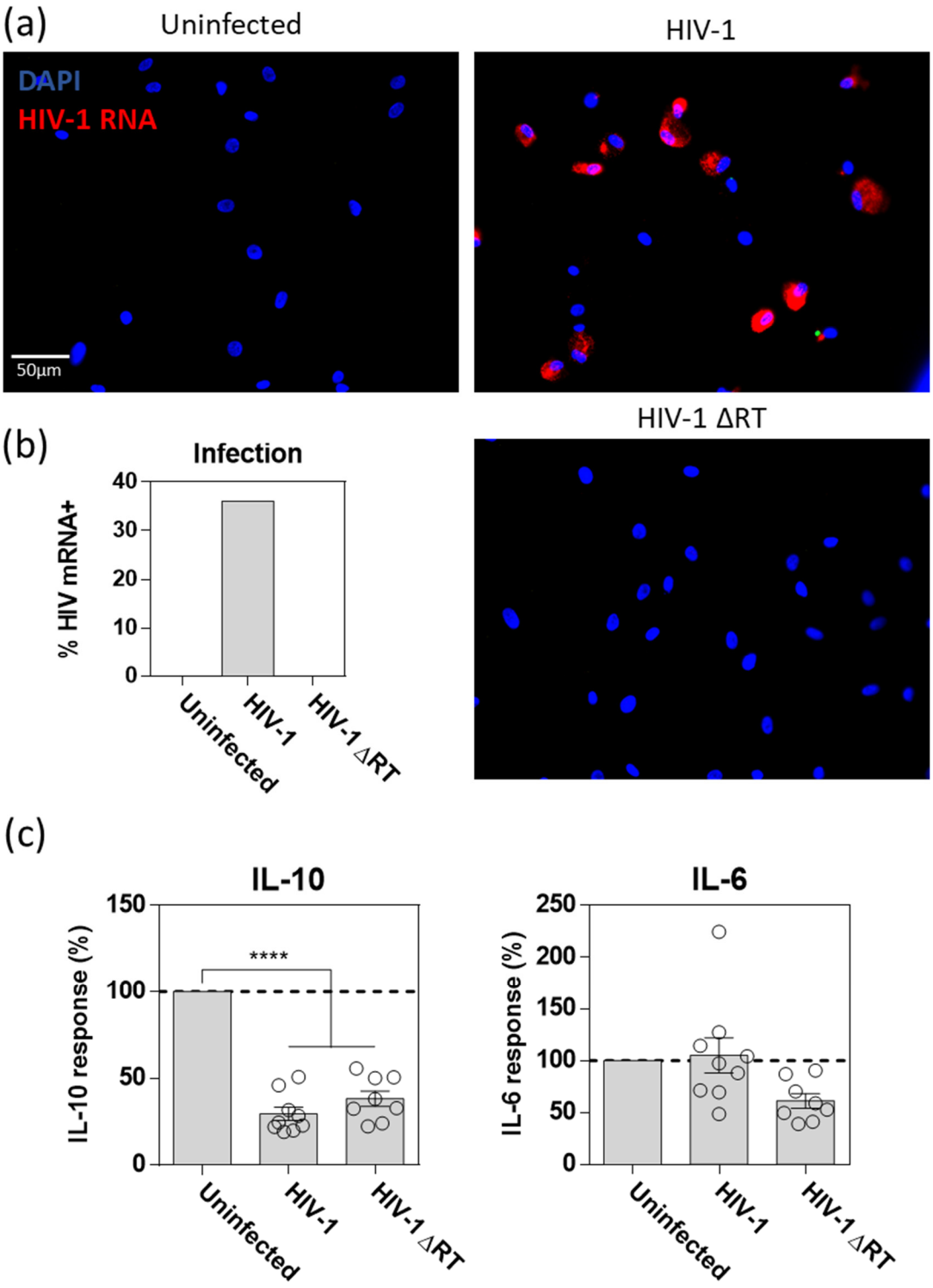

Figure 4.14: An RT mutant of HIV-1 fails to attenuate IL-10.

MDMs were infected with a reverse transcriptase knockout mutant of HIV-1 and tested for attenuation of IL-10 responses. **(a-b)** Staining for HIV-1 RNA at 48h post-infection and quantitation of productively infected cells. Representative of 3 donors. **(c)** ELISA on supernatants for cytokine responses to zymosan. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 8 donors.

4.2.13. Active viral entry is not necessary for IL-10 attenuation in macrophages

Macrophages express cytoplasmic and endosomal RNA sensors. Both groups of sensors have been reported to be capable of sensing HIV-1 RNA [404], [406], [421]. I therefore aimed to determine whether cytosolic or endosomal sensing is the mechanism responsible for attenuated IL-10 responses. HIV-1 with its natural envelope or pseudotyped with VSV-G can enter into endosomes and the cytoplasm [367], [479]. Based on the literature I reasoned that "bald" HIV vectors may be taken up by the endosomal pathway, but without an envelope to mediate fusion these vectors would fail to penetrate the endosomal membrane to enter the cytoplasm [480], [481]. I used RNA FISH to confirm that "bald" vectors were successfully taken up into macrophages (**Figure 4.15a**). Both the pseudotyped and the "bald" vectors attenuated IL-10 in my model, indicating that active fusion using a viral envelope protein is not required for IL-10 attenuation (**Figure 4.15b**). As anticipated, no productively infected cells were seen with the "bald" virions (**Figure 4.15c**). These results are most consistent with the hypothesis that endosomal sensing of viral RNA is sufficient to cause attenuation of macrophage IL-10 responses.

It is possible that as these virions cannot enter the cytosol they will rapidly be degraded if lysosomal fusion occurs. To investigate this, I stained for HIV-1 RNA at 2h, 6h and 48h post-infection (**Figure 4.15c**). As has previously been reported in the literature, the "bald" vectors were taken up at a reduced rate compared to VSV-pseudotyped virions, which is likely to be due to the lack of active fusion and inability to escape degradation. While no substantial loss of vector was seen between 2h and 6h after infection, by 48h the "bald" population degraded significantly. In contrast, productively infected cells were visible with the pseudotyped virions at this timepoint.

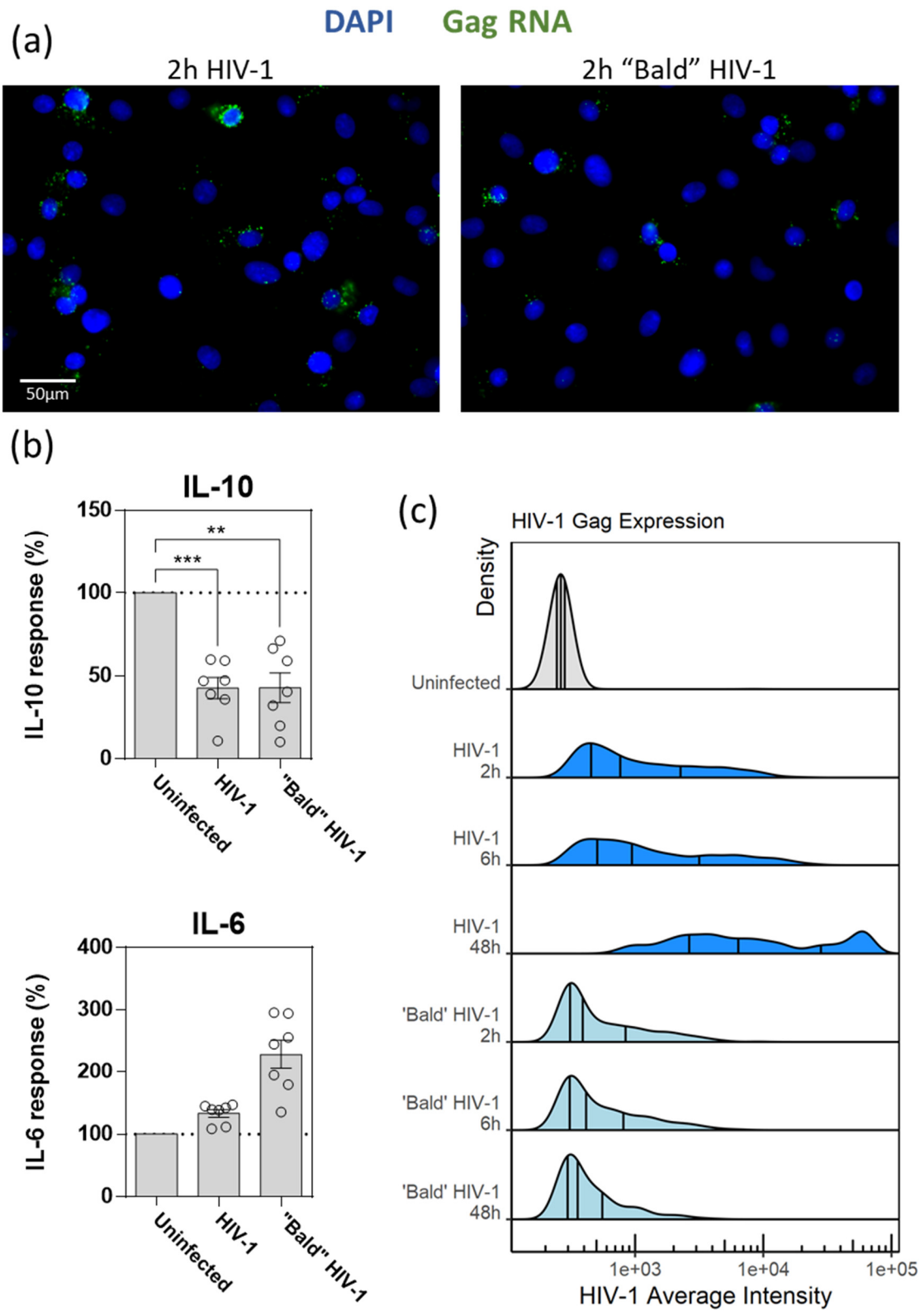

Figure 4.15: Active fusion is not required for IL-10 attenuation

"Bald" virions were produced by packaging HIV-1 virions in the absence of a genome plasmid. **(a)** RNA FISH was used to detect viral uptake after infection. **(b)** Treated cells were stimulated with zymosan for 4h and the supernatants tested for cytokine secretion by ELISA. **(c)** Viral uptake was quantified over a time course using MetaMorph 7. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 7 donors.

4.2.14. Delivery of viral RNA is sufficient to attenuate IL-10 responses.

In view of my findings with 'bald' viruses, I tested the hypothesis that HIV-derived RNA motifs are sufficient to attenuate macrophage IL-10 responses. ssRNA40, also known as R-1075, is a short U-rich 20-mer derived from the HIV-1 LTR sequence [403]. This oligonucleotide is known to activate murine TLR7 and human TLR8 in myeloid cells. A control variant, ssRNA41, was derived from ssRNA40 by replacing all stimulatory U residues with adenosine, which significantly reduced its potency as an agonist. These short RNA molecules were delivered in LyoVec to test their effect on IL-10 responses in macrophages.

I observed a dose-dependent inhibition of IL-10 responses in macrophages treated with ssRNA40, but not ssRNA41 (**Figure 4.16**). This demonstrated that HIV-derived RNA alone is sufficient to induce the phenotype. The absence of any effect from the modified ssRNA41 variant also strongly suggests that TLR8 may be the host sensor which is involved. It was not possible to quantify the uptake of the ssRNA itself due to the 20-mers being too short for labelling.

In order to support the hypothesis that specific stimulation of TLR8 was sufficient for attenuation of macrophage IL-10 responses, I tested the effect CL075. CL075 is a synthetic TLR8 agonist which is not an ssRNA [482]. This stimulates both TLR7 and TLR8, but we know that the macrophages used in this model significantly downregulate expression of TLR7 after differentiation from monocytes [414]. I tested the effects of both CL075 and ssRNAs by RNA FISH (**Figure 4.17a**). As expected CL075 replicated the effect of HIV-1 vectors and ssRNA40, while the ssRNA41 control had no effect on IL-10 expression (**Figure 4.17b**). Both synthetic agonists produced a stronger effect than HIV-1, although this is likely to be dose-dependent. Together these results demonstrate that TLR8 activation is sufficient to attenuate IL-10 responses, and that such activation can replicate the effects of HIV-1.

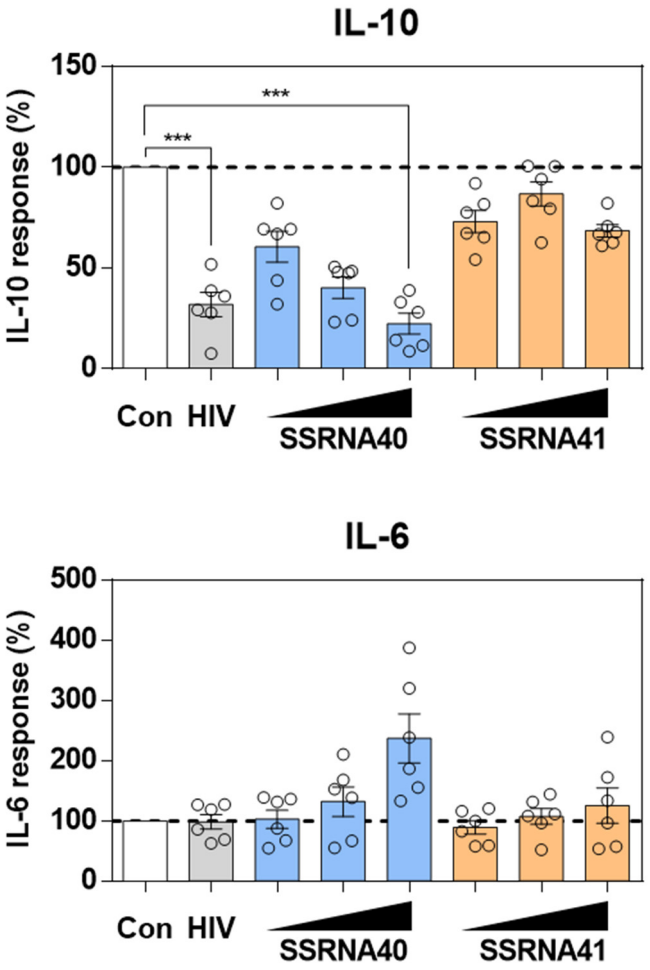

Figure 4.16: HIV-1-derived ssRNA is capable of attenuating IL-10 responses.

MDMs were treated with a dose titration of ssRNA in LyoVec (0.25-5µg/ml) or HIV-1 for 24 hours prior to stimulation. Supernatants were assayed by ELISA for cytokine expression after 4 hours of stimulation with zymosan. Graphs show means ± SEM. Significance was determined using one-way ANOVA. Results from 6 donors.

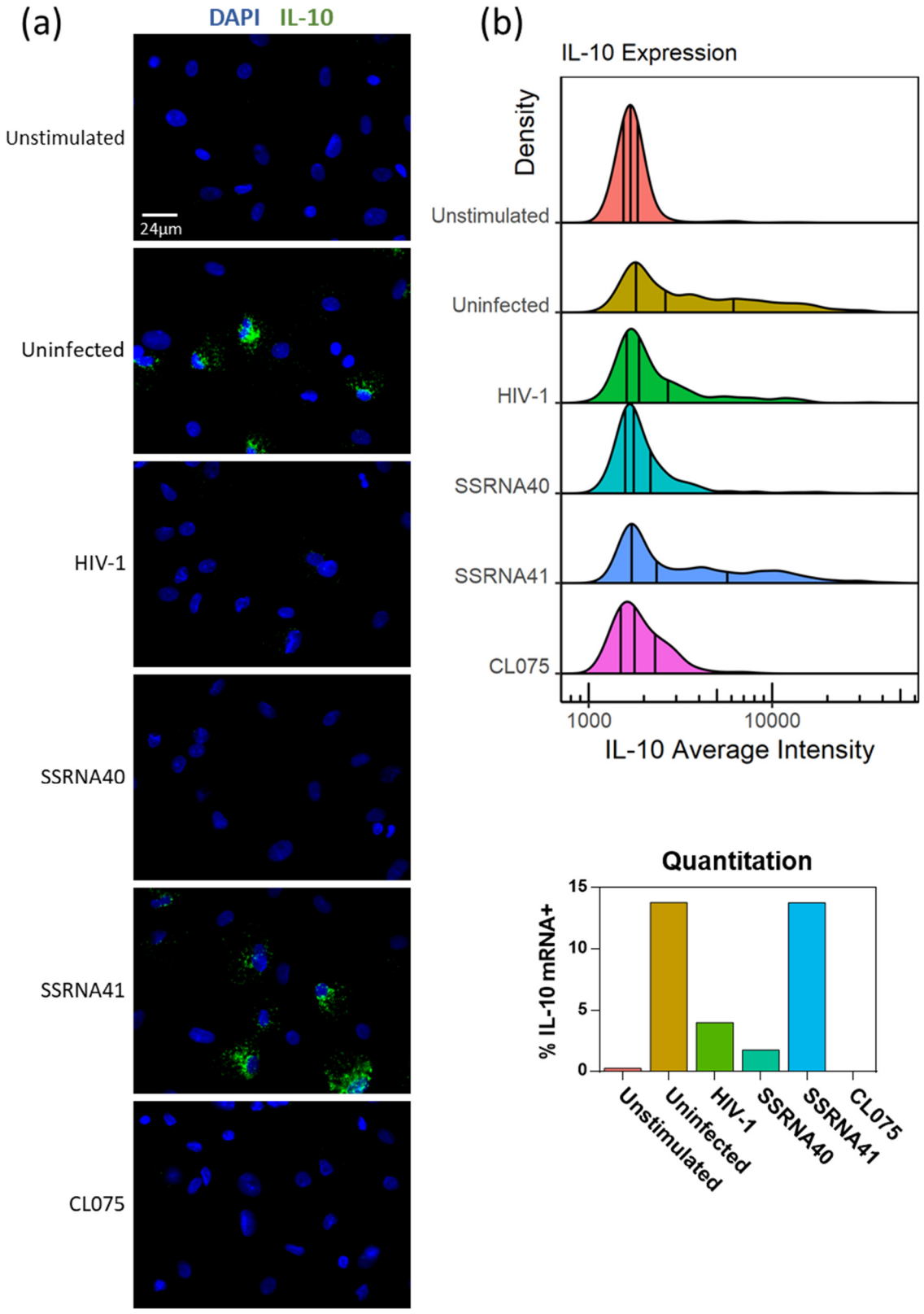

Figure 4.17: TLR8 agonists attenuate IL-10 at the single cell level.

MDMs were treated with HIV-1, ssRNA (5µg/ml) or CL075 (1ug/ml) for 24h prior to stimulation with zymosan for 4h. **(a)** Cells were fixed and stained for IL-10 RNA expression by RNA FISH. **(b)** IL-10 expression was quantified using Metamorph 7. R was used to determine the proportion of cells with staining above a threshold of 10,000. Representative of 2 donors.

