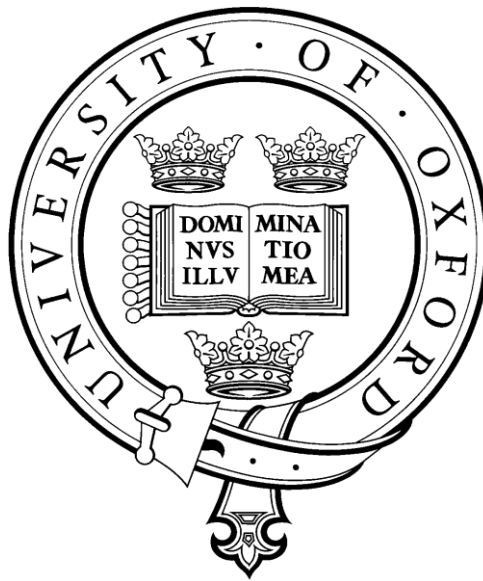


**Investigation into sub-cellular CD4 distribution in
human Embryonic Stem Cell derived macrophages
and its role in HIV-1 infection**



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Michaelmas Term 2012

A thesis submitted for the degree of Doctor of Philosophy
in the University of Oxford

Abstract

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Human macrophages are one of the main targets for HIV-1 infection, despite their moderately low surface expression levels of the main HIV-1 receptor, CD4. The site of HIV-1 fusion can occur at the surface or following uptake through an endosomal pathway and it might be anticipated that the site would affect the progress of HIV-1 through the cell to the nucleus. Previous pharmacological studies provide one line of evidence for an endosomal entry route which is dependent on Detergent Resistant Membranes (DRMs). However, these findings need confirmation using a genetic approach, as small molecules may have multiple non-specific effects. For this study, a novel genetic approach was developed to manipulate sub-cellular CD4 distribution and investigate whether it determines the HIV-1 entry pathway in macrophages. This was achieved by transducing human embryonic stem cells (hESC) with lentiviral vectors and differentiating these cells into homogeneous genetically modified macrophages. This cellular system by-passes the challenges posed by the refractoriness to direct genetic manipulation of heterogeneous primary macrophages. Firstly, as proof of principle, a short hairpin RNA targeting CD4 was expressed in hESC-macrophages, resulting in knockdown of CD4 and, as anticipated, strong inhibition of HIV-1 infection. Secondly, expression of LCK in hESC-macrophages effectively tethered CD4 at the cell surface, and sequestered HIV-1 into an unproductive pathway, presumably through surface fusion, rather than progressing successfully to the nucleus. Thirdly, endogenous CD4 was substituted with CD4 mutants designed to be excluded from DRMs, which resulted in reduced successful HIV-1 entry versus substituted control CD4. The results support the model in which the productive entry pathway of HIV-1 in macrophages occurs via fusion after a raft-dependent endocytic uptake pathway, and requires CD4 localization to lipid rafts.

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"Weet of rust"

"Schubert String Quintet"

"Dvorak Cello Concerto"

Abbreviations

°C	Degrees Celsius
~	approximately
<	is less than
>	is more than
≤	is less than or equal to
α	anti
Ψ	psi; HIV-1 packaging RNA sequence
3'	3 prime
5'	5 prime
A	Adenine
aa	Amino acids
aDMEM	Advanced Dulbecco's Modified Eagle's Medium
AF	Auto-fluorescence
AIDS	Acquired Immune Deficiency Syndrome
AMP(R)	Ampicillin Resistance Gene
ANOVA	ANalysis Of VAriance
AO	Acridine Orange
AP-2	Adaptor Protein 2
APC	Antigen Presenting Cell or Allophycocyanin
APOBEC3G	Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
BlaM	Beta-lactamase
bMDM	blood Monocyte-Derived Macrophages
BMP-4	Bone Morphogenetic Protein 4
C	Cytosine
CCF2-AM	Coumarin Cephalosporin Fluorescein / AcetoxyMethyl ester
CCR-	Chemokine (C-C motif) Receptor-
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CMV _{minimal}	minimal Cytomegalovirus
Dox	Doxycycline
cPPT	central polypurine tract
DAPI	4',6-Diamidino-2-Phenylindole/
DC	Dendritic Cells
det	Detergent

DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
DRM	Detergent Resistant Membrane
EB	Embryoid Body
EDTA	Ethylenediaminetetraacetic Acid
EF1 α /EF1a	Elongation Factor-1 alpha
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscopy
esMC	human Embryonic Stem Cell derived Monocytes
esMDM	human Embryonic Stem Cell derived Monocyte Derived Macrophages
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FCDR	Flow Cytometric Detergent Resistance
FCS	Fetal Calf Serum
Flt3-L	Fms-related tyrosine kinase 3 ligand
FRET	Förster (Fluorescence) resonance energy transfer (FRET)
FSC	Forward Scatter
G	Guanine
G418	Geneticin
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GM-CSF	Granulocyte Macrophage-colony Stimulating Factor
GOI	Gene Of Interest
GPI	Glycosylphosphatidylinositol
HIV-1	Human Immunodeficiency Virus-1
hES	human Embryonic Stem Cell
hESC	human Embryonic Stem Cell
hu	human
HUES-2	human Embryonic Stem Cell-2 line
I	IRES
IFN-	Interferon-
Ig	Immunoglobulin
IN	Integrase
Interleukin	IL
iPSC	human induced Pluripotent Stem Cells
kDa	Kilo Dalton

LCK	Lymphocyte-specific protein tyrosine kinase
LPS	Lipopolysaccharide
LTR	Long Terminal Repeat
Luc	Luciferase
LV	Lentivirus
M-CSF	Macrophage-Colony Stimulating Factor
mAb	monoclonal Antibody
max	maximum
MDM	Monocyte-Derived Macrophages
MEF	Mitomycin C-inactivated mouse embryo fibroblasts
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	minutes
miRNA	microRNA
mL	Millilitre
MOI	Multiplicity of infection
mRNA	messenger RNA
n	number
Nef	negative factor
nM	Nano-Molar
NCBI	National Centre for Biotechnology Information
ns	Non Significant
O/N	Overnight
p	plasmid
P	Puromycin
PA	polyadenylation
PIC	Pre-integration complex
PBMC	Peripheral Blood Mononuclear Cell
P(uro) ^R	Puromycin ^{Resistance}
Puro	Puromycin
R	Repeat region
RFP	Red Fluorescent Protein
RPMI	Roswell Park Memorial Institute
RQ	Relative Quantity
RRE	Rev Responsive Element
RT	Reverse Transcription or Reverse Transcriptase

RT	Room temperature
RTC	Reverse Transcription Complex
rtTA3	Reverse Transactivator 3
SD	Splice Donor or Standard Deviation
SCF	Stem Cell Factor
sec	seconds
SEM	Scanning Electron Microscopy or Standard Error of the Mean
SFK	Src Family Kinase
SH	Src homology
SIN	Self-inactivating
SOC	Super Optimal broth with Catabolite repression
shRNA	short hairpin RNA
shRNA-miR	microRNA-adapted shRNA
SSC	Side Scatter
SV40	Simian Virus 40
SV40 INT	SV40 intron
T	Thymine
TAR	Transactivation response region
TEM	Transmission Electron Microscopy
TM	Transmembrane
TX-100	Triton X-100
U	Uracil
UBC	Ubiquitin C
UD	Unique Domain
v/v	percentage volume per volume
VEGF	Vascular endothelial growth factor
Vpr	Viral protein
Vpu	Viral protein U
vRNA	viral RNA
VSV	Vesicular Stomatitis Virus
w/v	percentage weight per volume
WPRE	Woodchuck Posttranscriptional Regulatory Element
WT	Wild-Type

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Chapter 1: Introduction

1.1 HIV-1

Human immunodeficiency virus type 1 (HIV-1) is one of the most serious global health challenges. In 2011 ~34 million people were living with HIV/AIDS, and there were approximately 1.7 million deaths from the disease and 2.5 million new infections (UNAIDS 2012). Although anti-viral treatments can achieve life-long suppression of viral replication, major challenges remain, including improvement of anti-viral treatment and its side-effect, eradication of HIV-1 from infected patients and HIV-1 prevention (Volberding et al. 2010).

1.1.1 HIV-1 genome

HIV-1 belongs to the genus of lentiviruses of the Retroviridae family (retroviruses). This family is characterized by their use of viral reverse transcriptase (RT) to generate DNA from its RNA genome and their use of integrase (IN) for incorporation of the viral DNA copy into the host's genome. Lentiviruses are further characterized by their ability to infect non-dividing cells and cause slowly progressing diseases (Bukrinsky et al. 1993; Freed et al. 1994). The HIV-1 genome consists of a pair of single-stranded positive-sense RNAs of ~9 kb that contains 9 genes, encoding a total of 15 proteins (Freed et al. 2007). There are 3 essential structural genes (*gag*, *pol* and *env*), 2 regulatory genes (*tat* and *rev*) and 4 accessory genes (*vif*, *vpr*, *vpu* and *nef*). The viral genome is flanked by LTR (long terminal repeat) sequences that include the promoter for HIV-1 transcription and elements required for reverse transcription and integration. Adjacent to the 5'LTR, the genomic transcript contains a RNA packaging signal (ψ). Full length HIV-1 transcripts can be used as genomic RNA for packaging into new virions. Alternatively, transcripts can be translated into Gag and Gag-Pol or spliced to serve as mRNA for the other HIV-1 proteins. Gag and Pol are initially expressed as polyproteins, which are digested by the viral protease during budding. Env, also expressed as a polyprotein, is cleaved by a

cellular protease. *Gag* encodes the viral core proteins which drive viral assembly: matrix, capsid, nucleocapsid, p6, and spacer peptides SP1 and SP2. *Pol* encodes a set of enzymes required for viral replication: protease, reverse transcriptase and integrase. *Env* encodes the surface glycoprotein, gp120, and the transmembrane glycoprotein, gp41. Tat activates viral transcription by binding to the transactivation response region (TAR) at the 5' end of the viral mRNA. Rev controls the nuclear export of unspliced viral transcripts by binding to the Rev responsive element (RRE) in the viral mRNA. The accessory proteins affect the cellular environment to facilitate HIV-1 replication, but are dispensable. The exact role of Vpr (viral protein) remains unclear (Schaller et al. 2012). Vpr can induce cell-cycle arrest, apoptosis, and nuclear import (Freed et al. 2007). Vif (viral infectivity factor) enhances virus infectivity by counteracting the cytidine deaminase host restriction factor APOBEC3G, which would otherwise prevents productive infection by conversion of C to U during HIV-1 reverse transcription (Sheehy et al. 2002; Malim 2009). Vpu (viral protein U) induces CD4 degradation in the endoplasmic reticulum (ER) by recruiting an ubiquitin ligase complex (Malim et al. 2008). This is advantageous for the viral replication, as otherwise CD4 would retain HIV-1 Env in the ER (Malim et al. 2008). Vpu also enhances the release of budded particles from the infected cells, by counteracting the restriction factor Tetherin (Neil et al. 2008). Nef (negative factor) has several functions, including causing downregulation of CD4 (further described in Chapter 1.2), as well as downregulation of major histocompatibility complex I (MHC-I), and contributes to viral pathogenesis (Kirchhoff 2010).

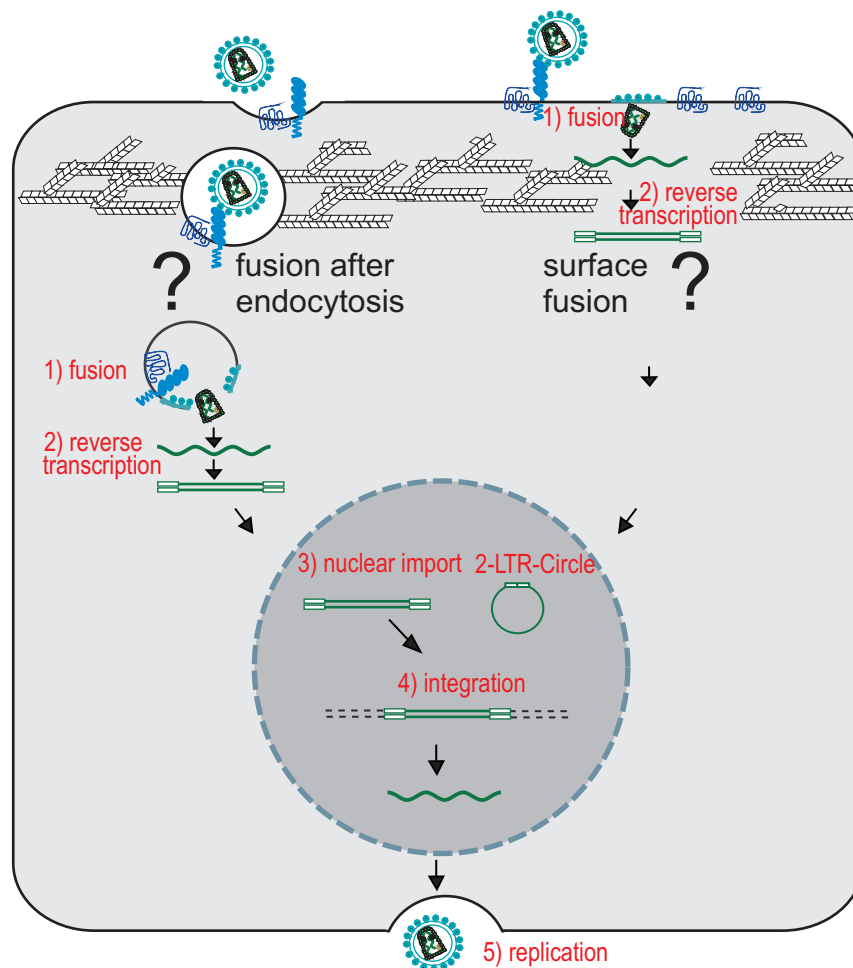
1.1.2 HIV-1 Life Cycle

The life cycle of HIV-1 can be divided into several phases (Figure 1.1): viral fusion, reverse transcription, nuclear import, integration of the viral DNA into the host cell genome and replication (expression of viral proteins and assembly and budding of new progeny virions).

Figure 1.1

Figure 1.1 Studying HIV-1 entry in MDM.

HIV-1 can enter cells via fusion after endocytosis (left) or plasma membrane fusion (right). However, in order to allow HIV-1 fusion, the presence of its receptor CD4 (shown in cyan blue) and a co-receptor CCR5 (shown dark blue) or CXCR4 (not shown) are required. The viral life cycle can consist of several stages, which can be studied using a panel of assays. 1) Fusion occurs when the HIV-1 envelope fuses with a cell-limiting membrane, delivering the virions to virus core to the cytosol. This is measured by the “HIV virion fusion assay”. 2) The genome is reverse transcribed into double-stranded DNA, which can be quantified by “qPCR of HIV-1 late reverse transcripts”. 3) The HIV-1 DNA copy is imported into the nucleus, measured by the “2-LTR circles assay”, considering the formation of unintegrated 2-LTRs circles is directly proportional to the amount of viral DNA imported into the nucleus. 4) the linear HIV-1 DNA copy is integrated into the host's genome, where it can be expressed and measured using the “HIV-1 integration (luciferase) assay”, which is an indicator of productive infection. 5) Once integrated, new viral particles are produced. “Quantification of p24 antigen by ELISA” measures HIV-1 replication.



In order to initiate fusion, HIV-1 requires CD4 expression, which functions as its main receptor, and expression of a co-receptor: CCR5 (for R5-tropic viruses), or CXCR4 (for X4-tropic viruses) (Dalgleish et al. 1984; Collin et al. 1993; Deng et al. 1996; Feng et al. 1996). Following binding to CD4, gp120 undergoes a conformational change that allows its binding to the co-receptor. This interaction triggers a further conformational change, which exposes the fusion peptide gp41. Next, gp41 inserts into the cellular membrane and mediates fusion of the HIV-1 envelope with a cell-limiting membrane. Three CD4 binding events and multiple coreceptor binding events are thought to take place in order to activate the necessary formation of trimolecular HIV-1 Env complexes (Layne et al. 1990; Lapham et al. 1996; Wu et al. 1996; Doms 2000). The affinity between gp120 and CD4 is relatively low and HIV-1 attachment to the cell surface may be stabilized by surface molecules such as lipids, syndecans, C-type lectins and heparan sulphate proteoglycans and adhesion molecules, such as ICAM-1 (Ugolini et al. 1999; Fuki et al. 2000; Vives et al. 2005; Miyauchi et al. 2009; Kondo et al. 2012). Originally, HIV-1 entry was thought to result from fusion at the plasma membrane (depicted on right side in Figure 1.1.), however, more recent studies reveal that in a range of cell types, including T-cells and macrophages, HIV-1 may be taken up through endocytosis and fuse with an endosomal membrane (depicted on left side in Figure 1.1.) (Fackler et al. 2000; Marechal et al. 2001; Daecke et al. 2005; Miyauchi et al. 2009; Carter et al. 2011). Yet, it remains disputed which fusion location leads to productive entry and whether this is cell-type specific.

Following fusion, either at the plasma membrane or following endocytosis, virion cores are delivered to the cytosol. The core consists of the capsid shell, which is associated to a layer of matrix proteins and contains the viral RNA (vRNA) genome as well as viral replication proteins, which are coated by the nucleocapsid (Gelderblom et al. 1987; Miller et al. 1997; Wilk et al. 2001; Briggs et al. 2003). After fusion, the core re-arranges and

matures into the reverse transcription complex (RTC), which is responsible for reverse transcribing the single-stranded vRNA genome into double-stranded DNA (Warrilow et al. 2007).

The RTC contains viral RNA (vRNA), a host cell tRNA^{lys} primer (which is packaged in the virion) and reverse transcriptase (RT), which form intermolecular interactions. Reverse transcription is initiated by binding of the primer to the primer binding site located about 180 nt from the 5' of the vRNA and reverse transcription is catalysed by RT (Basu et al. 2008). In short, the plus strand vRNA is first copied by RT a short distance to its 5' end, resulting in minus strand strong-stop DNA (plus/minus strand RNA/DNA hybrid). The portion of the vRNA template which is bound to the DNA is then degraded by RNase H activity of RT. To complete the synthesis of the remaining minus strand DNA, the strong-stop DNA that was generated at the 5'-end of the viral RNA, switches and hybridizes to the 3'-end of the vRNA (first strand transfer). As the vRNA is copied, the RNase H activity of RT degrades all of the viral RNA, except for the 3'-polypurine tract (3'-PPT) and the central PPT (cPPT), which are RNase H-resistant and remain hybridized to the minus strand DNA and function as primers for the generation of plus strand DNA. Plus strand DNA is synthesized, including part of the primer binding site. During the second strand transfer the primer binding site of the plus strand hybridizes to the complementary 3'-end of the minus strong-stop DNA and double stranded DNA is further synthesized. During termination at the plus strand cPPT, which contains a termination signal at its 3'-end, a 99-nucleotide DNA flap is generated, termed the central DNA flap, which acts as signal for nuclear localization (Zennou et al. 2000). The full length dsDNA, which contains duplicated LTR ends, is referred to as proviral DNA.

The RTC associates with the actin cytoskeleton during its transport to the nucleus (Bukrinskaya et al. 1998; Arhel 2010). Upon maturation of the RTC and completion of reverse transcription, the pre-integration complex (PIC) is formed which can enter the

nucleus to subsequently integrate into the host genome (Warrilow et al. 2007). Although some models suggest that viral cores uncoat (partially) immediately after fusion, recent studies suggest that uncoating occurs at the nuclear pore and is associated with the transition between RTC and PIC (Dismuke et al. 2006; Warrilow et al. 2009; Arhel 2010; Zhou et al. 2011). The integration process is driven by the viral protein integrase, which cleaves the 3'-ends of the provirus which then attack phosphodiester bonds in the host's genomic DNA (Pauza 1990; Vincent et al. 1990).

The ends of unintegrated linear HIV-1 DNA in the nucleus may join up to form a 2-LTR circle, which can further recombine into a single LTR circle (Farnet et al. 1991). Both 1-LTR and 2-LTR circles are considered by-products of HIV-1 infection which do not integrate into the host genome, and unlike integrated HIV-1 DNA, unintegrated HIV-1 DNA only allows limited gene expression, likely due to transcriptional silencing (Farnet et al. 1991; Kantor et al. 2009). The concentration of 2-LTR circles in the cell is directly proportional to the amount of viral DNA imported into the nucleus, and consequently used as a useful marker for nuclear import (Bukrinsky et al. 1993; Mandal et al. 2009).

After integration of the provirus and HIV-1 gene expression, new viral particles can be produced. Expression of integrated HIV-1 is a marker of productive infection, considering a large proportion of HIV-1 virions can also enter non-productively (Bourinbaïar 1994; Marechal et al. 1998).

During HIV-1 replication, HIV-1 genes are transcribed by the host RNA polymerase II, which is orchestrated by the viral LTR sequences (U3, R and U5). The core promoter elements are located in the U3 sequences and consist of a TATAA box for initiation and transcription factor binding sites. After synthesis of full length vRNA and spliced viral mRNAs, RNA is exported from the nucleus to the cytoplasm, viral proteins are synthesized and new virions assemble and bud through the host cell membrane, as recently reviewed by *Sundquist et al.* (Sundquist et al. 2012)

1.2 CD4

1.2.1 Expression, structure and domains

CD4 is a 55 kDa type I integral membrane glycoprotein that is part of the Immunoglobulin Superfamily. CD4 is mainly expressed by lymphoid and myeloid cells, at high levels and moderate expression levels, respectively and acts as a marker for Dendritic Cells, but can also be expressed by B cells, eosinophils, megakaryocytes and mast cells (Basch et al. 1990; Collman et al. 1990; Li et al. 2001; Lynch et al. 2006). The CD4 molecule consists of a signal peptide containing 25 amino acids (residues -25-0) and a chain containing 433 amino acids. CD4 is largely extra-cellular (residues 1-350) and contains a transmembrane domain (residues 350- 393) and a cytoplasmic tail (residues 394-433) (Ryu et al. 1990; Wang et al. 1990; Wu et al. 1997; Halaby et al. 1999). The extracellular region is formed by four tandem immunoglobulin domains (Domain 1-4). IgG Superfamily domains are designated as V-set (resembling immunoglobulin variable domains) or C2-set (resembling immunoglobulin constant domains) on the basis of sequence analysis (Williams and Barclay, 1988). D1 + D3 are similar to IgV domains and D2 + D4 are similar to IgC domains. Domain 1 has 13 sheets and 3 complementarity-determining regions (CDRs) loops (Ryu et al. 1990; Wang et al. 1990). CDR2 (C'C'' ridge) in CD4 Domain 1 contains the residues (40-60) that are critical for HIV-1 binding, although residues 29, 59-64, 77-81 and 85 may also be important (Jameson et al. 1988; Peterson et al. 1988; Arthos et al. 1989; Brodsky et al. 1990; Capon et al. 1991; McDougal et al. 1991; Sweet et al. 1991; Moebius et al. 1992; Simon et al. 1993; Kwong et al. 1998). The short cytoplasmic tail of CD4 contains domains that mediate various protein interactions and are important for its cellular distribution (see section 1.2.2, 1.2.3 and 6.1).

1.2.2 CD4 recycling, function and protein interactions

In macrophages, ~45 % of CD4 is expressed at the surface and CD4 undergoes constitutive endocytosis via clathrin-coated pits with turnover rates in the order of 4-5 % per min (Pelchen-Matthews et al. 1991; Pelchen-Matthews et al. 1998). Although the largest proportion of CD4 is thought to recycle back to the cell surface, 15 % of internalized CD4 is degraded, presumably in late endosomes or lysosomes involving clathrin-associated molecules (Pelchen-Matthews et al. 1993; Pelchen-Matthews et al. 1998; Pitcher et al. 1999). CD4 recycling is mediated by the di-leucine motif in CD4 at position L413/L414 which interacts with the clathrin adaptor complex AP2 (see section 1.3.6) (Pitcher et al. 1999; Bentham et al. 2003). CD4 recycling is further enhanced by phosphorylation of CD4 serines S408, S415, which flank the dileucine motif (Pitcher et al. 1999; Bentham et al. 2003). CD4 sub-cellular distribution in macrophages is distinct from lymphoid cells. Unlike myeloid cells, lymphoid cells express the kinase LCK which binds two cysteine residues at positions 420/422 in CD4, as such masking the di-leucine motif in CD4 at position L413/L414 and reducing the rate of CD4 endocytosis (0.2-0.5 % per min) and tethers CD4 to the cell surface (~90 % of CD4 is at the surface) (Turner et al. 1990; Pelchen-Matthews et al. 1991; Pelchen-Matthews et al. 1992; Pelchen-Matthews et al. 1998; Laguette et al. 2009).

In lymphoid cells, CD4 has several regulatory functions (Konig et al. 2004). For example, CD4 is expressed at high levels on the cell surface of T helper cells and acts as a T cell co-receptor (Konig et al. 2004). CD4 domain 1 binds to major histocompatibility complex (MHC) class II molecules on antigen-presenting cell, as such stabilizing the interaction between the T-cell receptor and antigen (Doyle et al. 1987; Wang et al. 2001). CD4 also acts as an essential part of signal transduction through its interaction with LCK, a kinase of the Src family (Veillette et al. 1989). Src Family Kinases are membrane-bound tyrosine kinases and act as signaling molecules that integrate and transmit signals important for

cell growth, differentiation, and survival, influence cellular adhesion, migration and invasion, and also regulate synaptic transmission (Engen et al. 2008).

Unlike T-cells, it is currently not understood whether CD4 has a functional role in macrophages. Nonetheless, the evidence does suggest that it is not redundant. Firstly, CD4 expression is specific and restricted to only a subset of hematopoietic cells, including both T-cells and macrophages, whereas in mouse macrophages, the CD4 transcript is truncated and CD4 is not expressed (Crocker et al. 1987; Foti et al. 1995). Secondly, several observations are suggestive of physiological activities of CD4 on human macrophages. For example, tyrosine kinase activity has been reported after stimulation of CD4 with T4-4 rabbit serum to sCD4 on the monocytic THP-1 cell line, indicating that CD4 can be active in macrophages (Graziani-Bowering et al. 2002). CD4 is continuously recycled, which may indicate that CD4 requires, or plays a role in, endocytosis (Pelchen-Matthews et al. 1991; Marsh et al. 1996). Also, CD4 expression can be controlled by soluble factors secreted by CD4⁺ T cells, suggesting that regulation of this protein is important to the biology of macrophages (Raposo et al. 2011). Domain 4 in CD4 can bind IL-16, and may act as a chemoattractant to CD4-expressing cells, although CD9 also acts as an IL-16 receptor (Krautwald 1998; Liu et al. 1999; Qi et al. 2006; Yadav et al. 2010). Further, a study in Jurkat cells point to a possible role of CD4 in actin-cytoskeletal assembly, as residues Q427 and K428 in the cytoplasmic tail of CD4 can interact with the actin-binding protein filamin-A (Vadlamudi et al. 2002; Jimenez-Baranda et al. 2007). Studies using various cell types, suggest that CD4 could signal indirectly, via association with other surface receptors (Wu et al. 1996; Toyo-oka et al. 1999; Xiao et al. 1999; Deneka et al. 2007). In macrophages, this could for example be via its association with CD9 or CCR5, as shown by coimmunoprecipitation studies (Xiao et al. 1999; Raposo et al. 2011).

1.2.3 CD4 internalization during HIV-1 infection

CD4 expression is required for HIV-1 entry into a target cell, but once inside the cell, it is critical for the HIV-1 life cycle to down-regulate CD4, and this is mediated by the viral proteins Nef, Vpu and gp160 (Wildum et al. 2006). Reduction of the expression of a virus' own receptors soon after entry is a mechanism used by many viruses to avoid superinfection and to promote viral release (Salmon et al. 1988; Hrecka et al. 2005; Michel et al. 2005). Superinfection, which occurs when multiple virions infect the same cell, decreases the rate of viral replication because large amounts of unintegrated viral DNA accumulate and cause the host-cell to undergo apoptosis, prematurely ending the production of new virions (Pauza et al. 1990; Robinson et al. 1990; Bergeron et al. 1992; Daniel et al. 1999). Viral budding and release can be hampered if CD4 would remain expressed, as it can capture newly-produced HIV-1 (Marshall et al. 1992; Bour et al. 1999; Ross et al. 1999). Viruses that fail to reduce CD4 expression are less efficient in viral replication and CD4 down-modulation correlates with pathogenesis of AIDS (Deacon et al. 1995; Lundquist et al. 2002; Stoddart et al. 2003).

During the early stages of the HIV-1 life cycle, Nef is expressed and accelerates CD4 internalization and degradation in the lysosomes through a clathrin/AP-2 pathway by binding to both the CD4 and AP-2 (see section 1.3.6) (Piguet et al. 1999; Preusser et al. 2001; Faure et al. 2004; Chaudhuri et al. 2007; Laguette et al. 2009). Nef-induced CD4 down-modulation is mediated through direct interactions of Nef and is independent of CD4 phosphorylation (Garcia et al. 1991; Piguet et al. 1999). During late stages of HIV-1 infection, Vpu and Env are expressed and hamper the transportation of newly synthesized CD4 to the cell surface. The viral envelope glycoprotein precursor gp160 binds to CD4 in the ER, forming gp160-CD4 complexes that block transport (Jabbar et al. 1990). At this stage, Vpu binds to the CD4 cytoplasmic tail (amino acids 418 to 425) and induces CD4

degradation via an ubiquitin/proteasomal pathway (Lenburg et al. 1993; Margottin et al. 1998; Schubert et al. 1998; Levesque et al. 2003)

1.3 Macrophages

Macrophages contribute to tissue homeostasis, have intrinsic anti-inflammatory functions and play a crucial role in the first line of defence to infection (Unanue et al. 1987; Murray et al. 2011; Shi et al. 2011). Macrophages can recognize, phagocytose and kill pathogens, as well as secrete specific cytokines to direct other immune cells and suppress inflammation. Upon activation, macrophages can function as antigen-presenting cells and as such also play a role during the early stages of the adaptive immune response (Gorczynski 1976). Macrophages have recently been reviewed in a set of articles published in the journal 'Nature Reviews Immunology' (Chow et al. 2011; Lawrence et al. 2011; Murray et al. 2011; Shi et al. 2011).