4.3. Chapter Discussion

In this chapter I found that that destruction of the virion by boiling or destruction of the genome by UV treatment abolished IL-10 attenuation, suggesting the involvement of viral genomic material in this phenotype. Delivery of virus-like particles without viral genome failed to attenuate IL-10, meaning that viral genome is necessary. This indicated that viral RNA or DNA was likely to be the causative factor in this model.

To further assist in investigating IL-10 attenuation, I visualised IL-10 expression at the single-cell level. Since available antibodies were unable to detect IL-10 at the protein level, I implemented RNA FISH as a means of staining for IL-10 mRNA. This assay could be multiplexed with other RNA targets which would be beneficial to our understanding of the phenotype. An analysis strategy involving a ring-shaped mask as an estimate of cytoplasmic staining was developed to overcome limitations in the microscopy hardware available.

RNA FISH revealed that a subset of MDMs express the majority of IL-10 mRNA, at least at the 4-hour timepoint. Similar heterogeneity was seen in response to other stimuli. The M-CSF-based differentiation protocol used in this study is not intended to drive differentiation towards any specific monocyte-derived macrophage subset, so it is likely that the resulting cells may exhibit a range of different activation phenotypes which contribute to this heterogeneity. IL-10 expression was seen to be attenuated by HIV-1 infection, recapitulating the results seen at the protein level by ELISA. Further work could focus on additional profiling of the IL-10-expressing subset to determine whether other functional characteristics such as phagocytic capacity may be affected by HIV.

During development of the RNA FISH assay I opted to substitute zymosan for curdlan as the secondary stimulus to minimise autofluorescence when using probe sets which exhibit weaker fluorescence. Both zymosan and curdlan displayed strong IL-10 attenuation without effects on inflammatory cytokine expression. While zymosan is known to activate TLR2 and Dectin-1, curdlan is more specific and only activates Dectin-1 [483]. This pathway is known to enhance the secretion of cytokines induced by TLR2 and TLR4 [484]. Unlike the TLRs, Dectin-1 signalling does not involve the MyD88 cascade as a downstream mediator [485]. That reduced IL-10 responses were seen in response to the Dectin-1-specific curdlan as well as TLR-specific agonists such as LPS and Pam_2CSK_4 [447] suggests that any potential effect of HIV-1 may be acting far downstream of the secondary stimulus.

The RNA FISH staining protocol was also damaging to fluorophores such as GFP, and so staining of HIV-1 RNA was evaluated as an alternative means of quantifying infection when

performing these experiments. High levels of viral RNA within a cell are suggestive of productive infection. Quantitation of such cells gave a similar percentage of infected cells to that seen when quantifying using GFP expression. This indicated that RNA FISH can be used to accurately determine infection levels within a culture. Moreover, I was also able to detect viral RNA from incoming virions. This would potentially be valuable for further investigation into viral entry by providing a secondary use for this stain when analysing early time points after infection. These experiments revealed that most cells in the cultures take up virus, indicating that host restriction or defective virions may be the limiting factor in macrophage infection, rather than entry into cells. Interestingly, while treatment with the RT inhibitor efavirenz robustly inhibited infection, there was no significant change in the amount of RNA present within cells at 6 hours post infection. Detection of virus by RNA FISH should be lost after viral RNA is reverse transcribed into DNA, so these results may suggest that either most virions are defective in reverse transcription or that RT in macrophages does not progress significantly until at least 6 hours after infection.

Sensing of HIV may occur primarily in cells which restrict infection, which would explain how large effects on IL-10 production were seen with only a limited proportion of infected cells. Staining of HIV-1 and IL-10 by RNA FISH revealed IL-10 attenuation in both the productively infected and uninfected “exposed” cells within an infected culture. The attenuation of IL-10 expression in uninfected cells explains how a substantial effect can still be seen in donors with only a small fraction of productively infected MDMs. The diverse array of described host restriction factors provide ample opportunity for targeted cells to block infection and/or detect the presence of virus [486]. Staining for viral uptake at early time points revealed that at the dosage used almost all cells in the culture receive at least one copy of the virus. This would support the notion that most cells which do not become productively infected in my experiments have been exposed to and restricted the virus. Interestingly there was substantial variance in the amount of virus within each cell at these early time points. Since phagocytic capacity varies between individual cells [63], it is likely that some cells take up large quantities of virions by endocytosis, while other cells may only experience active fusion at the plasma membrane.

It is plausible that cells which take up large quantities of virus would sense this and exhibit IL-10 attenuation, but this is difficult to test experimentally due to the potential for viral degradation before a second stimulus could be applied. At present commercially available RNA staining protocols are only compatible with fixed cells, although new technologies may allow RNA tracking in live cells in the future [487]. Other groups investigating HIV-1 entry have

achieved live virion tracking by labelling HIV capsid and/or integrase proteins [376], but this would not necessarily track RNA which has dissociated from the viral core.

Virions without an envelope protein still produced a phenotype, which suggests sensing at the cell membrane or within endosomes as likely mechanisms. Since without fusion HIV-1 proteins cannot be delivered to the cytoplasm, this eliminated cytosolic RNA and DNA sensing as a potential mechanism. Experiments with RT mutants and RT inhibitors confirmed that viral DNA was not required to induce IL-10 deficiency, leaving RNA sensing as the primary hypothesis. In agreement with this, delivery of short HIV-derived ssRNA molecules was sufficient to replicate the effects of full length virus on MDM cytokine responses. The ssRNA used has previously been reported as a TLR8 agonist, and so I tested the synthetic TLR8 agonist CL075 in the assay. This potently phenocopied the effects of HIV-1, confirming that TLR8 activation can specifically induce IL-10 attenuation.

It is worth noting that the ssRNA reagents are commercially formulated in LyoVec to stabilise the RNA and facilitate uptake, but this may preferentially deliver them into the cytosol rather than endosomes. Macrophages are likely to endocytose some material despite this not being the primary route of delivery, which may explain why relatively high doses were required to phenocopy the effects of HIV-1.

In summary, these results suggest a model in which phagocytic uptake and degradation of HIV-1 virions by endosomal proteases leads to viral RNA being exposed to TLR8 sensing, which then primes MDMs to express less IL-10 during subsequent activation. Enhancement of inflammatory TLR2/4 responses by pre-treatment with HIV-derived TLR8 agonists has previously been reported in cell lines [488], but this had not been demonstrated with infectious virus or linked to an IL-10 deficit at early timepoints during stimulation. Dendritic cells have also been reported to sense HIV-1 RNA via TLR7/8, although this induced interferon responses which have not been seen in the monocyte-derived macrophage model [404]. Future work is required to test the hypothesis that TLR8 is necessary for HIV-1 attenuation of macrophage responses.

5. Results 3 – Host factors in IL-10 attenuation.

5.1. Introduction

Regulation of IL-10 is known to be complex and cell type specific. Mechanisms have been described at the epigenetic, transcriptional and post-transcriptional levels [154]. This complexity may reflect the role of IL-10 in balancing antimicrobial activity and autoimmunity during inflammatory responses. In myeloid cells the MAPK/ERK and p38 pathways are reported to activate expression of IL-10, alongside numerous other cytokines and cellular processes (see section **1.4.3**) [489]. There have also been reports of PI3K signalling providing more specific regulation of IL-10 production by activating only the CREB and AP1 transcription factors downstream of the ERK and p38 pathways [490]. In addition to this, the GSK3 and mTOR pathways have been implicated in the epigenetic modification of the IL-10 promoter [491]. How these pathways cooperate to specifically regulate expression of IL-10 remains unclear.

Previous experimental data from our group suggested that HIV inhibits MAPK/ERK signalling in the context of coinfection with *Mycobacterium tuberculosis*, visualised as a defect in ERK1/2 and p38 phosphorylation in infected wells [445]. Additional experiments using the single round infection model with zymosan found that only small molecule inhibition of the PI3K-Akt-mTOR pathway replicated the specific effect of HIV-1 on IL-10 [447]. Investigation of these pathways is complicated by the fact that most described signalling mechanisms are involved in other cellular processes, which makes their inhibition by small molecules prone to off-target effects and/or toxicity. I sought to expand on these previous findings by testing whether inhibition of ERK1/2 phosphorylation by HIV could be recapitulated in the single round model, and whether HIV altered phosphorylation of the PI3K-Akt-mTOR axis during stimulation.

In the previous chapter I have shown that HIV RNA and TLR8 stimulation are sufficient to attenuate IL-10 responses. The canonical response to TLR8 recognition of viral RNA is the induction of type I IFNs [492], [493]. However, in our primary macrophage model productive infection with HIV-1 has not been found to significantly activate the interferon pathway [414]. While no IFN or IFN-inducible gene signature had previously been observed, it remains possible that the virus does trigger IFN responses below the level of detection to impact the regulation of IL-10. Alternatively, TLR8-mediated effects on IL-10 regulation may occur independently of IFN responses. To investigate this, I aimed to resolve whether IFN signalling would phenocopy the effects of HIV-1, and then whether blocking IFN activity would rescue IL-10 responses. The RNA FISH assays developed previously also provide the opportunity to test for interferon induction at the single cell level.

It has long been known that innate immune responses adapt to prior stimulation, typically by increasing anti-inflammatory regulation. This is exemplified by endotoxin tolerance in which repeated stimulation leads to attenuated responses to further stimuli [494]. A key mechanism for innate immune adaptation is via long lasting epigenetic modifications, which are increasingly referred to as 'trained immunity' to distinguish them from conventional adaptive immunity [495]. In my experimental model, TLR8 stimulation leads to attenuation of anti-inflammatory IL-10 responses to subsequent innate immune stimulation. Therefore, I sought to test the hypothesis that modification of IL-10 responses following TLR8 stimulation may also be long-lasting.

The objectives of this chapter are as follows:

1) Determine whether altered phosphorylation can be detected in pathways reported to regulate IL-10.
2) To investigate whether interferon activity can attenuate or enhance IL-10 in macrophages.
3) Confirm whether interferon induction could play a role in the phenotype seen with HIV-1.
4) Examine the duration of attenuation of IL-10 responses.

5.2. Results

5.2.1. Zymosan-induced Akt phosphorylation is unchanged by HIV-1.

To investigate the host mechanisms behind IL-10 attenuation I utilised Western blotting to investigate phosphorylation of signalling pathways reported to be involved in the regulation of IL-10. Akt (Protein Kinase B) is a serine/threonine kinase which is involved in signalling for a multitude of pathways, including IL-10 expression. Previous experimental data from our group revealed that small molecule inhibition of the PI3K-Akt-mTOR pathway replicated the effects of HIV-1 [447]. Akt can be activated by phosphorylation at S473 or T308. It was found that HIV-1 infection did not alter phosphorylation at Akt S473 in response to zymosan but T308 was not tested [447]. To consolidate and build on the previous experiments, I tested whether phosphorylation of Akt T308 in response to zymosan was altered by infection with HIV-1.

To assess this, lysates from HIV infected and uninfected MDMs were collected during a time course of zymosan stimulation and Western blotting was used to assess phosphorylation of Akt. Stimulation with zymosan induced phosphorylation at the T308 site, but no significant difference was evident at any time point in HIV infected macrophage cultures in two separate donors (**Figure 5.1a**). In the same experiments I was able to confirm HIV attenuation of macrophage IL-10 responses by ELISA (**Figure 5.1b**). I concluded that HIV-1 is does not alter any aspect of Akt activation in my model.

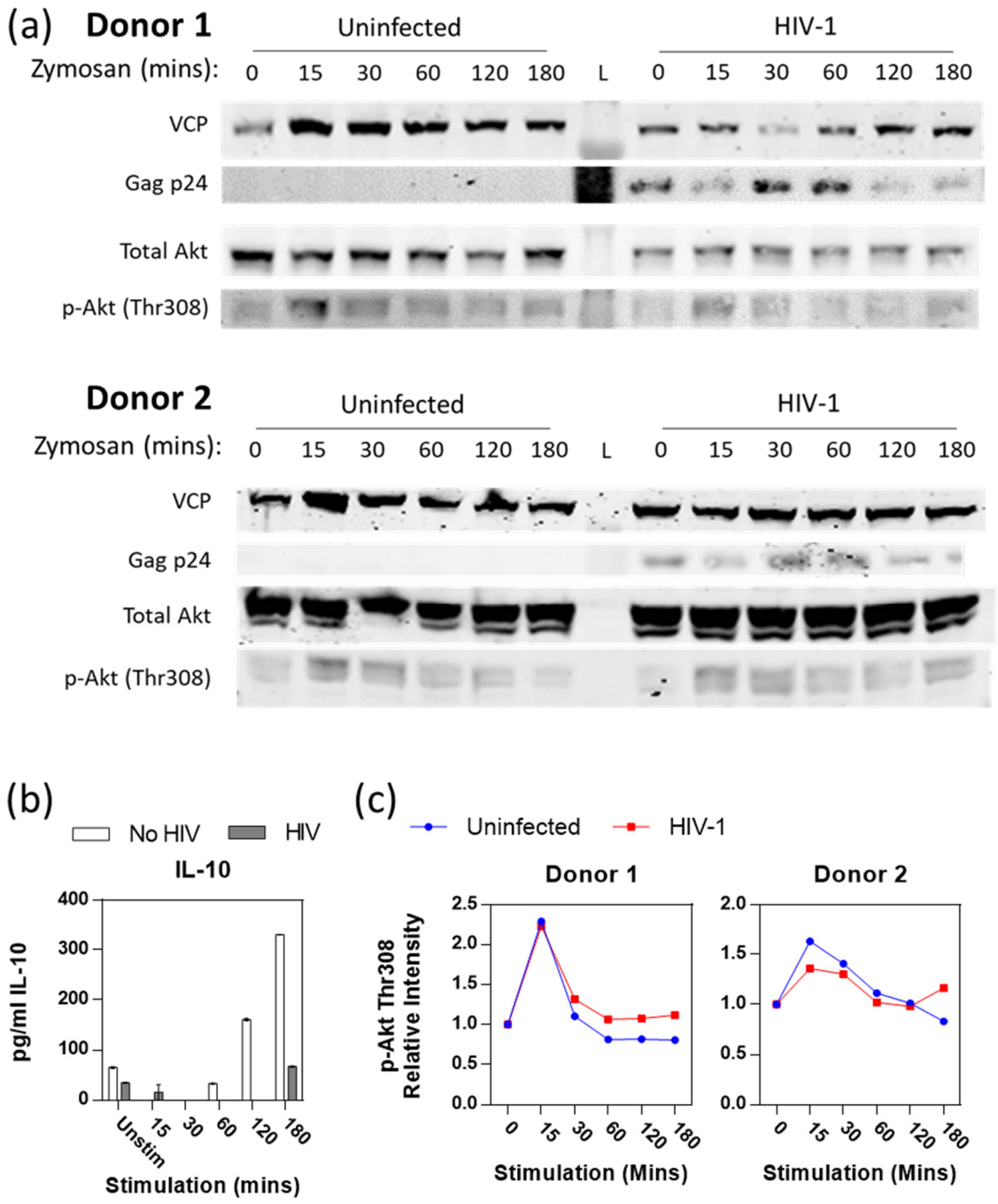

Figure 5.1: Timecourse of phosphorylation of Akt at T308 upon zymosan stimulation.

(a) Western blot on lysates from MDMs stimulated with zymosan over a time series, with and without HIV-1 infection. **(b)** IL-10 attenuation was confirmed by ELISA. Bars show mean +/- SEM. **(c)** Quantitation was performed with Image Studio Lite, normalising to p-Akt levels prior to stimulation.

5.2.2. Phosphorylation of ERK1/2 during stimulation of zymosan

TLR-mediated Extracellular Signal–Regulated Kinase (ERK) activation has been reported to influence IL-10 production in some cell types [496], [497]. The Ras-Raf-MEK-ERK signal transduction cascade has been implicated in numerous cellular functions and it's activation involves phosphorylation of ERK at residues T202 and Y204 [498]. Western blotting was performed to determine whether HIV-1 influences phosphorylation of ERK1/2 during zymosan stimulation. Phosphorylation of this pathway indicative of activation occurred in a short burst in the first hour of stimulation before returning to baseline (**Figure 5.2a**). Densitometry suggested a relative deficiency in ERK phosphorylation in HIV-infected samples from two of the three donors (**Figure 5.2b**).

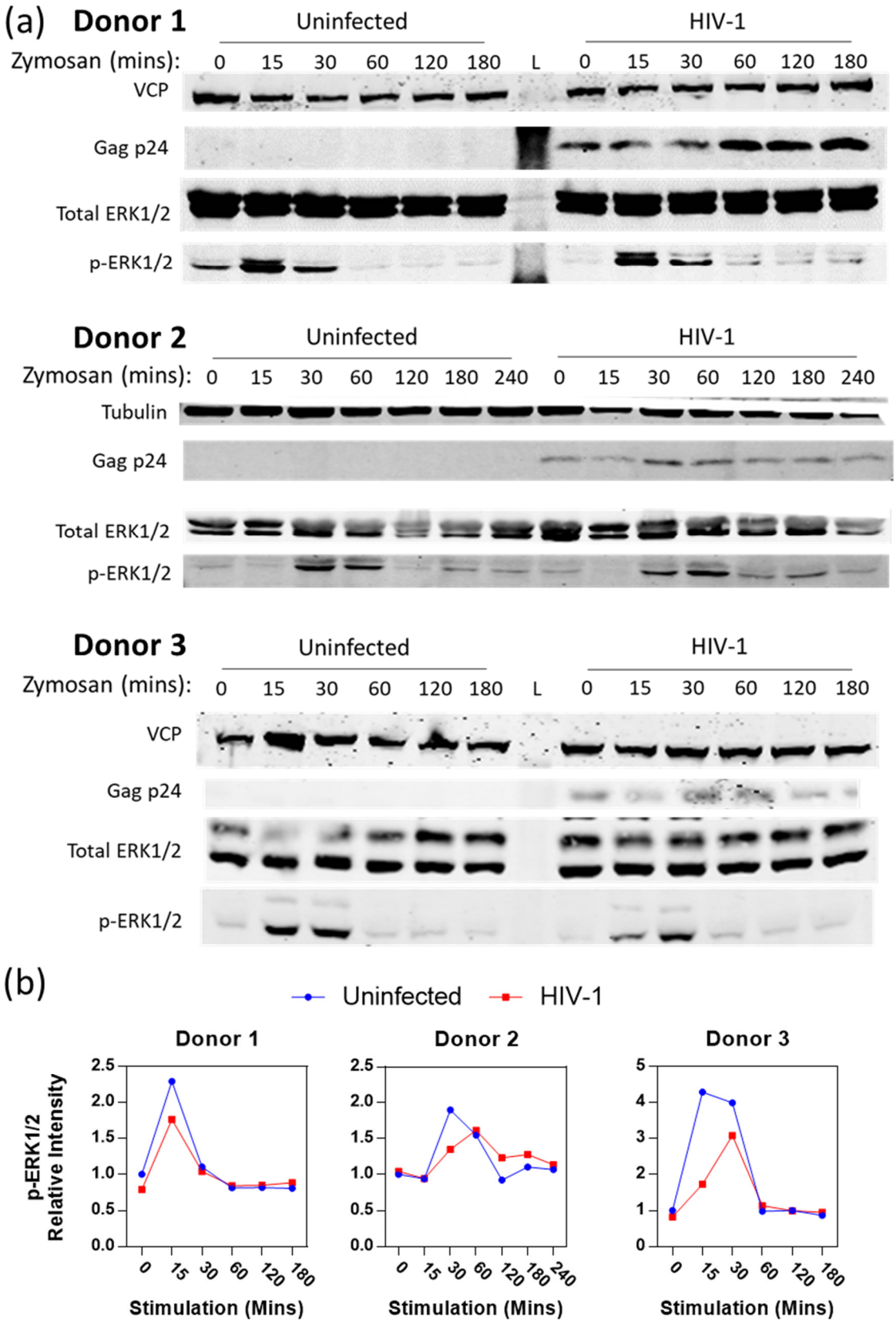

Figure 5.2: Phosphorylation of the ERK pathway during zymosan stimulation.