1.3.1 Macrophage origins

All human cells ultimately originate from human embryonic stem cells (hESC). hESC are pluripotent cells, present in the inner cell mass of blastocysts, that have the potential to differentiate into all cell types found in the human body (Thomson et al. 1998). As hESC differentiate, their developmental and self-renewal potential becomes increasingly restricted (Hudnall 2011). Macrophages are terminally differentiated cells, derived from the yolk sac or from multi-potential haematopoietic precursor cells (HSC) (van Furth et al. 1984; Fogg et al. 2006; Schulz et al. 2012). Recent research based on murine models suggest that the majority of tissue macrophages that can persist in the adult arise in the embryo, before the development of HSCs (Hoeffel et al. 2012; Schulz et al. 2012; Yona et al. 2013). In mice, such yolk sac-derived macrophages require the transcription factor PU.1 for their development, but are independent of the HSC transcription factor Myb (Schulz et al. 2012). It is thought that a smaller population of macrophages are derived from circulating monocytes precursors. Monocytes can be found in the bone marrow, spleen and circulating in the blood (Auffray et al. 2009; Swirski et al. 2009). Depending

on micro-environmental cues, monocytes are recruited to tissue, where they differentiate into monocyte-derived macrophages (MDM) or Dendritic Cells (DCs)(Gordon et al. 2005; Shi et al. 2011). Monocytes are derived from the monoblast (the precursor of monocytes, macrophages and dendritic cells) and the common myeloid progenitor (Tavian et al. 2001; Laiosa et al. 2006).

To direct differentiation into a certain cell type, specific growth factors are required. The hematopoietic hierarchy and required growth factors have recently been discussed in more detail by *Seita et al.* and *Hudnall* (Seita et al. 2010; Hudnall 2011). For example, the survival/growth factor IL-3 is required for the differentiation of all myeloid cells, but not for the differentiation of lymphocytes, and M-CSF is specifically required for the differentiation of monocytes and macrophages (Guilbert et al. 1980; Barreda et al. 2004; Seita et al. 2010; Hudnall 2011). Monocytes are heterogeneous and two major subsets have been described, a classical/major population of classical monocytes and a non-classical/minor population of monocytes.(Gordon et al. 2005). After stimulation, classical monocytes rapidly mobilize from reservoirs found in the bone marrow and spleen to inflamed tissues and lymph nodes, whereas non-classical monocytes patrol and monitor blood vessels (Palframan et al. 2001; Geissmann et al. 2003; Auffray et al. 2007). Monocytes can also be recruited to a site of inflammation where they can differentiate into inflammatory MDM (van Furth et al. 1972) (Gordon et al. 2005). Although monocytes, macrophages and dendritic cells have different morphologies, they are commonly referred to as the mononuclear phagocytic system as they can be derived from a common committed progenitor found in the adult bone marrow and they display a spectrum of functions such as phagocytosis, cytokine secretion and antigen presentation (van Furth et al. 1972; Murray et al. 2011).

Myeloid lineage cells can be generated *in vitro* from hESC. *In vitro*, aggregation of hESC allows the formation of Embryoid bodies (EBs) and spontaneous differentiation toward the three germ lineages similar to normal embryogenesis: endoderm (gut epithelium, lung, liver and pancreatic cells), ectoderm (neural and skin cells) and mesoderm (mesenchymal connective tissue, heart, blood, vascular, cartilage, bone, skeletal muscle cells) (Itskovitz-Eldor et al. 2000). Due to the similarities with *in vivo* embryogenesis, *in vitro* EB differentiation can be further directed using growth factors, as demonstrated by the production of monocytes from EBs, using M-CSF and IL-3 (Karlsson et al. 2008; Murry et al. 2008). Monocytes, in the presence of M-CSF (the key regulator for monocyte proliferation and differentiation) further differentiate into monocyte-derived, terminally differentiated macrophages, or, in the presence of GM-CSF and IL-4 differentiate into monocyte-derived DCs (Metcalf 1985; Spivak et al. 1985; Lopez et al. 1987; Sallusto et al. 1994; Geissmann et al. 2008). The developmental origin of *in vitro* hESC-derived myeloid lineage cells remains to be further characterized.

1.3.2 Macrophage function and heterogeneity

Macrophages are plastic and heterogeneous and can adjust their phenotype according to the cellular milieu (Lawrence et al. 2011). Most organs throughout the body contain large populations (sometimes up to 20% of total cells) of mature tissue macrophages (Racanelli et al. 2006; Hume 2008). Tissue macrophages are often long-lived (months to year or life-long) and found in for example the intestine, spleen, lymph node, brain (microglia), eye (intraocular macrophage), lung (alveolar macrophage), liver (Kupffer cell) and bone (osteoclast) (Gordon et al. 2005; Hume 2008; Murray et al. 2011). Similarly, tissue resident Dendritic Cells can be described, for example in the skin (Langerhans cells). In different tissues, macrophages are morphological, phenotypic, and functional distinct (Racanelli et al. 2006; Hume 2008). Nonetheless, tissue macrophages share central functions in tissue homeostasis and immune regulation (Murray et al. 2011). These

macrophages can phagocytize foreign material, recruit additional immune cells, and remodel and repair tissue during infection or after inflammation or injury (Gurtner et al. 2008; Murphy et al. 2008; Lucas et al. 2010; Murray et al. 2011). Macrophages monitor their surrounding via a range of membrane receptors, such as pattern recognition receptors which can recognize pathogen-associated specific molecular patterns and trigger phagocytosis (Taylor et al. 2005). Also cytokines and chemokines can recruit macrophages and lead to removal of extracellular and intracellular pathogens.

1.3.3 Macrophage activation

As resting macrophages encounter tissue injuries or pathogens, macrophages become activated and can, depending on the type of activation stimulus, upregulate specific cytokines and chemokines. Macrophages can be activated in various ways. The two major activation pathways are known as 'classical' and 'alternative' activation (Goerdts et al. 1999; Mosser 2003). Classical activation can be induced *in vitro* by IFN- γ (antiviral, immunoregulatory, and anti-tumor), and a further microbial stimulus like LPS (a lipoglycan expressed on Gram-negative bacteria). Classical activation leads predominantly to an increase in pro-inflammatory cytokine and chemokine production and the induction of a cellular immune response (Mosser 2003; Gordon et al. 2005). Alternative activation, can be induced *in vitro* by IL-4 (key regulator in humoral and adaptive immunity) or IL-13 (mediator of allergic inflammation)(Gordon et al. 2010). Alternative activation can lead to both anti- and pro-inflammatory cytokine and chemokine production, and is involved in tissue repair, humoral immunity, innate activation, allergic responses and parasitic infections (Gordon et al. 2005; Gordon et al. 2010). *In vivo* macrophages are exposed to a variable range of cytokines and chemokines and therefore can be described as being in a continuum of activation states (Mantovani et al. 2004; Martinez et al. 2006).

1.3.4 Macrophages and HIV-1

Because macrophages are among the first defence cells to be encountered by microbes, successful pathogens have to have evolved methods for dealing with macrophages, including strategies of infection. As such, macrophages can also contribute to the pathogenesis infectious, inflammatory and degenerative diseases (Murray et al. 2011). For example, they are specialised hosts for *Leishmania*, *Mycobacterium tuberculosis*, *Mycobacterium leprae*, human Dengue and have long known to be susceptible to infection with HIV-1 and play an important role in pathogenesis (Gartner et al. 1986; Gartner et al. 1986; Gendelman et al. 1989; Shi et al. 2011). Macrophages can be amongst the first cell types to become infected by HIV-1 and can carry HIV-1 in semen and in vaginal secretions (Mermin et al. 1991; Goulston et al. 1998; Martin et al. 1999). Macrophages are found adjacent to the mucosal epithelium and may be able to migrate between epithelial cells and possibly come into contact with HIV-1 (Tan et al. 1996; Lapham et al. 1999). As HIV-1 infection of macrophages is generally non-cytopathic, and considering infected macrophages may survive for several years, macrophages can act as a reservoir for viral persistence, maintaining the chronic state of HIV-1 infection, and further disseminate HIV-1 (Gendelman et al. 1989; Herbein et al. 2002; Cassol et al. 2006). Tissue macrophages (including Kupffer cells in the liver, microglia in the brain and alveolar macrophages in the lung) can become latently infected at early time points post-transmission with HIV-1 and evade immune recognition (Chayt et al. 1986; Koenig et al. 1986; Salahuddin et al. 1986; Tschachler et al. 1987; Hoda et al. 1991; Embretson et al. 1993). Latently infected macrophages may contribute to the development of neurological complications, AIDS-related dementia, and pulmonary conditions in the later stages of disease (Koyanagi et al. 1987; Cheng-Mayer et al. 1989; Meltzer et al. 1993; Ghorpade et al. 1998). HIV-1 replication in latently infected macrophages can be modified by macrophage polarization and tissue micro-environment and co-infection with opportunistic pathogens (Orenstein et al. 1997; Cassol et al. 2009; Herbein et al. 2010). As such,

macrophages can act as an important source for increasing viraemia and be induced to produce more viruses. Cytokine and chemokine secretion in infected macrophages are deregulated, causing immune dysfunctions (Marshall et al. 1999). Macrophages are also important in propagation of infection. Macrophages can spread HIV-1 directly to T cells via the formation of a supramolecular structure termed the virological synapse (Groot et al. 2008).

Macrophages express the HIV-1 receptor (CD4) and both co-receptors (CCR5 and CXCR4). Despite expressing both co-receptors, macrophages are preferentially infected by R5-tropic viruses and do not support productive infection by lab-adapted X4-tropic viruses, although can be infected, albeit less efficiently by primary X4 isolates (Di Marzio et al. 1998; Schmidtmayerova et al. 1998; Collman et al. 2000; Bakri et al. 2001). Dual-tropic isolates can infect macrophages more efficiently compared to X4-tropic isolates (Kedzierska et al. 2003). Advanced HIV-1 or AIDS has been associated with increased replication of R5-macrophage-tropic viruses (Li et al. 1999; Tuttle et al. 2002; Richards et al. 2010).

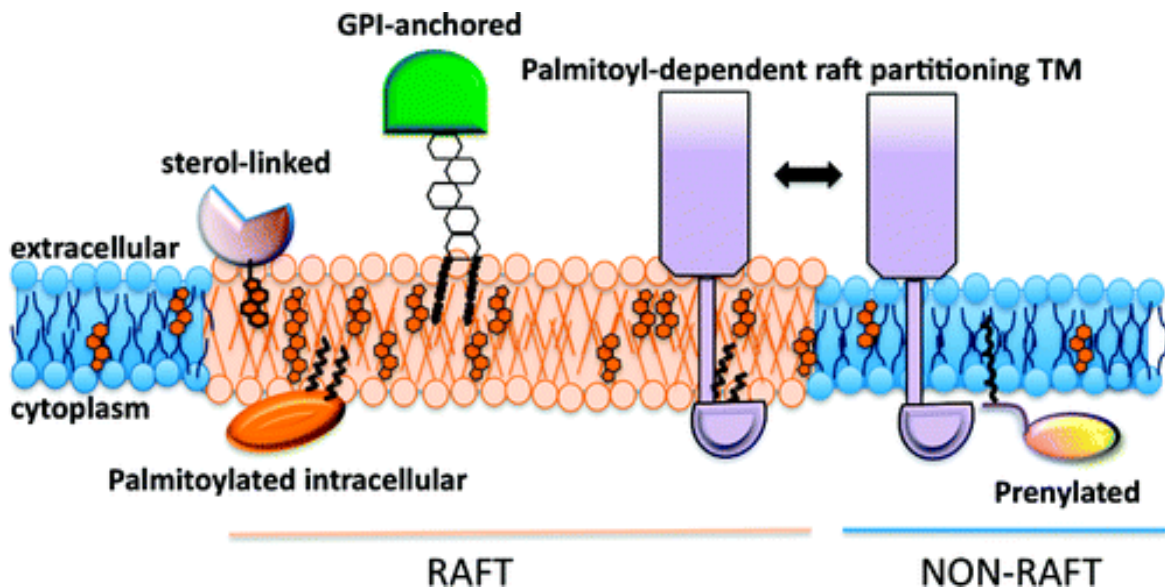
As mentioned above, HIV-1 entry may result from fusion at the plasma membrane or through up take up through endocytosis. Pharmacological studies using primary macrophages provide evidence for a raft-dependent uptake pathway, as further discussed in Chapter 6 (Carter et al. 2009; Carter et al. 2011). Lipid rafts and endosomal pathways are further described below.

1.3.5 Lipid rafts

The plasma membrane consists of wide range of lipids, which can form organised lipid raft microdomains within the lipid bilayer of cellular plasma membranes to which a specific subset of membrane proteins partition, as determined by their lipid modifications (Figure 1.2, adapted from *Levental et al*) (Simons et al. 1997; Levental et al. 2010).

Figure 1.2 Lipid Rafts and lipid modifications of proteins.

Lipid rafts are specialized lipid raft microdomains within the lipid bilayer of cellular plasma membranes. Lipid rafts are enriched in lipids and specific proteins. Raft associated proteins contain lipid modifications and domains that facilitate their partitioning to rafts. For example, glycosylphosphatidylinositol (GPI)- and sterol-anchors, as well as, palmitoylation targets proteins to the liquid-ordered lipid rafts in the lipid bilayer, whereas unsaturated and branched hydrocarbon chains generally localize to liquid-disordered phases of the plasma membrane.



Adapted from *Levental et al.* (Levental et al. 2010).

Rafts are rich in lipids such as sphingolipids and cholesterol, which partition away from other lipids, forming a small platform in the outer layer connected to phospholipids and cholesterol in the inner layer of the lipid bilayer (Simons et al. 2002). Sphingolipids are lipids with a ceramide core structure, composed of a 18-carbon amino alcohol chain (sphingosine) and a long, saturated acyl chain, that allows the formation of a compact structure in the plasma membrane (Ferguson et al. 2009). Cholesterol preferentially localizes to lipid rafts between the chains of the sphingolipids, forming a liquid-ordered conformation with substantial lateral movement of lipids and proteins (Simons et al. 2000). This is in contrast to phospholipids, which do not form raft domains and usually

contain unsaturated acyl chains, forming a loose structure in a liquid-disordered membrane with rapid movement of lipids and proteins (Manes et al. 2003). By depleting cell plasma membranes from cholesterol, for example by using the drug methyl- β -cyclodextrin, lipid rafts are disrupted and the surface expression of raft associated proteins reduces specifically, thereby confirming the association of such proteins to lipid rafts (Simons et al. 2000; Carter et al. 2009).

The name lipid rafts originates from the observation that the liquid-ordered microdomains can float in the liquid-disordered phospholipid plasma membrane (Manes et al. 2003). Another characteristic of rafts is their insolubility to cold, non-ionic detergents, such as Triton X-100 (TX-100), due to the tightly packed acyl chains and stable lipid-lipid interactions. As such, raft proteins can be separated from other proteins after treatment with non-ionic detergents, which dissolves other cellular material, followed by density gradient centrifugation, which allows the buoyant detergent-resistant lipid raft membrane to float away from the 'heavy' soluble and non-membranous material which remains in the bottom fraction containing ~45% sucrose (Brown et al. 1992; Foster et al. 2003). This commonly used method to isolate "Detergent Resistant Membranes" is frequently used as raft nomenclature as well, however, the definition of "lipid rafts" requires additional evidence, such as evaluation of size, mobility, and identification of presence of raft markers, such as sphingolipids, cholesterol and raft-associated proteins (Simons et al. 2000) (Prof. Tony Magee, personal communication). Nonetheless, despite technological advances to identify the presence of lipid rafts and raft-associated proteins, detergent resistance is widely used to operationally define lipid rafts (Harder et al. 1998; Munro 2003; Henderson et al. 2004; Ostrom et al. 2007).

In addition to lipids, rafts are enriched in glycosylphosphatidylinositol (GPI)-linked- and sterol-linked-, as well as, double-acylated proteins, whereas proteins with branched, unsaturated hydrocarbon chains, such as an isoprenyl group, are excluded from rafts

(Rajendran et al. 2005; Levental et al. 2010). GPI-anchored proteins are ubiquitously expressed and functionally diverse proteins. The GPI anchor is found in the outer leaflet of the lipid bilayer, facing the extracellular environment. It has a long, saturated acyl chain that interacts with sphingolipids and cholesterol in lipid rafts and a core glycan that links to the carboxyl termini of proteins, as such attaching proteins to the cell surface (Pike 2004; Ferguson et al. 2009). Protein acylation is a post-translational modification that attaches a functional group, commonly the fatty acids myristate or palmitate, through acyl linkages (Resh 1999). Myristate is a 14-carbon saturated fatty acid, and is attached to proteins that begin with the sequence Methionine–Glycine, whereas palmitate is a 16-carbon saturated fatty acid that generally attaches to Cysteine groups (Resh 2006). Protein prenylation is a lipid modification that covalently attaches either farnesyl or geranylgeranyl isoprenoids to proteins (Levental et al. 2010). Some proteins are permanently associated with rafts, whereas other can move in and out the rafts, depending on activation and co-clustering of rafts, as protein–protein, protein–lipid and lipid–lipid interactions take place (Simons et al. 2000; Hancock 2006; Lingwood et al. 2010; Simons et al. 2011). Potential raft proteins often have a weak affinity for rafts, but upon interaction with a ligand or other protein, can change its structure or become oligomerised, which increases their raft affinity (Harder et al. 1998; Simons et al. 2002). Raft association can be regulated by palmitoylation, which is a reversible modification (Simons et al. 2002; Zacharias et al. 2002; Linder et al. 2007). DRM are dynamic, possibly short-lived and involved in a range of cellular processes, including signaling, cell morphogenesis, cell adhesion, protein trafficking, cytoskeletal organization as well as endocytosis and pathogen entry (Simons et al. 1997; Simons et al. 2000; Manes et al. 2003; He et al. 2010).

1.3.6 Endocytic and lysosomal pathways

Endocytosis is a general term used to describe mechanisms by which cells take up a wide range of cargo by invaginations of the plasma membrane and internalization of membrane

vesicles (Ewers et al. 2011). Cargo that can be taken up by endocytosis includes surrounding particles, receptors, ligands, nutrients, solutes, fluids, membrane components, as well as toxins and pathogens (Ewers et al. 2011). Endocytosis is involved in a wide range of cellular functions, including cellular nutrition, cell communication, regulating cell dynamics and homeostasis (Collinet et al. 2010; Ewers et al. 2011). The term 'phagocytosis' is used to specifically describe the endocytosis of large particles such as apoptotic cells, certain pathogens and debris, whereas the term 'macropinocytosis' is used to specifically describe the uptake of extracellular cargo (especially fluid) by forming ruffled extensions of the plasma membranes (Le Roy et al. 2005; Doherty et al. 2009). A wide range of different endocytic pathways can exist alongside each other, as extensively reviewed by *Doherty et al.* (Doherty et al. 2009). The precise components of endocytic pathways are likely cell type and cargo-specific and in many cases remain hard to define. Two key pathways (clathrin-mediated and lipid raft-dependent endocytosis) present in many cell types, and the fate of internalized cargo are shortly discussed below. Extensive and comprehensive studies investigating endocytic pathways in macrophages specifically are currently lacking.

Clathrin-mediated endocytosis is a continuously on-going endocytic pathway, involving the packaging of receptors with or without their ligand into clathrin-coated vesicles. The pathway relies on the recognition of signals in the cytoplasmic domains of plasma membrane proteins by adaptor proteins, such as AP-2, and the assembly of soluble clathrin "triskelions" at the plasma membrane, to form coated pits that bud and pinch off from the membrane in a dynamin-dependent manner and give rise to clathrin-coated vesicles containing surface receptors with or without their ligand (Doherty et al. 2009).

Raft-dependent endocytosis can be defined by its clathrin-independence and cholesterol-sensitivity and is thought to involve the binding of ligands to membrane lipids, often leaving little space between the ligand and plasma membrane, thereby inducing

membrane curvatures and inwards budding of endocytic vesicles (Ewers et al. 2011). Scission of the vesicles likely involves lipid-binding proteins and activation of signaling pathways (Ewers et al. 2011). The involvement of various components, in a range of raft-dependent pathways, makes the further classification and characterization of raft-dependent endocytosis difficult. Instead, several unique, raft-dependent pathways have been described (Gold et al. 2010; Saeed et al. 2010; Zhao et al. 2011; Haspot et al. 2012; Iversen et al. 2012).

Internalized cargo, independent of its mode of uptake, is delivered to an interconnected endosomal network. First, cargo is delivered to the main sorting compartment (early endosome), from where it can follow various routes. Cargo may be delivered to a late endosomes, which subsequently fuses with lysosomes to form an endolysosomes, which lead to endosomal cargo degradation. Alternatively, cargo may be delivered to the trans-Golgi network or recycling endosomes, which transports components back to the cell surface (Ewers et al. 2011). Unlike the endolysosomal network, the ubiquitin–proteasome system degrades mostly intracellular, soluble proteins, although the ubiquitin–proteasome system may also degrade membrane proteins (Ciechanover 2006). The ubiquitin–proteasome system involves the attachment of ubiquitin moieties to protein substrates, followed by proteasomal-mediated degradation of the ubiquitinated protein (Ravid et al. 2008).

1.3.7 Limitations associated with the study of macrophages

There are limitations associated with studying macrophages using monocyte-derived macrophages from the blood (bMDM, using animal models, or using myeloid cell lines). The variability between experiments using bMDM may be high and many replicates are often necessary to ensure that one has a representative sample pool (Bennett et al. 1994; Elkord et al. 2005; Gordon et al. 2005; Bol et al. 2009). However, as macrophages are

terminally differentiated cells, the number of experiments possible with cells obtained from one blood donor may be limited.

Animal models do not offer a representative system to study human macrophages, as the species specific results may not be relevant to human immunopathology. This is especially important in HIV-1 studies, as only a limited number of primate species can be infected with HIV-1 and different species express species-specific restriction factors. For example, Old World monkeys cannot be infected with HIV-1 because they express TRIM5alpha, which blocks HIV-1 uncoating (Stremlau et al. 2004). This is not achieved by human TRIM5alpha due to protein differences (Stremlau et al. 2004). Also, there are species-specific differences in the functional capacity of the CD4 receptor to mediate entry (Moore et al. 1992; Humes et al. 2012).

Myeloid cell lines also don't offer an appropriate experimental system, as they are not terminally differentiated like MDM, which affects the way HIV-1 may enter the nucleus for integration (Ross et al. 2000; Mannioui et al. 2004; Fassati 2006). Indeed, myeloid cell lines do not closely represent the HIV-1 entry pathway of MDM, which involves a range of cell-specific cellular components likely to be distinct from cell lines (Cassol et al. 2006; Carter et al. 2011). Compared to primary cells, cell lines show abnormal changes in genetics, transcription, post-transcriptional regulation, protein expression, and signaling networks (Ross et al. 2000; Johnson et al. 2003; Roschke et al. 2003; Irish et al. 2004; Sandberg et al. 2005; Pan et al. 2009). Furthermore, myeloid-specific restriction factor SAMHD1, which hydrolyses the majority of cellular dNTPs and thereby limits HIV-1 reverse transcription specifically in these cells, can be counteracted by the HIV-2 protein Vpx in MDM, a characteristic process that cannot be replicated in most cell lines (Dr. Moore, personal communication) (Hrecka et al. 2011; Laguette et al. 2011, Goldstone, 2011 #802). Above all, MDM-tropic HIV-1 isolates, such as HIV-1 BaL, cannot replicate in myeloid cell lines such as THP-1, whereas T-tropic isolates can (Meylan et al. 1993).

Another key problem associated with the study of primary macrophages is their particular refractoriness to both stable transfection and transduction (Eisert et al. 2001; Kaushik et al. 2009). The phenotype and function of bMDM, as well as their HIV-1 infectability, are affected by genetic manipulations, and this might be anticipated, considering MDM are specialized to detect foreign DNA and can become activated upon transfection or transduction (Honda et al. 1998; Zybarth et al. 1999). Similarly, mouse macrophages are activated by bacterial DNA (Sester et al. 1999; Gao et al. 2002; Roberts et al. 2005). RNA transfection of bMDM is also highly inefficient and cytotoxic (Fan Cheng, University of Oxford, FHS Report 2009). Lentiviral transduction of MDM can be achieved without activating the cells, but is very inefficient at least in part due to the expression of the myeloid-specific restriction factor SAMHD1 (Goldstone et al. 2011; Laguette et al. 2011).

It is important to develop ways to achieve genetic modification in macrophages, as it offers a powerful tool to study cell function and cell-pathogen interactions. Antibiotic selection may be used to achieve a higher proportion of genetically modified bMDM, although the phenotype of selected bMDM would be affected due to their phagocytosis of surrounding dying cells. Currently, the best solution to achieve higher transduction efficiencies of bMDM, is achieved by co-expressing the HIV-2 or SIV protein Vpx (Sunseri et al. 2011). Vpx relieves the inhibition of lentiviral transduction mediated by the SAMHD1, by targeting SAMHD1 to the proteasome (Hrecka et al. 2011; Laguette et al. 2011). However, Vpx-expressing MDM would not be suitable for HIV-1 infection or immunology studies, as MDM expressing artificially high level of dNTPs would not be functionally normal and would demonstrate altered HIV-1 infection kinetics.

The use of human Embryonic Stem Cell (hESC) derived MDM (esMDM) could overcome the problems associated with working with bMDM and would offer a representative system to study macrophage biology (Karlsson et al. 2008; Cowley SA 2012). Using lentiviral vectors could be used as a relatively easy method for gene-delivery and achieve

stable vector integration into the genome, so that transgenic hESC could then be differentiated into transgenic esMDM, as tested in Chapter 3. In addition to hESC, CD34⁺ haematopoietic stem cells isolated from cord blood or bone marrow, also offer a tractable system for genetic modification. However, unlike hESC, CD34⁺ haematopoietic stem cells are challenging to expand ex-vivo and do not self-renew (Aggarwal et al. 2010). Although CD34⁺ haematopoietic stem cells could be characterized, they are mostly used without characterization of their patient background, whereas many hESC lines have been extensively characterized (Bhattacharya et al. 2012).

1.4 Lentiviral vectors

As described in section 1.1.1, lentiviruses can stably insert a DNA copy of their genome into the host genome in both dividing and non-dividing cells. Due to these key characteristics, lentiviruses have been exploited in biomedical research as a method of gene delivery. Genetically modified HIV-1 is most commonly used for the construction of lentiviral vectors, as recently reviewed (Sakuma et al. 2012). Originally such vectors were replication-competent, but improved designs contain various safety features so that viral proteins are not expressed after vector transduction. For example, to increase safety and reduce the chance of recombination, genes required for lentiviral transduction are split into three separate plasmids (packaging-, envelope-, and transfer- plasmids). This results in a replication-deficient HIV-1 based lentiviral vector, which originally was referred to as 'first-generation' lentiviral vectors. The packaging plasmid allows expression of HIV Gag, Pol, Rev, and Tat. The 'first generation' packaging plasmids also encode for the accessory proteins (Vif, Vpu, Vpr and Nef). The envelope plasmid allows expression of a viral envelope to facilitate cell entry. The VSV-G envelope is commonly used as it can enter a range of cell types in many different species (Aiken 1997). As a safety measure, the packaging and envelope plasmid do not contain a packaging signal or LTRs, so that no viral proteins are expressed in transduced cells. The transfer plasmid does contain elements required for packaging, reverse transcription and integration (LTRs, ψ and RRE), but doesn't contain any HIV-1 genes. Instead the transfer plasmid encodes for the transgene, driven by an internal promoter. Two elements, the central polypurine tract sequence (cPPT) and the woodchuck post-regulatory element (WPRE), are frequently included in the transfer vector. The cPPT enhances the transduction efficiency and WPRE enhances transcript stability (Donello et al. 1998; Zufferey et al. 1999; Follenzi et al. 2000).

A 'second generation' of HIV-1 based lentiviral vectors has been developed to further increase safety. The accessory proteins, which are dispensable for HIV-1 infection, are removed from the 'second generation' packaging plasmid (Zufferey et al. 1998). Moreover, in the 'second generation' transfer plasmid, part of the 3'-LTR is deleted, so that during reverse transcription deletions are incorporated in both the 5' and 3' LTR, resulting in transcriptional inactivation from the LTR promoter in the integrated lentiviral vector. Transfer plasmids containing the 3'LTR deletion are referred to as self-inactivating (SIN) lentiviral vectors. A 'third generation' of HIV-1 based lentiviral vectors, which use an additional packaging plasmid, have been developed to increase safety further, although vector yields are typically lower (Sakuma et al. 2012). In this thesis a 'second generation' system was used.

1.5 Scope of this thesis

The general hypothesis of this thesis was that sub-cellular CD4 distribution determines the HIV-1 entry pathway of macrophages. To this end, a method for producing large numbers of genetically modifiable, functional macrophages was developed and tested. This then allowed the generation of genetic methods to manipulate the sub-cellular distribution of CD4, including expression levels, location and recycling-rate, in macrophages. Macrophages with altered sub-cellular CD4 distribution were infected with HIV-1 and a panel of assays were used as readouts for sequential stages of HIV-1 infection. These methods would test whether the productive entry pathway of HIV-1 in macrophages occurs via fusion after endocytosis, as opposed to fusion at the cell surface and whether CD4 association with DRMs affects productive infection by HIV-1.

Chapter 2: Materials and Methods

2.1 Cell culture

2.1.1 Cell counts

Cells were counted and assessed for viability using NC-3000 Viability and Cell Count Assays (chemometec). 1 μ L of solution 13, containing AO and DAPI (chemometec) and 19 μ L of cell suspension were mixed and from this 9 μ L was loaded onto a chamber of a A8-slide (chemometec). All viable cells are stained with AO and non-viable cells are stained with DAPI. Alternatively, cells were counted and assessed for viability manually using trypan blue dye and a haemocytometer.

2.1.2 Cell lines

Jurkat or J.CaM cells were cultured in RPMI (PAA). 293T and TZM-bl cells were cultured in DMEM (PAA). Both media were supplemented with 10 % FCS (PAA) and 2 mM Glutamax-I (Gibco), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen). Cells were cultured at 37 °C with 5 % CO₂.

2.1.3 hESC culture

Culture of the hESC line, HUES-2 (originally from Douglas Melton in Harvard) was approved by the UK Stem Cell Bank Steering Committee (Cowan et al. 2004). hESC were generally cultured at 37 °C with 5 % CO₂ on Mitomycin C-inactivated mouse embryo fibroblasts (MEFs) in hESC media. hESC media consisted of knock-out DMEM (Invitrogen), 20 % knock-out-Serum Replacement (Invitrogen), 2 mM Glutamax-I (Gibco), 100 U/mL penicillin (Invitrogen), 100 μ g/mL streptomycin (Invitrogen), 1% non-essential amino acids (Invitrogen), 0.055 mM 2-mercaptoethanol (R&D), 10 ng/mL bFGF (R&D).

MEF's were prepared the day before on 0.1 % gelatin-coated tissue culture plates (Corning) at a density of 50,000 cells/cm² in hESC media. The following day, hESC were plated at a density of 5,000-10,000 cells/cm² and supplemented with 1 mM Rock-inhibitor (Y27632; Calbiochem). Medium was changed daily by removing 50 % of the media and replacing with fresh hESC medium. Colonies were passaged every 5 days by manual micro-dissection. Alternatively, cells were passaged by enzymatic dissociation by first washing hESC with PBS and incubating the cells for 5 min at 37 °C with 1 mL of warm TrypLE Express (Gibco by Life Technologies). The cells and TrypLE were well mixed into a single cell suspension by pipetting up and down and collected in a 15 mL centrifuge tube and diluted 1:10 with PBS and spun down and plated as described above,

Alternatively, hESC were cultured feeder-free according to one of two methods. Firstly, this was achieved where indicated using Synthemax™ plates (Corning). hESC were maintained in 2 mL mTeSR™-1 medium (Stem Cell Technologies) and passaged when colonies covered approximately 80 % of the culture surface. To passage cells, the media was removed, PBS was added to wash cells, and 1 mL Collagenase IV (1 mg/mL, 200 U, Life Technologies, Invitrogen) was added and incubated for 4 min at 37 °C. Next, the collagenase IV was removed, cells were washed with PBS and 2 mL of warm mTeSR™-1 medium was added. To lift the cells, the cells were gently scraped and pipetted up and down to break up clumps to pieces 0.5 -1mm (not single cells) which were plated at ~10,000 cells/cm² (corresponding to ~1:4 split). The passaged hESC was returned to incubator and not fed for the first 48 hours, but thereafter fed daily with fed 2 mL media (full media change). Secondly, feeder-free hESC culture was achieved where indicated using Matrigel™ coated plates. 1-48 h before hESC were plated, tissue culture plates (Corning) were coated with a basement membrane matrix Matrigel™ (BD Biosciences) at 37 °C. Matrigel™ is mainly composed of laminin, collagen IV, entactin and heparin sulphate proteoglycan, secreted from a mouse tumour cell lines. Next, Matrigel™ was aspirated and hESC were plated at a density of 5,000-10,000 cells/cm² mTeSR™-1

medium (Stem Cell Technologies) and supplemented with 1 mM Rock-inhibitor (Y27632; Calbiochem). Medium was changed daily by removing 100 % of the media and replacing with fresh mTeSR™-1 medium. hESC were passaged enzymatically every 3-5 days, using TrypLE Express (Gibco by Life Technologies), as described previously.

2.1.4 EB formation by mechanical dissociation

hESC were mechanically dissociated by scoring each 10 cm² well into a grid of approximately 100 patches with a 23G needle. Next, the patches were lifted with a cell scraper. Patches were transferred into a well of a 6-well ultra-low adherence plate (Corning) in hESC culture medium and cultured for 4 days in hESC media (contents listed in section 2.1.3).

2.1.5 EB formation by Spinning

Spin-EBs were formed using either a 96-well ultra-low adherence plate (Costar 7007) or AggreWells™800 (Stemcell Technologies). In both cases, hESC were washed with PBS and harvested by incubating the cells for 5 min at 37 °C with 1 mL of warm TrypLE Express (Gibco by Life Technologies). The cells and TrypLE were well mixed into a single cell suspension by pipetting up and down and collected in a 15 mL centrifuge tube and diluted 1:10 with PBS. Cells were counted and spun down. After centrifugation PBS was aspirated and the cell pellet was tapped loose and resuspended in mTeSR™-1 spin-EB medium. mTeSR™-1 spin-EB medium consisted of mTeSR™-1 (Stem Cell Technologies), supplemented with 1 mM Rock-inhibitor (Y27632; Calbiochem); 50ng/mL BMP-4 (Peprotech EC Ltd.), 20 ng/mL SCF (Miltenyi Biotec Ltd), 50 ng/mL VEGF (Peprotech EC Ltd.), and where specified with 50 ng/mL GM-CSF (R&D). For generating EBs in 96-well ultra-low adherence plates (Costar 7007), cells were resuspended at 10 µL/mL to give a final cell concentration of 1×10^5 cells/mL and 100 µL was added per well. The 96-well ultra-low adherence plate was centrifuged at 800 rpm for 3 min and the plate

was gently put into the incubator and left for 4 days. EBs were fed at day 2 by gently aspirating 50 μ L medium and very gently adding 50 μ L of fresh EB medium dripping down the side of the well so the EBs are not disturbed. To harvest EBs at day 4, the contents of the wells were pipetted up and down several times to dislodge the EBs from the micro-wells. The EBs were transferred to a tissue culture plate, which was then tilted to remove media without removing EBs (which settle to the edge). Differentiation media was added, as described below.

For AggreWells™800 (Stemcell Technologies, 300 micro-wells), plates were first prepared by rinsing each AggreWell with PBS and aspirating the PBS. Next, 1 mL of mTeSR™-1 spin-EB medium was added to the AggreWell (which not yet contained cells). The AggreWell plate with medium, but no cells, was centrifuged at 3000g for 1-2 min (insuring centrifuge is balanced), to remove microscopic air bubbles. After preparing the plate, 1 mL of 4×10^6 hESC were added per well. 4×10^6 hESC are equivalent to 2-3 just-confluent wells of a 6-well plate grown without feeder cells, on Matrigel in mTeSR™-1. The plate containing hESC and 2 mL of spin-EB medium per well was centrifuged at 800 rpm for 3 minutes. The plate was examined under the microscope to verify cells were evenly distributed among the micro-wells. Very gently the plate was put into the incubator and left for four days. The EBs were fed daily with spin-EB medium (first brought to RT), by gently aspirating 1 mL medium using a p1000 Gilson and very gently adding 1 mL fresh spin-EB medium in a drop-wise manner down the side of the well so the EBs were not washed out of the microwells. This wash was repeated to achieve a 75 % medium change overall.

To harvest EBs at day 4, the contents of the wells were pipetted up and down several times using a 5 mL serological pipette to dislodge the EBs from the micro-wells. The contents was taken up and transferred onto a 40 μ M cell strainer inverted over a 50 mL centrifuge tube. The contents were washed out of the well several times with PBS and

transferred onto the same strainer to collect all EBs and wash the EBs on the strainer. This way, the EBs were on top of the inverted strainer and the media/PBS passed through the inverted strainer into the 50 mL collection tube. The inverted strainer, with the EBs balanced on top, was carefully inverted over onto a new 50ml centrifuge tube so that the EBs were now at the bottom of the strainer and could be collected into the new 50 mL tube, by passing through differentiation media (contents of media described in section 2.1.6). The strainer was held at an angle to facilitate the collection of EBs. EBs could be counted by transferring 50 μ L sample to a well of a 96-well plate and using a light microscope.

2.1.6 Directed differentiation of EBs into esMC

For differentiation, ~10 EBs were transferred into one well of a six-well tissue culture plate in 3 mL medium or 1 EB was transferred into one well of a 24-well tissue culture plate in 1 mL medium. 2/3 of the medium was replaced every 5 days. Culture medium consisted of Advanced Dulbecco's modified Eagle's medium (aDMEM) (Invitrogen) and 10 % fetal calf serum (Biosera) or X-VIVOTM15 (Lonza), supplemented with 100 ng/mL M-CSF (Invitrogen), 25 ng/mL IL-3 (R&D), 2 mM glutamax (Invitrogen), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen), and 0.055 mM β -mercaptoethanol (Invitrogen). Once esMC were visible in the supernatant of the cultures (from 2-3 weeks onwards), non-adherent esMC were harvested weekly from the supernatant of EB cultures.

For characterization of surface antigen during monocytopoiesis, adherent EB cultures were harvested using Collagenase B (Roche). Collagenase B was obtained as a powder and dissolved in PBS at 0.4 U/mL and sterilised by passing through a 0.22 μ M filter. The media of cell cultures was removed and cultures were washed with 2 mL PBS. 1 mL of Collagenase B was added per 9.5 cm² (1 well of a 6-well plate) and incubated at 37 °C for 1 – 1.5 hours. Next, with a pair of syringe needles (26g x 1/2") cell cultures were pulled apart and another 0.5 mL collagenase B was added. The cell suspension was pipetted up

and down to further break up clumps and returned to the incubator for another 30 min. Next, the cell suspension, was passed through a 100 μ M cell strainer, followed by PBS, and cells were counted and prepared according to the methods for flow cytometry (section 2.5).

2.1.7 Blood-derived monocytes isolation and monocyte purification

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE healthcare) density gradient centrifugation from donated blood. After centrifugation (2000 rpm for 20 min, RT, brake off), the layer of PBMCs were isolated and washed (1500 rpm for 7 min, 4 °C, brake low) five times in PBS (PAA) and until essentially free of platelets. PBMCs were used for CD14⁺ cell isolation. Where specified also hESC-derived monocytes were further purified using anti-human CD14 magnetic beads (Miltenyi Biotec, 130-050-201) according to the manufacturer's instructions.

2.1.8 Macrophage culture and maturation

Monocytes were plated onto tissue culture-treated 6-well plates (Corning) at a density of 1.5×10^6 cells/well; or at 6×10^5 cells/well of a 12-well plate (Greiner Bio-One); or at a density of 3×10^5 cells/well of a 24-well plates (Corning), or 1×10^5 cells/well of a 96-well plates (Corning). Unless specified differently, cells were maintained in MDM medium ($.375 \times 10^6$ /mL), consisting of RPMI 1640 (PAA) with 10% FCS (PAA), 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 μ g/mL streptomycin (PAA). The medium was supplemented with 100 ng/mL (approx. 1.7×10^4 units/mL) recombinant human M-CSF (R&D Systems). Cells were incubated at 37 °C, with 5% CO₂. Monocytes were differentiated for 5-7 days prior to use. For activation of macrophages for cytokine profile experiments, IFN γ (R&D, 100 U/mL) and LPS (Sigma, 100 ng/mL) were added to the culture medium during the last 16 hours of culture. For MDM analysis, cells were detached using cold PBS containing EDTA (5 mM).

2.1.9 Dendritic cell culture and maturation

Monocytes were plated onto tissue culture-treated 6-well plates (Corning) at a density of 1.5×10^6 cells/well in 4 mL medium, consisting of RPMI 1640 (PAA) with 10 % FCS (PAA), 2 mM L-glutamine (PAA), 100 U/mL penicillin and 100 µg/mL streptomycin (PAA) and supplemented with 100 ng/mL GM-CSF (R&D) and 25 ng/mL IL-4 (R&D) for four days to generate hESC-derived immature DC's, and for an additional 3 days with the maturation cocktail, as described by *Tseng et al.* (10 ng/mL IFN- γ , R&D; 10 ng/mL IL-1- β , R&D; 50 ng/mL TNF- α , R&D; 50 ng/mL GM-CSF, R&D; 1 µg/mL PGE₂, SIGMA) to generate mature DC's (Tseng et al. 2009).

2.1.10 Freezing and thawing

Cells were harvested by the enzymatic dissociation method as described above. They were suspended in either mTeSR™-1 or regular hESC medium at a density of 2×10^6 cells/mL. Afterwards, an equal volume of ice-cold 2X hESC Freezing Medium (20 % DMSO, Sigma; 60 % Embryonic Stem Cell Qualified Fetal Bovine Serum, Life Technologies; 20 % KO-DMEM; Gibco) was added in a drop-wise manner with constant stirring and the cells were diluted down to a density of 1×10^6 cells/mL. Cell aliquots were transferred into cryovials and quickly put into cryo-bombs in a -80°C freezer (Nalgene, Mr Frosty, containing Isopropanol to achieve a -1 °C/min cooling) where they were kept for no more than 24 hours, before transferring them to liquid nitrogen for long term storage.

For thawing, vials of frozen cells were incubated in 37 °C water bath until the ice just started to melt. Cells were then washed twice with ice-cold PBS to remove as much DMSO as possible. Finally, cells were re-suspended in either regular hESC or mTeSR™-1 medium, containing 1 mM Rock-inhibitor (Y27632; Calbiochem). Plating density after thawing depended on the culture technique: for feeder-based culture, the regular density

of 5,000-10,000 cells/cm² was used; whereas for feeder-free culture, cells were initially plated at a higher density of 1×10^5 cells/cm² to prevent cell death.

2.2 Antibodies

Western Blotting antibodies					
<i>Antibody</i>	<i>Host</i>	<i>Isotype/ conjugate</i>	<i>Clone/ number</i>	<i>Supplier</i>	<i>Concentration / Dilution</i>
αhuCD4	rabbit	IgG	H-370	Santa Cruz	1:200
αhuLCK	mouse	IgG2b	3A5	Santa Cruz	1:200
αhuGAPDH	mouse	polyclonal	2275- PC-100	R&D Systems	1:5000

Flow cytometry isotype controls					
<i>Antibody</i>	<i>Host</i>	<i>Isotype/ conjugate</i>	<i>Clone/ number</i>	<i>Supplier</i>	<i>Concentration / Dilution</i>
IgG control	rabbit	IgG/none	DA1E	Cell Signaling	1:25
IgG2a control	mouse	IgG2a/APC	133303	R&D Systems	1:10
IgG2b control	mouse	IgG2b/APC	400322	Biolegend	1:10
IgG2a control	mouse	IgG2a/FITC	SFL929F	AbD Serotec	1:2.5
IgG1 control	mouse	IgG1/APC	21275516	Immunotools	1:10
IgG1 control	mouse	IgG1/FITC	21335013	Immunotools	1:10
IgG1k control	mouse	IgG1k/APC	17-4714	eBioscience	1:10
IgG1k control	mouse	IgG1k/PE	555750	BD UK	1:10
IgG2b control	mouse	IgG2b/PE	130-092- 215	Miltenyi Biotec Ltd	1:10
IgG1k control	mouse	IgG1k/PE	555749	BD UK	1:50
IgG1k control	mouse	IgG1k/PE	12-4714	eBioscience	1:10
IgG2b control	mouse	IgG2b/PE	IC0041P	R&D	1:10
Secondary antibodies					
αrlgG	goat	FITC	sc-2012	Santa Cruz	1:250

Flow cytometry primary antibodies					
<i>Antibody</i>	<i>Host</i>	<i>Isotype/ conjugate</i>	<i>Clone/ number</i>	<i>Supplier</i>	<i>Concentration / Dilution</i>
αhuLCK	rabbit	IgG/none	73A5	Cell Signaling	1:25
αhuCD4 (D1)	mouse	IgG2a/APC	11830	R&D Systems	1:10
αhuCD4 (D3)	mouse	IgG2b/APC	OKT4	Biolegend	1:10
αratCD4 (D3)	mouse	IgG2a/FITC	OX68	AbD Serotec	1:2.5
CD45	mouse	IgG1/APC	MEM-28	Immunotools	1:10
CD14	mouse	IgG1/APC	MEM-15	Immunotools	1:10
CD34	mouse	IgG1κ/APC	4H11	eBioscience	1:10
Thy1	mouse	IgG1κ/PE	5E10	BD UK	1:10
CD38	mouse	IgG2b/PE	IB6	Miltenyi Biotec Ltd	1:10
CD33	mouse	IgG1κ/APC	WM-53	eBioscience	1:10
CD16	mouse	IgG1/APC	LNK1	Immunotools	1:10
CD163	mouse	IgG1κ/PE	GHI/61	BD UK	1:50
CD86	mouse	IgG1/FITC	BU63	Immunotools	1:10
MHCII	mouse	IgG1/FITC	MEM-12	Immunotools	1:10
CD206	mouse	IgG1κ/PE	19.2	eBioscience	1:10
CD71	mouse	IgG1/FITC	MEM-75	Immunotools	1:10
CCR5	mouse	IgG2b/PE	45531	R&D	1:10
CXCR4	mouse	IgG2b/PE	44717	R&D	1:10

2.3 Microscopy

2.3.1 Light

To take phase contrast and bright field images of cells, a Zeiss Axiovert 40 inverted microscope was used. To capture images, a digital camera (Colour CMOS UXGA [2.0 megapixel] USB 2.0 Camera; XL imaging) and QImaging software was used.

2.3.2 SEM

Samples were prepared with Michael Shaw or Errin Johnson (Sir William Dunn School of Pathology, University of Oxford). Monocytes were plated onto 13 mm coverslips and differentiated into macrophages, as described above or monocytes were settled onto coverslips coated with 1 % polyethyleneimine. Cells were fixed with 2.5 % glutaraldehyde, 2 % paraformaldehyde in 100 mM cacodylate buffer (pH 7.2) O/N at 4 °C. Samples were post-fixed with 1 % osmium tetroxide in 100 mM cacodylate buffer (pH 7.0) for 1 h at RT. Next, cells were washed with distilled water and dehydrated with 50 % ethanol for 15 min.; 70 % ethanol O/N; 90 % ethanol for 15 min.; and finally 100 % ethanol for 3 times 30 min. Next, samples were dried using liquid CO₂ in an Autosamdri®-815 (Touismis) critical point dryer and mounted onto double-sided carbon tabs on aluminium SEM stubs and coated with gold in an E5100 Series II sputter coater (Bio-Rad). Cells were examined using a JOEL JSM 6390 scanning electron microscope.

2.3.3 TEM

Samples were prepared with Michael Shaw or Errin Johnson (Sir William Dunn School of Pathology, University of Oxford). Monocytes were plated onto 13 mm coverslips and differentiated into macrophages, as described above or monocytes were used directly. Cells were fixed with 2.5 % glutaraldehyde, 0.1 % tannic acid in 100 mM cacodylate buffer (pH 7.2) for 16 h at 4 °C. Cells were washed by incubating the samples with 200 mM

cacodylate buffer (pH 7.2) for three times 10 min. Samples were post-fixed with 1 % osmium tetroxide in 100 mM cacodylate buffer (pH 7.0) for 1 h at RT. Next, cells were washed with distilled water. Cells were washed with distilled water and dehydrated with 50% ethanol for 15 min.; 70% ethanol for 15 min.; 90% ethanol for 15 min.; and finally 100% ethanol for 3 times 30 min. Cells were pelleted by centrifugation. Samples were embedded using Epoxy resin (Agar 100, Agar Scientific). To allow the Epoxy resin to infiltrate the sample, samples were incubated for 1h with ethanol and resin (added in a 2:1 ratio); 1h with ethanol and resin (added in a 2:1 ratio); 1 h with ethanol and resin (added in a 1:2 ratio); O/N with resin. The next morning, the resin was replaced with fresh resin and incubated for 8 h. Next, the samples were put into embedding moulds with fresh resin. Samples were polymerised for 24 h at 60 °C and sectioned using a Reichert Ultracut E microtome and post-stained with 2 % uracil acetate and Reynold's lead citrate (Reynolds 1963). Samples were examined using a FEI Tecnai 12 electron microscope at 120 kV.

2.3.4 Histochemistry

1.0×10^5 cells were centrifuged (Shandon Cytospin) at 400 rpm for 5 min to make cytopins. For eosin-methylene blue staining, slides were fixed in methanol for 30 sec, stained with methylene blue for 30 sec and stained with eosin for 1 min, and washed with water. Slides were mounted with round cover slips using DPX and dried overnight. Images were taken using a Nikon Coolscope.

2.4 Western Blotting

Media was removed and adherent cells were washed with ice-cold PBS and harvested using a cell scraper. Cells were pelleted and stored at -80°C for later lysis, or lysed directly in ice-cold lysis buffer (1 % v/v n-Dodecyl beta-D-maltoside , 20mM Tris / HCl pH 8; 150 mM NaCl, containing and phosphatase inhibitors (Sigma)) to a final concentration

of 2×10^5 cells/ μL for 15 min on ice. Lysates were centrifuged for 10 min at 4°C , 13,000 g and the cleared lysate was mixed with 4 x NuPAGE LDS (Lithium dodecyl sulphate) sample buffer and 10 x NuPAGE sample reducing agent (Invitrogen) and heated for 10 min at 70°C . Lysates were electrophoresed at 180 V at RT through NuPAGE® Bis-Tris Precast Gels (Invitrogen). The electrophoresed proteins were electroblotted for 1 h at 75 V (at 4°C) onto PVDF transfer membranes. The membranes were blocked with 3 % (w/v) low-fat dry milk powder in PBS O/N on a rocking platform. Blocked membranes were incubated with primary antibodies diluted in 3 % (w/v) low-fat dry milk powder in PBS-T (1x PBS, 0.1 % (v/v) Tween-20) O/N at 4°C on a rocking platform. After incubation with primary antibodies membranes were washed 3 times with 1X PBS-T and then incubated with the appropriate LI-COR secondary antibody (goat anti-mouse IgG IR Dye 800/680 or goat anti-rabbit IgG IR Dye 800/680) diluted in 3 % (w/v) low-fat dry milk powder in PBS-T for 30 min at RT. Washed membranes were scanned using the quantitative western blotting imaging system Odyssey (LI-COR).

2.5 Flow cytometry

For analysis of cell surface molecules, $0.5\text{--}1 \times 10^6$ cells were washed and stained in flow cytometry buffer consisting of PBS, human IgG (10 $\mu\text{g/mL}$ Sigma), FCS (1 % Hyclone) and sodium azide (0.01 %), with an antibody or an isotype-matched control (with same fluorophore, from the same manufacturer) on ice for 30 min. For two-colour staining, two antibodies or two isotype controls (attached to different fluorophores) were added together, except for the LCK and CD4 double staining (which is described below). Antibodies used are described in the main text or the table in section 2.2. After the primary staining, cells were washed three times and if unconjugated antibodies were used, stained with a secondary antibody on ice for 30 min and washed another three times. Cells were fixed with 4 % formaldehyde in PBS. Fluorescence was measured using

a FACS Calibur (Becton Dickinson), and data was analysed using FlowJo software on marked cell populations on FSC-SSC dot plots.

For the two-colour staining with CD4 and LCK, cells were first stained for surface CD4 (APC conjugated, clone 11830, R&D systems) and fixed with 4 % formaldehyde in PBS and permeabilised using 0.2 % saponin (Sigma) in flow cytometry buffer. Next, cells were stained for total LCK expression (unconjugated, clone 73A5, santa cruz) and stained with a FITC conjugated secondary antibody (Cell Signaling), as described above, except 0.2 % saponin was kept present during the washes and staining incubation.

2.6 Flow Cytometric assay of differential detergent resistance

Cells were labelled according to the flow cytometry protocol as described above. Unlabelled cells were used to measure auto-fluorescence (AF). The detergent, Triton X-100 (Sigma) was diluted using PBS to achieve 0.025 %, 0.030 %, 0.040 % (v/v) solutions and cooled on ice. The labelled or unlabelled control cells were mixed with ice-cold detergent (det) or ice-cold PBS (as a control). Fluorescence was immediately measured in the time-resolved mode for 4 min using a FACS Calibur (Becton Dickinson). Also, the end-point mode was used to obtain the MFI before or 4 min after adding the detergent or PBS. Data was analysed using FlowJo software. In order to calculate the FCDR index, the mean fluorescence intensity (MFI) of the following conditions was obtained: labelled, detergent-treated cells (MFI_{det}); unlabelled detergent-treated cells (MFI_{AFdet}); labelled, PBS-treated cells (MFI_{max}); unlabelled PBS-treated cells (MFI_{AF}). The extent of detergent resistance was calculated as follows: $FCDR = (MFI_{det} - MFI_{AFdet}) / (MFI_{max} - MFI_{AF})$.

2.7 Multiplex assay for cytokine and chemokine secretion

Media from 7 day differentiated macrophages (either unactivated, or activated for 16 h with 100 U/mL IFN γ (R&D) and 100 ng/mL LPS (Sigma) (added into the existing medium)

were spun down at 400 *g* to remove cells and the supernatant was frozen at -20 °C until analysis. Proteome Profiler Human Cytokine Array (Panel A, R&D Systems) was used to detect the presence of 36 different cytokines using a membrane antibody array, with biotinylated detection antibodies followed by streptavidin conjugated to horse radish peroxidase (HRP) and development with a chemiluminescent substrate (Pierce, performed according to the manufacturer's instructions). The pixel density of spots on the image (captured with Gene Snap software) was quantified using Gene Tools software, with background subtracted, and individual cytokine pixel density was expressed as a percentage of the mean positive control spot density.

2.8 Phagocytosis assay of zymosan particles

S.cerevisiae Zymosan A BioParticles® (Alexa Fluor 488 conjugated, Invitrogen) were reconstituted according to the manufacturer's instructions, using PBS with 2 mM sodium azide, and sonication to disrupt particle aggregates. Fluorescent zymosan particles were carefully counted, and added to adherent macrophages at a ratio of 2 particles per cell, in serum-free RPMI. Phagocytosis was allowed to occur for 30 min at 37 °C, followed by 1 wash with PBS, 1 wash with 250 µg/mL trypan blue in PBS to quench particles bound to the outside of the cell, and a further wash with PBS. Cells were then incubated with 0.25% trypsin/0.5 mM EDTA (in Hanks' Balanced Salt Solution; Sigma) at 4°C for 1h to detach the cells. Detached macrophages were centrifuged at 400 *g* and fixed with 4 % formaldehyde in PBS. Uptake of zymosan was quantified using a Becton-Dickinson FACS Calibur flow cytometer and data analysed using FlowJo software. Fluorescence negative cells (not fed zymosan) were used to establish a threshold for quantifying the percentage of positive cells having taken up one or more zymosan particle.

2.9 qPCR of endogenous and chimeric CD4 transcripts and integrants

Macrophages were harvested by scraping and pelleted by centrifugation. Total RNA and DNA was extracted using All-Prep RNA/DNA kit (Qiagen) according to the manufacturer's instructions, with on-column DNase digestion. cDNA was produced from the extracted RNA using the Ambion RETROscript kit (1 µg of RNA per reaction with Oligo-dT primer). The cDNA or DNA was used in a SYBR green real-time qPCR reaction using (Applied Biosystems) with the following primers and probes.

Name	Sequence (5'→3')
huCD4 fwd DNA	TGGAGTCGCAAGCTGAACTA (Thanakiatkrai et al. 2012)
huCD4 rev DNA	CAGAGTGAGAACCTGTCTTGAAAA (Thanakiatkrai et al. 2012)
huCD4 fwd cDNA	GCTGGAATCCAACATCAAGG (Zhou et al. 2011)
huCD4 rev cDNA	CTTCTGAAACCGGTGAGGAC (Zhou et al. 2011)
chCD4 fwd	TTTAGAGAGCCCACCCGGTA
chCD4 rev	GCTGGCTCACACTTAGGGTT
fwd: forward; rev: reverse; if not specified, same primers for DNA and cDNA was used.	

To determine relative expression levels (using the $\Delta\Delta C(t)$ method (Livak et al. 2001)), β -actin DNA and cDNA was measured in parallel using primers from the β -actin control kit (Eurogentec). Reactions contained 12.5 µL *Power SYBR® Green qPCR Mastermix* (Applied Biosystems), 100 nM forward primer, 100 nM reverse primer and 1 ng DNA or cDNA, in a total reaction volume of 25 µL (volume made up with water). The qPCR programme was as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 57.5 °C for 1 min, with a plate read after each cycle, followed by a melting curve stage: 15 sec 95 °C and 1 min 60°C and reading the plate every 0.3 °C to 95 °C, where it was hold for 15 sec and performed on an Applied Biosystems StepOne Plus Real Time PCR machine, with StepOne software. StepOne software (Applied Biosystems) was used to determine

relative expression levels. Error bars represent the SEM based on the relative quantification (RQ)_{min}/RQ_{max} confidence set at 95 %.

2.10 Cloning techniques

Details regarding the design, cloning and sequence of plasmids are described in the appendices and plasmid maps and sequence data can be found on the CD-ROM included with this thesis. General cloning techniques are described below.

Digestions were performed by generally using 2 µg of donor plasmid and 1 µg of recipient plasmid for 4h-O/N at 37 °C, according to New England BioLabs recommendations. Digested plasmids were separated using 1 % agarose gels electrophoresis and were run alongside a 1 Kb DNA ladder (Invitrogen). The bands were visualized using ethidium bromide and UV light. Bands were extracted from the gel according to the instructions from the QIAquick gel extraction kit (Qiagen) and eluted with water. Ligations were generally performed by incubating 5 µL of insert, with 3 µL of linearized backbone, using 2 uL T4 ligase and 10 uL 2X rapid buffer for 10 min at RT. Alternatively, 6 µL of insert, 3 µL of linearized backbone, using 1 uL T4 ligase and 10 uL 10x buffer were mixed and incubated for 10 min-2 h at RT. To grow up plasmid DNA, competent *Escherichia coli* (*E.coli*) cells transformed. *E.coli* cells were thawed on ice and 1 µL of plasmid (or 10 µL of ligation product) was added and mixed gently. The mixture was incubated on ice for 30 min.; heat-shocked at 42 °C for 45 sec.; returned to ice and 250 µL of SOC medium (Invitrogen) was added and left on ice for 2min. The transformation mixture was then incubated at 37°C for 1 h, 200 rpm. Next, the transformed bacteria were plated onto LB agar plates containing 100 µg/mL ampicillin and incubated O/N at 37°C. The next day, colonies were picked and used to inoculate 3 mL LB medium containing 100 µg/mL ampicillin and in preparation for a Miniprep (QIAprep Spin Miniprep Kit; Qiagen) grown overnight. Alternatively, in preparation of a Midiprep (Sigma GenElute™) the LB culture

was grown for 6 h, after which 300 μL was transferred to 50 mL LB medium with ampicillin, which was cultured O/N. Minipreps and Midipreps were performed according to the manufacturer's instructions.

2.11 Lentivirus preparation

To generate pseudotyped virus capable of a single round of replication, 293T cells were seeded 24 h prior to transfection onto a well of a 6-well plate at 1×10^6 cells/well in 2 mL to achieve 90 %-100 % confluency on the day of transfection. Cells were transfected with the transfer plasmid (as described in the main text and appendices), together with plasmid pCMVdR 8.91 (1.5 μg /well) encoding the structural lentiviral genes (gag and pol) and a plasmid pMD2.G encoding VSV-G pan-tropic envelope (0.5 μg /well), in the ratio 3:3:1 (Zufferey et al. 1997). Transfection was carried out using FuGENE HD Transfection Reagent (Roche), according to manufacturer's protocol. In short, for transfection of a confluent well of a 6-well plate, 3.5 μg DNA was prepared in 175 μL OptiMeM (Gibco), which was first brought to RT, in an Eppendorf tube and 10.5 μL FuGene (first briefly vortexed) was added without touching the sides of the tube, corresponding to a ratio of 6:2 of FuGene and DNA. The mix was vortexed vigorously for 2 sec and left for 5-10 min at RT before adding the mixture to the cells. 184 μL transfection mix was added to one well of 293T cells in a 6-well plate in a dropwise manner and the plate was carefully swirled. The plate was incubated for 4 h at 37 °C followed by a full medium change (DMEM (PAA), supplemented with 10 % FCS (PAA) and 2 mM Glutamax-I (Gibco), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen)). The virus stocks were harvested after 48 h, centrifuged at 514 g, passed through a 0.45 μm filter and frozen. Pseudotyped virus was titrated on 293T cells to allow the addition of equal infectivity of pseudotyped viruses. For titrations, cells were seeded at 4×10^4 cells per well in a 24-well plate 24h prior to transduction. On the day of transduction, cells in one representative well were counted and cells were infected with a series of 10-fold dilutions of pseudotyped

virus using a total volume of 200 μL , from neat to 1 in 1×10^5 . The infection was left to proceed for 2 h, after which the inoculum was replaced with 1 mL fresh medium. Two days post transduction Puromycin (5 $\mu\text{g/mL}$) was added and 5 days post transduction colonies were counted and the multiplicity of infection (MOI) was calculated.