(**a**) Western blot on lysates from MDMs stimulated with zymosan over a time series, with and without HIV-1 infection. (**b**) Quantitation was performed with Image Studio Lite, normalising to p-ERK levels prior to stimulation.

5.2.3. Exogenous interferon induces IL-10 attenuation

Type I interferons can be produced by macrophages and have been reported to influence IL-10 responses in other cell types [204]. I therefore aimed to test whether exogenous interferon could induce IL-10 attenuation in monocyte-derived macrophages. Cells were treated with recombinant IFNβ for 24 hours prior to stimulation with zymosan. Stimulation with IFNβ phenocopied the specific effect of HIV-1 without altering IL-6 responses (**Figure 5.3**). This may indicate that some of the pathways induced by IFNs could be involved in the phenotype induced by HIV-1.

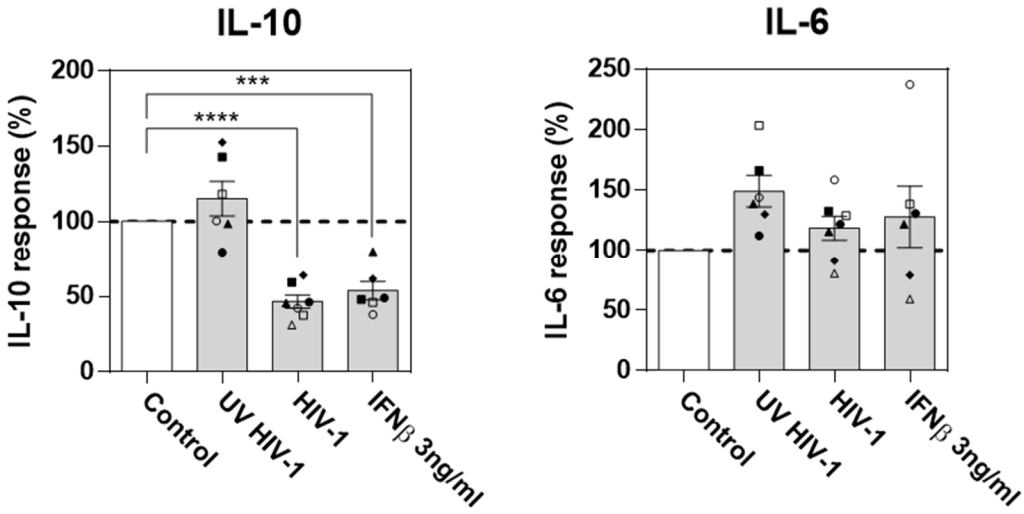

Figure 5.3: Exogenous interferons attenuate IL-10 responses

MDMs were treated with HIV-1, UV-inactivated HIV-1 or recombinant human interferon beta for 24h prior to stimulation with zymosan for 4h. ELISA was used to measure cytokine secretion in the supernatant. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 6 donors.

5.2.4. Interferon-stimulated genes are not induced by HIV-1 or ssRNA prior to stimulation

In view of our previous findings that HIV-1 infection of macrophage cultures did not lead to upregulation of IFN or ISGs, I utilised single cell RNA FISH staining to test the hypothesis that there may be IFN responses at the single cell level, below the level of detection in previous measurements in bulk cultures. Transcription of secreted interferons is transient and so may be missed [499], I therefore stained for expression of selected ISGs as a surrogate for IFN activity [94]. Exogenous IFNβ induced strong expression of CXCL10 in >25% of the cells. The same was not evident in macrophage cultures infected with HIV-1 or treated with the synthetic TLR8 agonist, CL075, in which no CXCL10 upregulation was evident (**Figure 5.4a**). HIV-1-derived ssRNA40 induced expression of CXCL10 a much smaller fraction of cells (<2%) (**Figure 5.4b**). These results suggest that HIV-1 does not induce significant interferon during infection and that stimulation of TLR8 may not result in canonical IFN responses in our model.

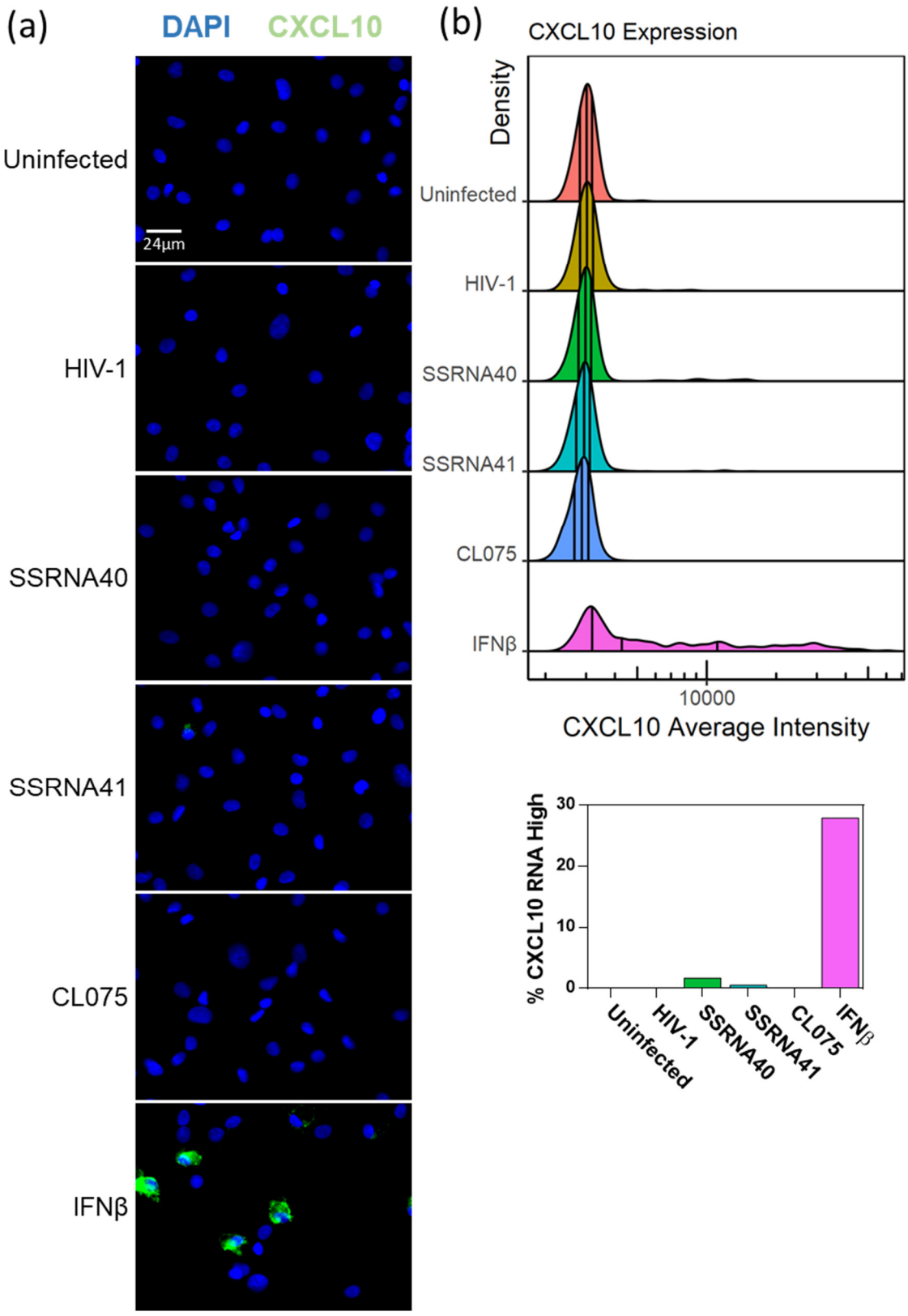

Figure 5.4: ISG expression is not induced by HIV-1 or ssRNA

MDMs were treated with HIV-1, TLR8 agonists or exogenous interferon for 24h prior to fixation. **(a)** ViewRNA FISH was used to detect expression of CXCL10. **(b)** Staining was quantified using Metamorph 7. High expressing cells were evaluated based on a threshold MFI of 10,000 using R. Representative of 2 donors.

5.2.5. ISG expression is not induced at early timepoints during infection with HIV-1

While my previous experiment found no evidence of significant ISG induction at 24h after infection, it is possible that any ISG expression may only be short lived and would no longer be evident at later time points. To assess this, RNA FISH was performed in a time series after HIV-1 infection. This allowed for the visualisation of incoming virus at the same time as any interferon signalling. CXCL10 expression was not significantly induced during HIV-1 infection at any time point 2-48 hours after infection (**Figure 5.5a**).

Although no ISG induction was seen during primary infection, CXCL10 expression was upregulated by stimulation with zymosan (**Figure 5.5b**). This would be consistent with the interferon component of the response to TLR stimulation which has been reported in the literature [500]. As previously described, HIV-1 uptake was seen across the majority of cells in the culture, but with wide variation in the amount of virus per individual cell (**Figure 5.5c**). By 24h post-infection the productively infected population could be detected. Together, these results suggested that HIV-1 infection in this monocyte-derived macrophage model does not trigger interferon signalling.

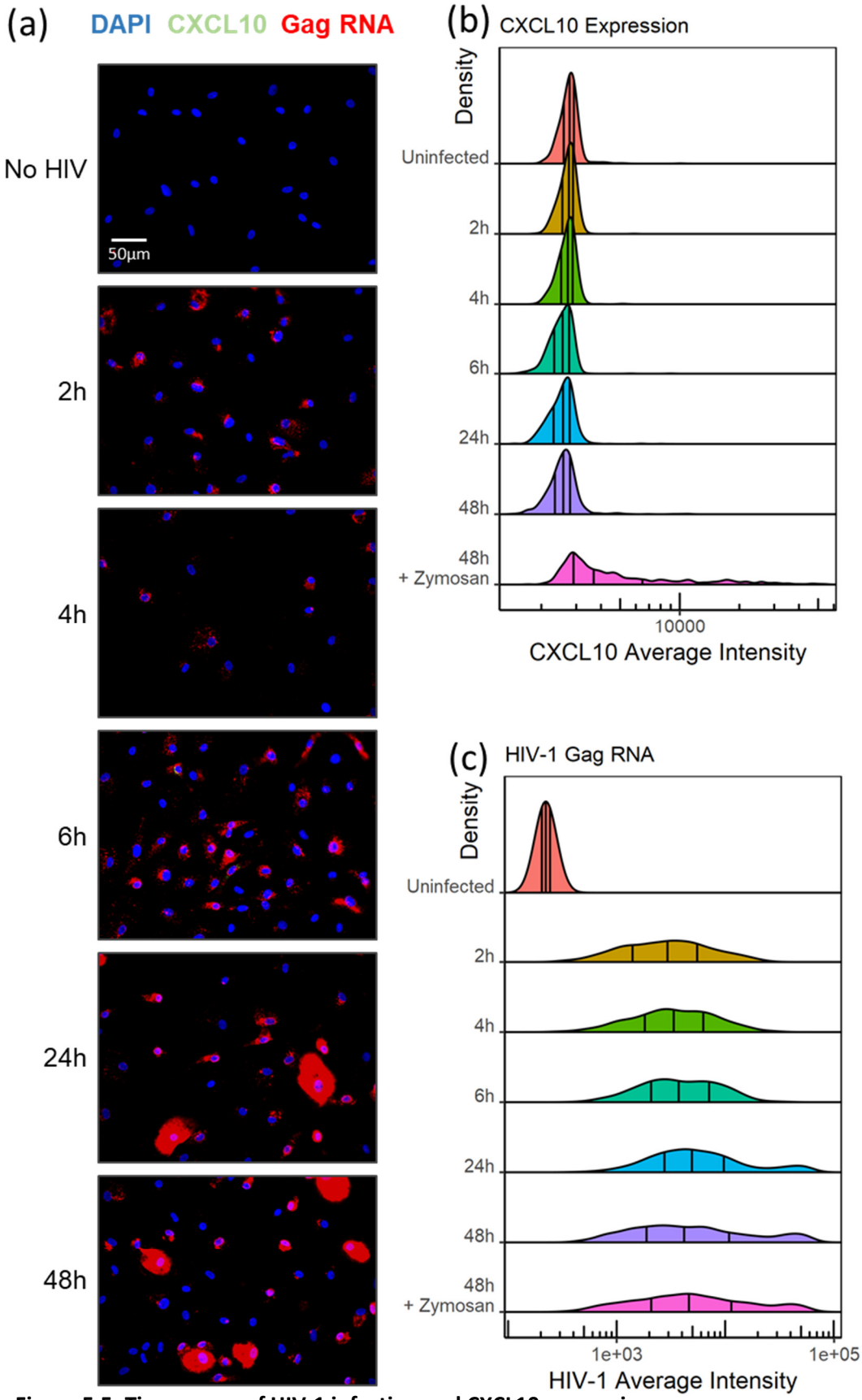

Figure 5.5: Timecourse of HIV-1 infection and CXCL10 expression

MDMs were infected with HIV-1 and fixed at a series of timepoints post-infection, with an additional sample stimulated with zymosan for 4h. **(a)** ViewRNA FISH was used to visualise expression of HIV-1 and CXCL10 RNA. Metamorph 7 was used to quantify CXCL10 **(b)** and HIV **(c)** RNA content. Representative of 3 donors.

5.2.6. ISG responses to zymosan stimulation are enhanced by HIV-1

Incidentally, I observed that although strong CXCL10 induction was observed in response to zymosan in cultures infected with HIV-1, expression was largely absent during stimulation of uninfected cultures (**Figure 5.6**). Multiplexed staining for IL-10 confirmed that IL-10 expression was also attenuated in these cells. Staining for IFIT1 expression, another ISG, showed induction by zymosan in the absence of HIV-1. Despite this the presence of HIV-1 still significantly enhanced IFIT1 expression (**Figure 5.7**). This may suggest that HIV-1 promotes interferon secretion in response to zymosan, or that stimulation with zymosan promotes interferon expression in response to the presence of HIV-1.

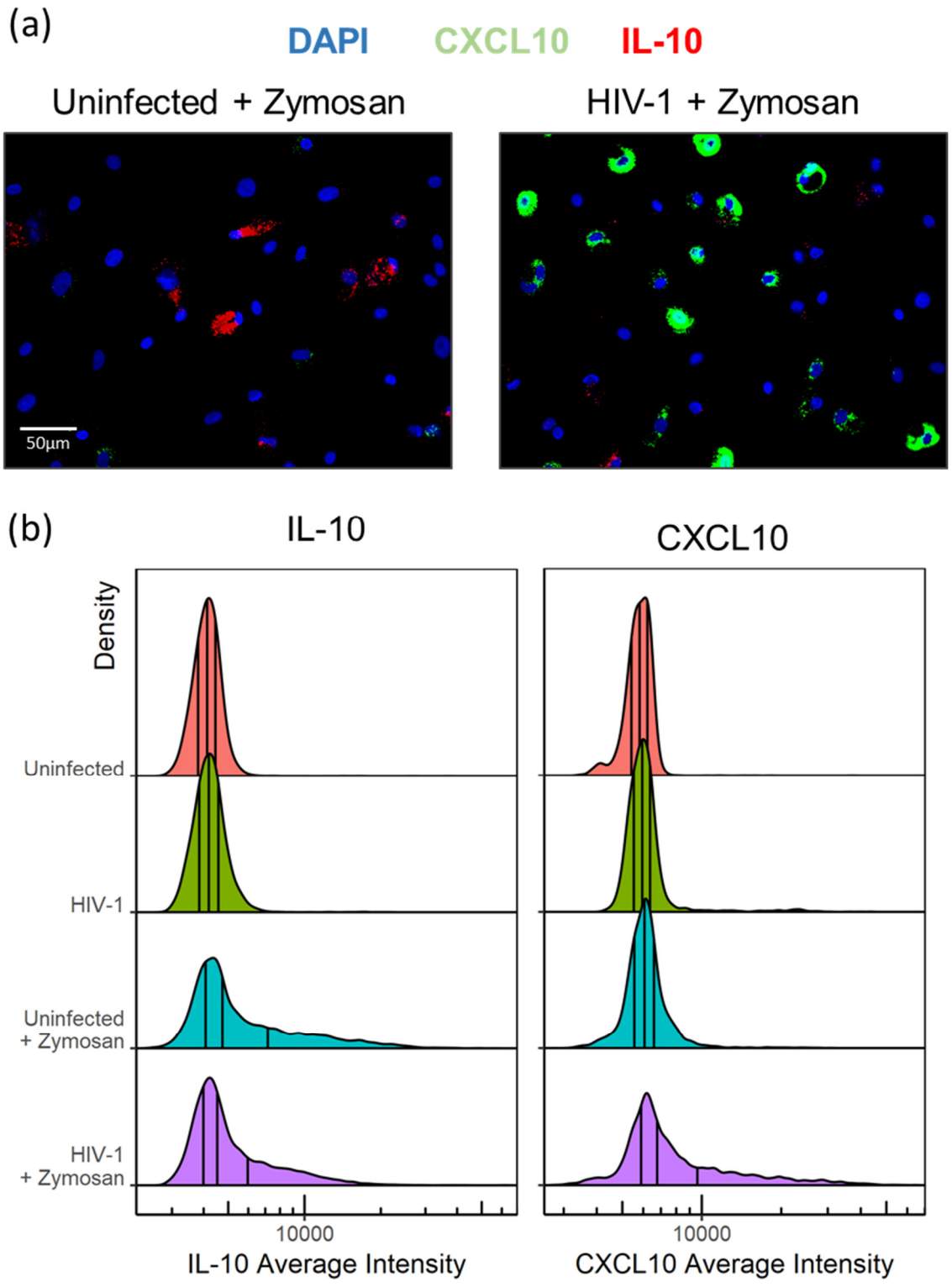

Figure 5.6: CXCL10 expression in response to zymosan is enhanced by HIV-1.