2.12 Lentiviral transduction of hESC

Lentivirus was added to hESC (~70% confluence) with a MOI of approximately 0.08 in a total volume of 1mL, supplemented with polybrene (5 $\mu\text{g/mL}$, Sigma) onto one well of a 6-well plate and centrifuged for 1h 37 °C at 1600 *g*. Cells were incubated at 37 °C for 3 h after which the inoculum was replaced with fresh media. Cells were fed as normal for 2 days, and on day 3 puromycin (5 $\mu\text{g/mL}$, Sigma) was added. Surviving colonies were fed with MEF-conditioned medium until ready to passage (as described above) onto fresh MEFs. Puromycin was added to cells for at least two days before each subsequent (manual) passage and cells were fed with MEF-conditioned medium.

2.13 HIV-1 assays

2.13.1 replication-defective pseudotyped HIV-1

A replication-defective pseudotyped HIV-1 allows the examination of single-cycle HIV-1 infection and was used in the HIV virion fusion assay and HIV-1 integration assay. A commonly used replication-defective virus is NL4-3.Luc.R-E- (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau) that is negative for Env and Vpr, due to two frameshifts (5' Env and Vpr aa 26) and has the firefly luciferase gene inserted into the NL4-3 nef gene, allowing it to be used as a reporter gene for infection (Connor et al. 1995; He et al. 1995). The NL4-3.Luc.R-E can be pseudotyped with different viral envelopes, such as the HIV-1 R5-tropic viruses JRFL and BaL or with VSV-G. VSV-G enveloped viruses use an ubiquitous membrane

component (phospholipid) to enter cells, through clathrin-mediated endocytosis and pH dependent fusion (Matlin et al. 1982; Yamada et al. 1986). Because entry of VSV-G pseudotyped viruses is independent of CD4 expression levels and distribution, it can be used as a control to normalise to the number of cells present.

2.13.2 HIV virion fusion assay

BaL and VSV-G pseudotyped HIV-1 NL4.3.Luc.R-E containing β -lactamase-Vpr chimera (BlaM-Vpr), capable of a single round of replication were prepared using Fugene transfection on 293T cells as described in section 2.11, except the following amounts of plasmid were used per well of a 6-well plate: 0.6 μ g pVpr-rBlaM, 1.8 μ g pNL4.3luc and 1.2 μ g VSV-G or BaL (1:3:2). esMDM were plated on a 24-well plate and spinoculated (1.5 h, 1800 *g*, 37 °C) with NL4.3 BaL (Vpr-BlaM) or NL4.3 VSV-G (Vpr-BlaM) and incubated for 90 min-2 h at 37 °C with 5 % CO₂. Cells were washed with CO₂-independent media (Gibco) and loaded with 600 μ L of the fluorogenic β -lactamase substrate CCF2-AM loading solution (Invitrogen). The loading solution was prepared by diluting solution A (prepared by adding 46 μ L of supplied DMSO), 10x with solution B (GeneBLAzer™ *In Vivo* Detection Kit, 12578-134, Invitrogen). The mixed solution A+B was diluted 16.7x with CO₂-independent media (18045-054, Invitrogen), resulting in 6x CCF2-AM loading solution. This was further diluted with CO₂-independent media to create 1x CCF2-AM loading solution. The plate was covered with aluminium foil and incubated for 1h in the dark at RT. 600 μ L of CO₂-independent media supplemented with 2X probenecid (P-8761, Sigma, final concentration of 2.5 mM) and 2X M-CSF (R&D Systems, final concentration of 100 ng/mL) was added to the well and left for 16 h at RT in the dark. The lipophilic, esterified form of this substrate CCF2-AM readily enters the cell and gets cleaved by endogenous cytoplasmic esterases to convert it to its negatively charged form, CCF2 which is retained in the cytosol. Probenecid is a non-specific inhibitor of anion transport that can increase the amount of CCF2-AM retained in the cell. esMDM were detached by

incubating with 0.25 % trypsin (PAA) for 30 min-1 h at 37 °C, and fixed at for 30 min 4 °C in 4 % formaldehyde in PBS. Fluorescence was measured using Dako CyAn™ (Beckman Coulter), and data was analysed using FlowJo software. Gates were set based on the fluorescent signal of uninfected samples loaded with CCF2-AM. Viral fusion was detected by the fluorescence shift associated with FRET-based cleavage of the BLaM substrate. The CCF2 substrate consist of a cephalosporin core linking two fluorophores, coumarin and fluorescein, that form an efficient FRET pair. In the absence of beta-lactamase activity, exciting the coumarin at 405 nm in the intact CCF2 molecule results in FRET to the fluorescein, which emits a green fluorescence signal at 518 nm (Violet FL-2). In the presence of β -lactamase activity, however, cleavage of the cephalosporin core of CCF2 spatially separates the two dyes and disrupts FRET, so that exciting the coumarin at 405 nm produces a blue fluorescence signal at 447 nm

2.13.3 qPCR of HIV-1 late reverse transcripts

HIV-1 BaL was obtained from *Gartner et al.* from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and amplified in PBMCs for 12-21 days before harvesting (Gartner et al. 1986). To remove any proviral DNA viral stocks were treated with 100 μ g/mL DNase I (Sigma). Macrophages were spinoculated with 500 μ L DNase I treated HIV-1 BaL by centrifugation at 2000 *g* for 90 min at 37 °C and incubation for an additional 30 min at 37 °C. The inoculum was removed and replaced with fresh media. After incubating the cells for another 28 h in the incubator, media was removed and the cells (still on tissue culture plates) were stored at -20 °C or used directly to extract DNA. DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions, except PBS and proteinase K were directly added onto the cells on the plates and cells were harvested from plates by pipetting up and down. DNA was eluted in AE buffer, supplied with the kit and used in a Taqman real-time qPCR

reaction using Brilliant QPCR Core Reagent Kit (Agilent Technologies) with the following primers and probes.

Name	Sequence (5'→3')
forward (R5pol)	TGGGTTATGAACTCCATCCTGAT
reverse (R5pol)	TGTCATTGACAGTCCAGCTGTC
probe (RT)	FAM- TTTCTGGCAGCACTATAGGCTGTACTGTCCATT -TAMRA

The primer set used detects a conserved HIV-1 cDNA sequence, corresponding to a 81-bp fragment from the HIV-1 reverse transcriptase gene (*pol*). Reactions contained 10 µL BrilliantqPCR Mastermix (Agilent Technologies), 375 nM forward primer, 375 nM reverse primer, 125 nM probe, 300 nM reference dye and ~80 ng DNA from infected cells (generally 2 µL), in a total reaction volume of 20 µL. Standards were prepared in duplicate using the pNL4.3.Luc.R-E- HIV-1 backbone plasmid diluted ranging from 10 to 1×10^7 copies. To allow normalisation of the number of copies of HIV-1 DNA to the number of cells, β -actin DNA was quantified (β -actin control kit, Eurogentec) in parallel, according to the manufacturer's instructions. β -actin standards were prepared using human Xsomal genomic DNA (Eurogentec) at 10 fold dilutions ranging from 60 ng to 0.006 ng (30 ng corresponds to 5000 cells). The fast qPCR programme was as follows: 95 °C for 3 min, and 40 cycles of 95 °C for 5 sec, 60 °C for 10 sec, and performed on an Applied Biosystems StepOne Plus Real Time PCR machine, with StepOne software.

2.13.4 qPCR 2-LTR circles

HIV-1 cDNA of infected macrophages was obtained as described under “qPCR of HIV-1 late reverse transcripts” and used in a Taqman real-time qPCR reaction using Brilliant QPCR Core Reagent Kit (Agilent Technologies) with the following primers and probes.

Name	Sequence (5'→3')
forward (MH535)	AACTAGGGAACCCACTGCTTAAG

reverse (MH536)	TCCACAGATCAAGGATATCTTGTC
probe (MH603:5)	FAM-ACACTACTTGAAGCACTCAAGGCAAGCTTT-TAMRA
(Butler et al. 2001)	

To determine relative expression levels (using the $\Delta\Delta C(t)$ method (Livak et al. 2001)), β -actin DNA was quantified (β -actin control kit, Eurogentec) in parallel, according to the manufacturer's instructions. β -actin standards were prepared using human Xsomal genomic DNA (Eurogentec). Reactions contained 10 μ L Brilliant qPCR Mastermix (Agilent Technologies), 375 nM forward primer, 375 nM reverse primer, 125 nM probe, 300 nM reference dye and ~80 ng DNA from infected cells (generally 2 μ L), in a total reaction volume of 20 μ L. The fast qPCR programme was as follows: 95 °C for 3 min, and 40 cycles of 95 °C for 5 sec, 60 °C for 10 sec, and performed on an Applied Biosystems StepOne Plus Real Time PCR machine. StepOne software (Applied Biosystems) was used to determine relative expression levels. Error bars represent the SEM based on the relative quantification (RQ)min/RQmax confidence set at 95 %.

2.13.5 HIV-1 integration (luciferase)

Replication-defective pseudotyped HIV-1 was used to quantitate the stages of the viral life cycle up to and including integration into the genome of the target cell. HIV-1 pseudovirus Envelope proteins (HIV-1 JRFL, HIV-1 BaL, obtained from the NIH/AIDS reagents programme and the control envelope protein VSV-G) were expressed from the vector pcDNA3.1/Zeo (Invitrogen). For production of pseudotyped virus capable of a single round of replication, 293T cells were transfected as described in section 2.11, except the following ratios of plasmid were used: pNL4.3.Luc.R-E- (2.8 μ g per well of 6-well plate) and pEnv or pVSV-G (0.7 μ g per well of 6-well plate) in the ratio of 4:1. Macrophages were spinoculated in 96-well plates with 100 μ L of pseudotyped virus at 2000 g for 90 min at 37°C. Cells were incubated for 2h in the incubator, before the virus inoculum was removed and replaced with 200 μ L of medium. Three days later, viability was assessed

using CellTiter 96® AQueous One Solution Cell Proliferation (Promega), according to manufacturer's protocol. Cells were washed with PBS and lysed with 60 µL/well of ONE-Glo™ luciferase substrate (Promega). Luciferase expression from the viral genome was measured by transferring 50 µL of lysed cells into a black 96 well plate. The luminescence (in arbitrary units termed relative light units; RLU) was quantified using SpectraMax M5 with SoftmaxPro version 5 software.

2.13.6 Quantification of p24 antigen by ELISA

HIV-1 BaL was obtained from *Gartner et al.* from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH and amplified in PBMCs for 12-21 days before harvesting (Gartner et al. 1986). To measure multiple rounds of HIV infection, macrophages were infected in 96-well plates with HIV-1 BaL for 6 h at 37 °C. The inoculum was removed and replaced with RPMI + 10 % FCS + 100 ng/mL M-CSF. Over a 2-3 week period, supernatant samples were taken at intervals and kept at -20°C. 96-well flat-bottomed high binding plates (Greiner) were coated with 100 µL /well of 10 µg/mL anti-p24 (D7320, Aalto) in coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6, Sigma) O/N at RT, rocking. Plates were then washed 4 times in PBS with 0.05 % Tween 20 and blocked with 200 µL 2 % BSA (Sigma) in TBS (Tris buffered saline, 0.144 M NaCl, 0.05 % Tween 20 (Sigma), 25 mM Tris-HCl, pH 7.5) for 30 min at RT, and the washing steps were repeated. A standard curve was created with dilutions of recombinant HIV-1 p24 protein (Aalto) in TES (TBS + 1 % empigen (Fluka), 10 % FCS) to provide concentrations ranging from 200 ng/mL to 0.23 ng/mL. Samples from infected cells were diluted 1 in 4 in TES to give a final concentration of 1 % empigen and heated to 56 °C for 30 min to inactivate the virus.

100 µL of the sample or standard was applied to the plates in triplicate. The plate was covered and incubated for 2 h at 37 °C with gentle agitation (75 rpm). Plates were washed 5 times as before and 100 µL of biotinylated anti-p24 antibody (Aalto BC1071-BIOT)

diluted in TT/SS (TBS, 20% FCS, 0.05% Tween 20) was added for 2h. Plates were washed as before, and 100 μ L of streptavidin-HRP (Pierce) diluted 0.1 μ g/ml in TT/SS applied for 1 h. Plates were washed and then developed with 100 μ L 1-step Ultra TMB-ELISA (Pierce, #34028) according to the manufacturer's instructions. The reaction was stopped with 100 μ L 1M H₂SO₄ (Sigma) and the absorbance was read at 450 nm using a Molecular Devices Emax precision microplate reader and SoftMax Pro software version 4.0. The data were analysed using GraphPad Prism software to create standard curves (2-site binding equation) and to calculate unknown values.

2.14 Statistical analysis and software

All data are represented as mean $\pm$ SEM of independent biological replicates is shown and statistical analysis was carried out with GraphPad Prism software. Statistical significance was reported as ns $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. For comparisons of means from multiple groups against one control group the one-way ANOVA using the Holm-Šídák post-test for multiple comparisons, with $\alpha=5.000\%$.. For p24 ELISA, the repeated measures one-way ANOVA with the Holm-Šídák post-test for multiple comparisons was used, as well as unpaired t test per time point correcting for multiple comparisons with the Holm-Šídák method, with $\alpha = 5 \%$.

Chapter 3: esMDM to study HIV-1 infection of human macrophages

Introduction

The differentiation of human monocyte-derived macrophages (MDM) from human embryonic stem cells (hESC) offers a simple, cellular system to study macrophage biology and overcomes problems associated with working with blood-derived MDM (bMDM) (Karlsson et al. 2008; Cowley SA 2012). hESC derived macrophages (esMDM) are phenotypically and functionally similar to bMDM, and most importantly, they accurately replicate the HIV replication kinetics seen in bMDM (Karlsson et al. 2008; Cowley SA 2012). The protocol for the differentiation of esMDM was created in our laboratory by *Karlsson et al.* (Karlsson et al. 2008). However, this original protocol is not very robust and is sensitive to procedural variability, such as variable quality of starting cells and density of cells in the differentiation culture. The main aim of the work described in this Chapter was to further optimize and define the differentiation of esMDM to achieve higher, as well as, more consistent yields of esMDM and to test the use of a lentiviral vector to achieve stable gene expression in esMDM.

3.1 Improved method for increased production of viable esMDM from hESC

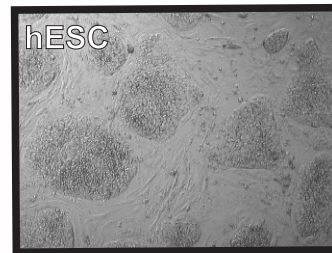
The method for producing functional macrophages from hESC, as developed by *Karlsson et al.* can yield over 1×10^7 monocytes from a 6-well plate of differentiation cultures (Karlsson et al. 2008). However, such high yields are generally only achieved once a week, for 1-3 weeks, after which yields dramatically decrease. Serum is the most likely component of the medium to cause the culture to become less productive over time, as it likely contains many growth factors, not all of which are necessarily optimal for myeloid lineage maintenance. Therefore, to optimize the differentiation, it was sought to remove the serum. X-VIVO™ 15 media are optimized for cellular maintenance without serum, so X-VIVO™ 15 was tested as the basal medium in an improved protocol ('X') in place of aDMEM + 10 % FCS in the original protocol (version 1, 'v1'). Protocol X and v1 are outlined in Figure 3.1A. Firstly, hESC spontaneously differentiate into EBs after mechanical dissociation and transferred to ultra-low adherence plates. Secondly, ~20 EBs are seeded into a well of a 6-well plate and myeloid differentiation is directed by supplementing the media with IL-3 and M-CSF. In protocol v1, the culture media base is aDMEM + 10 % FCS, whereas in protocol X, the culture media base is X-VIVO™ 15. EBs adhere and produce a homogeneous population of monocytes (esMC) that can be harvested routinely from the supernatant of the differentiation cultures. Thirdly, harvested monocytes can be cultured in M-CSF for 5-7 days to allow the differentiation into macrophages (esMDM).

Figure 3.1

Figure 3.1. Improved method for increased production of viable esMDM from hESC.

A) Diagram, using light microscopy images of human Embryonic Stem Cells (hESC, 10x), Embryoid Bodies (EBs, 10x), differentiating EBs (20x) and hESC-, monocyte-derived macrophages (esMDM, 10x), outlining the original and improved differentiation method. hESC colonies cultured on inactivated feeder cells were mechanically dissociated and transferred to low adherence plastic for 3 days in hESC media for spontaneous EB formation. EBs were transferred to regular tissue culture plates and differentiated in X-VIVO™15 media (improved protocol, X) or aDMEM media (protocol version 1, v1) and supplemented with M-CSF and IL-3. Non-adherent esMC were harvested from the supernatant of cultures and plated in RPMI media + 10 % serum + M-CSF to generate esMDM.

A day 0



mechanical dissociation

day 4

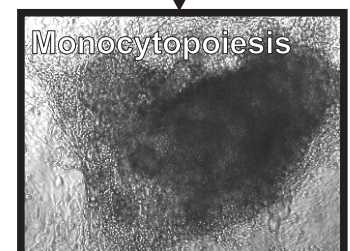
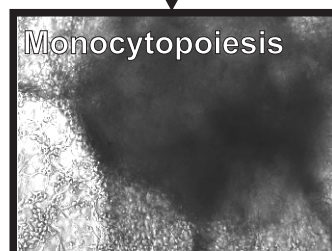


myeloid differentiation

improved protocol (X):
culture in X-VIVO™15
+ M-CSF + IL-3

protocol version 1 (v1):
culture in aDMEM
+ M-CSF + IL-3

day 14⁺



*harvest/plate monocytes
in RPMI + M-CSF*

*harvest/plate monocytes
in RPMI + M-CSF*

day 14⁺+7

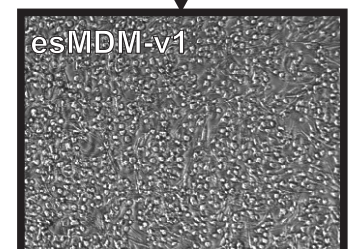
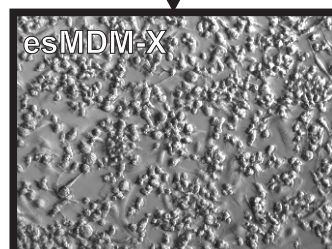
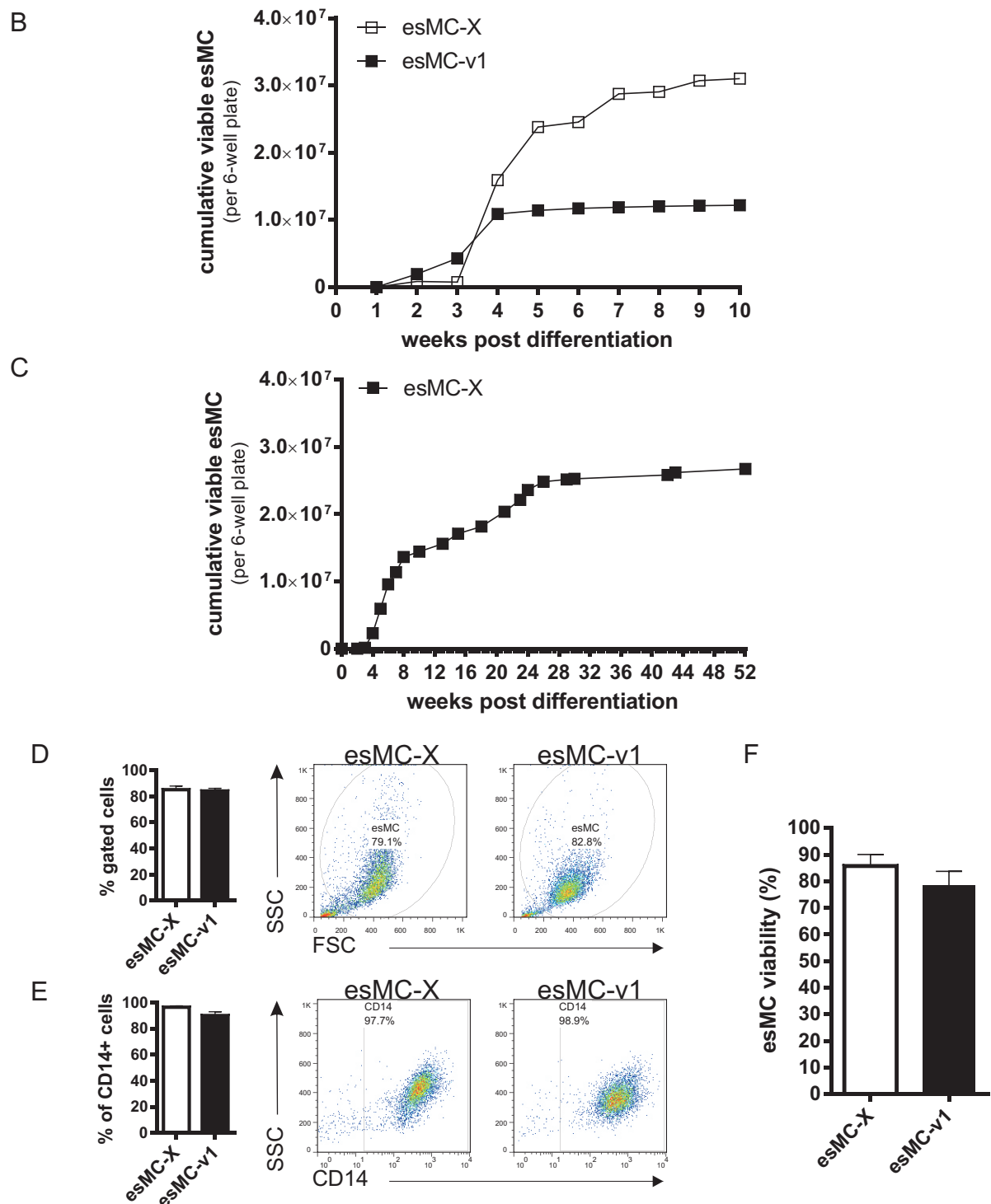


Figure 3.1

Figure 3.1. (continued) Improved method for increased production of viable esMC from hESC.

B+C) Non-adherent esMC were harvested from the supernatant of cultures that had been prepared according to protocol X or v1, and counted using a cell counter (Chemometec). The media were replaced for repeated esMC harvests over a period of 10 weeks (B) or 52 weeks (C). Data represent the cumulative number of viable esMC from 6 wells per condition. D) Forward Scatter (FSC) and Side Scatter (SSC) dot plot of harvested esMC showing a gate around the homogenous cell population. Bars represent the mean percentage $\pm$ SEM of gated cells of esMC-X (n=7) and esMC-v1 (n=11). E) CD14 and FSC dot plot showing percentage of CD14+ cells within the homogenous cell population. Bars represent the percentage of CD14+ gated cells $\pm$ SEM of esMC-X (n=16) and esMC-v1 (n=14). F) Viability of esMC-X (n=7) and esMC-v1 (n=7) were determined by an AO-DAPI stain (Chemometec). Bars represent the mean viability $\pm$ SEM.



esMC were harvested weekly over a period of 10 weeks. Figure 3.2B shows the cumulative counts of viable esMC per 6-well plate. Both esMC produced according to protocol X (esMC-X), and esMC produced according to protocol v1 (esMC-v1), were produced after a lag period of 2-3 weeks, followed by a rapid production phase and subsequently a period of continuous lower level production. The rapid production phase only lasted 2-3 weeks using the original protocol v1, but continued for longer using protocol X. After 5 weeks of monocytopoiesis, only 0.5×10^6 esMC-v1 could be collected, whereas yields were 20-fold higher for esMC-X. Cumulatively, after 10 weeks of monocytopoiesis 2.6-fold more esMC were harvested using protocol X. To monitor the long-term esMC production using protocol X, esMC-X were harvested for the duration of 1 year (Figure 3.1C). After 52 weeks of myeloid differentiation, 5×10^5 esMC-X were still being produced per plate. esMC produced using both protocols were homogeneous and pure, based on cell scatter properties and the distribution of CD14 (Figure 3.3D+E). In addition, AO-DAPI stains confirmed that the esMC were viable (Figure 3.1F).

Taken together, the results showed that compared to protocol v1, protocol X (which uses chemically defined and serum-free basal media for the differentiation of EBs) produces higher yields of homogenous, viable monocytes for a longer duration.

3.2 Monocytopoiesis characterization

Protocol X resulted in longer term production of esMC compared to protocol v1, suggesting that the X-cultures undergoing monocytopoiesis may contain a higher proportion of precursor cells or may support production of precursor cells for a longer duration. Therefore, monocytopoiesis cultures were further characterized for markers of haematopoiesis.

In order to produce esMC from hESC, hESC undergo monocytopoiesis and may first differentiate into a multi-potential haematopoietic precursor cells (HSC) and various haematopoietic precursors. Differentiation of HSC is associated with changes of gene and cell surface receptor expression (Szilvassy 2003). Flow cytometric analysis of surface marker expression is commonly used to identify and isolate HSC (Szilvassy 2003). A single surface marker to identify human HSC has not been reported, instead, combinations of markers have been described which are thought to be enriched on HSC. CD34 is the most commonly used marker for HSCs and progenitor cells, although CD34 may also be expressed by endothelial cells (Gangenahalli et al. 2006; Nielsen et al. 2008). CD34 is a highly glycosylated surface antigen involved in adhesion to bone marrow stroma and cell homing. In addition to CD34 expression, HSC are marked by their co-expression of Thy1 and lack of CD38 expression (Baum et al. 1992; Craig et al. 1993; Hao et al. 1995; Bhatia et al. 1997; Doulatov et al. 2010; Notta et al. 2011). CD34⁺Thy1⁺ cells possess *in vitro* and *in vivo* long-term hematopoietic activity and do not express any lineage markers (Murray et al. 1995). Thy-1 (CD90) is a glycoprotein expressed by HSC, but can also be found on fibroblasts, neurons, endothelial cells, smooth muscle cells and thymocytes (McKenzie et al. 1981; Baum et al. 1992; Craig et al. 1993). In humans Thy-1 may be involved in inflammatory responses and cell-cell or cell-ligand interactions (Mason et al. 1996). CD34⁺ CD38⁻ HSC do not express lineage markers and are almost

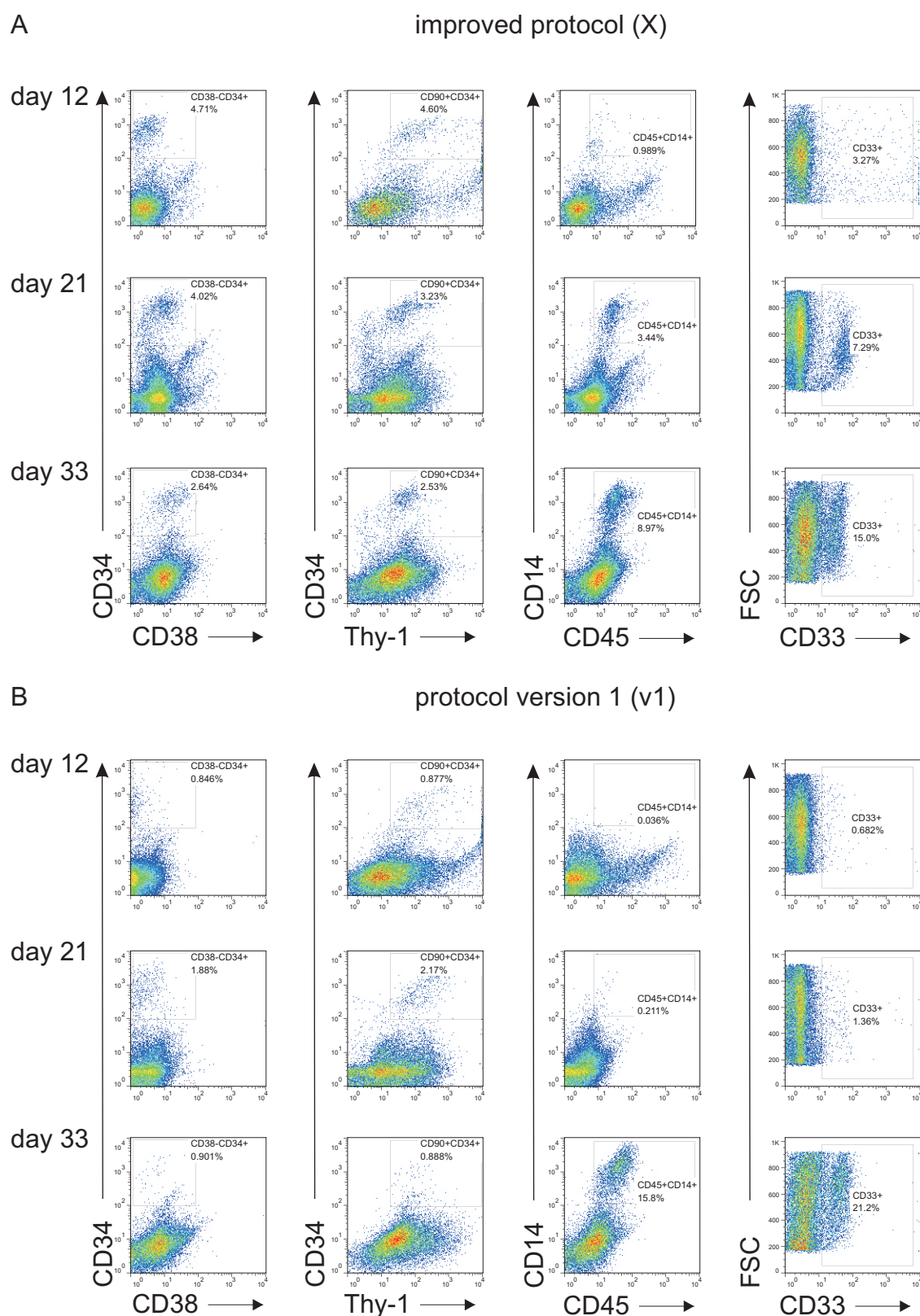
exclusively quiescent (Hao et al. 1995; Bhatia et al. 1997). CD38 ubiquitously is expressed and a multifunctional, glycoprotein, that can catalyse the metabolism of two Ca^{2+} messengers (Lee 2006). Expression of CD38 is associated with increased differentiation (Hao et al. 1995). Co-expression of CD45 and CD34 may also serve as a marker for HSC. CD45 is a glycoprotein involved in regulating development, adhesion, and apoptosis (Penninger et al. 2001). CD45 is expressed on all nucleated hematopoietic cells, however, it is also expressed at high levels on both lymphocytes and monocytes and alternatively can be used as a pan-leukocyte marker (Shah et al. 1988). As such, CD45 co-expression with CD14, which is expressed on non-erythroid myeloid cells (MC and macrophages especially), can be used as a marker for monocyte precursors (Manz et al. 2002; Kuwana et al. 2003; Kodama et al. 2005; Dominici et al. 2006; Pufe et al. 2008). The cell adhesion molecule CD33 is a myeloid cell marker, highly expressed on progenitor cells and monocytes, but weakly expressed on other myeloid cells such as granulocytes and absent on hematopoietic stem cells or lymphoid cells (Andrews et al. 1983; Freeman et al. 1995; Crocker et al. 2001).

To characterize HSC and monocytopoiesis using protocol X (Figure 3.2A), as well as using protocol v1 (Figure 3.2B) in parallel, adherent EB cultures were harvested at 12, 21 and 33 days after spontaneous EB formation and stained with CD34 and CD38 or CD34 and CD90. Cultures were also stained with CD14 and CD45 or CD33. Both protocol X and protocol v1 produced HSC based on the identification of CD34⁺CD38⁻ and CD34⁺CD90⁺ cell populations. The highest proportion of CD34⁺CD38⁻ cell population found in cultures prepared according to protocol X and protocol v1, were 4.71% and 1.88%, respectively. The highest proportion of CD34⁺CD90⁺ cells found in cultures prepared according to protocol X and protocol v1, were 4.60% and 2.17%, respectively. The putative HSC population was consistently higher and present over a greater timespan in protocol X cultures, compared to protocol v-1 cultures.

Figure 3.2

Figure 3.2. Monocytopoiesis characterization.

Time-course analysis of cell surfaces marker expressed during monocytopoiesis. Adherent EBs, cultured according to protocol X (A) or protocol v1 (B), were harvested at 12, 21 and 33 days after spontaneous EB formation and stained for various cell surface markers. Immunofluorescence dot plot analysis of CD38 and CD34; Thy-1 and CD34; CD34 and CD14; CD33, and Forward Scatter (FSC) are shown. Gates were determined by using the relevant isotype control antibodies.



Monocyte precursors present in the cultures were also characterized. Whereas HSC were detected at day 12 in protocol X, Monocyte progenitors were only detected at day 21 (Figure 3.2A). Similarly, there was a delay in detection of monocyte progenitors using protocol v1, though HSC were detected at day 21 and monocyte progenitors at day 33 (Figure 3.2B). At day 21, there were 3.44 % CD45+CD14+ and 7.39 % CD33+ cells in the protocol X culture, whereas only 0.21 % CD45+CD14+ and 1.36 % CD33+ cells were present in the protocol v1 culture. By day 33 both protocol X and v1 cultures contained a substantial proportion of monocyte progenitors.

Overall, these data indicate that both protocol X and protocol v1 produce cultures which contain both putative HSC and monocyte progenitor cells. X-cultures undergoing monocytopoiesis contain a higher proportion of haematopoietic precursor cells which is in agreement with the increased esMC yield that was achieved using protocol X.

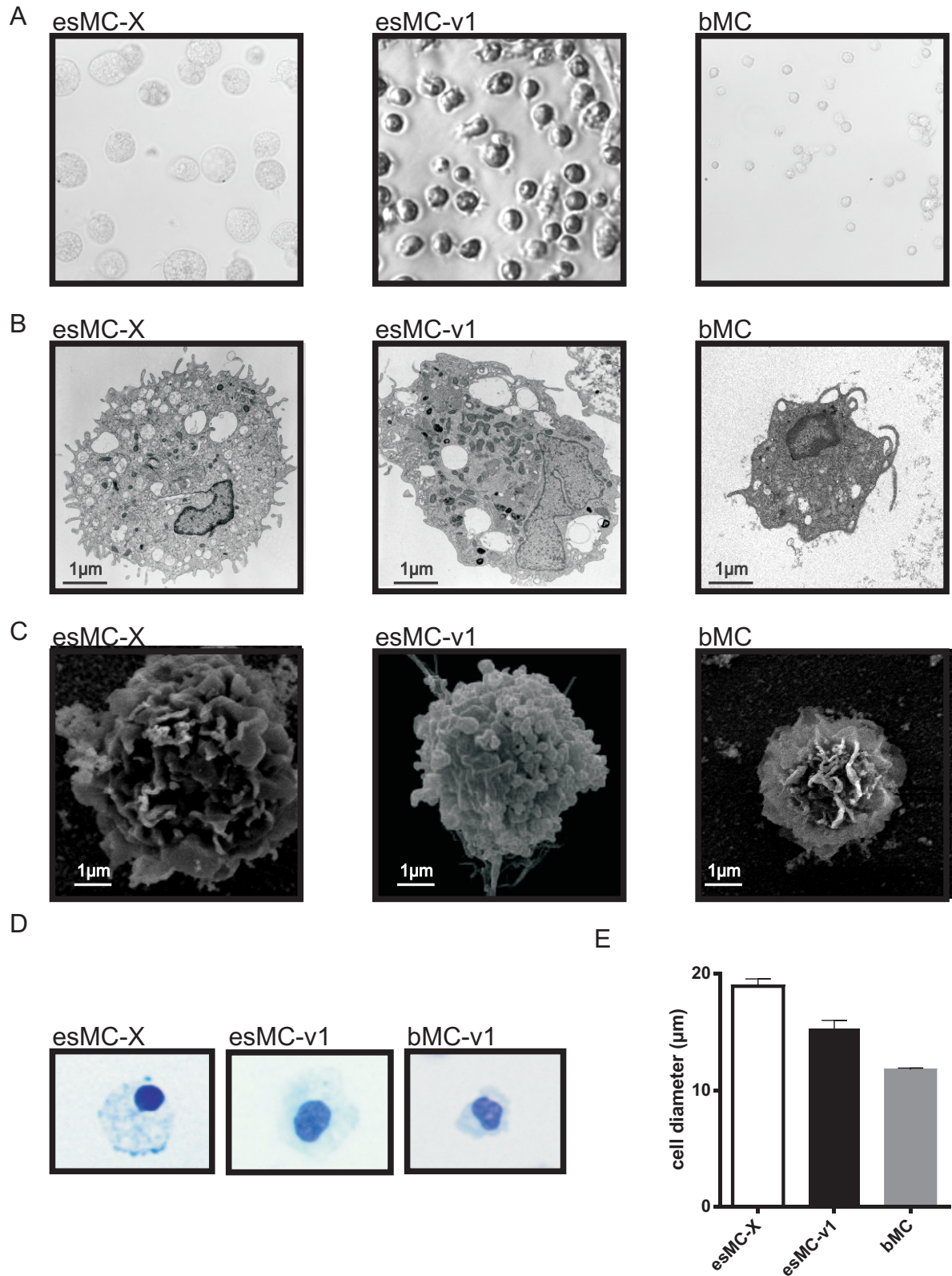
3.3 esMC morphology

Having shown the improved production of esMC-X compared to esMC-v1, the morphology of monocytes was assessed and compared to bMC (Figure 3.3). All esMC had a single indented nucleus and a high cytoplasm-to-nucleus ratio. The voluminous cytoplasm of the monocytes contained various organelles and was surrounded by a ruffled membrane. All monocytes contained vesicles, but these were particularly large in esMC-X accounting for their relatively large diameter (mean $\pm$ SEM, esMC-X: 18.9 ± 0.70 ; esMC-v1: 15.3 ± 0.61 ; bMC: 11.8 ± 0.14). Despite the large number of vesicles, esMC were viable, as shown in Figure 3.1. Eosin-methylene blue staining showed that the population of stem cell-derived cells had typical monocyte morphology and was largely free of other myeloid cells. In summary, analysis of MC morphology confirmed that the improved protocol X produces high yields of a pure monocyte population.

Figure 3.3

Figure 3.3. esMC morphology.

Morphology of stem-cell derived monocytes prepared according to the improved protocol X (esMC-X) or original protocol v1 (esMC-v1) or monocytes derived from blood (bMC). A) Light microscopy images (40x magnification; esMC-X and b-MC brightfield; esMC-v1 phase-contrast). B) Scanning electron microscopy images. C) Transmission electron microscopy images. D) Eosin-methylene blue staining of MC from cytopins. E) Diameter of esMC-X (n=7), esMC-v1 (n=7) and (bMC, n=3) were determined by an AO-DAPI stain (Chemometec). Bars represent the mean diameter $\pm$ SEM.



3.4 esMC phenotype

MC express a range of different proteins which can be used to characterize the MC phenotype and validate protocol X. The CD14, CD16, CD163, CD86 and MHCII receptor expression profile of esMC-X was assessed and compared to esMC-v1 and bMC (Figure 3.4).

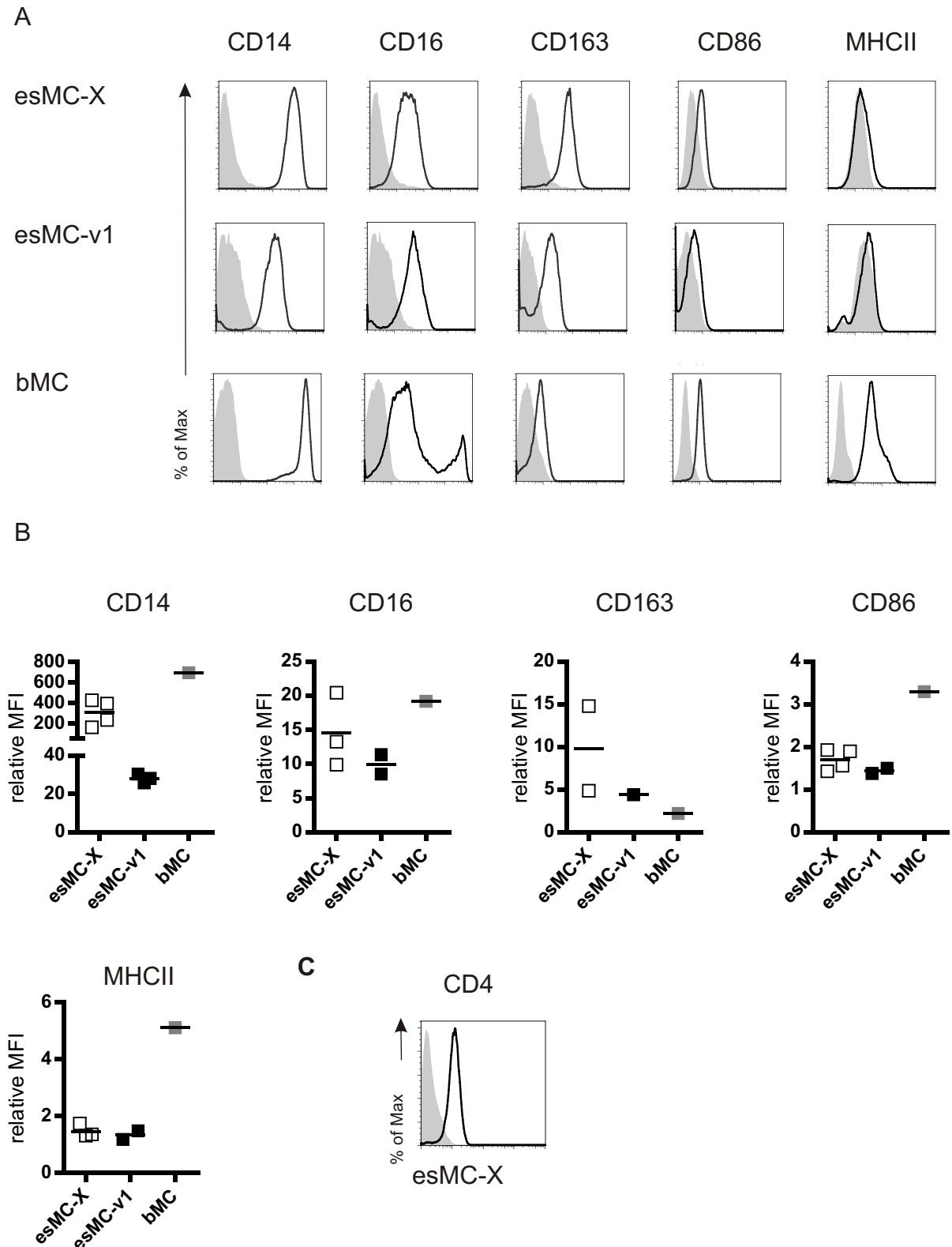
CD14 is a glycoprotein that is characteristically expressed on MC and MDM, but is also present at lower levels on neutrophils, granulocytes and various DC populations (Schutt 1999; Grage-Griebenow et al. 2001). MC CD14 expression levels depend on the cell maturation, as well as activation state, and can be characterized as either high or low, which corresponds to two distinct monocyte populations: major bMC and minor bMC, respectively (Passlick et al. 1989; Ziegler-Heitbrock 2007). CD14 is part of the receptor for LPS, as well as the LPS-binding protein, and functions to enhance cell activation and to initiate cytokine and chemokine production. CD14 was expressed at high levels on esMC-X, similar to the major bMC. Although CD14 was also expressed on esMC-v1, expression levels were lower compared to esMC-X and bMC.

CD16 (FcyRIII) is a glycoprotein that is expressed in two isoforms: CD16A and CD16B (Ravetch et al. 1989; Selvaraj et al. 1989). CD16A is anchored through a transmembrane domain, whereas CD16B is GPI-anchored. CD16A is expressed by MC and MDM as well as NK cells. CD16B is expressed by neutrophils. CD16 expression levels can be characterized as being either high or low, corresponding to the minor bMC and major bMC, respectively (Passlick et al. 1989). CD16 binds to the Fc portion of IgG and acts as the low affinity IgG receptor. CD16 receptor binding can lead to antibody-dependent cell-mediated cytotoxicity, phagocytosis, release of inflammatory mediators and cytokine production (Ravetch et al. 2001). CD16 was expressed at low levels on esMC-X, similar to major bMC and esMC-v1.

Figure 3.4

Figure 3.4. esMC phenotype.

Phenotype of stem-cell derived monocytes prepared according to the improved protocol X (esMC-X) or original protocol v1 (esMC-v1) or monocytes derived from blood (bMC). Surface expression of CD14, CD16, CD163 and CD86 were measured by flow cytometry. A) Histograms represent surface staining (black line) compared to the isotype control (shaded gray). B) The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control. C) CD4 staining on esMC-X.



CD163 is predominantly expressed on MC and MDM and is a member of the scavenger receptor cysteine-rich family of proteins (Hogger et al. 1998). CD163 plays a homeostatic role in clearing haemoglobin, but can also regulate cytokine production in MDM (Kristiansen et al. 2001). CD163 was expressed on all monocytes

CD86 is a glycoprotein expressed on myeloid cells and upregulated on activated macrophages and dendritic cells. It is the natural ligand for the T cell co-stimulation receptor CD28 (Freedman et al. 1991). MHCII is expressed on antigen presenting cells, such as MDM, and mediates the presentation of exogenously derived peptides to T cells (Schulze et al. 2012). CD86 and MHCII were expressed on all monocytes, though at lower levels in esMC compared to bMC.

As in later experiments esMDM-X would be used to study CD4 distribution in the context of HIV-1 infection. CD4 expression on esMC-X was confirmed (Figure 3C).

In summary, analysis of the receptor expression profile of esMC-X showed that the improved protocol X produces MC that are phenotypically similar to bMC and esMC-v1.

3.5 esMDM morphology

In vivo, MC account for 5-10 % of peripheral blood leukocytes in the blood and circulate through the blood before migrating into other tissues where the MC can differentiate into resident macrophages, according to the mononuclear phagocyte system (van Furth et al. 1972). M-CSF is an essential cytokine that is responsible for regulating monocyte survival and differentiation into tissue macrophages (Brugger et al. 1991; Pixley et al. 2004; Liu et al. 2008; Irvine et al. 2009). *In vitro*, the most common method for differentiating MDM is achieved by plating MC on tissue-culture treated plastic in the presence of M-CSF for 7 days (Becker et al. 1987; Barreda et al. 2004). Comparisons of *in vivo* and *in vitro* MDM suggest that *in vitro* MDM are a relevant model for tissue MDM (Xu et al. 2007).

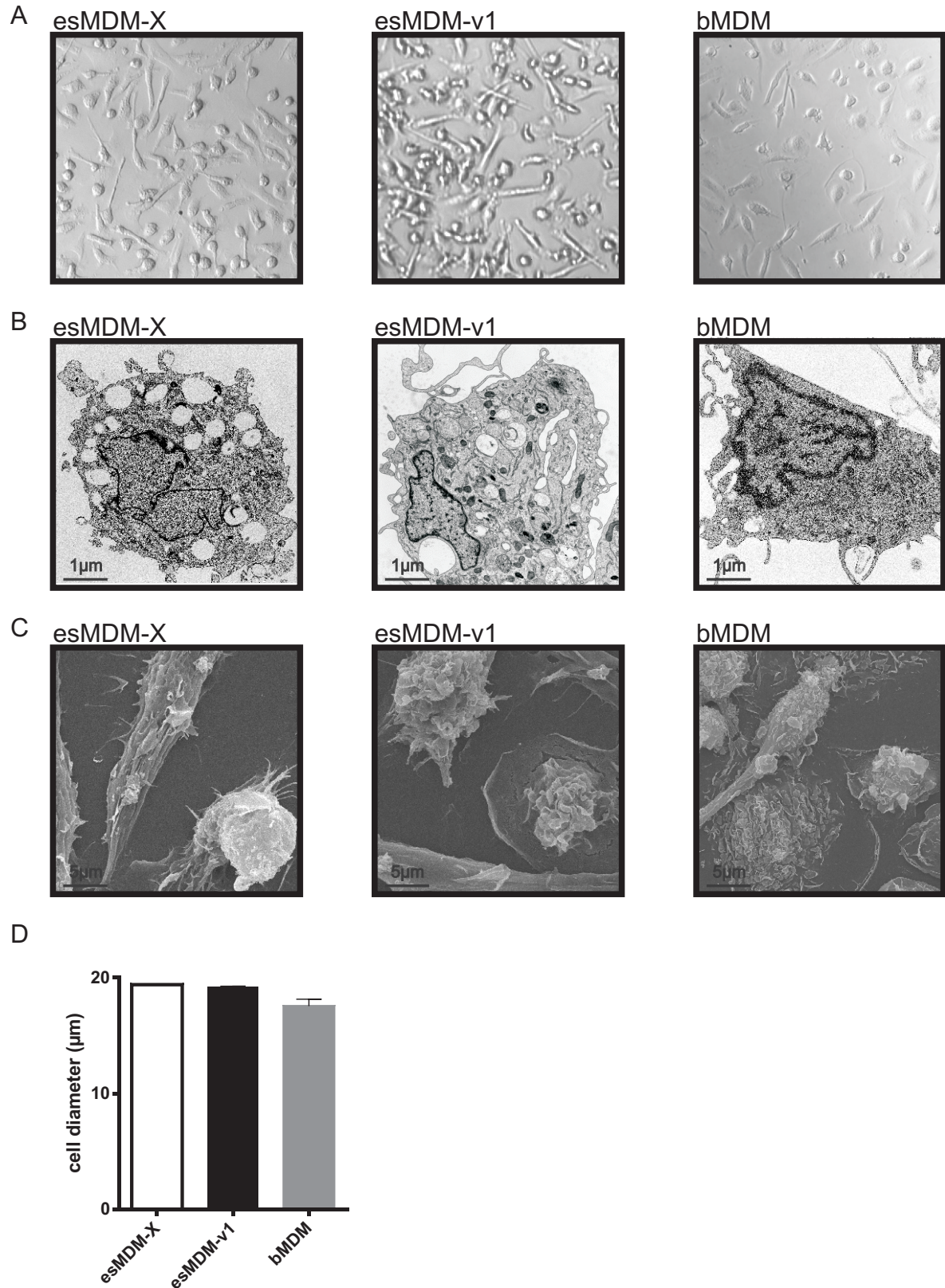
To test whether esMC-X can differentiate into MDM similar to esMC-v1 and bMC differentiation, monocytes were harvested and differentiated for 7 days in the presence of M-CSF and the morphology of monocytes were assessed and compared to bMDM (Figure 3.5). esMC-X differentiated into an adherent esMDM-X population with characteristic membranes blebs and ruffles, similar to esMDM-v1 and bMDM. MDM were elongated (spindle-shaped) or round-shaped. These two morphologically distinct MDM are also known as M- and G-MDM (Young et al. 1990; Hashimoto et al. 1999; Akagawa et al. 2006). The cytoplasm of esMDM-X were particularly abundant in vesicles, although the diameter of esMDM-X was similar to bMDM (mean $\pm$ SEM, esMDM-X: 19.4 ± 0.06 ; esMDM-v1: 19.2 ± 0.03 ; bMDM: 17.6 ± 0.60).

In summary, analysis of MDM morphology confirmed that the improved protocol X produces high yields of a pure monocytes population that can differentiate into MDM that are morphologically similar to bMDM and esMDM-v1.

Figure 3.5

Figure 3.5. esMDM morphology.

Morphology of stem-cell derived macrophages prepared according to the improved protocol X (esMDM-X) or original protocol v1 (esMDM-v1) or macrophages derived from blood (bMDM). A) Light microscopy images (20x magnification). B) Scanning electron microscopy images. C) Transmission electron microscopy images. D) Diameter of esMDM-X (n=3), esMDM-v1 (n=3) and (bMDM, n=6) were determined by an AO-DAPI stain (Chemometec). Bars represent the mean diameter $\pm$ SEM.



3.6 esMDM phenotype

To further characterize esMDM-X, the expression of surface receptors of esMDM-X was assessed and compared to the surface receptor expression of esMDM-v1 and bMDM-1 (Figure 3.6).

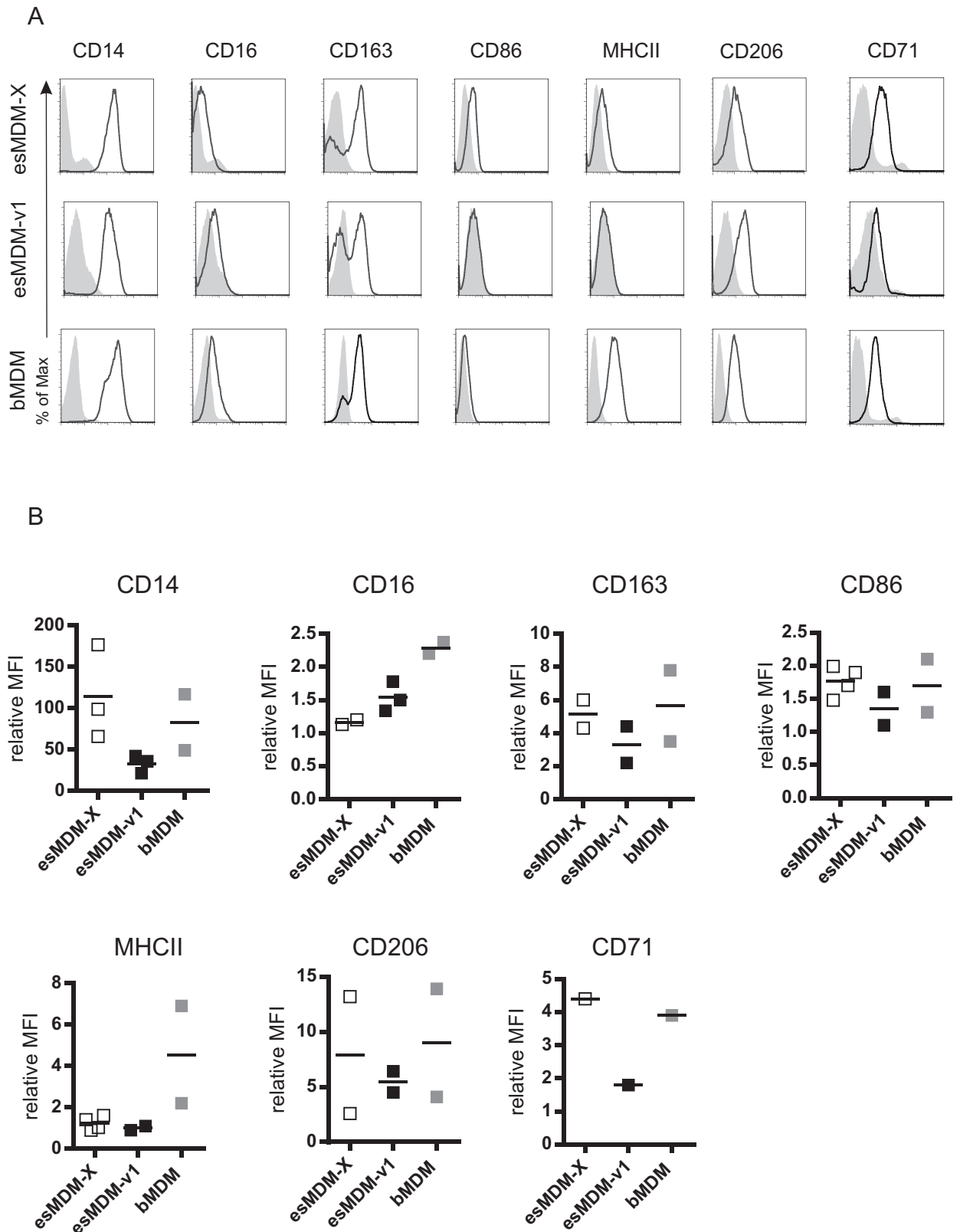
The LPS receptor CD14 and the IgG receptor CD16 were expressed on esMDM-X, similar to bMDM and esMDM-v1. MDM had reduced CD14 and CD16 expression levels compared to MC, although CD14 expression levels were still high. The scavenger receptor CD163 was expressed on esMDM-X, similar to esMDM-v1 and bMDM. CD163 is a suitable marker to distinguish between M-type and G-type MDM, as it is expressed on M-type MDM, but not on G-type MDM (Verreck et al. 2004; Verreck et al. 2006). This expression pattern corresponds with the suggested functional differences between the two MDM types. M-type MDM are thought to be anti-inflammatory and/or regulatory and the involvement of CD163 to clear haemoglobin is thought to reduce inflammation (Graversen et al. 2002). In contrast, GM-type MDM are characterized by pro-inflammatory functions (Grisotto et al. 2001). MHCII was expressed at low levels on esMDM-X and bMDM. On esMDM-v1, MHCII expression was undetectable, in agreement with previous results, although MHCII can be upregulated on esMDM-v1, as well as esMDM-X, upon activation (Dr. K Karlsson, thesis 2008 and data not shown).

The co-stimulatory molecule CD86 was downregulated upon differentiation of MDM, irrespective of monocyte origin (hESC or blood) and protocol (v1 or X). CD206, also known as the Mannose Receptor, is a C-type lectin carbohydrate binding protein, expressed on MDM and DC, that is involved with pathogen recognition and homeostasis (Linehan et al. 2000). CD206 was expressed on the surface of esMDM-X, esMDM-v1 and bMDM. CD206 expression was down-regulated upon classical activation with LPS and IFN γ and up-regulated upon alternative activation with IL-4 in all MDM (data not

Figure 3.6

Figure 3.6. esMDM phenotype.

Phenotype of stem-cell derived macrophages prepared according to the improved protocol X (esMDM-X) or original protocol v1 (esMDM-v1) or macrophages derived from blood (bMDM). Surface expression of CD14, CD16, CD163, CD86, MHCII, CD206 and CD71 were measured by flow cytometry. A) Histograms represent surface staining (black line) compared to isotype control (shaded gray). B) The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control of independent experiments.



shown). CD71, also known as the transferrin receptor, is a membrane glycoprotein that binds the plasma protein transferrin and mediates the cellular uptake of iron from transferrin into the cell (Harel et al. 2011). CD71 is expressed on all cell types and are elevated on activated MDM (Tacchini et al. 2008). As such, it is surprising that *Karlsson* found no CD71 expression on esMDM-v1 (Dr. K Karlsson, thesis 2008). Using a different monoclonal antibody to detect CD71, low levels of CD71 were detected on esMDM-v1. Compares to esMDM-v1, higher levels of CD71 were detected on esMDM-X and bMDM. CD71 expression was up-regulated upon classical activation on all MDM (data not shown).

In summary, the surface expression profile of MDM differentiated according to protocol X indicated that esMDM-X are phenotypically similar to bMDM.

3.7 esMDM function

It has previously been shown that esMDM-v1 are functionally similar to bMDM: esMDM-v1 can be activated, secrete chemokines and cytokines, endocytose through scavenger receptors, phagocytose yeast particles, recognise LPS, and suppress proliferating PBL and T-cells (Karlsson et al. 2008; Cowley SA 2012). Since protocol X for the production of esMDM is a modification of protocol v1, the functionality of esMDM-X is expected to be similar to esMDM-v1 and bMDM. Nonetheless, to confirm esMDM-X function, two key functional features shared by MDM *in vivo* and *in vitro* were assessed (Gordon et al. 2005). Firstly, the cytokine profile upon activation of MDM was analysed, and secondly, MDM were assayed for their ability to phagocytose yeast particles. These key features (cytokine/chemokine secretion and phagocytosis) are characteristic for all tissue resident MDM, despite bMDM heterogeneity caused by factors such as tissue distribution and exposure to various stimuli.