(a) Cells infected for 24h with HIV-1 were stimulated with zymosan for 4h prior to fixation and staining for IL-10 and CXCL10 expression by RNA FISH. **(b)** Expression was quantified using Metamorph 7. Representative of 3 donors.

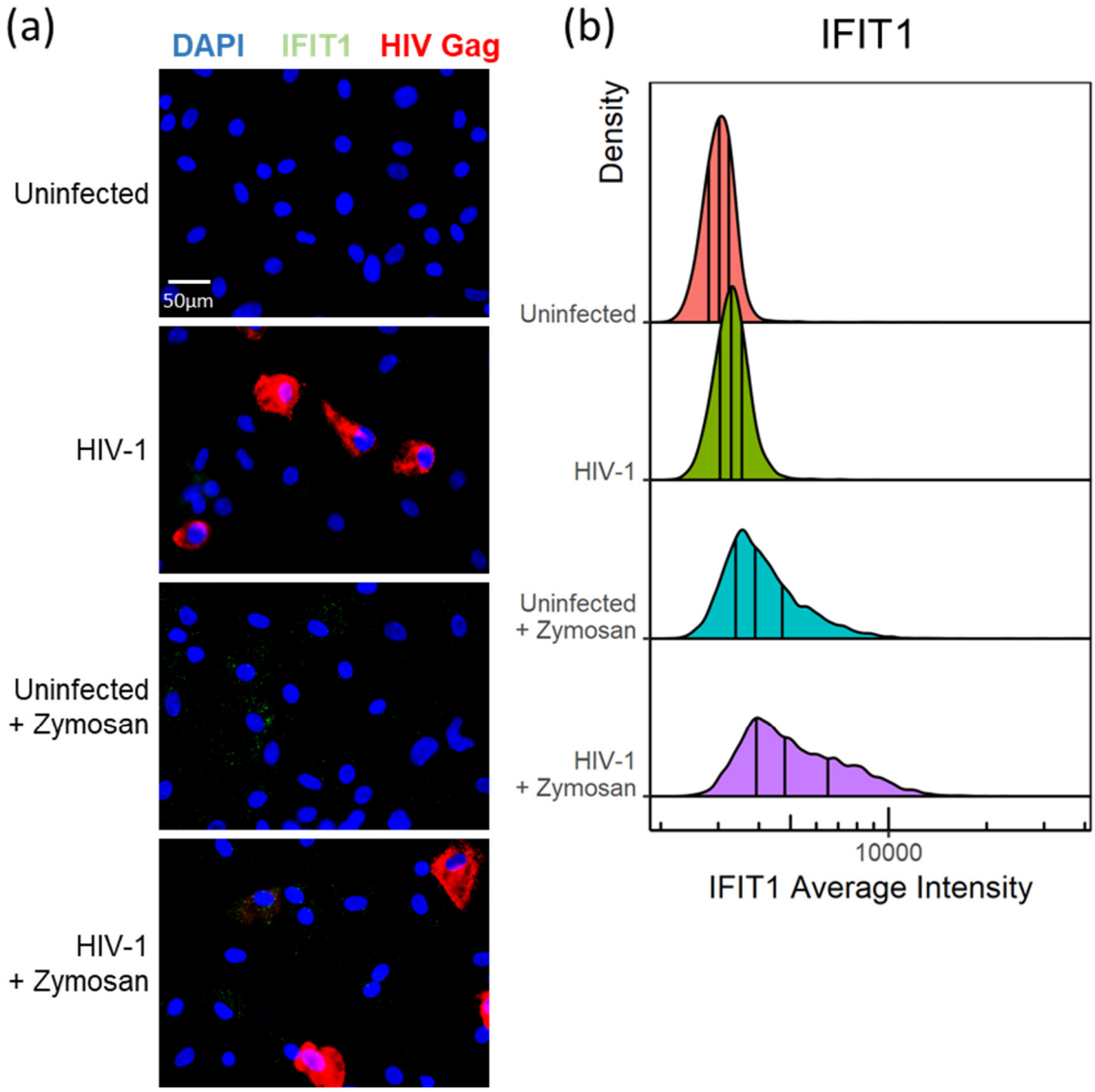

Figure 5.7: IFIT1 expression in response to zymosan is enhanced by HIV-1.

(a) Cells infected for 24h with HIV-1 were stimulated with zymosan for 4h prior to fixation and staining for IFIT1 and HIV RNA expression by RNA FISH. **(b)** Expression was quantified using Metamorph 7. Representative of 3 donors.

5.2.7. Single cell analysis of ISG expression during stimulation

My previous results had demonstrated that while infection with HIV-1 does not induce ISG expression, the presence of virus enhanced ISG expression during subsequent stimulation. This would suggest that HIV somehow primes cells to generate an exaggerated IFN response to a second stimulus. This could be mediated in some way by the same mechanism which is responsible for attenuation of IL-10 expression. I therefore hypothesised that the same cells which exhibit attenuated IL-10 responses would also express IFN upon stimulation. To test this, I utilised RNA FISH to analyse expression of multiple targets in response to zymosan or curdlan. Quantitation of gene expression across the entire population demonstrated IL-10 attenuation and ISG enhancement with both zymosan and curdlan, but no change in IL-6 expression (**Figure 5.8**). This was consistent with previous results.

Analysis of expression of CXCL10 against HIV-1 RNA content suggested that ISG induction was biased towards cells which did not become productively infected but do contain virus (**Figure 5.9**). This may suggest that sensing and restriction of virions in this population primes cells to express ISGs upon restimulation. Alternatively, *de novo* expression of viral proteins in infected cells may inhibit interferon signalling as HIV-1 Vpr and Vif have been reported to interfere with these pathways [424], [501].

I also investigated whether cells which express ISGs in response to zymosan would not express IL-10, reflecting an inflammatory profile. Comparing expression of CXCL10 and IL-10 at the single cell level showed that, while a small proportion of cells expressed both transcripts, the majority of cells exclusively express one target (**Figure 5.10**). This may indicate a shift in these cells towards expressing ISGs instead of IL-10, possibly representing a different activation phenotype. The same shift was visible when analysing IFIT1 expression (**Figure 5.11**).

Together, these results may reveal a shift in signalling away from regulatory IL-10 production and towards inflammatory interferon signalling in cultures infected with HIV-1. A key question is whether HIV-1 RNA needs to be present during stimulation to drive antiviral signalling instead of IL-10, or if exposure to virus primes cells towards inflammatory signalling which persists after virus has been degraded.

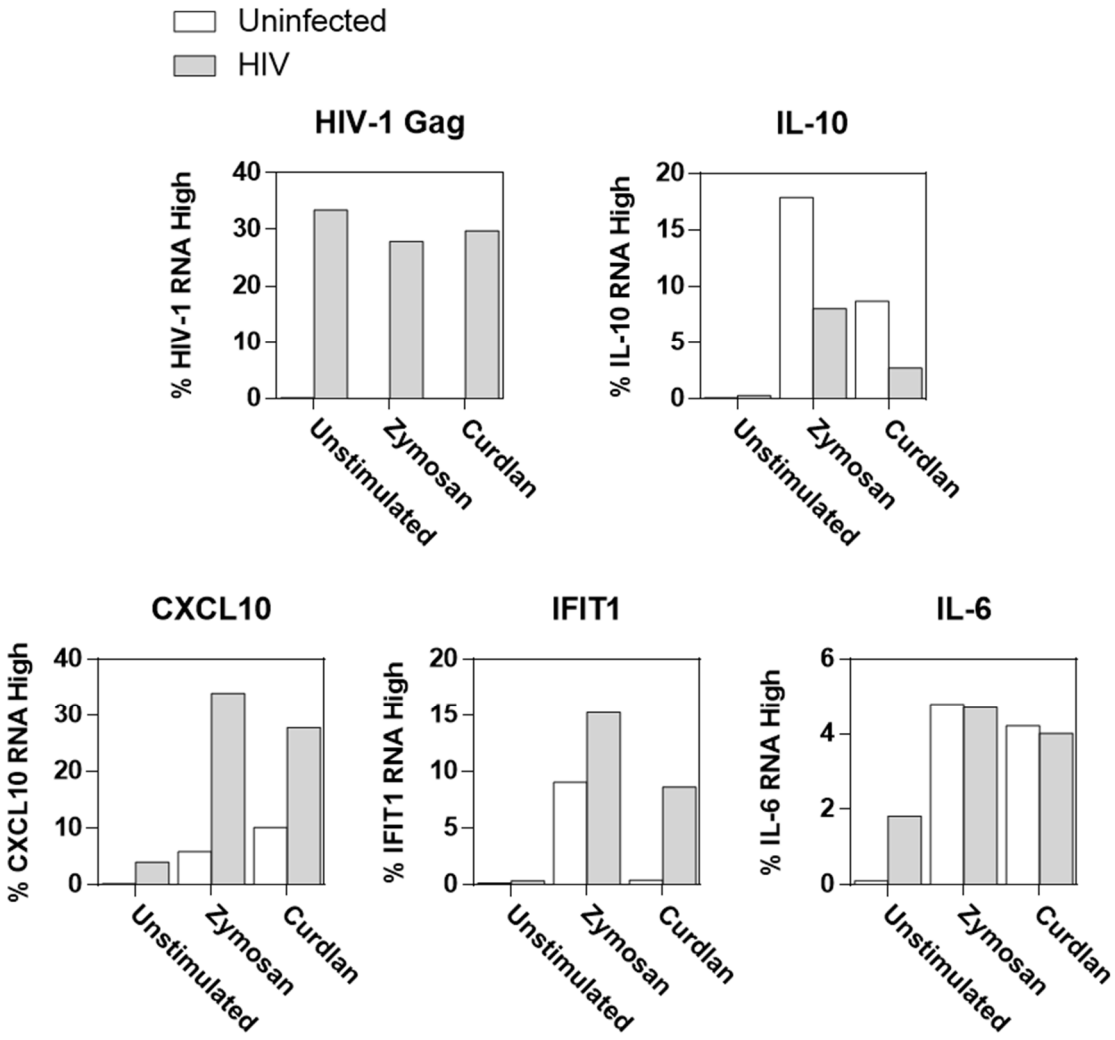

Figure 5.8: HIV-1 enhances ISGs and attenuates IL-10 in response to zymosan and curdlan.

Cells infected for 24h with HIV-1 were stimulated with zymosan or curdlan for 4h prior to fixation and staining for IL-10, CXCL10, IFIT1 or IL-6 expression. Staining was quantified using Metamorph 7. Proportions of cells above a threshold were quantified using R. Measurements were performed on at least 10,000 cells per condition.

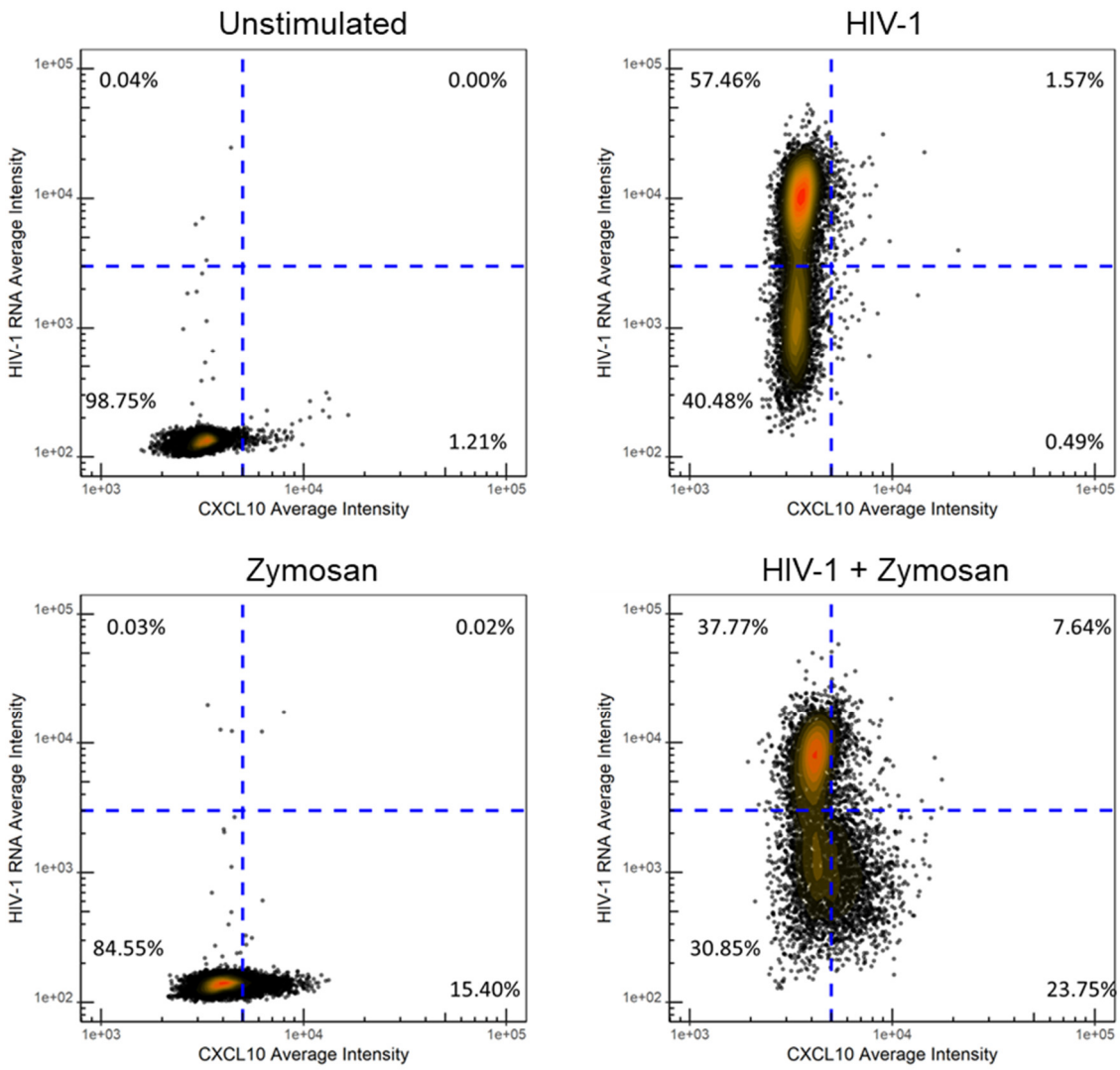

Figure 5.9: Relationship between HIV-1 RNA content and CXCL10 expression

Cells infected for 24h with HIV-1 were stimulated with zymosan or curdlan for 4h prior to fixation and staining for HIV-1 RNA and CXCL10 expression. Staining was quantified using Metamorph 7. Staining above/below marked thresholds was determined using R.

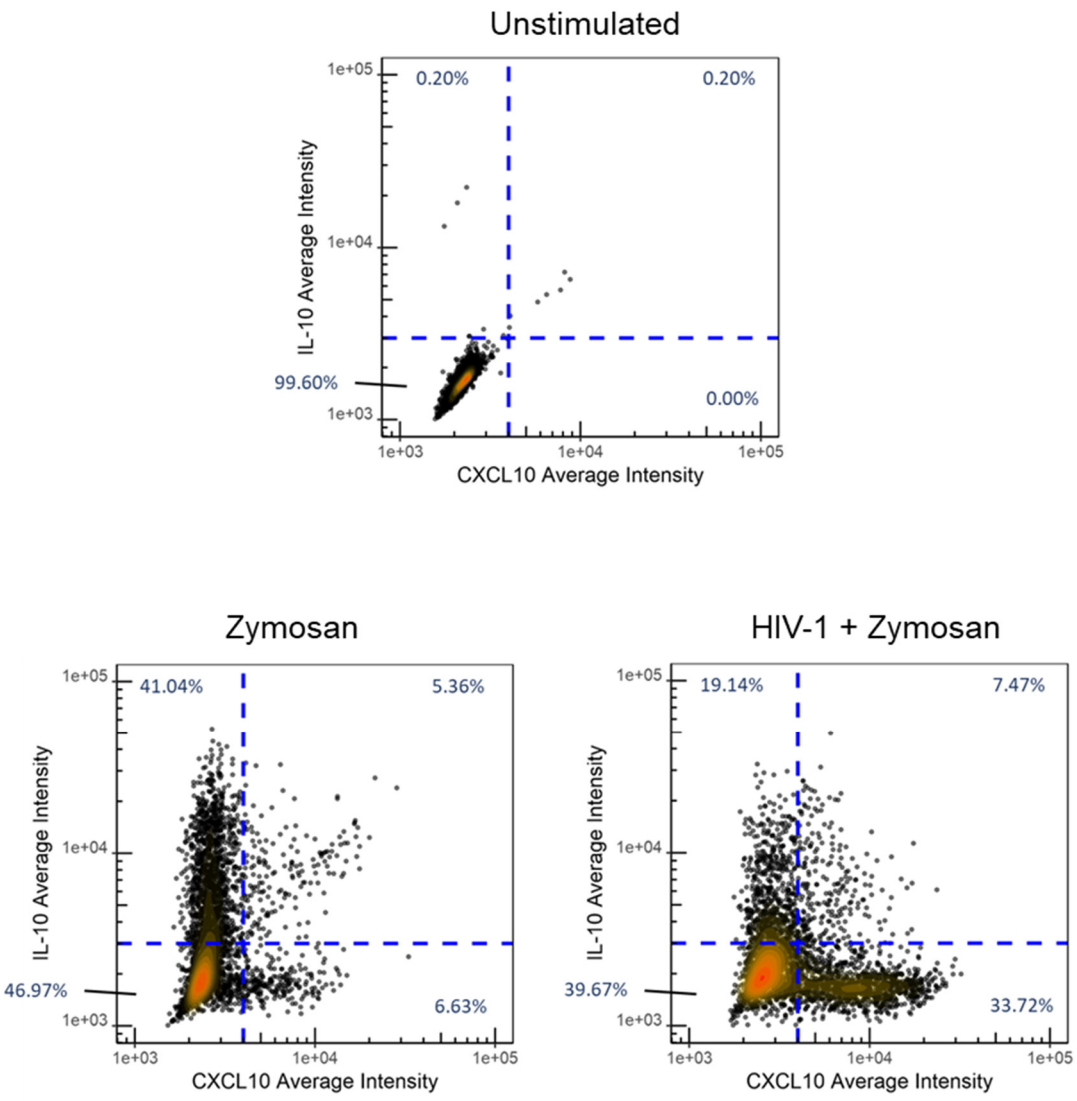

Figure 5.10: Relationship between IL-10 and CXCL10 expression

Cells infected for 24h with HIV-1 were stimulated with zymosan or curdlan for 4h prior to fixation and staining for IL-10 and CXCL10 expression. Staining was quantified using Metamorph 7. Staining above/below marked thresholds was determined using R.