The first key feature, secretion of cytokines and chemokines, is used by MDM to modulate the immune response and mediate MDM immune functions, such as pathogen clearance and wound healing (Mantovani et al. 2004). Different cytokines and chemokines are upregulated depending on the type activation stimulus. Classic activation can be induced *in vitro* by IFN- γ and a further microbial stimulus like LPS, leading to predominantly increased pro-inflammatory cytokine and chemokine production and triggers a cellular immune response (Mosser 2003; Gordon et al. 2005). To assess the cytokine and chemokine profile in resting esMDM-X and test whether the esMDM-X could be classically activated, the expression of 36 different cytokines in resting (Figure 3.7A, top) and classically activated (Figure 3.7A, bottom) MDM-X were measured using a membrane antibody human cytokine array and a chemiluminescent substrate. Similarly, cytokine expression profiles for esMDM-v1 and in bMDM were assessed. Resting esMDM-X had

remarkably similar profile of cytokine production as esMDM-v1 and bMDM (Figure 3.7B left panel). Although, unlike bMDM, esMDM-X also expressed low levels of I-309 (chemoattractant) and did not express Serpin E1 (protease inhibitor). As expected, cytokine secretion was upregulated upon classical MDM activation (Mantovani et al. 2004). As in resting MDM, the pattern of production was very similar between esMDM-X and esMDM-v1 or bMDM (Figure 3.7B right panel), but distinct from expression profiles reported for other cell types, such as T-cells (Schade et al. 2008; Wang et al. 2011). All MDM upregulated mostly the same cytokines, albeit at variable levels. Exceptions included IL-10 (anti-inflammatory), which was secreted by bMDM, but not by esMDM-v1 and esMDM-X. This is in contrast to previous findings by *Karlsson et al.* who found particularly high levels of IL-10 secretion in activated esMDM-v1 using a tailored multiplex cytokine assay system (Bio-Rad Laboratories Hercules, CA), presumably detecting a different isoform (de Jager et al. 2003; Karlsson et al. 2008). Secretion of I-309 and Serpin E1 were dissimilar between in the three forms of resting MDM, but similar in activated MDM. As expected, IFN- γ , which was used to activate the cells, was detected in the supernatant by all activated MDM, but not by any of the resting MDM. TNF- α (pro-inflammatory) was secreted by the activated MDM. TNF- α is expressed predominantly by classically activated MDM and induced by LPS, which was also used to activate cells. Other key pro-inflammatory cytokines were also upregulated by esMDM-X, such as IL-1 β (unexpectedly, this was, not expressed by bMDM), and IL-6 (Duffield 2003; Gordon 2003; Mosser 2003). As expected, esMDM-X upregulated key chemokines involved in attracting neutrophils, immature dendritic cells, natural killer cells, and activated T cells, such as IL-8, IP-10, MIP-1 α , MIP-1 β and RANTES (Luster 2002). Overall, the results provide evidence of the functional similarity of esMDM-X to esMDM-v1 and bMDM regarding cytokine production and response to classic activating ligands.

Figure 3.7

Figure 3.7. esMDM function.

Functional analysis of stem-cell derived macrophages prepared according to the improved protocol X (esMDM-X) or original protocol v1 (esMDM-v1) or macrophages derived from blood (bMDM). A+B) The presence of secreted cytokines from MDM was detected using a Proteome Profiler Human Cytokine Array Kit, Panel A from R&D systems. Pixel density was quantified using Gene Tools software and normalised to the pixel density of the positive control spots. A) Representative film following development. The table shows the position of each cytokine on the array (not to scale).

A



PC				PC
PC				PC
RANTES	IL-32α	IL-10	IL-1α	C5a
RANTES	IL-32α	IL-10	IL-1α	C5a
SDF-1	IP-10	IL-12 p70	IL-1β	CD40L
SDF-1	IP-10	IL-12 p70	IL-1β	CD40L
TNF-α	I-TAC	IL-13	IL-1ra	G-CSF
TNF-α	I-TAC	IL-13	IL-1ra	G-CSF
sTREM-1	MCP-1	IL-16	IL-2	GM-CSF
sTREM-1	MCP-1	IL-16	IL-2	GM-CSF
	MIF	IL-17	IL-4	GROα
	MIF	IL-17	IL-4	GROα
	MIP-1α	IL-17E	IL-5	I309
	MIP-1α	IL-17E	IL-5	I309
	MIP-1β	IL-23	IL-6	sICAM-1
	MIP-1β	IL-23	IL-6	sICAM-1
	Serpin E1	IL-27	IL-8	IFNγ
	Serpin E1	IL-27	IL-8	IFNγ
NC				PC
NC				PC

PC = positive control NC = negative control

Figure 3.7. (continued) esMDM function.

B

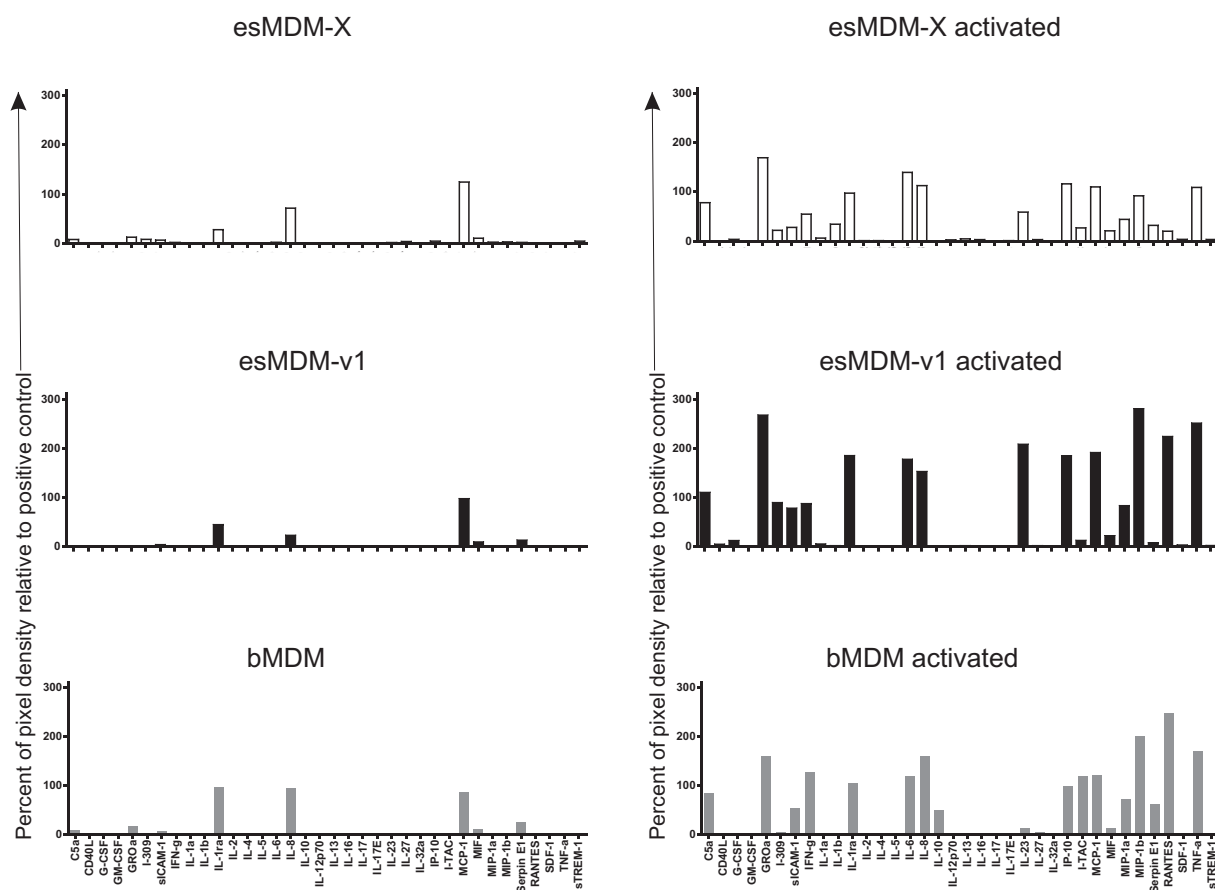
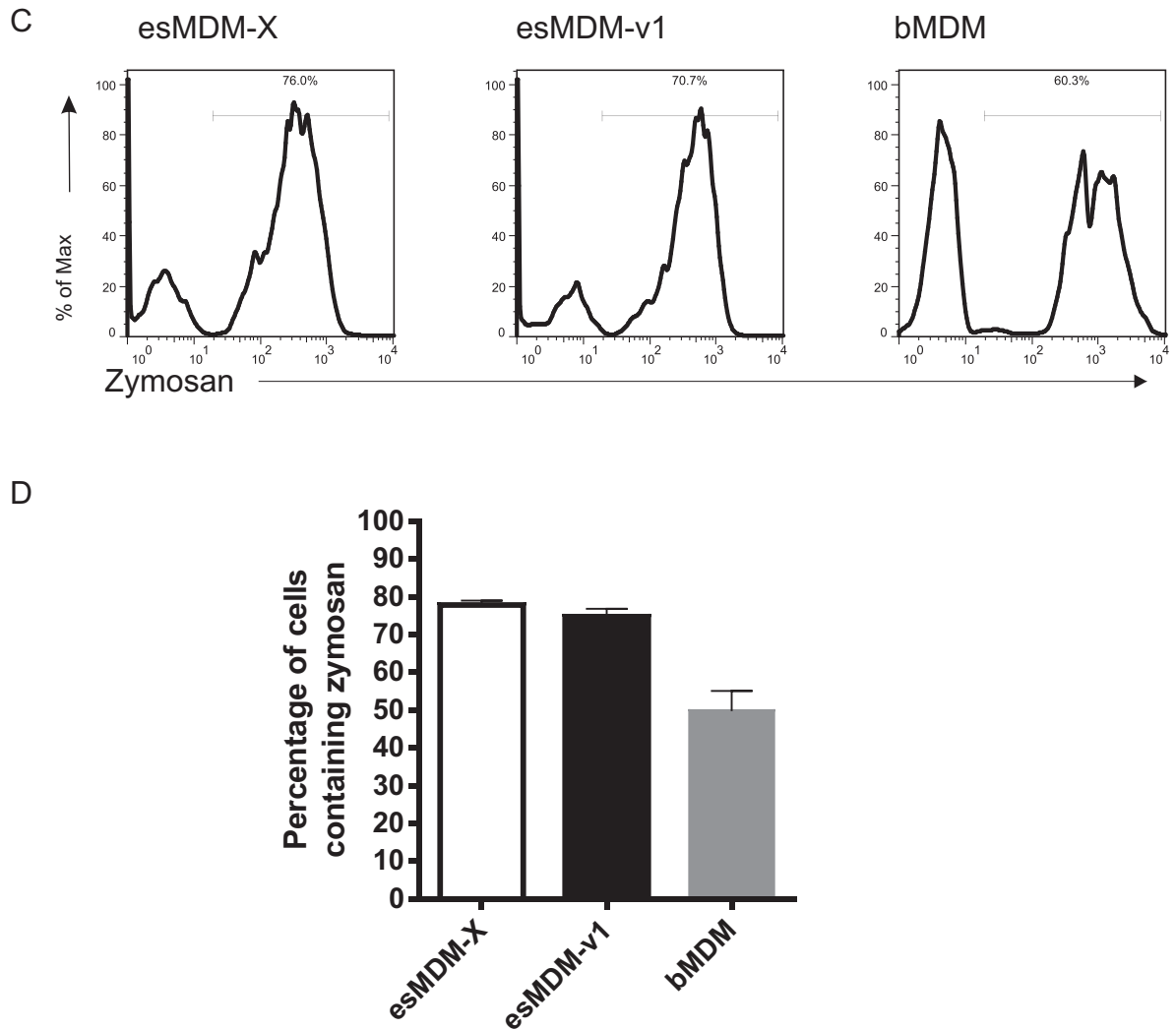


Figure 3.7

Figure 3.7. (continued) esMDM function.

C+D) Phagocytosis of fluorescent zymosan particles by MDM. C) Representative histogram showing percentage of zymosan-positive cells. D) Bars represent the the mean percentage of zymosan-positive cells of three replicate wells for esMDM and from 7 donors from bMDM.



The second key feature of MDM (phagocytosis) is used by MDM to internalize and clear pathogens, infected cells or apoptotic cells and elicits an adequate immune response (Aderem et al. 1999). MDM (as well as neutrophils) are particularly efficient in phagocytosis and as such are also known as 'professional phagocytes'. A range of receptors, such as dectin-1, CD206, complement receptors and Fc receptors can stimulate phagocytosis (Aderem et al. 1999; Brown et al. 2002). Dectin-1 is a pattern-recognition receptor and the predominant receptor involved in phagocytosis of zymosan (Brown et al. 2002). Zymosan is a yeast-derived particle (*Saccharomyces cerevisiae*) consisting of protein-carbohydrate complexes, widely used to study phagocytosis.

Fluorescent zymosan particles were used to assess the phagocytic capacity of esMDM-X, esMDM-v1 and bMDM. The MDM were incubated with zymosan particles for 30 min at 37 °C, surface-bound zymosan was quenched with trypan blue, and the percentage of cells that had taken up zymosan was quantified by flow cytometry (Figure 3.7 C+D). esMDM were highly phagocytic, with an average (mean $\pm$ SEM) of 77.8 % $\pm$ 1.2 and 74.8 % $\pm$ 2.1 of esMDM-X and esMDM-v1, respectively, phagocytising at least one particle, compared to 49.8 % $\pm$ 5.3 of bMDM. These results indicate that esMDM-X are capable phagocytosis.

All the results presented so far suggest that esMDM-X are functionally similar to esMDM-v1 and to bMDM, suggesting that protocol X could be used as a more efficient, representative *in vitro* model to study MC and MDM functions.

3.8 HIV-1 infection of esMDM

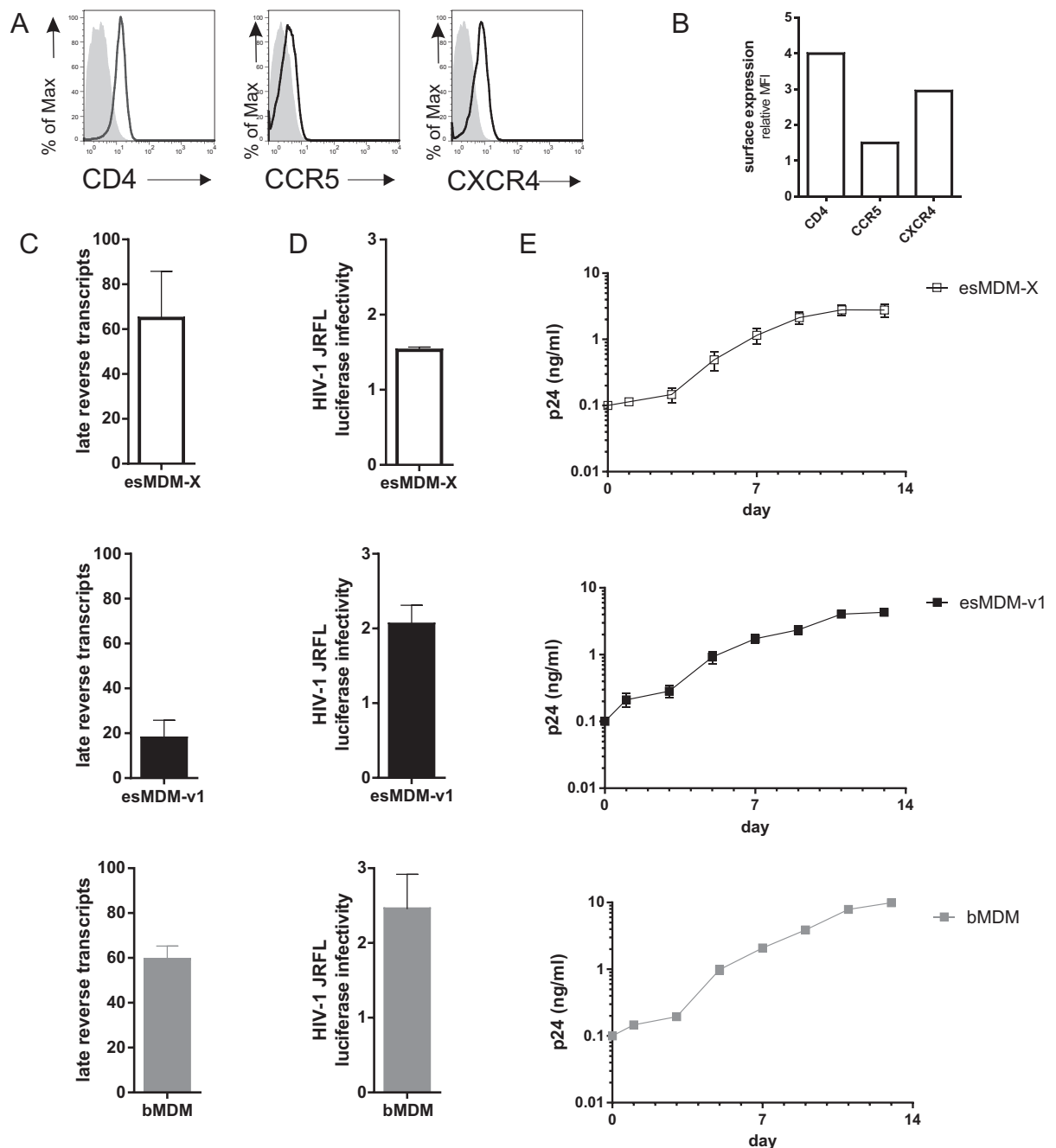
In order to confirm that esMDM-X can be used as an accurate *in vitro* model to study HIV-1 infection of MDM, HIV-1 infection and replication kinetics were tested. HIV-1 infection requires CD4 expression, which functions as its main receptor. Depending on virus tropism, CCR5 or CXCR4 is required as the HIV-1 co-receptor. MDM are preferentially infected by R5-tropic viruses (Di Marzio et al. 1998; Schmidtmayerova et al. 1998; Bakri et al. 2001). Firstly, CD4, CCR5 and CXCR4 expression on esMDM-X was confirmed by flow cytometry (Figure 3.8A+B). Next, HIV-1 entry was assessed using two single cycle assays: “qPCR of HIV-1 late reverse transcripts” (Figure 3.8C) and “HIV-1 integration (luciferase)” (Figure 3.8D). Finally, HIV-1 replication was measured by “quantification of p24 antigen by ELISA” (Figure 3.8E). All three HIV-1 assays demonstrated that esMDM-X (Figure 3C-E, top) are permissive to HIV-1 infection and allow HIV-1 replication, like esMDM-v1 (Figure 3C-E, middle) and bMDM (Figure 3C-E, bottom). HIV-1 replication kinetics in esMDM-v1 have previously been extensively characterized and also found to be very similar to HIV-1 infection in bMDM (Cowley SA 2012).

Taking all results together, esMDM-X offer the most efficient and representative *in vitro* model to study MC and MDM functions, as well as, MDM HIV-1 infection.

Figure 3.8

Figure 3.8. HIV-1 infection of esMDM.

HIV-1 infection of stem-cell derived macrophages differentiated according to the improved protocol (esMDM-X) or protocol version 1 (esMDM-v1) and blood-derived macrophages (bMDM) were assessed. A+B) Surface expression of the HIV-1 (co-)receptors CD4, CCR5 and CXCR4 were measured by flow cytometry. A) Histograms represent surface staining (black line) compared to isotype control (shaded gray). B) Bars reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control. C) Reverse transcription of HIV-1 was measured by detecting late (pol) products by qPCR after 30 h of infection. Symbols represent the relative mean number of copies of HIV-1 DNA $\pm$ SEM of independent experiments (esMDM-X, n=3; esMDM-v1, n=5; bMDM, n=4), normalised to the number of cells using a β -actin control. D) HIV-1 integration was measured by detecting luciferase activity in MDM infected with HIV-1 NL4.3.Luc.R-E- virus pseudotyped with JRFL or VSV-g envelope. Symbols represent the relative mean luciferase detection of JRFL infected cells normalised to VSV-g infected cells $\pm$ SEM of independent experiments (esMDM-X, n=3; esMDM-v1, n=3; bMDM, n=12). E) HIV-1 replication in esMDM was measured by p24 ELISA. Symbols represent the mean p24 concentration at different time points $\pm$ SEM of independent experiments (esMDM-X, n=3; esMDM-v1, n=4; bMDM, n=2).



3.9 esMDM can be genetically modified

To further explore the usefulness of esMDM-X to study MDM biology and pathogen interactions using a genetic approach, transgene expression in esMDM-X was tested. Lentiviral transduction, as well as DNA and RNA transfection, are inefficient in MDM, whereas sustained transgene expression can be achieved in hESC using lentiviral vectors (Salmon et al. 2000; Ma et al. 2003; Ben-Dor et al. 2006). This relatively easy method of gene-delivery allows stable vector integration into the genome, so that transgenic hESC can then be differentiated into transgenic esMDM.

Effective transgene expression in hESC has previously been achieved using a second-generation, self-inactivating (SIN) lentiviral vector, using the elongation factor-1 α (EF1) promoter to control high-level gene expression in hESC and human hematopoietic progenitors cells (Salmon et al. 2000; Gropp et al. 2003; Ben-Dor et al. 2006). Previous published studies, supported by findings in our laboratory, showed that antibiotic selection, for example through an IRES-Puromycin cassette, is required to maintain transgene expression and prevent transgene silencing (Yao et al. 2004; Ben-Dor et al. 2006; Bernstein et al. 2006; Cowley SA 2012)(Ahmed Salman Bodla, thesis under preparation). An internal ribosome entry site (IRES) allows the coordinate expression of two genes, such as a transgene and puromycin^R (Puro^R) from a single mRNA, so expression of one of the genes should ensure expression of the other. IRES is a nucleotide sequence commonly found in RNA viruses that allows translation in a cap-independent manner (Adam et al. 1991; Mokrejs et al. 2006). Optimal transgene expression is achieved when the transgene is located upstream rather than downstream of the selectable marker (Ben-Dor et al. 2006). Based on the above findings, a second generation, SIN lentiviral vector, referred to as pEF1-GOI-IRES-Puro^R ("control P") was designed and used to test transgene (Gene Of Interest, GOI) expression in esMDM-X

(see Appendix II for cloning details). To easily monitor transgene expression, the RFP gene was cloned into this SIN lentiviral vector, downstream of the EF1 α promoter (in the place of the GOI), resulting in a plasmid referred to as pEF1-RFP-IRES-Puro^R (see Appendix for cloning details). A schematic diagram of the design for the RFP vector is shown in Figure 3.9A and a map is shown in Figure 3.9B.

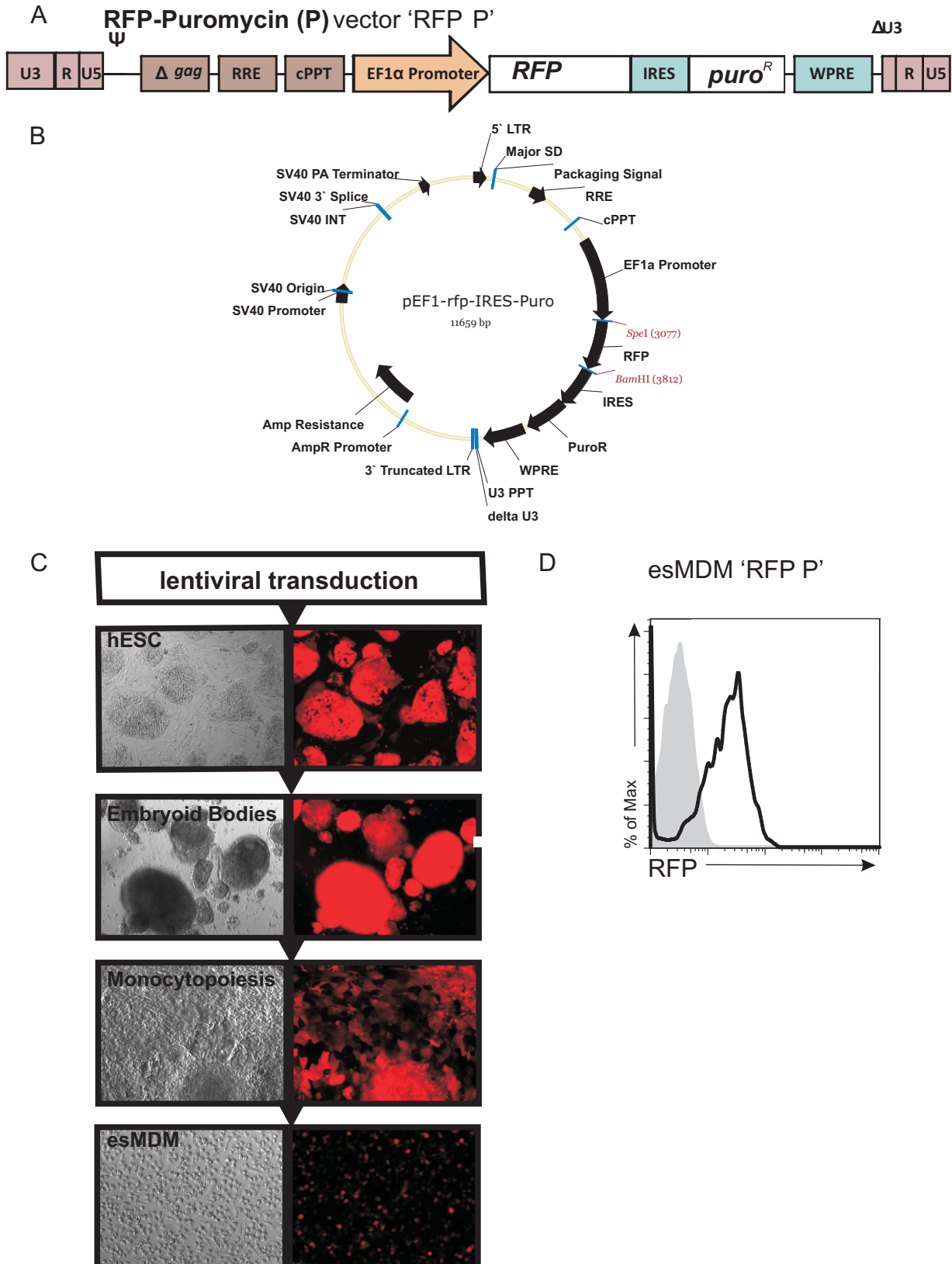
In order to produce RFP-encoding lentiviruses (LV), 293T cells were transfected with the pEF1-RFP-IRES-Puro^R along with a packaging plasmid and an envelope plasmid. The supernatant, containing newly produced LV were harvested 48 h post-transfection. hESC were transduced with LV-EF1-RFP-IRES-Puro^R and differentiated into esMDM, according to protocol X. Cells were kept under continuous puromycin selection (5 μ g/mL: a concentration at which untransduced cells died), as such selecting a pure population of cells containing the lentiviral vector. Fluorescent microscopy analysis showed RFP expression of hESC, of EBs, during monocytopoiesis and in esMDM (Figure 3.9C). Flow cytometric analysis of RFP expression confirmed that a pure population RFP expressing esMDM was obtained (Figure 3.9D).

These results demonstrate that a transgene can be expressed in esMDM-X and that esMDM-X can be used to study genetically modified MDM.

Figure 3.9

Figure 3.9. esMDM can be genetically modified.

A) Schematic diagrams of a self-inactivating lentiviral construct used to express *RFP* and *puromycin^R* (RFP⁺ P). B) Plasmid map. C) hESC were transduced with the lentiviral vector 'RFP⁺ P' to generate genetically modified, RFP positive, hESC-derived MDM according to protocol X. Light and fluorescent microscopy images of transduced hESC (10x), EB (10x), differentiating EB (20x) and esMDM (10x) are shown. D) RFP expression was measured by flow cytometry. The histogram represents RFP expression by esMDM differentiated from hESC transduced with 'RFP⁺ P' (black line) or control esMDM (shaded gray).



3.10 Summary and discussion

In this chapter the method to produce MDM from hESC was successfully optimized. Higher, as well as, more consistent yields of homogenous, viable esMC were produced over a longer period of time. esMDM differentiated according to the improved protocol X were similar to bMDM. Successful HIV-1 infection and genetic modification of these esMDM demonstrated that protocol X is the most efficient representative *in vitro* model to study MC and MDM functions and HIV-1 infection.

Expression of surface antigens during differentiation was used to confirm the differentiation of hESC into MC. CD34⁺, Thy1⁺ and CD38⁻ were used as markers for HSC. The HSC population was detectable at all time points in cultures prepared according to protocol X, but was transient in cultures prepared according to protocol v1. To further characterize HSC progenitors during monocytopoiesis and compare protocol X and protocol v1, cultures could be harvested at later time points, at which esMC-X are still being produced, but esMC-v1 are not. The percentage of CD34⁺CD38⁻ cells present in the cultures were similar to the CD34⁺ population found *in vivo* in the bone marrow, cord blood and fetal liver, where it forms 0.5-5% of all hematopoietic cells (Krause et al. 1996; Kondo et al. 2003). Also in mice, where CD34 was first identified as an HSC marker, CD34 is expressed on less than 5% of all blood cells (Civin et al. 1984). CD33 expression or CD45 and CD14 expression were used to identify monocyte precursor cells, and suggested that these cells were present in both cultures. The cell populations expressing monocyte precursor markers constituted up to 15% of the protocol X cultures. In addition to myeloid cells, earlier HSC progenitors also express CD45. Accordingly a CD14-CD45⁺ cell population was present early during differentiation. Although the cell cultures contained monocyte-precursors and HSC cell populations, other cell population were present as well. These likely represent supporting tissue that is spontaneously formed

during differentiation, such as stromal cells that originate from mesoderm (Friedenstein et al. 1976; Caplan 1991). Although CD34⁺, Thy1⁺ and CD38⁻ were used as markers for HSC, Thy1⁻ cell populations may also have extensive self-renewal potential (Notta et al., 2011). Interestingly, a small population of CD34⁺Thy1⁻ cells were present in protocol X cultures, but not in protocol v1 cultures. To further delineate HSC, expression of the integrin CD49f has recently been identified as an additional HSC marker (Notta et al. 2011; Yu et al. 2012). Furthermore, the HSC population can be subsequently delineated by their characteristically low rhodamine-123 dye retention (McKenzie et al. 2007; Notta et al. 2011). Rhodamine-123 is a mitochondrial dye that binds to active mitochondria, as such the metabolically inactive, quiescent HSC fluoresce dimly (Bertoncello et al. 1988). Identifying multipotent cell populations that have the capacity for self-renewal is currently an important area of investigation with experimental and therapeutic applications. For example, identification and isolation of HSC would allow the generation of long-term multilineage grafts or may improve *in vitro* production of monocytes (Priddle et al. 2006; Notta et al. 2011).

Monocyte differentiation was achieved by culturing EBs with IL-3 and M-CSF. Although IL-3 can support the development of other myeloid cells, including megakaryocytes, erythrocytes, neutrophils, eosinophils and mast cells, M-CSF is specifically required for the differentiation of monocytes (Guilbert et al. 1980; Barreda et al. 2004; Seita et al. 2010; Hudnall 2011). Based on morphological features and CD14 expression, as well as, FSC and SSC properties, a homogenous population of monocytes was obtained. esMC were phenotypically similar to bMC, which were isolated by positive selection for CD14. esMC-X expressed various monocyte markers, including CD14, CD16, CD163 and CD86. Based on their high CD14 expression and low CD16 expression, esMC were similar to the major bMC population, which represent ~85% of blood monocytes and are characterized by higher phagocytic activity (Auffray et al. 2009). esMC could differentiate into functional esMDM, with high phagocytic activity. Flow cytometry analysis of cell surface receptors

showed that esMDM expressed MDM markers, similar to bMDM, although MHC II expression was notably low on esMDM, which may suggest that esMDM resemble immature macrophages (Murphy et al. 1996; Hoeffel et al. 2012; Yona et al. 2013). Recent research based on murine models suggest that the majority of tissue macrophages arise in the embryo, before the development of HSCs and independent of the supply of blood-derived monocytes and the esMDM described here may represent an embryonic stage of development. (Hoeffel et al. 2012; Schulz et al. 2012; Yona et al. 2013). Further work will address the ability of the esMC/esMDM to be directed to differentiate to tissue-specific macrophages, such as microglia and Kupffer cells, by coculture/codifferentiation with neural or hepatic cell types respectively. In spite of the low MHC II expression, esMDM were functional and could be activated in response to LPS and IFN γ , upregulating expected key cytokines and chemokines. Morphological analysis showed that esMDM were round or spindle in shape, with membrane ruffles. Although a large number of vesicles were detected in esMC-X and esMDM-X, consistent with observations by other laboratories, it didn't appear to affect their functional properties compared to esMC-v1, esMDM-v1, bMC or bMDM (Choi et al. 2011; Klimchenko et al. 2011).

The method optimized in this Chapter was based on a robust, simple protocol, as first described by *Karlsson et al.*, which does not require a complex mix of cytokines as used in other published protocols, and has been widely used across multiple hESC and human induced Pluripotent Stem Cell (hiPSC) lines to study MDM function (Karlsson et al. 2008; Zhou et al. 2011; Jerebtsova et al. 2012; Jiang et al. 2012; Panicker et al. 2012). The main difference between the original and improved protocol, is the use of X-VIVOTM 15 during continuous monocytopoiesis. Unlike aDMEM, used in the original protocol, X-VIVOTM 15 is a chemically defined, hematopoietic cell medium. aDMEM cultures require supplementation with Fetal Calf Serum, whereas X-VIVOTM 15 is serum-free. Serum contains unknown factors that influence cell growth and differentiation and shows great

batch-to-batch variability. The use of serum-free and defined media during monocytopoiesis minimizes variability. Despite this improvement, another step in the improved protocol -the differentiation of esMC into esMDM-, as described in this Chapter and used for the experiments in this thesis, does still require the use serum-containing media. However, further optimization to create a serum-free and chemically defined, improved protocol showed that MC can also be successfully differentiated in X-VIVO™ 15 supplemented with M-CSF to derive MDM under serum-free conditions (Appendix I). Nonetheless, animal-derived feeder-cells may also secrete undefined factors and potentially transfer animal pathogens and are a source of variability (Heng et al. 2004; Mallon et al. 2006). Therefore, feeder-free and defined versions of protocol X for the differentiation of esMDM was developed (Appendix I). The defined, serum- and feeder-free versions of the improved protocol, use enzymatic dissociation and centrifugation to create uniform EBs of a defined size. This is a further improvement of protocol X, considering mechanical dissociation, as described in this chapter, generates EBs of varied size resulting in variable production kinetics and esMC yield (Dr. Karlsson, thesis 2008). Accordingly, *Ng et al.* found that the number of hESC that form the EB affects hematopoietic differentiation (Ng et al. 2005). Another advantage of the improved protocol is the potential of esMC-X to differentiate into dendritic cells, as described in Appendix I (Dr. Karlsson, thesis 2008).

The optimized protocol X is currently the preferred protocol in our, as well as in collaborating, laboratories for MDM research. In this Chapter one hESC cell line was used, however, other hESC and hiPSC cell lines can differentiate into functional esMC and esMDM using the improved protocol (unpublished results from our laboratory). Using multiple hESC cell lines can be used to increase the reproducibility and statistical power of the results. The method to derive esMDM according to the improved protocol, together with the lentiviral vector system shown in Figure 3.9, is now routinely used for HIV-1

research and expression of transgenes as well as hairpins in esMDM in our laboratory. In addition, this methodology is used for all the subsequent Chapters of this thesis.

Chapter 4: An effective strategy for knockdown of CD4 in esMDM, resulting in correspondingly reduced levels of HIV-1 infection

Introduction

Macrophages play a central role in HIV-1 immunopathogenesis, by acting as latent reservoirs and by disseminating virus throughout the course of HIV-1 infection (Cassol et al. 2006; Coleman et al. 2009). Many aspects of the molecular biology of HIV-1 infection in macrophages and their role in AIDS disease progression are still unclear and under investigation (Kuroda 2010). However, macrophage HIV-1 studies are limited, because blood monocyte-derived macrophages (bMDM) are a difficult cell-type to study. Also, there are several problems associated with using animal models, as well as with cell lines, for the study HIV-1 infection and host factors in relevant macrophages, as described in more detail in Chapter 1.3.7. For example, macrophages are inherently refractory to genetic manipulation, an important technique for uncovering the basic functions of different genes. In contrast, human macrophages which have been derived simply and efficiently from human embryonic stem cells (hESC) can easily be genetically modified, as demonstrated in Chapter 3.9. hESC-derived MDM (esMDM) are phenotypically and functionally similar to bMDM (see Chapter 3.5-3.7), and most importantly, they accurately replicate the HIV-1 replication kinetics seen in bMDM (see Chapter 3.8). Therefore, an advanced lentiviral-based dual expression system for the genetic modification of human embryonic stem cell derived MDM (esMDM) was developed to further facilitate the study of HIV-1 infection in macrophages. The advanced lentiviral-based dual expression system allows stable and effective knockdown of endogenous genes, as well as expression of exogenous Genes Of Interest (GOI) The usefulness of this system is demonstrated here for the first time by knocking down the main HIV-1 receptor (CD4) in esMDM and measuring the effect on HIV-1 infection.

A major advance in studying host-pathogen interaction and protein function in MDM and other cell types has been by expressing short hairpins (shRNAs) to specifically knock down gene expression. For example, using genome-wide RNA interference (RNAi) screens, almost 1 000 host genes were shown to be involved in the HIV-1 life cycle (Brass et al. 2008; Konig et al. 2008; Zhou et al. 2008; Yeung et al. 2009). Also, a range of host-proteins in MDM that play a role in HIV-1 infection have been identified using proteomic and biochemical analysis (Chertova et al. 2006; Melendez et al.). The role of many of these proteins in HIV-1 infection remains unknown. Protein function can be determined by studying the effects of gene loss, using specifically targeted shRNAs. Moreover, using shRNAs to knockdown HIV-1 or host proteins forms an important strategy for potential RNA-based anti-HIV therapeutics. The concept of this therapeutic approach is to engineer hematopoietic stem cells or progenitor cells with an shRNA to create an HIV-resistant genotype and transplant the engineered cells into patients. For example, knocking down the HIV-1 co-receptors CXCR4 and CCR5 is currently being explored as HIV/AIDS gene therapy (Anderson et al. 2007; DiGiusto et al. 2010; Cannon et al. 2011). HIV itself could also be targeted, though extra measures, such using multiple or second-generation shRNAs need to be taken to address the problem of escape mutants (Das et al. 2004; ter Brake et al. 2008; Schopman et al. 2012; Shah et al. 2012).

shRNAs are artificial hairpins, transcribed under the control of RNA polymerase III (Pol III) and are part of a family of small, non-translated double-stranded RNAs (dsRNA), which guide the sequence-specific silencing of mRNA, in a process called RNA interference (RNAi) (Fire et al. 1998; Brummelkamp et al. 2002; Hannon 2002; Paddison et al. 2002). Other small RNAs that allow RNAi include: microRNAs (miRNAs), shRNA-miRs and small interfering RNAs (siRNAs). MicroRNAs, unlike shRNAs, are endogenously expressed and transcribed by RNA polymerase II (Pol II) to form a long primary miRNA (pri-miRNA) precursor (Cullen 2004; Lee et al. 2004). shRNA-miR are artificial pri-miRNA transcripts that can be transcribed under either a Pol II or Pol III promoter (Cullen 2005; Dickins et al.

2005; Silva et al. 2005). RNA polymerase III promoters, including H1 and U6 are generally used to drive the expression of a hairpin, because of their natural function in the production of small cellular transcripts (Oler et al. 2010). Moreover, they allow high expression and use precise initiation and termination sites, consisting of 5–6 nucleotide poly(U) tract (Liu et al. 2011). shRNAs, as well as, shRNA-miRs can be introduced into the cell by a vector. The RNase III enzyme Dicer cleaves double stranded RNA in microRNAs, shRNAs and shRNA-miRs into fragments containing 21 to 24 nucleotides, called small interfering RNA's (siRNA) (Bernstein et al. 2001). siRNAs can also be produced synthetically and directly delivered into the cell (Elbashir et al. 2001). Synthetic siRNAs are less suitable to generate knockdowns *in vitro*, because knockdown is transient, whereas shRNAs and shRNA-miRs allow stable and efficient knockdown *in vitro* (Fewell et al. 2006). Endogenous and exogenous double-stranded siRNAs are unwound and the sense strand is cleaved, whereas the anti-sense strand is incorporated into a multisubunit ribonucleoprotein complex, called RNA-inducing silencing complex (RISC). The anti-sense strand of the siRNA guides RISC to mRNA transcripts that have a high degree of sequence complementarity with the siRNA (Martinez et al. 2002). If the anti-sense strand of the siRNA only is partially complementary with the mRNA, translational repression occurs (as is often the case with miRNA mediated RNAi), whereas near-perfect complementary leads to mRNA cleavage (Lim et al. 2005).

Considering the importance of RNAi, the development of a lentiviral-based system for knocking down host genes in MDM was further investigated as part of this thesis. When using a knockdown system, it can be valuable to have the option to simultaneously express a Gene of Interest (GOI). For example, protein expression could be restored, or a mutant or corrected version of the silenced gene can be introduced. Knockdown-knock-in vectors have previously been designed, but are not necessarily suitable for expression in MDM, for example due to ineffective delivery or poor shRNA design. Also, when using stem-cell derived MDM, the lentiviral vector will have to fulfil additional requirements to

overcome problems related to stem cell differentiation, such as promoter silencing and the need for antibiotic selection (Cowley SA 2012). Previously, the most suitable knockdown-knock-in system tested in our laboratory was based on a vector that expressed a GOI (RFP in our case) co-transcribed with a shRNA-miR (targeting CD4 in our case): EF1 α -RFP-shCD4-IRES-Puromycin^R (Cowley SA 2012). The level of CD4 knockdown was shown to be functionally relevant and RFP was expressed, however, both the knockdown of CD4 and intensity of RFP expression were less effective compared to a simpler system (Cowley SA 2012). This is likely due to the fact that both the GOI and shRNAmir are located on the same transcript. The fate of the transcripts is expected to be divided between some that are translated at the ribosome, and some that are processed to make mature interfering RNAs, which could pose a problem in experimental design and interpretation. To improve knockdown and transgene expression, a new lentiviral expression system was developed, allowing stable, as well as, high hairpin and transgene expression from two separate promoters.

CD4 was chosen as the target to demonstrate the principle of using a shRNA to study HIV-1 infection in esMDM. CD4 is the main HIV-1 receptor in both MDM and T-cells (Dalglish et al. 1984; Hussey et al. 1988). In MDM this is supported by plenty of evidence using recombinant soluble CD4 and anti-CD4 monoclonal antibodies, which blocks infection by several macrophage-tropic HIV isolates, as well as, infection-related cytopathic effects (Collman et al. 1990; Harbison et al. 1990; Jordan et al. 1991; Ho et al. 1992; Collin et al. 1993). However, unlike in T-cells, inhibition of HIV-1 infection in MDM has not been demonstrated by the use of lentiviral expression of shRNAs targeting CD4 (Lee et al. 2005). In addition, the development of an effective CD4-knockdown vector that has the potential to knock-in CD4, could further facilitate the study of CD4 by knocking in CD4 mutants, as described in Chapter 6.

I hypothesised that MDM could be successfully genetically modified with the use of a simple, novel lentiviral vector which allows effective CD4 knockdown, as well as, transgene expression in MDM. I further hypothesised that CD4-knockdown MDM would be resistant to successful HIV-1 entry. The hypotheses were tested firstly by designing a lentiviral based vector and testing three different hairpins in TZM-bl cells to achieve complete CD4-downregulation. Next, hESC were transduced with this novel lentiviral based vector and differentiated into esMDM. CD4 knockdown was tested by measuring mRNA and protein expression levels. GOI knock-in was demonstrated by expression of a Puromycin resistance gene. CD4 knock-in, as well as, Puromycin resistance gene expression and CD4 knockdown from the same lentiviral vector was further tested in Chapter 6. Next, CD4-knockdown esMDM were infected with HIV-1 and a panel of assays were used as readouts for sequential stages of HIV-1 infection (shown in Figure 1.1). The first assay “HIV virion fusion assay” (described in more detail in material and methods), measures the fusion of HIV-1 envelope with a cell-limiting membrane, detecting release of β -lactamase from virion cores that have been delivered to the cytosol. Virions that either don't enter the cell or are degraded inside the cell produce no signal (Marechal et al. 1998). Once virions gain access to the cell cytoplasm, the single-stranded vRNA genome is reverse transcribed into double-stranded DNA, which can be quantified by the second assay, “qPCR of HIV-1 late reverse transcripts”. The first two assays measure steps that are necessary but not sufficient for productive entry. Only once the HIV-1 DNA copy is integrated into the host's genome, can it be expressed, and new viral particles produced. The third assay, “HIV-1 integration (luciferase)”, reports expression from HIV-1 proviruses, and is an indicator of productive infection. In contrast to the above assays, which measure a single cycle of HIV-1 infection, the final assay “quantification of p24 antigen by ELISA” measures HIV-1 replication kinetics. If knocking down CD4 leads to a decrease in infection as measured by at least the first three assays, it would provide direct evidence for the requirement for CD4 in HIV infection of MDM.

4.1 Design of lentiviral vectors encoding shRNA driven by U6 promoter

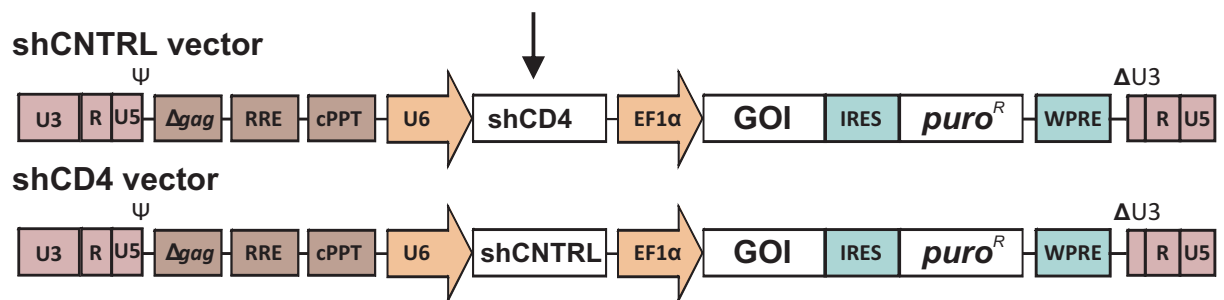
Designing a suitable system to knockdown gene expression in esMDM is not trivial. Previously a strategy was employed using an shRNA-miR co-transcribed with the GOI, which resulted in low expression levels. To improve expression, a system was created with two separate promoters: the U6 promoter to drive the expression of an shRNA and the EF1 α promoter to drive the expression of a transgene. The U6 promoter and a control shRNA (shCNTRL) or shRNA targeting CD4 (shCD4) were cloned upstream of the EF1 α promoter in a previously made plasmid pEF1-GOI-IRES-Puro^R (described in Appendix II). Earlier work had shown that a Gene Of Interest (GOI) can successfully be expressed from the EF1 α promoter in esMDM using pEF1-GOI-IRES-Puro^R (see Chapter 3). The puromycin resistance gene (Puro^R) allows selection of a 100 % pure cell population expressing GOI-IRES-Puro^R. The cloning steps of inserting the U6-shRNA elements are further described in Appendix III. The resulting plasmids were named: pU6-shCD4-EF1-GOI-I-Puro^R and pU6-shCNTRL-EF1-GOI-I-Puro^R. Figure 4.1A shows schematic diagrams of these plasmids and Figure 4.1B shows a representative vector map. The expected phenotype (presence or absence of endogenous human CD4) in cells expressing this lentiviral vector is indicated by the symbols shown in Figure 4.1C and used in subsequent figures.

Figure 4.1

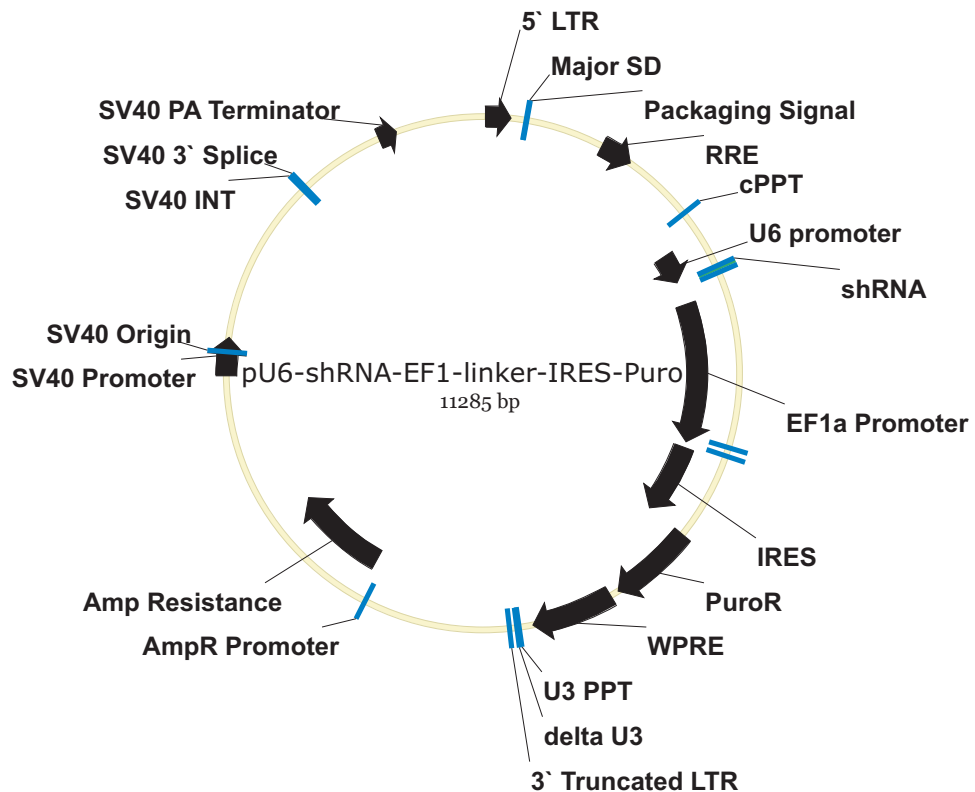
Figure 4.1. Design of lentiviral vectors encoding shRNA driven by U6 promoter

A) Schematic diagrams of self-inactivating lentiviral constructs used to express *puromycin^R* and a non-specific short-hairpin control (shCCTRL) or a short hairpin targeting CD4 (shCD4). A gene can easily be cloned downstream the EF1 α promoter into the place of the 'Gene Of Interest' (GOI) due to the presence of a linker (a set of unique restriction sites). B) Plasmid map for the lentiviral vectors. C) Symbols indicate the presence or absence of endogenous human CD4 in different experimental conditions ('control', 'shCCTRL' and 'shCD4').

A



B



C

			
	'control'	'shCCTRL'	'shCD4'
puromycin ^R	-	+	+
shCD4 _{human}	-	-	+
shCCTRL	-	+	-

4.2 Variable ability of three different hairpins to knockdown CD4 in TZM-bl cells

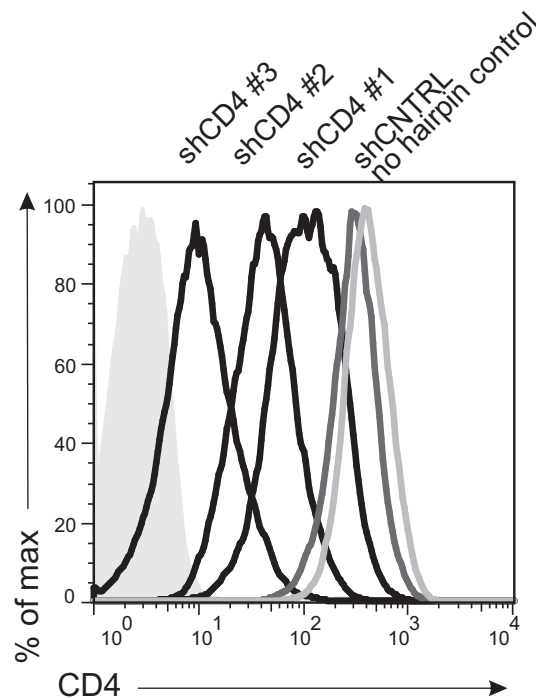
To obtain effective knockdown, three shRNAs targeting CD4 were designed (#1-3), as well as a control shRNA, based on previously published sequences. Target sequence #1, was based on a miR-shRNA target sequence from Open Biosystems that had been previously validated in our laboratory (Cowley SA 2012). Target sequence #2 was based on an siRNA used by *Novina et al.* (Novina et al. 2002). Target sequences #3 was identical to the siRNA sequence used by *García-Expósito et al.* (García-Expósito et al. 2011). The control shRNA target sequence was adapted from *Kamiya et al.* (Kamiya et al. 2011). More details about the design can be found in Appendix III. To test knockdown efficiencies, TZM-bl cells were transduced with lentiviral vectors. This cell line was chosen as it was readily available and expresses high levels of CD4. The TZM-bl (JC53-bl) cell line is derived from the HeLa cancer cell line, but has been genetically engineered to stably express proteins, including CD4 and CCR5. (Scherer et al. 1953; Platt et al. 1998). A pure population of TZM-bl cells containing the lentiviral vector was selected using Puromycin (5 µg/mL). To determine the expression levels of CD4 at the cell surface, the puromycin-selected, as well as control untransduced cells, were stained for surface CD4 expression and analysed by flow cytometry. High CD4 levels were detected in control cells and cells transduced with shCCTRL, but cells transduced with one of three shCD4-lentiviruses had variable, reduced CD4 expression levels. Comparing the ratio of the geometric mean fluorescence intensity (MFI) over the isotype control relative to the untransduced control cells showed the shCD4#1-3 resulted in a 73%, 87% and 97% reduction of surface CD4 expression, respectively. This shows that, by expressing a hairpin targeting CD4 under the control of the U6 promoter, effective knockdown can be achieved, in particular when using shCD4 #3. As such, shCD4#3 was used in subsequent experiments and referred to as shCD4.

Figure 4.2

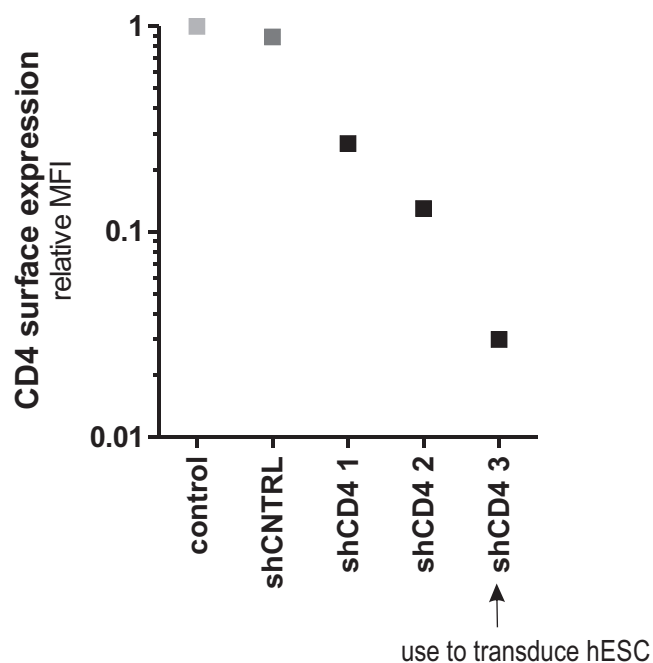
Figure 4.2. Variable ability of three different hairpins to knock-down CD4 in TZM-bl cells.

Three short hairpin interfering RNAs (shRNA) against human CD4 (shCD4 #1-3) and a non-specific shRNA (shCNTRL) were designed and placed under the control of the human U6 promoter. To test the knockdown efficiency, the lentiviral vectors were transduced into TZM-bl cells and CD4 expression was measured by flow cytometry using anti-CD4 antibody. A) Representative histogram showing CD4 surface staining with mAb clone 11830. Lines represent TZM-bl cells transduced with shCD4 design #1, #2 or #3 (black); shCNTRL (dark grey); or untransduced control (dark grey), compared to the matched isotype control (solid gray histogram). B) Quantification of CD4 expression relative to the control group. The symbols reflect the ratio of the geometric mean fluorescence intensity (MFI) over the isotype control.

A



B



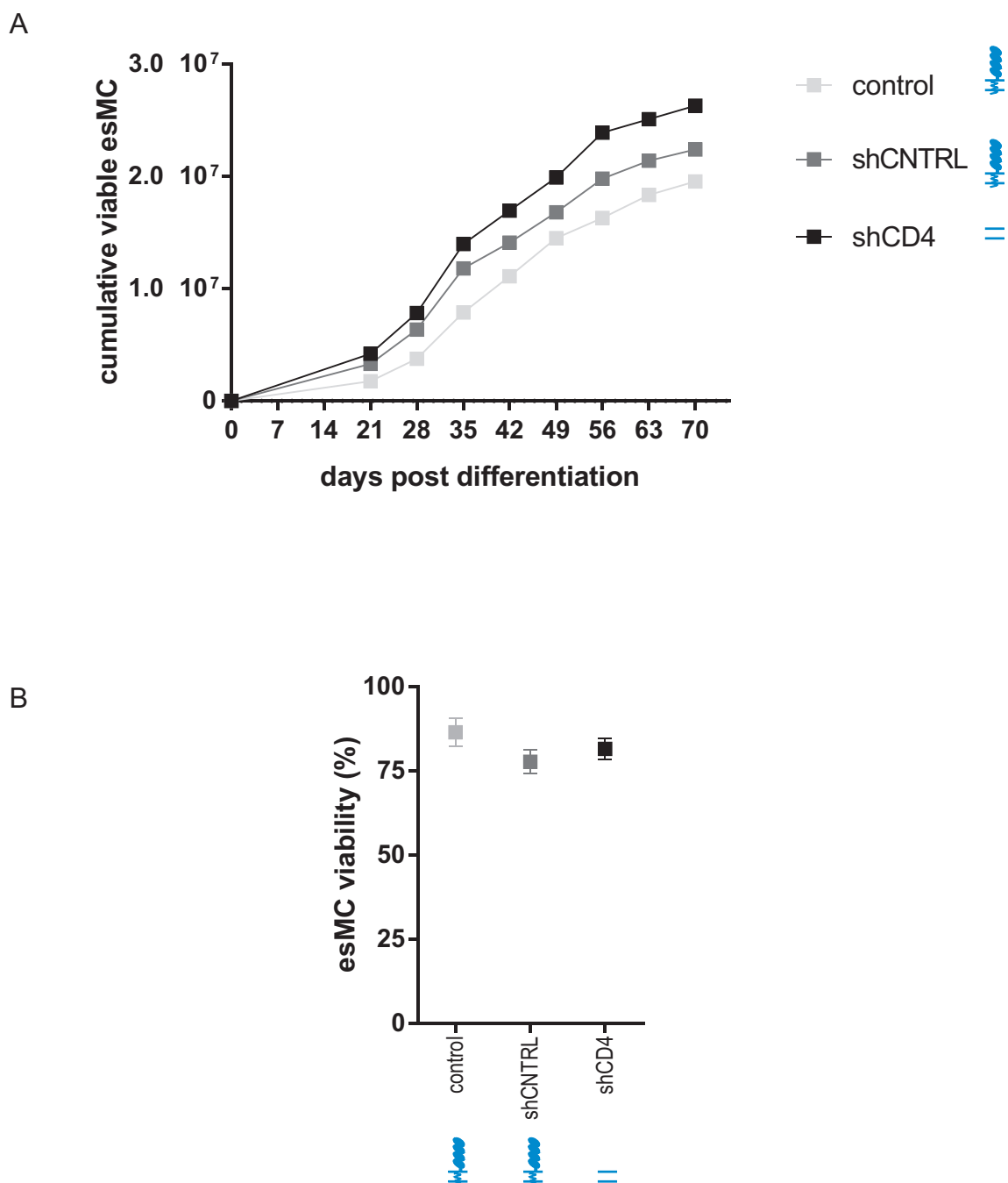
4.3 Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding a hairpin targeting CD4 (shCD4) or a control hairpin (shCNTRL)

hESC were transduced with lentiviral vectors encoding shCNTRL or shCD4 (#3). Control hESC and transduced hESC were differentiated into functional monocytes (esMC). Transduced hESC were kept under continuous puromycin (5 µg/mL) selection (a concentration at which untransduced cells died). esMC were harvested weekly over a period of 70 days. esMC were CD14⁺ selected to exclude any possible contamination of CD14⁻ esMC precursor cells present in the culture. Figure 4.3A shows the cumulative counts of viable esMC from >20 wells per condition. This shows that esMC were harvestable for several weeks from one differentiation culture. Figure 4.3B shows that the mean viability of CD14⁺ selected esMC, as determined by an AO-DAPI stain (Chemometec), was >75 % for all conditions. Normal differentiation and production of viable monocytes shows that the hairpins do not have any apparent toxic effects. The puromycin-resistance shows that the esMC contain the lentiviral vector and were able to drive the expression of the Puro^R-gene from the EF1α promoter.

Figure 4.3

Figure 4.3. Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding a hairpin targeting CD4 (shCD4) or a control hairpin (shCNTRL).

A) hESC were infected with shCNTRL or shCD4 (#3) encoding lentiviral vectors. These and untreated hESC ('control') were used to generate hESC-derived monocytes (esMC) using the Embryoid Body method. Non-adherent esMC were harvested from the supernatant of cultures and counted using a cell counter (Chemometec). The media were replaced for repeated esMC harvests over a period of 70 days. Data represent the cumulative number of viable esMC after CD14⁺ selection (Miltenyi) from >20 wells per condition. B) Viability of CD14⁺ selected esMC determined by an AO-DAPI stain (Chemometec).