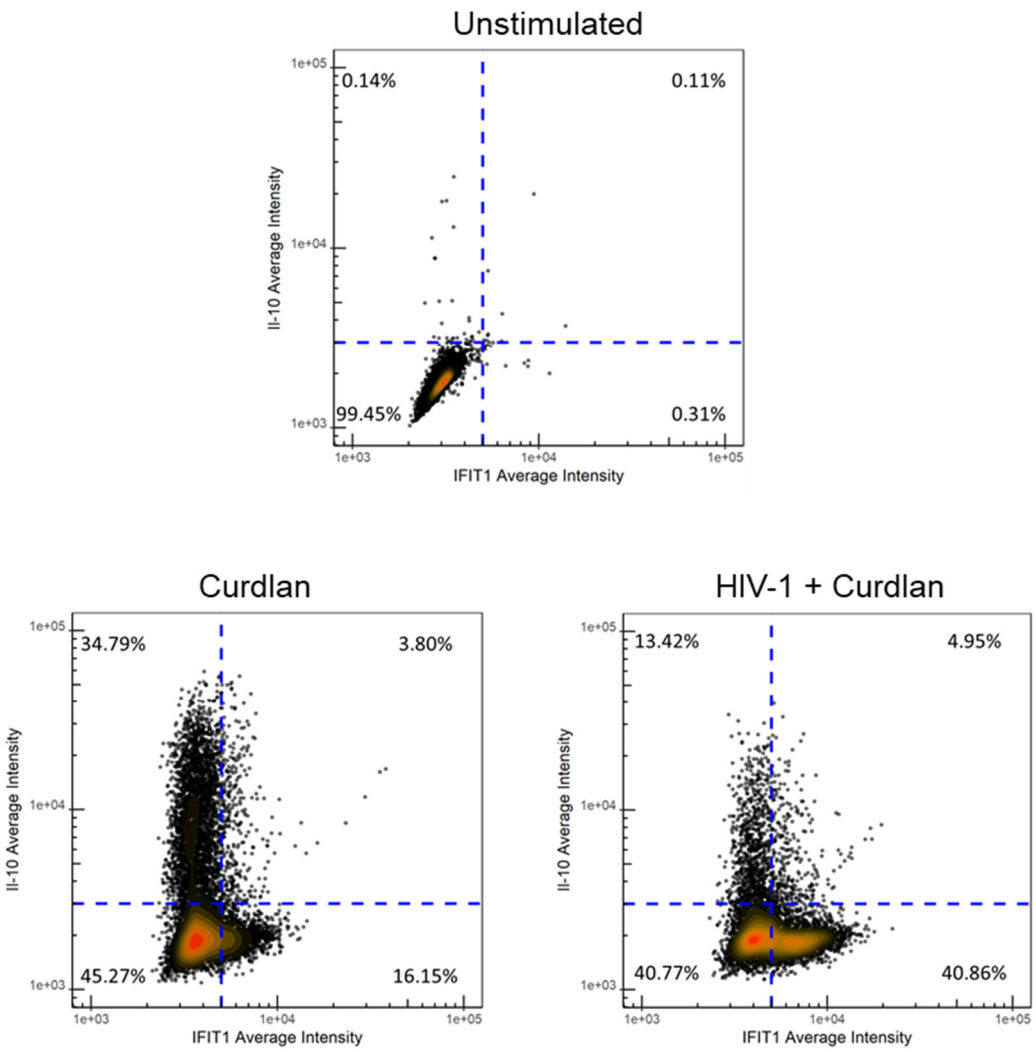

Figure 5.11: Relationship between IL-10 and IFIT1 expression

Cells infected for 24h with HIV-1 were stimulated with zymosan or curdlan for 4h prior to fixation and staining for IL-10 and IFIT1 expression. Staining was quantified using Metamorph 7. Staining above/below marked thresholds was determined using R.

5.2.8. Interferon secretion does not mediate IL-10 attenuation by HIV-1

Notwithstanding our observations that HIV-1 infection does not induce detectable IFN responses in my experimental model, it is possible that any IFN response may be below our limit of detection and inadequate to restrict virus infection. I sought to further test the hypothesis that the virus induces IFN responses that may function to attenuate macrophage production of IL-10. IFN exerts its function by being secreted and binding to the extracellular type I IFN receptor (IFNAR) at the cell surface. This interaction can be competitively blocked using antibody to the IFNAR. Therefore, I tested the effect of this antibody in my experimental model. The antibody itself had no impact on zymosan-induced IL-10 production by macrophage cultures, but as expected IFNAR blockade reversed attenuated IL-10 responses in cultures pre-treated with IFNβ. In contrast, the blocking antibody had no effect on attenuated IL-10 responses in macrophage cultures infected with VSV-pseudotyped HIV-1 or the HIV-1-derived CSGW vector which lacks the HIV-1 accessory genes (**Figure 5.12**).

I also repeated this experiment using a viral vector incorporating a gag-luciferase fusion protein, which was previously found to induce IFN responses. We speculate that this may be due to an unstable capsid resulting from the incorporation of fusion proteins (unpublished data). IFNAR blockade did not reverse attenuation of IL-10 responses in this case either. (**Figure 5.13**). Taken together, these data indicate that while interferon signalling can inhibit IL-10 responses, secreted interferon is not the mechanism by which HIV-1 induces this phenotype.

Interestingly, in contrast to IL-10, the expression of IL-6 following zymosan stimulation was consistently reduced in the presence of anti-interferon receptor antibody. This suggests that IL-6 responses are partially dependent on IFN responses to zymosan.

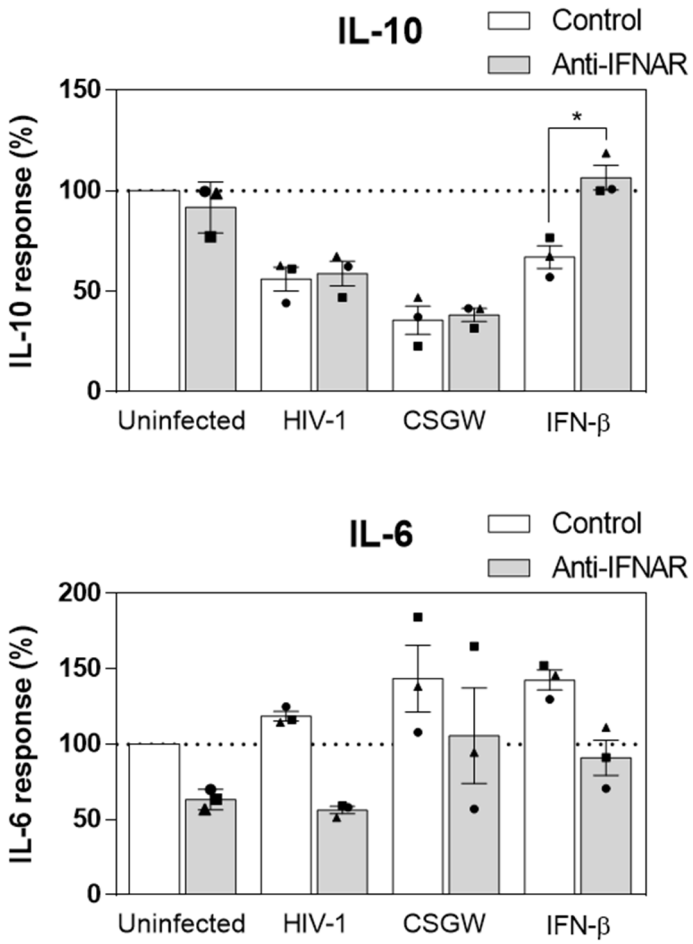

Figure 5.12: Blockade of the interferon receptor does not rescue IL-10 responses from HIV-1.

Interferon signalling was blocked using an IFNAR1 antibody for 2 hours prior to addition of HIV-1, the CSGW vector or recombinant interferon. After 24h cells were stimulated with zymosan and cytokine secretion in the supernatants was measured by ELISA. Graphs show means ± SEM. Significance was determined by paired t-test. Results from 3 donors.

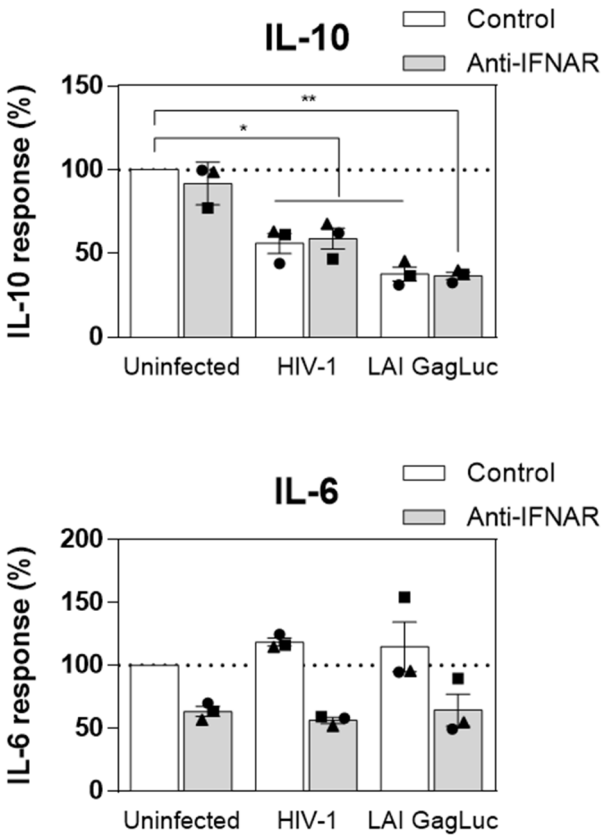

Figure 5.13: An interferon triggering mutant attenuates IL-10 independently of secreted IFN signalling.

Interferon signalling was blocked using an IFNAR1 antibody for 2 hours prior to addition of HIV-1 or the interferon-triggering GagLuc vector. After 24h cells were stimulated with zymosan and cytokine secretion in the supernatants was measured by ELISA. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 3 donors.

5.2.9. Interferon receptor blockade does not rescue IL-10 from TLR8 agonists.

My data suggested that HIV stimulation of TLR8 can lead to macrophage attenuation of IL-10 responses independently of IFN responses. In order to consolidate these data, I tested whether IL-10 attenuation is evident if TLR8 is stimulated specifically with synthetic agonists while the IFN receptor is blocked. As seen previously, IFNAR blockade rescued IL-10 responses after pre-treatment with exogenous interferon. However, the effect HIV-1-derived TLR8 ligand ssRNA40 on attenuation of IL-10 was not reversed (**Figure 5.14**). This confirmed that the effects of TLR8 stimulation on IL-10 responses do not require secreted interferon as a mediator.

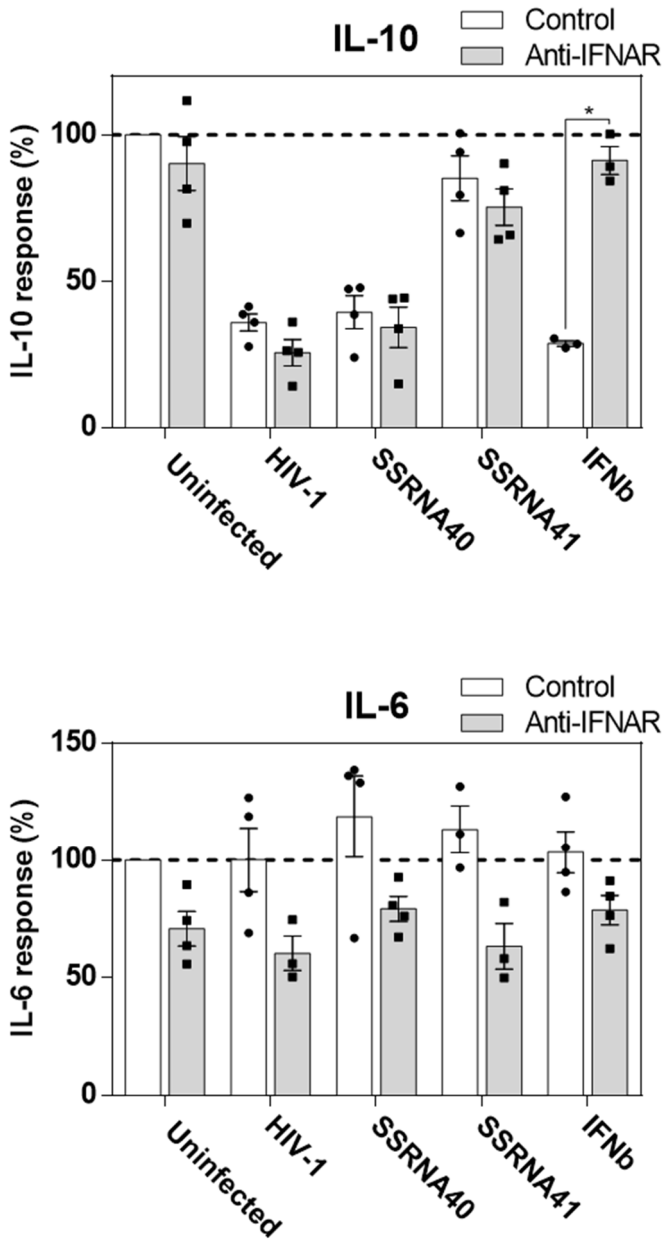

Figure 5.14: ssRNA attenuates IL-10 independently of secreted IFN signalling.

Interferon signalling was blocked using an IFNAR antibody for 2 hours prior to addition of ssRNA-based TLR8 agonists, HIV-1 or IFNβ. After 24h cells were stimulated with zymosan and cytokine secretion in the supernatants was measured by ELISA. Graphs show means ± SEM. Significance was determined using paired t-tests corrected for multiple comparisons using the Holm-Sidak method. Results from 4 donors.

5.2.10. IL-10 attenuation is not mediated by a secreted factor

Data from previous experiments revealed that IL-10 was attenuated in cells which are exposed to virus but do not become productively infected. One possible mechanism for this would be the secretion of a signalling factor from some MDMs, which then induces IL-10 attenuation in bystander cells. To assess whether such a secreted factor contributes to this phenotype, I generated conditioned media from cells infected for either 24 hours or 1 week. I conducted these experiments with the HIV RT mutant in order to ensure that the conditioned media was not contaminated with any nascent viral products released from the infected cells. Conditioned media was pooled from 4 donors to minimise the influence of donor variability.

While virus significantly attenuated IL-10 as expected, pooled conditioned media was unable to replicate this effect in the model, indicating that a secreted factor is not the mechanism by which HIV exerts this phenotype (**Figure 5.15**). Based on these findings I hypothesised that the effects on IL-10 are driven by events within each individual cell, most likely from cells taking up virions themselves. It remains possible that any bystander effect may only act locally upon neighbouring cells, meaning that in the entire supernatant a secreted factor would not be in sufficient quantities to recapitulate the phenotype under investigation.

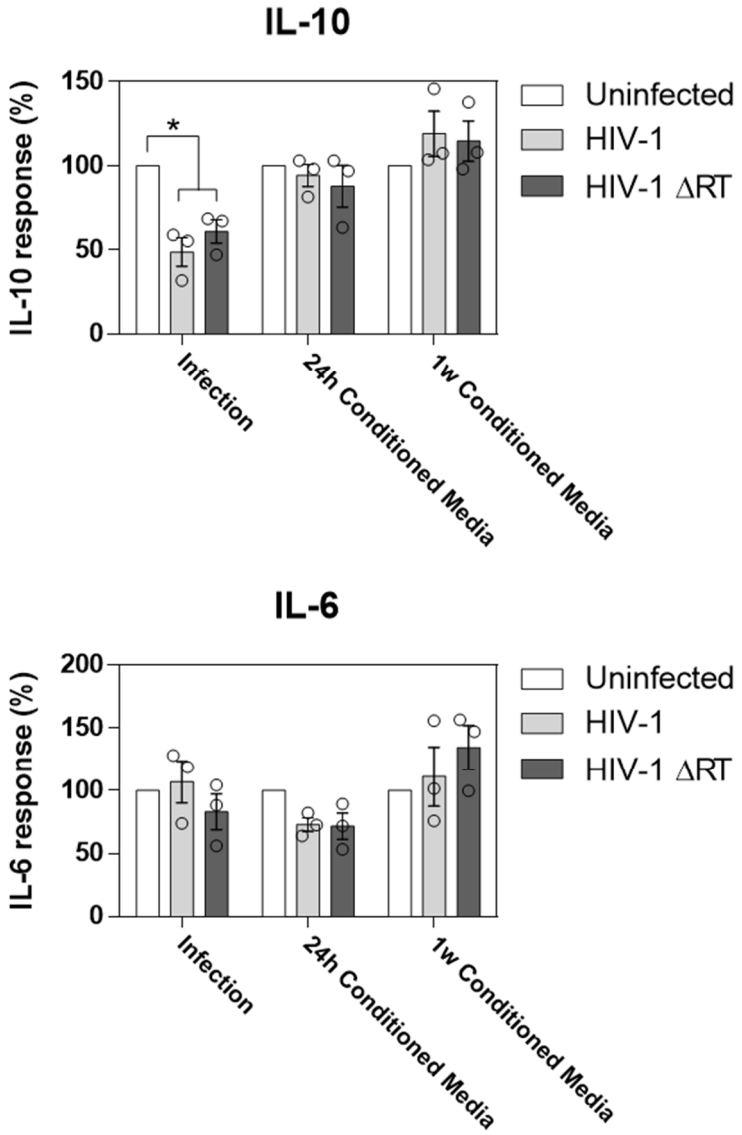

Figure 5.15: Conditioned media does not induce IL-10 attenuation.

Conditioned media was generated from cells infected for 24h or 1w with HIV-1 Δenv or HIV-1 Δenv ΔRT. Conditioned media was pooled from 4 donors and 0.22µm filtered prior to use in a 1:1 ratio with fresh media. MDMs were treated with virus or conditioned media for 24h prior to stimulation with zymosan. Cytokine secretion was measured by ELISA. Graphs show means ± SD. Significance was determined by one-way ANOVA. Results from 3 donors.

5.2.11. Attenuation of IL-10 responses is persistent

Based on my results, I hypothesised that the attenuation of IL-10 responses may result from innate immune adaptation due to viral RNA stimulating TLR8, which could modify the response to subsequent stimulation. Such adaptation is typically mediated by long-lasting epigenetic modifications and so should persist over time. To investigate this, cells were infected with HIV-1 or the RT deletion mutant for 24 hours or 1 week prior to stimulation. While the single round HIV-1 strain will infect cells and consistently produce "bald" vectors which were shown to induce IL-10 attenuation, the RT mutant is non-infectious and so should be degraded by the 1-week timepoint. Interestingly, at the protein level both the wild type and RT mutant viruses were capable of attenuating IL-10 1 week after infection (**Figure 5.16**).

To confirm whether viral RNA is still present in the RT mutant at 1-week post-infection, the experiment was repeated using the RNA FISH assay (**Figure 5.17a**). HIV-1 Gag RNA could be observed in WT and RT mutant virus at 24h post-infection, but after 1w there was no detectable RNA remaining from the RT mutant (**Figure 5.17b-c**). As anticipated, small quantities of viral RNA were observed in uninfected cells with the single round WT virus after 1 week, which is likely to represent the production of non-infectious virions by the infected cells within the culture.

IL-10 expression was also quantified at the single cell level, using both zymosan and curdlan as the secondary stimulus. With each stimulus IL-10 was still attenuated at the 1-week timepoint by both the WT and RT mutant virus (**Figure 5.18**). Based on this I concluded that the presence of HIV-1 RNA is not required at the time of stimulation, which would suggest that any sensing event occurs during exposure rather than during the secondary response.

In line with my hypothesis, the prolonged effects on IL-10 expression are suggestive of epigenetic modification, perhaps via changes in chromatin accessibility or methylation. Epigenetic control of both IL-10 expression and broader polarisation towards macrophage subtypes has been previously described [154], [502]. This would explain how effects persist in the absence of the original stimulus, so further work should focus on searching for alterations in epigenetic markers around the IL-10 locus.

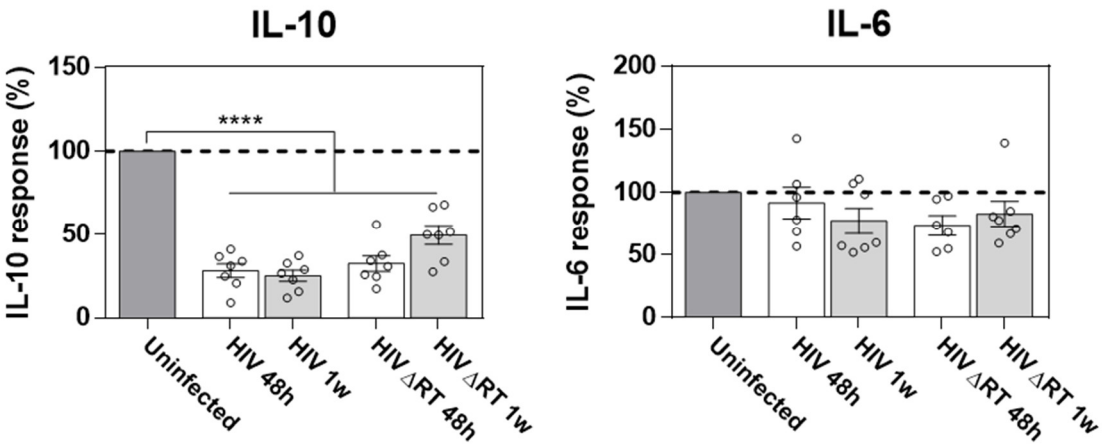

Figure 5.16: IL-10 responses are still attenuated at 1w post-infection.

MDMs were infected with single-round HIV-1 or an RT mutant for 24 hours or 1 week prior to stimulation with zymosan for 4h. Cytokine secretion was measured by ELISA. Graphs show means ± SEM. Significance was determined by one-way ANOVA. Results from 7 donors.

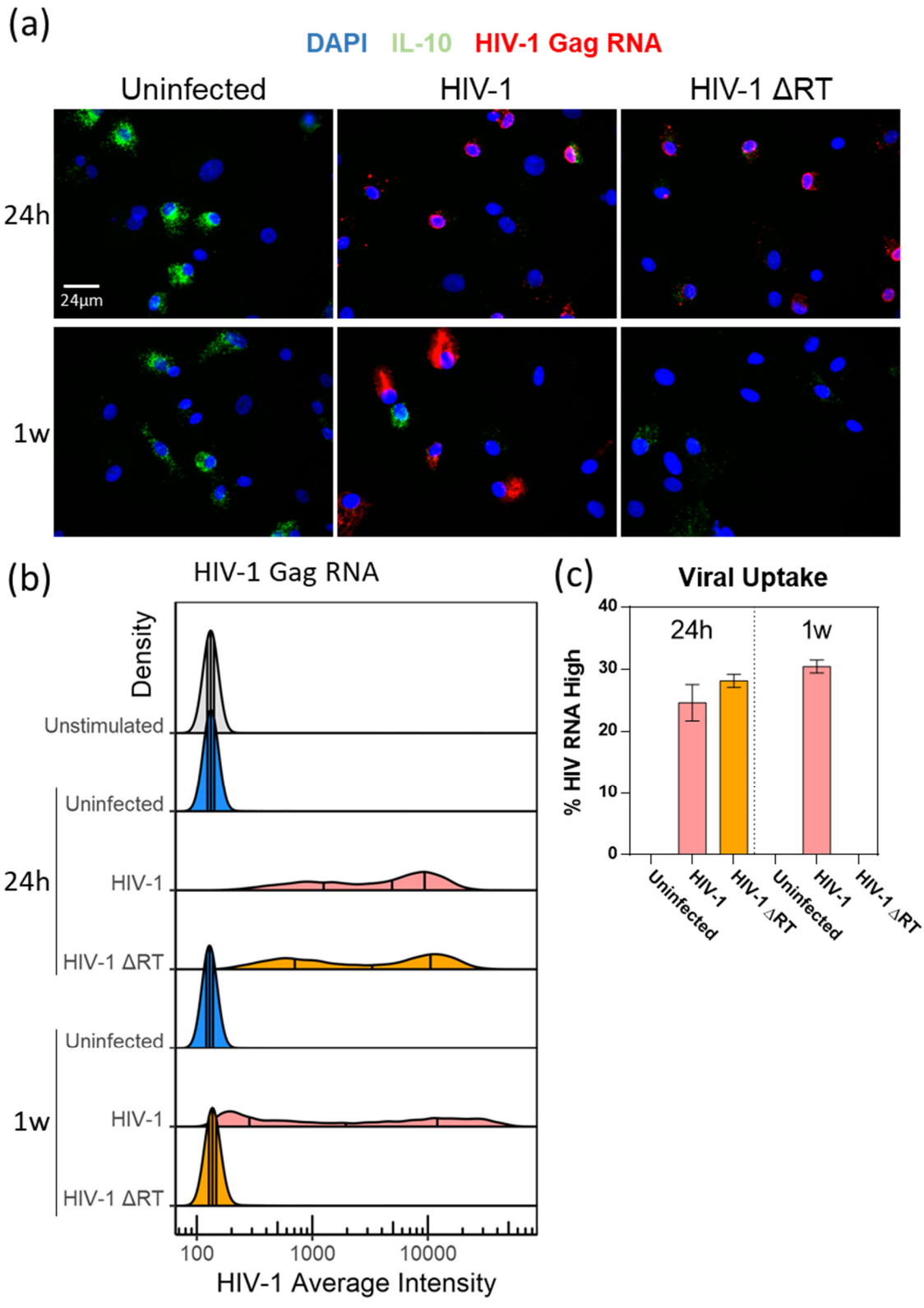

Figure 5.17: HIV-1 ΔRT RNA is degraded by 1w post-exposure.

MDMs were infected with single-round HIV-1 or an RT mutant for 24 hours or 1 week prior to stimulation with zymosan for 4h. **(a)** RNA FISH was used to visualise content of HIV-1 RNA and IL-10 expression. **(b-c)** Metamorph 7 was used to quantify RNA content. Graphs show mean +/- SEM from duplicate wells. Representative of 2 donors.

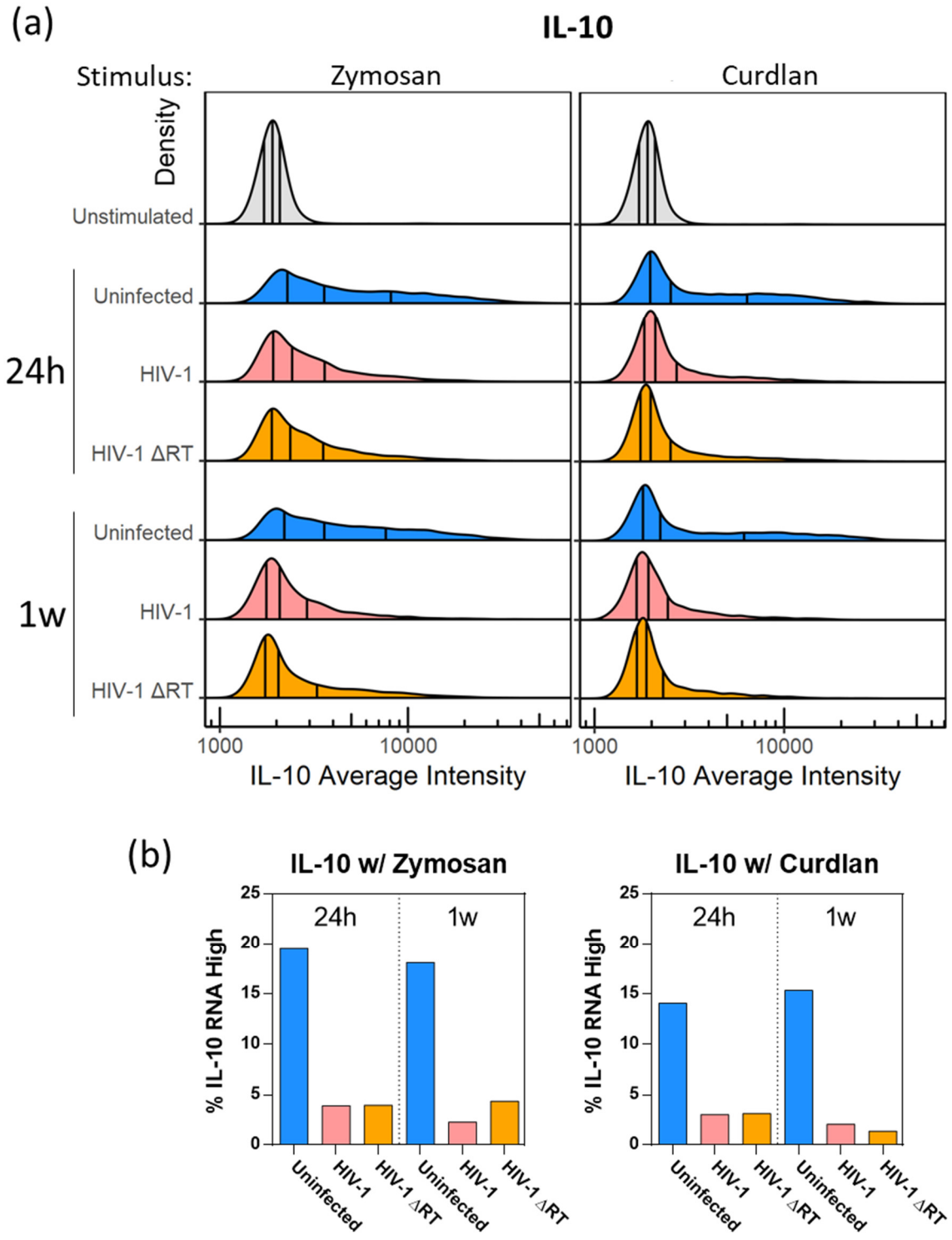

Figure 5.18: HIV-1 attenuates IL-10 at 1w post-exposure.

MDMs were infected with single-round HIV-1 or an RT mutant for 24 hours or 1 week prior to stimulation with zymosan or curdlan for 4h. **(a)** RNA FISH was used to detect IL-10 expression, which was quantitated using Metamorph 7. **(b)** An MFI threshold of 10,000 was used to classify cells expressing high levels of IL-10. Representative of 2 donors.

5.2.12. ISG induction is independent of IL-10 attenuation

I had previously hypothesised that the enhanced ISG induction observed during zymosan stimulation may be due to the presence of virus within cells at the time of stimulation. However, my results suggested that IL-10 attenuation does not require the presence of virus at the time of stimulation and may be a long-lived response mediated by epigenetic modification. I therefore aimed to test whether enhanced ISG responses also persist at later timepoints, even after viral material has been degraded.

ISG expression was visualised using RNA FISH at the single cell level using the two-timepoint experiment with wild-type and RT mutant single round vectors. Boosted CXCL10 expression was seen with both the WT and RT mutant vector, although the RT mutant induced a weaker response (**Figure 5.19a**). After 1 week the additional CXCL10 expression seen with zymosan stimulation was significantly reduced with wild type virus, while the weaker effects of the RT mutant persisted to a slightly greater extent (**Figure 5.19b-c**). The more potent effect of the wild-type virus may suggest that stimulation may promote DNA sensing of this strain, which could stimulate a greater interferon response than the viral RNA alone. The weaker phenotype at 1w post-infection even in the presence of RT mutant vectors is interesting, although CXCL10 is not totally specific to the interferon response and can be induced by cytokines such as TNFα in some cell types, which are also seen during the stimulation with zymosan [503].

To further confirm these results, I also stained for expression of IFIT1, which is a more specific marker of the interferon system [504]. As with CXCL10, IFIT1 expression was boosted with both HIV-1 vectors at the early timepoint (**Figure 5.20a**). However, at 1w post-infection ISG enhancement was absent, even in the wells infected with wild type single-round HIV-1 (**Figure 5.20b-c**). This would indicate that, unlike attenuation of IL-10 responses, enhancement of ISG expression is not a persistent phenotype which could be mediated by epigenetic modification.

"Bald" vectors are also still present at the 1w timepoint for wild type virus, so the absence of any enhancement of ISG expression suggests that endosomal sensing is not the mechanism behind ISG enhancement. This would mean that cytosolic DNA or RNA sensing may be the means through which this aspect of the phenotype occurs.

Together these results indicate that the enhancement of ISG responses seen in the presence of HIV-1 is not directly related to IL-10 attenuation. While ISG expression coincides with lower IL-10 production in a single cell, attenuation of IL-10 responses does not require ISG expression. Further work should investigate whether a second stimulus promotes sensing of HIV-1 genomic material, but this was beyond this scope of this thesis.

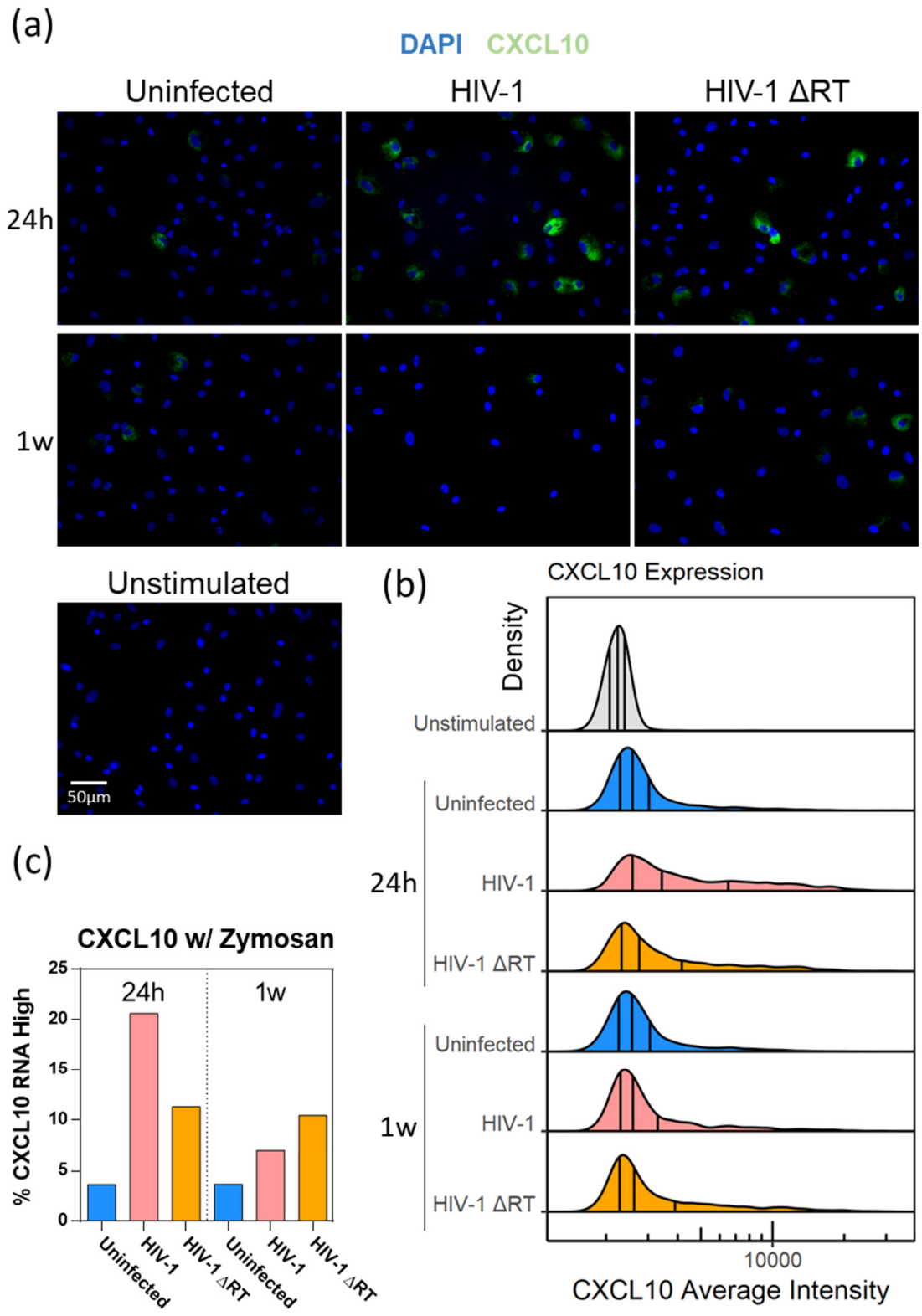

Figure 5.19: HIV-induced exaggeration of CXCL10 expression does not persist.

MDMs were infected with single-round HIV-1 or an RT mutant for 24 hours or 1 week prior to stimulation with zymosan or curdlan for 4h. **(a)** RNA FISH was used to detect CXCL10 expression. **(b)** Expression was quantified using Metamorph 7. **(c)** An MFI threshold of 10,000 was used in R to classify cells expressing high levels of CXCL10. Representative of 2 donors.

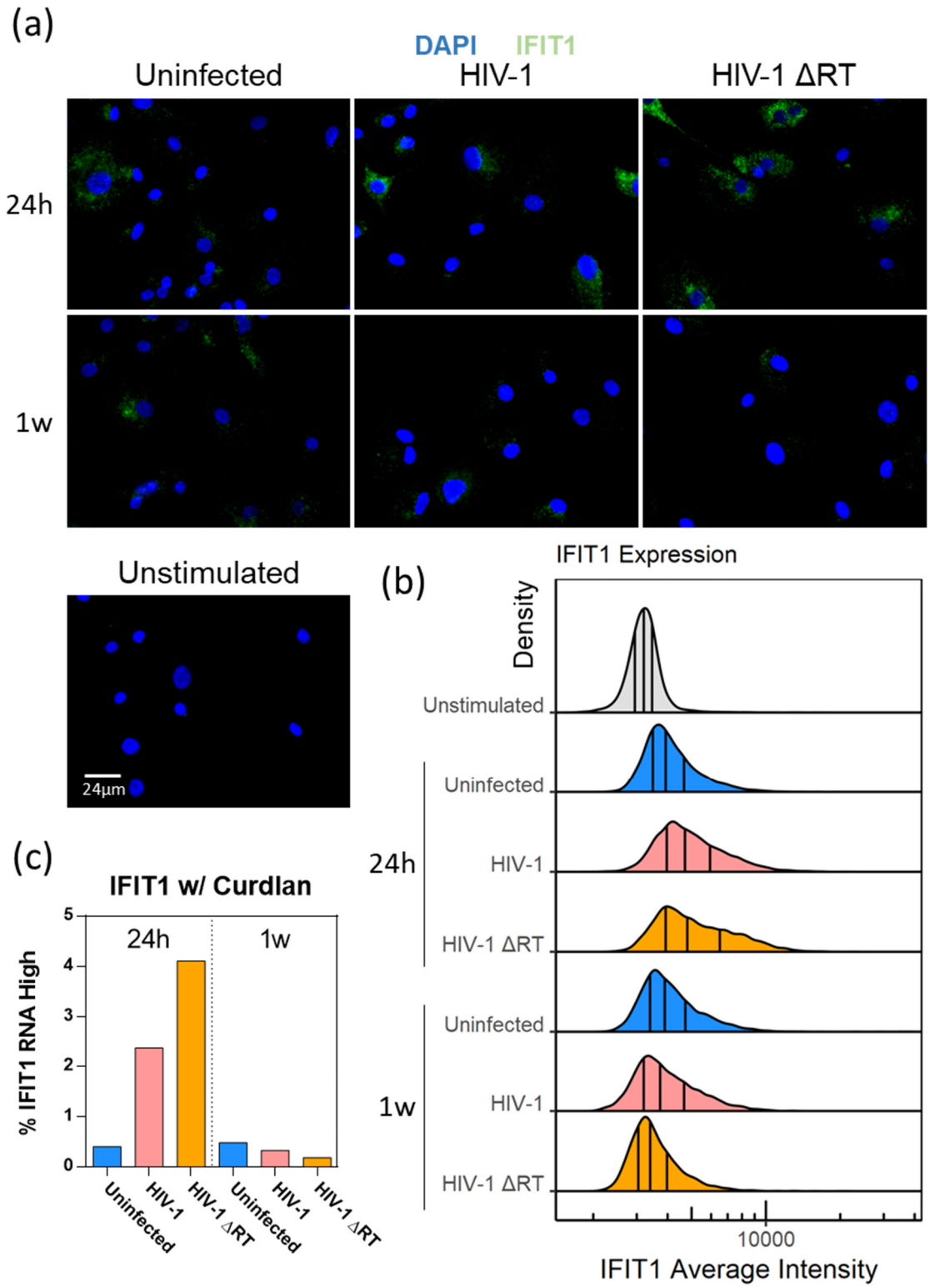

Figure 5.20: HIV-induced exaggeration of IFIT1 expression does not persist.

MDMs were infected with single-round HIV-1 or an RT mutant for 24 hours or 1 week prior to stimulation with zymosan or curdlan for 4h. **(a)** RNA FISH was used to detect IFIT1 expression. **(b)** Expression was quantified using Metamorph 7. **(c)** An MFI threshold of 10,000 was used in R to classify cells expressing high levels of IFIT1. Representative of 2 donors.

5.2.13. Persistence of IL-10 attenuation induced by TLR8 agonists.

To assess whether IL-10 attenuation was also persistent with TLR8 agonists and exogenous interferon treatment, MDMs were left for 48h or 1w after treatment before stimulation with zymosan. Quantitation of cytokine secretion by ELISA revealed that both ssRNA40 and IFNβ induced strong IL-10 attenuation at 48h, which begins to recover by the 1w timepoint (**Figure 5.21**). It is possible that over long periods of time IL-10 responses may recover completely, but the presence of the phenotype 1 week after the initial stimulus mirrors the effects of HIV-1. Further experiments at even later timepoints could further explore this issue.

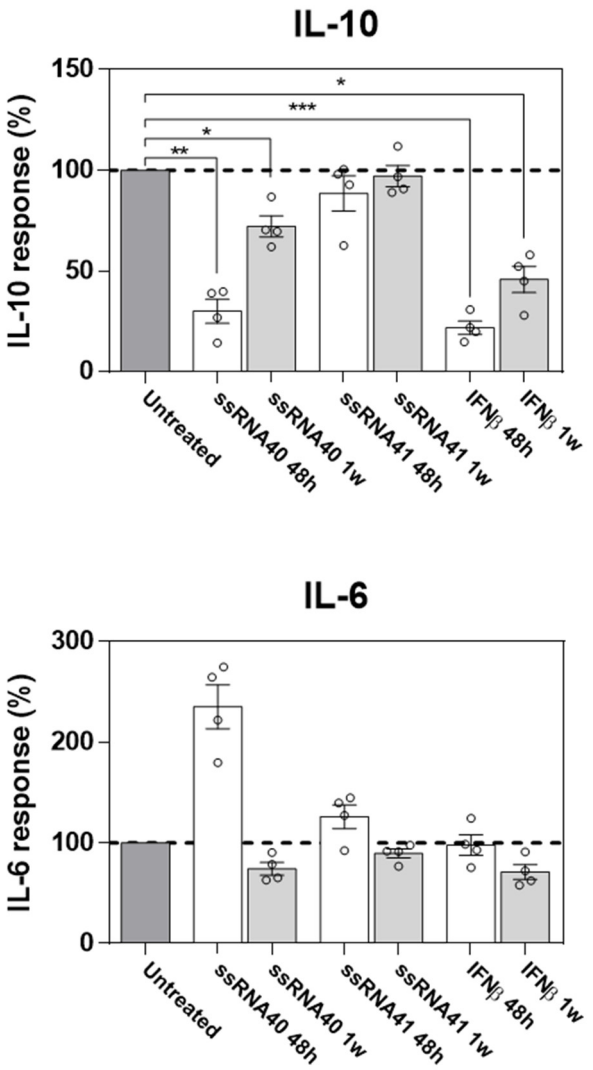

Figure 5.21: ssRNA and interferon induce long term IL-10 attenuation

MDMs were treated with ssRNA or exogenous interferon 48 hours or 1 week prior to stimulation with zymosan for 4h. ELISA was used to quantify cytokine secretion in the supernatant. Graphs show means ± SEM. Significance was determined using one-way ANOVA. Results from 4 donors.

5.3. Chapter Discussion

In this chapter I investigated the host factors involved in IL-10 attenuation in FCS MDMs. Western blotting demonstrated that phosphorylation of Akt during zymosan stimulation is not altered by HIV-1 infection, although this does not rule out changes further downstream in the PI3K-Akt-mTOR pathway. Phosphoblotting for ERK1/2 produced less consistent results suggestive of a defect in ERK phosphorylation in the presence of HIV-1. The initial peak in signalling was immediately seen at 15 minutes post-stimulation before phosphorylation decreased at subsequent timepoints. It may be possible to further resolve any differences in this rapid activation by using additional time points early after stimulation. This assay was also prone to significant background noise due to the involvement of these pathways in multiple cellular functions, alongside the substantial macrophage heterogeneity observed previously. Future work will utilise single cell immunofluorescent staining alongside RNASeq and pathway analysis to resolve the effects of HIV on these pathways. Single cell methodologies could produce more definite results by allowing analysis of phosphorylation to be restricted to cells which express IL-10.

Because interferon signalling has been reported to influence IL-10 production [204], I tested whether treatment with recombinant interferon could phenocopy HIV-1. Exogenous interferons were capable of inhibiting IL-10 responses in the same manner as HIV-1, raising the question of whether interferon signalling was being triggered in my model. RNA FISH was used to investigate whether ISG expression is induced by HIV-1 during infection. Neither HIV-1 or TLR8 agonists induced significant ISG signalling prior to stimulation with zymosan, and a time course of HIV-1 infection revealed no evidence of widespread ISG signalling at any point during the first 48h post-infection. These results suggested that while interferons can replicate the effects of HIV-1, this is not the mechanism through which the virus attenuates IL-10 in this model. It is possible that a shared pathway is activated by both TLR8 signalling and the antiviral response.

Blockade of the interferon receptor would not rescue IL-10 from the virus or HIV-derived ssRNA, confirming that interferon secretion is not the mediator for this phenotype. Conditioned media from infected cells was also unable to attenuate IL-10 responses, therefore indicating that a secreted factor is not responsible for this phenotype. An alternative hypothesis was that localised sensing of virus would induce IL-10 attenuation in cells without infection. While IFN-independent induction of ISGs and antiviral signalling has previously been described [505], there was no evidence of substantial ISG induction in my experiments.

Interestingly, while ISG induction was not seen during infection with HIV-1, infection significantly enhanced ISG expression during secondary stimulation with zymosan or curdlan.

Augmented TLR-inducible IFN expression in HIV-infected cultures has previously been described in dendritic cells [506]. This may be due to the additional stimulation sensitising cells to the presence of HIV-1 RNA or DNA, although enhanced ISG expression was primarily seen in uninfected cells containing HIV-1 RNA.

Innate immune adaptation has been reported to alter TLR responses after repeated stimulation, typically through epigenetic modification which persists over time. To assess this, experiments were carried out to determine whether IL-10 attenuation and/or ISG enhancement are persistent. At 1-week post-infection the incoming HIV-1 RNA from the RT deletion mutant virus had been degraded, but IL-10 attenuation was still present. This suggested a sensing mechanism and possibly epigenetic modification as the mediator for this phenotype rather than a direct effect of the presence of virus during secondary stimulation. In contrast, the ISG enhancement was substantially weaker at the 1-week timepoint, which may indicate that this is primarily mediated by a response to viral RNA or DNA during the zymosan response itself. The detection of IL-10 attenuation in the absence of ISG enhancement confirms that these two phenotypes are independent.

The induction of long-term IL-10 attenuation may have significant consequences for innate immunity to other pathogens. Genetic defects in IL-10 and the IL-10 receptor have been implicated in autoimmune conditions such as Inflammatory Bowel Disease [142], [143]. The persistence of this effect in the context of HIV-1 may be mediated by epigenetics. Chromatin modification and DNA methylation have both been described as mechanisms which can alter immune responses in the context of viral infection [507], [508]. Future work should focus on investigating epigenetic markers via techniques such as ATAC-seq [509] and methylation arrays [510].

Overall, these results suggest that HIV-1 exposure induces long-term IL-10 attenuation, possibly via epigenetic modification. The pathways responsible for this may be shared with antiviral signalling induced by interferon, but during infection the presence of HIV-1 RNA is not sufficient to induce interferon secretion or ISG expression in the absence of a secondary stimulus. This may indicate that the ability of HIV-1 to evade innate immunity also depends on whether additional sources of innate immune activation are present at the time of infection.

6. General Discussion

The attenuation of IL-10 responses in macrophage cultures infected with HIV-1 is a novel host-pathogen interaction with the potential to influence the immunopathogenesis of HIV-1 disease and of co-infecting pathogens. I have investigated the viral and host mechanisms involved in this phenotype using a single round model of HIV-1 with TLR2/Dectin-1 agonists as the secondary stimulus. This was studied in both cell lines and primary cells from healthy volunteers, and I extended conventional measurements in bulk cell culture to analysis at the single cell level by combining RNA FISH with high-throughput imaging and analysis.

6.1. Viral components involved in IL-10 attenuation

While THP-1 cells could be made to express IL-10 upon zymosan stimulation by differentiating them with a PMA treatment protocol which included a resting step, the resulting phenotype after infection with HIV-1 was less consistent than in primary cells. This may be due to differential regulation of innate immune pathways in this cell line, with the multiple levels of regulation of IL-10 production providing additional steps at which cytokine release could be affected. Nonetheless, this model could potentially be useful in future work due to the ability to generate knockout mutant lines. Further refinement and optimisation of the MDM-based model used in previous studies allowed me to remove SIV VLP supplementation while maintaining high levels of infection by growing cells in FCS, which eliminated a potentially confounding factor which could have interfered with results.

6.1.1. Viral factors responsible for the phenotype

My results demonstrated that IL-10 attenuation does not require *de novo* viral gene expression in infected cells. This indicated that any protein or immune stimulus is delivered alongside the virion itself. Testing with knockout mutants established that this factor was not a viral accessory protein, which was surprising given that the accessory genes are typically responsible for modulating host immunity [511]. Experiments using a gene therapy vector which does not encode viral proteins revealed that *de novo* viral gene expression is not required. This made the most likely hypotheses that an essential (structural or regulatory) viral protein carried in the virion interferes with IL-10 expression or that innate immune sensing of a viral component triggers a host-driven change.

RNA FISH staining demonstrated that a subset of macrophages express high levels of IL-10 in response to stimulation. Due to the fact that only a further subset become infected with HIV-1, single cell analysis was used to investigate the impact of infection at single cell resolution. This revealed that IL-10 attenuation is not restricted to infected cells within a culture, with a

substantial reduction in IL-10 expression being seen across all cells. This result further explained how a 6-fold decrease in cytokine production was possible in experiments featuring only ~25% infection. The RNA FISH also allowed the detection of incoming viral RNA prior to reverse transcription, which revealed that the vast majority of cells within a culture contained viral material. Additionally, a subset of cells appeared to take up large quantities of virus, perhaps reflecting uptake into endosomes. These results suggest that macrophages may potently restrict HIV-1 infection or that a substantial proportion of virions are not infectious, meaning that only a small fraction of virions successfully integrate to productively infect cells.

With the knowledge that productive infection is not necessary for IL-10 attenuation, I investigated the minimal components of a HIV-1 vector which are needed to induce the phenotype. Destruction of the virion by boiling or UV irradiation ablated the effects on IL-10, confirming the requirement for intact virus. Treatment with HIV VLPs lacking a viral genome also failed to influence IL-10 expression, indicating that viral genomic material is required for this phenotype. This would explain why IL-10 attenuation had not been seen in previous work using SIV VLPs alone, since these also lack a packaged genome [445]. Interestingly, active viral entry with an envelope protein was not required for the induction of IL-10 attenuation. This is likely to indicate that virions taken up by the host cell via endocytosis can mediate this effect [367]. In line with this, RNA from these “bald” viruses lacking envelope could still be detected within macrophages. Since in the absence of an envelope the virus cannot escape endosomes, this highlighted endosomal sensing rather than cytosolic detection as a potential mechanism for investigation. As productive viral entry and infection are not required, this may suggest that a similar effect could be seen in other phagocytic cell types.

Cells infected with a reverse transcriptase-deficient mutant of HIV still displayed IL-10 attenuation, indicating that production of viral DNA is not required. The same effect was seen when wild type virus was treated with reverse transcriptase inhibitor efavirenz. The persistence of IL-10 attenuation in the absence of reverse transcription eliminates cytosolic DNA sensing as a mechanism for this effect, which renders RNA sensing as the most likely explanation. Furthermore, in the absence of viral DNA integration cannot occur, meaning that DNA damage sensing arising from integration into the host genome is not a viable mechanism for this phenotype. In support of these findings, direct delivery of a short ssRNA derived from the HIV-1 LTR was able to phenocopy full length virus and inhibit IL-10 responses. This effect on IL-10 was rescued when nucleotides stimulatory to TLR8 were removed from the HIV-derived sequence [488]. These results suggest a mechanism in which detection of viral RNA mediates IL-10 attenuation through a host sensing pathway, rather than the activity of a viral protein.

6.2. The Host factors involved in IL-10 attenuation

Western blotting to investigate the pathways modulated by HIV-1 suggested a possible effect on ERK1/2 phosphorylation as seen in previous work with *M. tuberculosis* [445]. In contrast, no change in Akt phosphorylation was detected at either target residue. While previous work only found specific inhibition of IL-10 by small molecule inhibition of the PI3K-Akt axis, it is possible that other pathways such as ERK/P38 can regulate IL-10 specifically and that total inhibition is not representative of this process. Another hypothesis would be that effects on the PI3K axis may be further downstream of Akt. However, the primary signalling molecules downstream of Akt with relevance to IL-10 are GSK-3β and mTOR [512], [513]. GSK-3β is dephosphorylated upon activation of this pathway, and in previous work any change in activation driven by zymosan stimulation was not detectable compared to background noise, most likely due to the other cellular functions this pathway is involved in. In contrast, mTOR is only reported to influence IL-10 translation rather than transcription, which does not match our observations of IL-10 attenuation at the transcript level [514]. Therefore, small molecule inhibition of this pathway may impact IL-10 in a different way to HIV-1. The precise mechanism that might impact ERK activity in a way which specifically influences IL-10 production remains unclear and should be a focus for future work.

My results demonstrated that host sensing of HIV may be the mechanism behind this phenotype. The effects on both infected and uninfected cells in the culture also raised the possibility that this may be mediated by a secreted factor. However, conditioned media from infected cells was unable to induce IL-10 attenuation. While it is possible that such a factor may simply be very short-lived, it is more likely that sensing of individual virions occurs at a local level, with most cells in the culture being exposed to virus at the dosages used. Multiple innate sensing pathways capable of detecting HIV-1 RNA and DNA have been described [515]. Given that HIV-derived ssRNA was sufficient to inhibit IL-10, and that TLR8 agonists replicate this effect, I hypothesise that endosomal sensing of the viral genome mediates this effect in my model.

However, innate sensing is typically reported to trigger interferon expression, particularly in immune cells [408], [516]. Given the array of positive and negative effects of interferon on IL-10 regulation that have been reported, I hypothesised that the presence of HIV-1 virions may be triggering antiviral signalling. This could in turn promote inflammation as part of the antiviral response, which consequently may inhibit IL-10 expression. Interestingly, pre-treatment of MDMs with recombinant Type I IFNs phenocopied the effects of HIV-1, indicating that activation of antiviral signalling could influence IL-10 responses. However, blockade of interferon receptor

only rescued IL-10 from the effects of exogenous IFN, not HIV-1 or TLR8 agonists. This would imply that secreted interferon is not the mediator of the effects on IL-10 driven by HIV-1.

It was possible that localised ISG induction might be induced by cells which are producing interferon without needing secreted IFN as a mediator. RNA FISH was therefore used to search for ISG expression at the single cell level, yet no evidence of ISG expression was seen prior to secondary stimulation in infected wells. These results were in keeping with previous observations that HIV evades the triggering of innate antiviral responses in this model [374]. It is possible that an effector mechanism which regulates IL-10 can be activated by both broad IFN responses and an independent mechanism in this phenotype. A possible explanation is that stimulation of macrophages with TLR8 does not trigger antiviral signalling but does prime cells towards further inflammatory signalling during subsequent stimulation. This was supported by the finding that IL-10 attenuation persists for over 1 week even after RNA has been degraded.

These results may be suggestive of epigenetic regulation such as chromatin modification, which should be an area of future study. Because macrophages act as sentinel cells for innate immunity, repeatedly sampling the extracellular environment and clearing debris [27], it may be the case that the presence of extracellular viral RNA is not a sufficient stimulus to trigger the full antiviral response in this cell type. In contrast, DNA sensing indicative of active infection has been reported to trigger full IFN signalling [4]. Although TLR8 stimulation is typically thought to induce NF-κB and IRF7 activation [492], it has been reported that PMA-differentiated THP-1 cells lack the IRF-driven arm of this response [517]. While this has not been tested in primary cells, it is possible that differentiation of monocytes alters the cellular response to TLR8 activation to adapt for their role in maintaining homeostasis. There may be limited IRF7 expression in differentiated macrophages, which could explain the lack of an interferon component in the TLR8 response. The inability of such sensing to generate interferon responses may also explain why the virus does not appear to have a means to evade such detection, since the effects of TLR8 signalling in this context may not exert a selective pressure on the virus unless a secondary stimulus is present.

Interestingly, while ISG induction was not seen during infection, the presence of virus dramatically enhanced ISG expression as part of the zymosan response. This appears to coincide with a reduction in IL-10 expression within these cells. One possible explanation is that stimulation with zymosan prompts cells to respond to viral stimuli which are already present, resulting in interferon induction. This is supported by the finding that the effect on interferon does not persist until the 1-week timepoint at which virions have been degraded, whereas IL-10 attenuation is still detectable. This would suggest that either the effects of the virus are short-lived, or viral genomic material must still be present at the time of secondary stimulation to see

ISG enhancement. Furthermore, these results decouple IL-10 attenuation from ISG enhancement. Detection of virions may trigger both phenotypes, but their different duration suggests an independent mechanism of action. It remains possible that interferon expression could have an additive effect and further exaggerate IL-10 attenuation, which could be tested experimentally by supplementing with recombinant interferon during zymosan stimulation rather than during priming.

6.3. A model of macrophage IL-10 attenuation by HIV-1

My results suggest a model in which uptake and endosomal sensing of incoming HIV-1 RNA by TLR8 leads to attenuation of macrophage IL-10 transcription without induction of type I IFNs (**Figure 6.1**). These findings represent three novel discoveries. First, the discovery of a functional interaction between HIV-1 and TLR8 in human macrophages; second, the discovery that viral RNA can stimulate TLR8-dependent events without induction of type I IFNs that have hitherto been the canonical response to such a host-pathogen interaction; and third, the discovery that specific regulation of IL-10 responses can be mediated by TLR8.

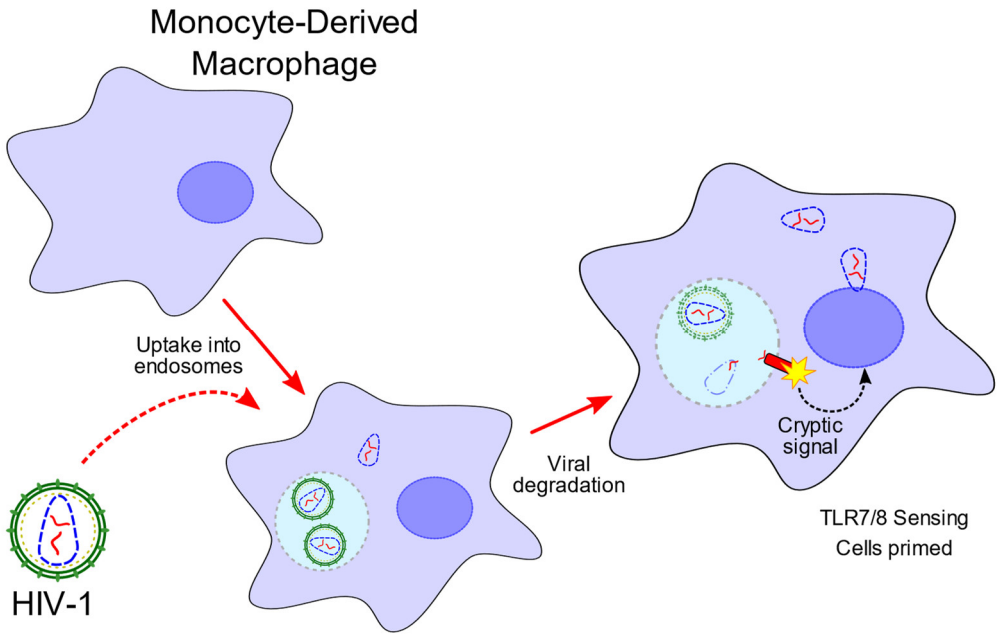

Figure 6.1: A model for IL-10 response attenuation by HIV-1.

HIV-1 virions are taken up into macrophages by endocytosis. Viral degradation results in HIV RNA being released, which can then bind to and activate TLR8. A resulting signalling cascade results in macrophages becoming primed to express less IL-10 upon subsequent stimulation, which persists after the original viral RNA has been degraded.

Although macrophages are unequivocally permissive to productive infection, there is still controversy about the frequency of HIV-1 infection of macrophages *in vivo* and the contribution

of this cellular niche to overall viral load [372]. In this context, it is particularly noteworthy that productive HIV-1 replication was not necessary for this phenotype and that RNA FISH analysis showed the presence viral RNA in most cells in the absence of productive infection. Therefore, any macrophage uptake of HIV-1 RNA may be sufficient to disrupt IL-10 immunoregulation. We speculate that this is likely to occur at high frequency in HIV-1 infected individuals.

I have not established the precise molecular mechanism by which TLR8 stimulation leads to attenuation of IL-10 responses. We have established that a time interval (4-24 hours) between exposure to virus and IL-10 stimulation is necessary for this phenotype, and that the effect of transient exposure to virus lasts for at least one week. Since innate immune signalling events are generally rapid and transient, it is unlikely that TLR8-dependent signalling intersects specifically with an IL-10 signalling pathway. Instead, our observations are most consistent with a model in which TLR8-dependent signalling leads to longer lasting epigenetic modifications that impact on IL-10 expression.

6.4. The physiological significance of my findings

In the model described above, HIV-1 infection can lead to widespread dysregulation of IL-10 immunoregulation by macrophages. Macrophages are not the only cells to produce IL-10 and their specific contribution to immunoregulation is not known [149]. Nonetheless, our findings raise the possibility that this mechanism contributes to chronic immune activation that in turn may contribute to progressive immunodeficiency and increased risk of cardiovascular or neoplastic disease [518]. Likewise, HIV-1 conditioning of macrophages may exacerbate acute immune reconstitution inflammatory syndromes in co-infected patients receiving antiretroviral treatment. The long-lasting effects of viral exposure may also suggest that defective IL-10 responses could persist in patients after antiretroviral therapy is initiated.

In the context of coinfection with *Mycobacterium tuberculosis*, which the IL-10 phenotype was originally observed in, these results may have implications for our understanding of disease pathology. While the incidence of HIV-1 and TB coinfection in the same cell is likely to be low, the finding that exposure to virus without productive infection is sufficient to attenuate IL-10 responses provides a pathway for widespread effects on macrophage function. In a viraemic patient it is possible that most alveolar macrophages encounter virions but restrict infection, which would result in altered innate immune responses which could interfere with control of TB infection. Further investigation into the effects of HIV-1 exposure on alveolar macrophage function could provide insight into the impact of the virus on innate immunity in the lung.

Our observations also raise the question of how attenuation of macrophage IL-10 responses may impact on HIV-1 itself. A plausible hypothesis is that this effect provides an advantage to the virus by counteracting the reported inhibition of HIV-1 replication by IL-10 and potentiates pro-inflammatory enhancement of HIV-1 replication [519]. In the context of dendritic cells HIV-1 has also been reported to exploit TLR8 signalling in order to activate NF-κB and initiate transcription of integrated provirus [421]. While activity of NF-κB was not examined in this thesis, if the effects of TLR8 stimulation in macrophages involve this transcription factor it may offer a pathway for the virus to promote transcription of itself without triggering an interferon response. Knockout mutants for viral accessory proteins had no effect on the phenotype, which may indicate a lack of any viral countermeasure to prevent such RNA sensing. This may further suggest that TLR8 sensing does not interfere with viral replication.

Finally, my findings provide compelling evidence of IFN-independent actions downstream of viral innate immune sensing and a novel example of adaptation of innate immune responses as a sequela of prior experience. The term 'trained immunity' has emerged to distinguish this effect from conventional definitions of adaptive immunity dependent on somatic gene recombination [520]. Like previous reports of trained immunity, the mechanism for HIV-1 attenuation of macrophage IL-10 responses is also most likely to be epigenetic. Importantly, other examples have described propagation of trained immunity via epigenetic modification of haematopoietic stem cells, raising the possibility that HIV-1 attenuation of IL-10 may extend beyond its direct effect on macrophages to other cell types if progenitor cells are affected in the same way [521].

6.5. The limitations of my study

The main limitation of my study is that I have not been able to show experimentally that TLR8 is necessary for HIV-1 attenuation of macrophage IL-10 responses. In addition, the hypothesis that the effect on IL-10 responses is mediated by epigenetic modifications remains untested. Likewise, it seems unlikely that epigenetic modifications will only lead to an effect on the IL-10 locus. The original observations leading to the discovery of this phenotype were based on genome-wide transcriptional responses assessed by microarray technology [445]. Any potential link between IL-10 and other transcriptional changes attributable to HIV-1 priming was not assessed.

Although my results identify TLR8 as a sensor for HIV RNA which can induce IL-10 attenuation, this research does not exclude the potential involvement of other pattern recognition receptors in the phenotype. Questions also remain on how viral RNA might become exposed to sensing within endosomes. It is possible that this occurs stochastically as capsids destabilise, but it is also possible that endosomal proteases are required to break apart viruses.

This could be investigated by using inhibitors of phagosome maturation to test whether IL-10 attenuation is induced without fusion of the phagolysosome. It is also not clear whether RNA from virions which successfully escape the endosome or enter directly into the cytosol can still be detected, possibly using cytosolic sensors or through autophagy.

Results with the ssRNA ligands suggested that there may be some recovery of IL-10 responses after 1 week, in contrast with HIV-1. However, further testing with additional timepoints may be necessary to further characterise the duration of IL-10 attenuation. It is possible that the ssRNA ligands degrade much faster than full virions, resulting in a shorter stimulus which may have effects which do not persist. It is also possible that multiple sensing mechanisms could contribute to IL-10 attenuation. It would be worthwhile to investigate whether a greater effect can be obtained by delivering full length viral RNA in isolation, rather than short segments which needed to be provided at large quantities. This has the potential to include additional secondary structures which may interact with other host sensing pathways [522]. Furthermore, the ssRNAs were delivered in LyoVec, which can fuse with the plasma membrane and deliver RNA directly into the cytoplasm rather than into endosomes. Further work may therefore be necessary to investigate whether cytosolic RNA sensors such as RIG-I can detect HIV RNA and induce changes in IL-10 expression. While evidence for attenuated IL-10 responses *in vivo* has been previously described, it is unclear how *in vitro* monocyte-derived macrophages compare to tissue macrophages [446]. The changes in cytokine secretion and permissivity to HIV infection induced by growth in FCS demonstrate the plasticity of these cells and highlight how changes in the environment can dramatically alter macrophage function. Given that stimulation with interferons can induce IL-10 attenuation, it is also possible that the elevated serum interferon levels observed in HIV patients could provide an additional mechanism for this phenotype *in vivo* [411], [523].

The RNA FISH assay allowed for the analysis of gene expression at the single cell level. Since this assay has the sensitivity to detect incoming virions, there are potentially broader applications for this technology for the investigation of viral entry and uptake. However, this assay does not reveal whether viral RNA remains associated with the viral capsid, which could potentially shield it from host sensors. RNA FISH is also unable to detect virus which has reverse transcribed into DNA. It is therefore difficult to establish which cells containing incoming virions would eventually become productively infected. These limitations could potentially be overcome by adding additional stains for viral proteins and DNA.

One key limitation in the RNA FISH experiments was that this assay does not reveal the uptake pathway utilised by virions, and it is therefore difficult to confirm whether virions reside in endosomal compartments or the cytosol. This could be resolved by staining for endosomal

markers, although the large numbers of such compartments typically seen in macrophages may make it difficult to accurately localise viral RNA. It is also possible that a fraction of virions which enter cells are rapidly degraded, limiting visualisation. Inhibitors of the proteasome or endosome maturation may provide further insight into the longevity of virions inside cells.

For IL-10 attenuation to be detected at the protein level, cultures had to be exposed to large doses of virus which delivers viral RNA into almost all cells. While this model may reflect exposure to virus in patients with viraemia, it is not clear what dosage of virus is required within a single cell to induce IL-10 attenuation. However, RNA FISH may make it possible to utilise lower doses of virus and compare IL-10 expression between cells which contain and do not contain virus. While it is known that macrophages exhibit heterogeneity in their capacity for phagocytosis [63], [524], how uptake compares to eventual productive infection would also be an interesting area to explore in future research.

Furthermore, investigation by Western blotting into which host pathways are affected by HIV was somewhat limited by the need to utilise protein samples from bulk cultures. My results established that the majority of cells do not express high levels of IL-10, which may limit the resolution of this technique when analysing protein phosphorylation in the context of a broader zymosan response. This is especially important due to the fact that the pathways which regulate IL-10 are also involved in other cellular functions [154]. Single cell techniques such as RNA FISH and fluorescent immunostaining may therefore be more informative when studying pathways which regulate IL-10 as they allow analysis to be restricted to cells of interest.

6.6. Summary and future work

Overall, my results suggest that uptake and sensing of HIV-1 RNA primes macrophages towards inflammatory signalling by inducing a defect in IL-10 responses to subsequent stimulation. Similar effects can be seen when cells are exposed to interferons, suggesting that this may be mediated by effector mechanisms shared with antiviral signalling pathways. However, the delivery of virus alone is not sufficient to trigger ISG expression in the monocyte-derived macrophages used in our model.

To build further confidence in this model, it will be important to try to rescue IL-10 responses from HIV-1 by inhibiting TLR8. TLR8 activation is not well understood, but several inhibitors are now commercially available. Inhibition of phagosome maturation may also provide an avenue for separating endosomal sensing from cytosolic RNA sensing. While primary macrophages are not compatible with gene editing, it may also be valuable to attempt to generate knockout THP-1 lines deficient in hypothetical sensing mechanisms. This would help to further investigate

whether RNA sensing is necessary or sufficient for IL-10 attenuation in response to complete vectors.

A key aspect of this phenotype which merits further investigation is to identify the signalling process which results in IL-10 attenuation after TLR8 stimulation. Future work will try to elucidate this process by using RNAseq to profile the cellular transcriptome in the presence or absence of zymosan stimulation and HIV infection. The transcriptional response to zymosan will be compared between infected and uninfected cells to identify genes which are significantly altered by HIV-1. Pathway and transcription factor binding site analysis would then potentially provide insights into which of the multitude of signalling pathways which influence IL-10 production are affected by infection.

Furthermore, the prolonged effects on IL-10 suggest a mechanism involving epigenetic modification. This can be investigated using techniques such as ATAC-seq to detect changes in chromatin accessibility and methylation arrays to test for alterations in DNA methylation patterns [509]. This may reveal modifications around the IL-10 locus which promote or inhibit expression, which could also provide an explanation for how IL-10 is specifically inhibited despite being primarily regulated by transcription factors shared between many pathways. This could also improve understanding of the epigenetic regulation of IL-10 as part of the immune response.

Whether this phenotype is specific to HIV-1 could also be an area of interest. Exaggerated inflammatory responses are often associated with enhanced pathology of disease. Given that interferon expression can also inhibit IL-10 production it is possible that a multitude of viral infections may have significant consequences for the response to co-infection with other pathogens. It would also be interesting to test whether non-viral stimuli can also exhibit a priming effect on macrophages, as the requirements for the primary stimulus are yet to be fully determined.

Further *in vivo* evidence for this phenotype could be obtained by acquiring bronchioalveolar lavage (BAL) samples from HIV patients and comparing the inducible IL-10 response from alveolar macrophages to that from healthy volunteers. My model would predict that substantial IL-10 attenuation would be seen in patients with viraemia even with a limited proportion of productively infected cells. A potentially interesting question to investigate is whether patients on long-term ART lose this phenotype after viral load is suppressed, or if the effects persist due to epigenetic factors until the macrophage populations are eventually renewed.

It may also be worthwhile to compare the strength of this phenotype with different strains of virus, particularly when expressing envelopes with different coreceptor tropism.

Macrophage-tropic viruses tend to utilise CCR5 as the coreceptor which is thought to be able to fuse in endosomes after uptake via macropinocytosis, providing the potential source of endosomal stimulation [525]. However, envelopes using CXCR4 may preferentially fuse at the cell surface, which could avoid any potential to influence IL-10 unless virions are actively taken up into endosomes by the cell [526]. Comparing the effects of viruses with these envelopes to those pseudotyped with VSV-G could provide further insight into the behaviour of virus *in vivo*. Another area for investigation may be the potential influence of the virological synapse, which could facilitate transfer of large quantities of virions to macrophages in a more localised manner [328].

Finally, additional physiological consequences of HIV RNA sensing may be revealed by further characterisation of macrophage function. There may be further effects of this mechanism impacting phagocytic capacity, differentiation and other important processes. There may also be implications for interactions between macrophages and T cells which could affect the development of the adaptive immune response. Transcriptional profiling of cells exposed to virus (but not infected) may provide further insights into any such effects of HIV exposure.

7. References

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