4.4 CD4 mRNA expression is reduced in esMDM expressing shCD4

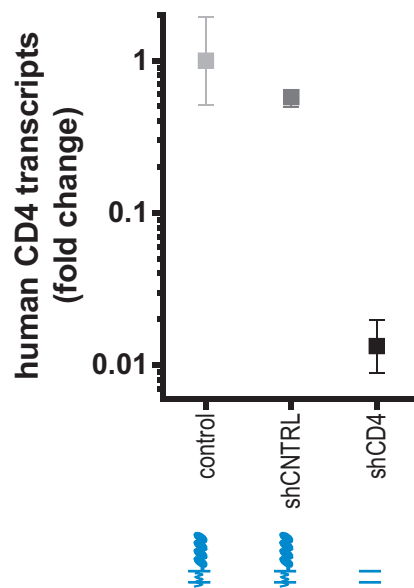
The pure population of viable esMC were further cultured in MDM medium supplemented with M-CSF for 4-7 days to direct the differentiation into esMDM. To find out whether esMDM derived from hESC transduced with shCNTRL or shCD4 maintained the expression of these hairpins throughout the differentiation process, RNA was isolated from control and transgenic esMDM and analysed by RT-qPCR using primers specific for CD4 mRNA. CD4 transcripts were reduced by a factor of 100 in shCD4-esMDM compared to control (Figure 4.4A). As a negative control, DNA was isolated from the same esMDM cells and analysed by qPCR using primers specific for CD4 integrants. Genomic CD4 integrants were expressed at similar levels in control, shCNTRL- and shCD4-esMDM (Figure 4.4B). This shows that hESC maintained the expression of shCD4 from the U6 promoter throughout differentiation and that this hairpin could effectively lead to the degradation of CD4 transcripts in esMDM.

Figure 4.4

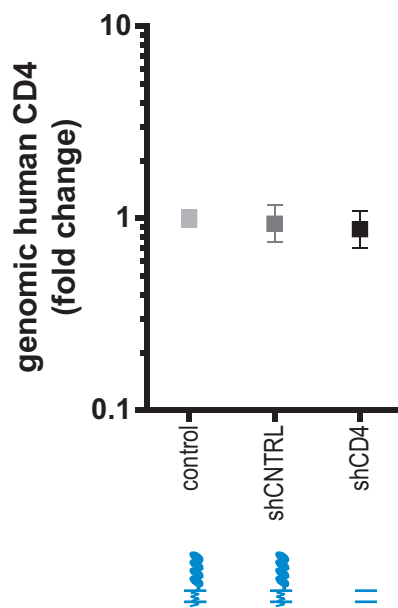
Figure 4.4. CD4 mRNA expression is reduced in esMDM expressing shCD4.

A) To detect CD4 transcripts, RNA was isolated from control and transgenic esMDM and analysed by RT-qPCR. Symbols represent the relative mean number of copies of CD4 mRNA $\pm$ SEM of technical replicates (n=3) using pooled RNA from three independent experiments. B) To detect genomic CD4, DNA was isolated from control and transgenic esMDM and analysed by qPCR. Symbols represent the relative mean number of copies of genomic CD4 $\pm$ SEM of technical replicates (n=3) using pooled DNA from three independent experiments.

A



B



4.5 CD4 protein expression is reduced in esMDM expressing shCD4

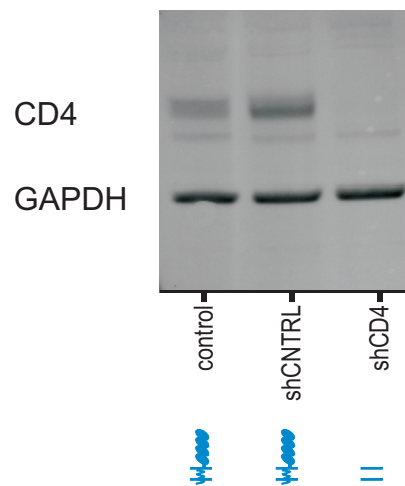
To confirm whether esMDM derived from stem cells transduced with shCNTRL or shCD4 maintained the expression of these hairpins throughout the differentiation process, esMDM lysates were analysed by western blotting using an anti-CD4 antibody. CD4 was detected in lysates from control and shCNTRL esMDM, but not in lysates from shCD4 esMDM, whereas GAPDH, used as a loading control, was detected in all conditions (Figure 4.5A). CD4 expression was normalised to GAPDH expression and showed that shCD4 esMDM (mean $\pm$ SEM: 0.08 ± 0.05 ; $P \leq 0.01$) reduced the amount of CD4 by 92 % compared to the control esMDM (mean set to 1), whereas shCNTRL esMDM (mean $\pm$ SEM: 1.0 ± 0.29 ; ns) did not have significantly different CD4 expression levels compared to control esMDM (Figure 4.5B). Together with the qRT-PCR results showing CD4 mRNA reduction, this validates the lentiviral expression system and shows that CD4 expression is knocked down in shCD4 esMDM.

Figure 4.5

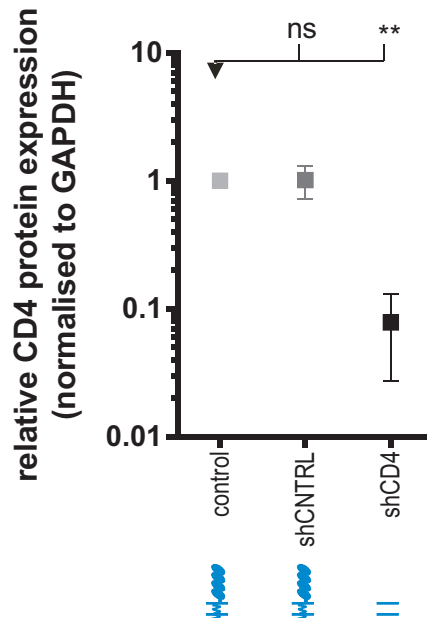
Figure 4.5. CD4 protein expression is reduced in esMDM expressing shCD4.

A) To detect protein expression of CD4, control and transgenic esMDM lysates were analysed by western blotting using anti-CD4 antibodies. GAPDH, a loading control, was detected using anti-GAPDH antibody. A) representative blot is shown B) Protein levels were measured with Odyssey software (Li-COR) and CD4 expression was normalised to GAPDH expression. Symbols represent normalised CD4 expression, relative to the esMDM control group, of three independent experiments $\pm$ SEM (n=3). ns: non-significant ($P>0.05$); $**P\leq 0.01$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.

A



B



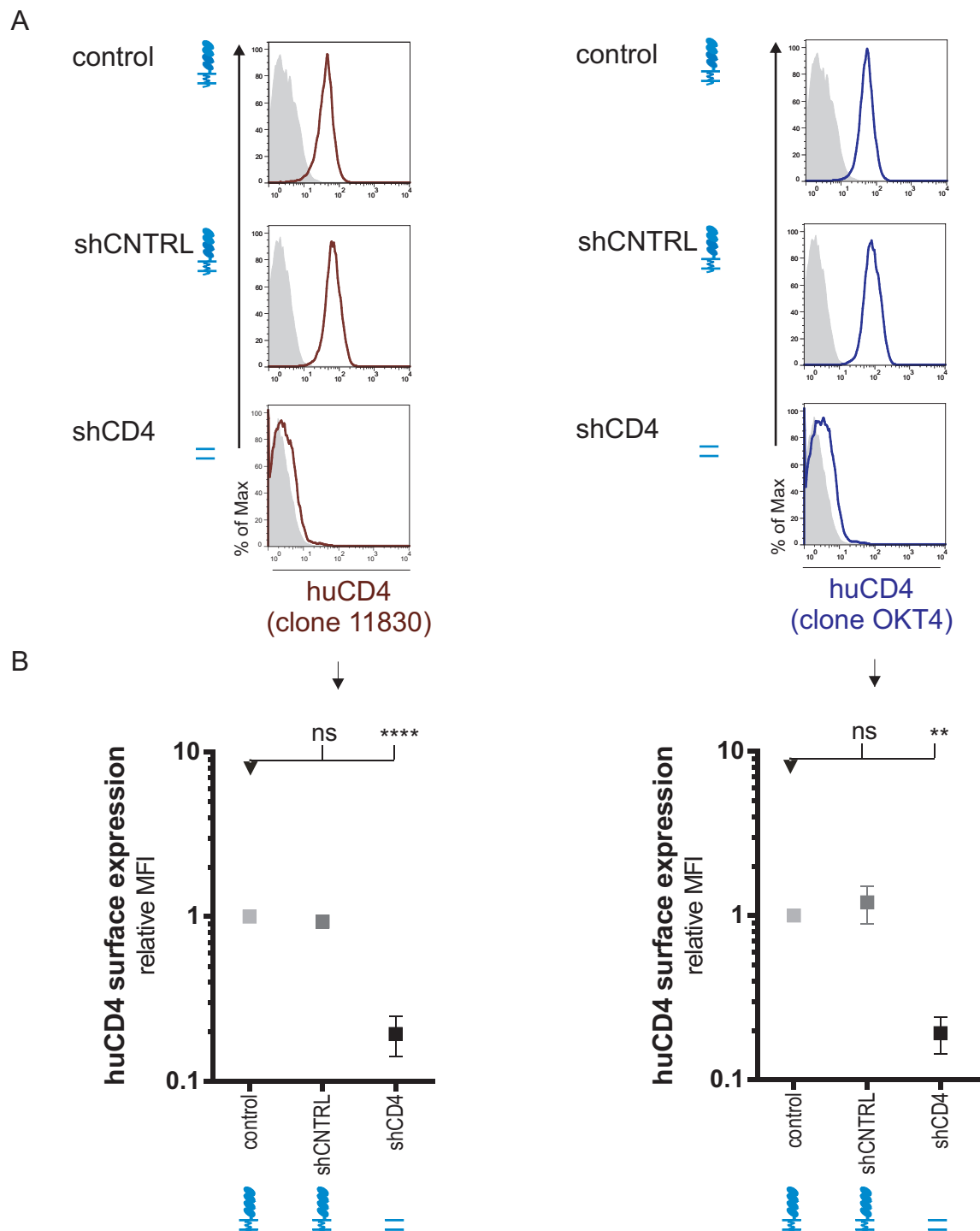
4.6 Surface CD4 protein expression is reduced in esMDM expressing shCD4

To further consolidate the results observed by western blotting, surface CD4 expression (as opposed to total cellular CD4) was measured using flow cytometric analysis using two different clones of anti-CD4 antibodies. The relative surface CD4 expression level, measured using mAb clone 11830, was reduced to background levels on shCD4 esMDM (mean $\pm$ SEM: 0.2 ± 0.05 ; $P \leq 0.0001$) compared to control esMDM (mean set to 1), but unaffected on shCNTRL esMDM (mean $\pm$ SEM: 1.2 ± 0.31 ; ns) (Figure 4.6, left panel). Similar results were obtained using mAb clone OKT4 (shCD4 mean $\pm$ SEM: 0.2 ± 0.05 ; $P \leq 0.01$; shCNTRL mean $\pm$ SEM: 0.9 ± 0.07 ; ns) (Figure 4.6, right panel). Together with the qRT-PCR and Western Blot results, the flow cytometry results validate the lentiviral expression system (LV-U6-shCD4-EF1-GOI-I-Puro^R) for delivery of constructs to hESC and effective knockdown in downstream esMDM. Specifically for this study, it also provided strong evidence that CD4 mRNA, as well as, CD4 surface and cellular total expression was reduced to background levels in esMDM expressing shCD4 #3 (from now on also referred to as CD4-knockdown esMDM).

Figure 4.6

Figure 4.6. Surface CD4 protein expression is reduced in esMDM expressing shCD4.

Control and transgenic esMDM were tested for surface CD4 expression by flow cytometry using two different clones of anti-CD4 antibodies. A) Representative histogram showing CD4 surface staining with mAb clone 11830 (red/brown line, left panel) and with mAb clone OKT4 (blue line, right panel), both compared to isotype control (gray). B) Quantification of CD4 expression with mAb clone 11830 (left) and with mAb clone OKT4 (right) relative to the esMDM control group. The bars reflect the ratio of the geometric mean fluorescence intensity (MFI) over the isotype control $\pm$ SEM of independent experiments (n=4). ns: non-significant ($P>0.05$); ** $P\leq 0.01$; **** $P\leq 0.0001$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



4.7 HIV-1 fusion is reduced in CD4-knockdown esMDM

Having shown that esMDM were successfully genetically modified with the use of a lentiviral vector expressing both a shRNA, to effectively knockdown CD4, as well as a puromycin resistance gene, to allow selection of a cell population that expressed the lentiviral construct, the impact this has on the different stages of HIV-1 infection was evaluated by a range of assays. This would demonstrate the principle of using shRNA to study HIV-1 infection in esMDM.

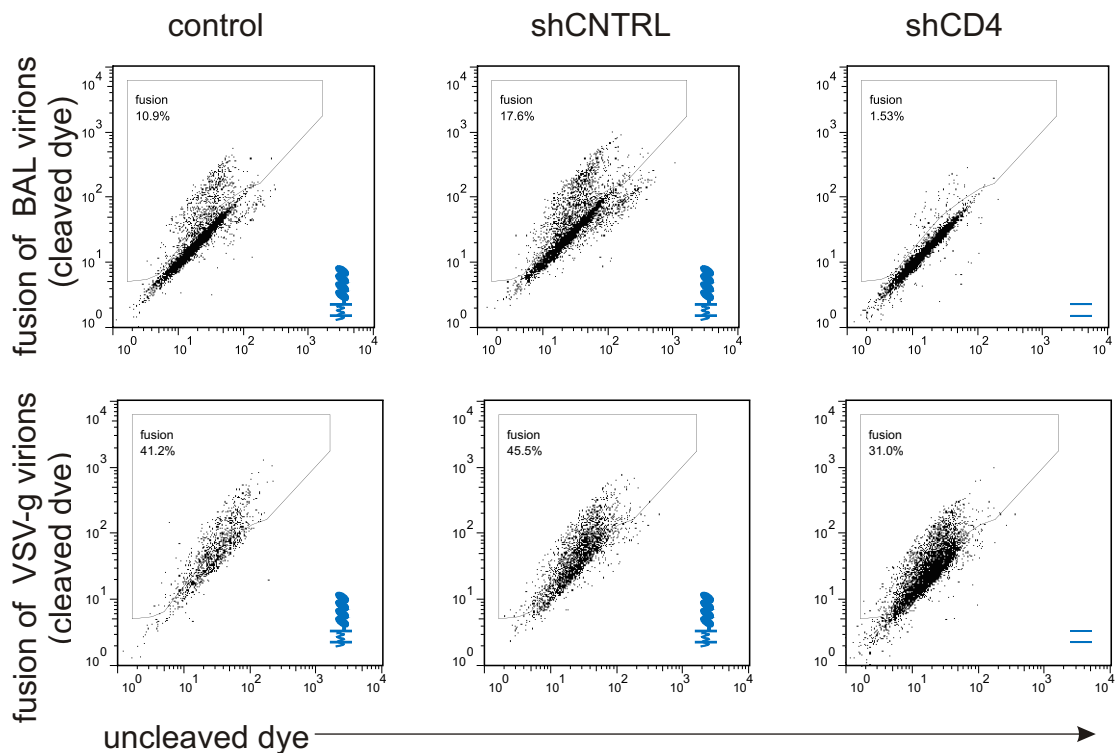
Firstly, fusion of HIV-1 virions to the cells was measured using a fusion assay. BaL or VSV-G pseudotyped HIV-1 virions containing a β lactamase-Vpr (BlaM-Vpr) fusion protein were used to infect esMDM. esMDM were loaded with a fluorogenic substrate. Following virion fusion, BlaM gains access to the substrate in the cytosol and cleaves the substrate, changing its fluorescence emission, as detected by flow cytometry (Figure 4.7A shows a representative flow cytometry plot). The percentage of BaL-infected esMDM in which fusion had occurred was normalised to the equivalent percentage in VSV-G infected esMDM (Figure 4.7B). VSV-G-pseudotyped virions would be expected to enter all cells with equal efficiency, independent of CD4 expression levels, as they do not require a cell surface receptor and mediate fusion after endocytosis and acidification of the endosome (Aiken 1997). Based on four independent experiments, compared to control esMDM (mean $\pm$ SEM: 0.8 ± 0.11), fusion was significantly lower in shCD4 esMDM (mean $\pm$ SEM: 0.2 ± 0.07 ; $P \leq 0.01$), but not in shCNTRL esMDM (mean $\pm$ SEM: 0.9 ± 0.16 ; ns). This shows that knocking down CD4 leads to reduced HIV-1 fusion in esMDM.

Figure 4.7

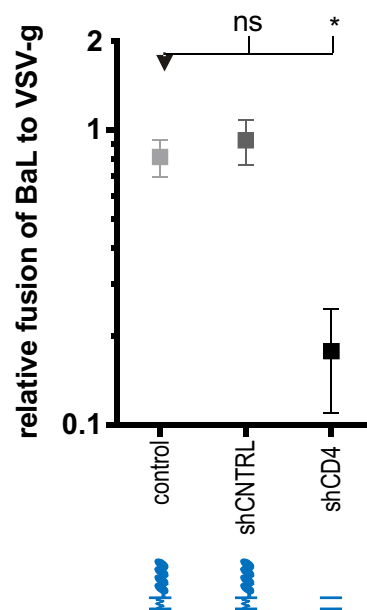
Figure 4.7. HIV-1 fusion is reduced in CD4-knockdown esMDM.

Viral fusion was detected by the fluorescence shift associated with cleavage of the BLaM substrate after 24h of infection of esMDM with NL4.3 BaL (BLaM-Vpr) or NL4.3 VSV-g (BLaM-Vpr), as described in material and methods. A) Representative plots of control and transgenic infected esMDM with NL4.3 (BLaM-Vpr) BaL (top) or VSV-g (bottom). Gates were set based on the fluorescent signal of uninfected samples loaded with the BLaM substrate. B) The percentage of fusion of BaL pseudotyped virions was normalised to the percentage fusion of VSV-g pseudotyped virions. Symbols represent the normalised mean fusion relative to the esMDM CD4_{WT} group (shown in Figure 6.9) of four independent experiments $\pm$ SEM (n=4). ns: non-significant ($P > 0.05$); $*P \leq 0.05$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.

A



B



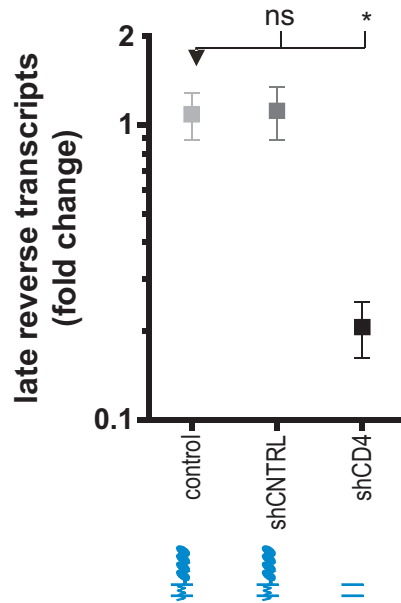
4.8 HIV-1 reverse transcription is reduced in CD4-knockdown esMDM

Post fusion, an essential step in the HIV-1 life cycle is reverse transcription: the conversion of vRNA into DNA. To determine whether this stage was affected by CD4 knockdown, reverse transcription was measured in esMDM-infected cells after spinoculation and 28h of infection. To quantify HIV-1 reverse transcription, the number of copies of HIV-1 late reverse transcription products was measured by qPCR, using β actin primers to normalise to the number of cells present. Compared to control esMDM (mean $\pm$ SEM: 1.1 ± 0.19), based on four separate experiments, fewer copies of late reverse transcripts per cell were detected in shCD4 esMDM (mean $\pm$ SEM: 0.2 ± 0.04 ; $P \leq 0.01$), but similar copies were detected in shCNTRL esMDM (mean $\pm$ SEM: 1.1 ± 0.23 ; ns) (Figure 4.8). This indicates that, in addition to decreased HIV-1 fusion, knocking down CD4 results in reduced HIV-1 reverse transcription.

Figure 4.8

Figure 4.8. HIV-1 reverse transcription is reduced in CD4-knockdown esMDM.

Reverse transcription of HIV-1 in esMDM was measured by detecting late (pol) products by qPCR after 30h of infection. Symbols represent the relative mean number of copies of HIV-1 DNA $\pm$ SEM of independent experiments ($n=4$), normalised to the number of cells using a β -actin control. ns: non-significant ($P>0.05$); $*P\leq 0.05$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



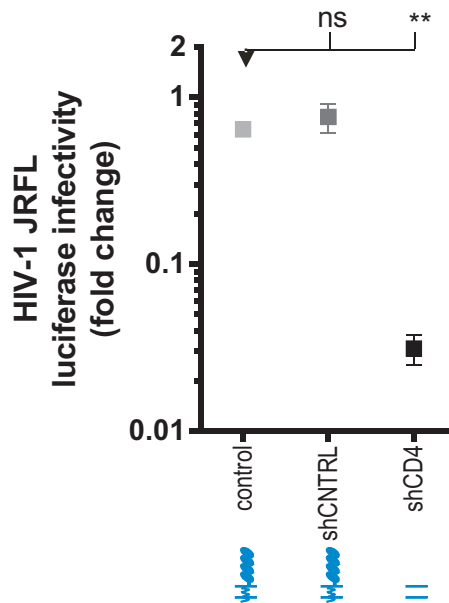
4.9 Reporter expression from HIV-1 integrants is reduced in CD4-knockdown esMDM

Once the vRNA has been reverse transcribed into a strand of cDNA and has been imported into the nucleus, the viral cDNA can be integrated into the host cell DNA. Only once the viral DNA (provirus) is transcribed, can new viruses be produced. Therefore, a reporter assay from HIV-1 integrants was used as measure of a single round of productive infection. This assay uses a replication-defective pseudotyped HIV-1 that has the gene for luciferase inserted in place of the *nef* gene, and can therefore function as a reporter gene for infection. esMDM were infected with JRFL-NL4.3 or VSV-G-NL4.3, for 3 days before cells were lysed and luciferase expression was measured. The luciferase activity of esMDM infected with JRFL-NL4.3 was normalised to esMDM infected with VSV-G-NL4.3. VSV-G-pseudotyped virions would be expected to enter all cells with equal efficiency, independent of CD4 expression levels, as they do not require a cell surface receptor for entry (Aiken 1997). Compared to control cells (mean $\pm$ SEM: 0.6 ± 0.05), detection of luciferase protein produced in lysates from shCD4 esMDM was reduced 20.6-fold (mean $\pm$ SEM: 0.03 ± 0.01 ; $P \leq 0.001$), but similar in shCNTRL esMDM (mean $\pm$ SEM: 0.76 ± 0.15 ; ns)(Figure 5.9). These results are in accordance with the previous result, suggesting that when CD4 is knocked down in esMDM, HIV-1 fusion, reverse transcription, as well as, productive infection are reduced.

Figure 4.9

Figure 4.9. Reporter expression from HIV-1 integrants is reduced in CD4-knockdown esMDM.

HIV-1 integration was measured by detecting luciferase activity in esMDM three days after transduction with HIV-1 NL4.3.Luc.R-E- virus pseudotyped with JRFL or VSV-g envelope. Luciferase expression was quantified using Promega One-Glo luciferase substrate and a luminometer. JRFL NL4.3.Luc.R-E- transduced cells were normalised to VSV-g NL4.3.Luc.R-E- transduced cells. Symbols represent the mean luciferase detection relative to the esMDM CD4_{WT} group (shown in Figure 6.11), $\pm$ SEM of independent experiments (n=6). ns: non-significant ($P>0.05$); $**P\leq 0.01$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



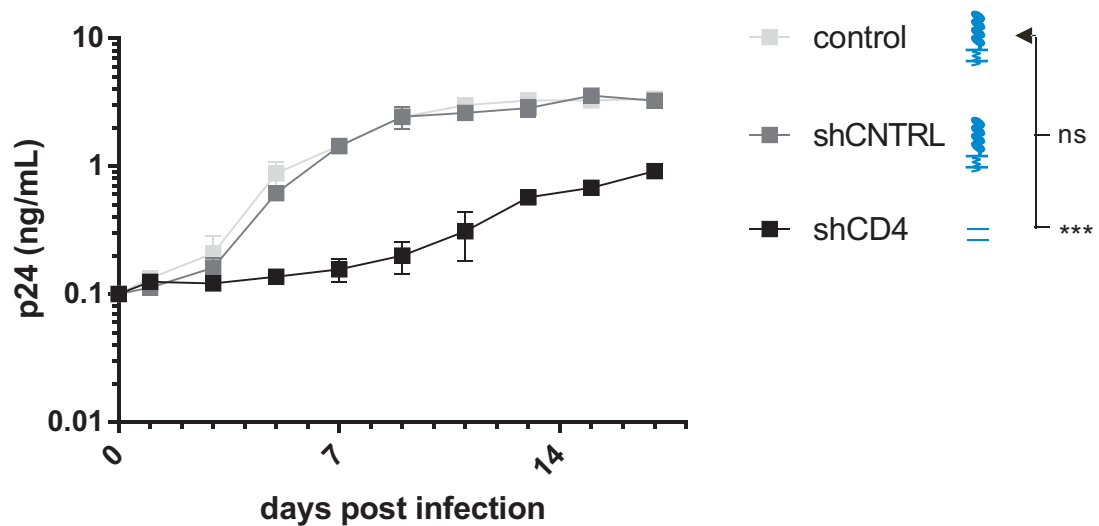
4.10 Multiple rounds of HIV-1 replication are significantly reduced in CD4-knockdown esMDM

The above assays measured events during a single cycle of HIV-1 infection. Once new viruses are produced and bud from the infected cell, the cycle repeats itself. A multi-cycle assay was, therefore, also performed, even though HIV-1 replication in MDM over multiple generations may change the phenotype of these cells, and this might complicate the interpretation of the results. HIV-1 replication kinetics in control, shCNTRL and shCD4 esMDM were measured by culturing HIV-1 BaL infected esMDM for 11-17 days. Samples of the supernatant were taken at intervals and assayed for viral p24 protein levels by ELISA. Compared to control cells, infection with HIV-1 BaL resulted in similar levels of virus being produced by shCNTRL esMDM, but up to 12-fold less by shCD4 esMDM. Although this assay probably does not give as accurate reflection of successful infection as the previous experiments, owing to amplification effects, the results are in accordance with the previous results. That is, when CD4 is knocked down in esMDM, there is reduced HIV-1 fusion, reverse transcription, productive infection and replication.

Figure 4.10

Figure 4.10. Multiple rounds of HIV-1 replication are reduced in CD4-knockdown esMDM.

HIV-1 replication in esMDM was measured by p24 ELISA. esMDM were infected with HIV-1 BaL. Samples of the supernatant were taken at regular intervals and assayed for p24 levels by ELISA. Symbols represent the mean p24 concentration at different time points $\pm$ SEM of independent experiments. n=3 for day 1-11; n=2 for day 13; and n=1 for day 15-17. ns: non-significant ($P>0.05$); *** $P\leq 0.001$. Repeated measure one-way ANOVA followed by Holm-Sidak test versus the esMDM control group.



4.11 Summary and Discussion

In this chapter, experiments were described in which, for the first time, CD4 was effectively knocked down in a hESC-derived macrophage, using a simple novel lentiviral vector (LV-U6-shCD4-EF1-GOI-I-Puro^R). When these cells were challenged with HIV-1, successful HIV-1 entry was strongly inhibited, confirming the essential role of CD4 in HIV-1 infection of MDM. The results confirmed the hypotheses that MDM can successfully be genetically modified with the use of a simple, novel lentiviral vector which allows effective CD4 knockdown, as well as, transgene expression in MDM and that CD4-knockdown MDM are resistant to successful HIV-1 entry.

The first desired property of the lentiviral dual expression vector (LV-U6-shRNA-EF1 α -GOI-IRES-P) was efficient knockdown. This was demonstrated using a hairpin targeting CD4 under the control of the Pol III U6 promoter. This mediated a complete knockdown of CD4 down to background expression levels in shCD4 esMDM by targeted degradation of CD4 mRNA. Significantly lower levels of mRNA, total cellular and surface CD4 protein were found compared to control esMDM, whereas a control hairpin did not significantly affect CD4 expression. This suggests that the expression levels from the lentiviral vector were sufficiently high to study the effects of loss of protein function. The shCD4 resulted in 92 % reduction of total CD4 protein expression in esMDM compared to control cells. This is almost a 10-fold more efficient knock down compared to what has been reported in human primary CD4⁺ T-cells that had been nucleofected with an siCD4 with the same target sequence (García-Expósito et al. 2011). The reduction of CD4 surface protein expression was higher in shCD4 TZM-bl cells (97 % reduction versus control) compared to shCD4 esMDM (80 % reduction versus control), nonetheless CD4 levels were reduced to background levels in esMDM. Two other shCD4 sequences were tested in TZM-bl cells, both of which resulted in a less effective CD4 knockdown. The first resulted in 73 %

reduction of surface CD4 in TZM-bl cells expression compared to control TZM-bl cells. A shRNA-miR with a similar target sequence, but a different expression system, as shown by Cowley *et al.*, resulted in a 40 % reduction of surface CD4 expression in esMDM (Cowley SA 2012). The reduced efficacy of the shRNA-miR compared to the shRNA is likely due to differences between the lentiviral based expression systems used to express the hairpins. The second hairpin that was tested in TZM-bl cells resulted in a 87 % reduction of surface CD4 expression. Novina *et al.* showed that a similar CD4-siRNA, reduced CD4 expression in Magi-CCR5 cells (Novina et al. 2002). However, unlike in the tested shCD4#2 TZM-bl cells, where all cells expressing the hairpin were selected due to the presence of the puromycin resistance gene, the CD4-siRNA only targeted CD4 in about 75 % of Magi-CCR5 cells (Novina et al. 2002). The above mentioned findings demonstrate that optimisation of the target sequence is important, and effective knockdown depends on both cell type and type of expression system.

The expression of the puromycin resistance gene, which resulted in a population of cells that were resistant to 5 µg/mL Puromycin, demonstrated the second key property of the lentiviral dual expression vector LV-U6-shRNA-EF1α-GOI-IRES-P: transgene expression. The puromycin resistance gene was expressed under the control of the EF1α promoter, located downstream of a putative Genes Of Interest (GOI), separated by an internal ribosome entry site (IRES). IRES allows two proteins to be co-ordinately expressed from a single mRNA, so that expression of one of the genes should ensure expression of the other (Adam et al. 1991). The lentiviral dual expression vector LV-U6-shRNA-EF1α-GOI-IRES-P was designed with restriction sites adjacent to the shRNA and GOI, to allow easy replacement by other shRNAs or GOI, making it a quick and accessible system to study a range of proteins. Insertion of a GOI downstream of the EF1α promoter and upstream of the IRES-Puro^R element is described in Chapter 6. The lentiviral vector can be modified so that it can knockdown expression of multiple genes, or target one gene with multiple shRNAs. This can be particularly relevant when HIV-1 is targeted, to overcome possible

escape mutants (ter Brake et al. 2008). To achieve this, a second Pol III promoter, such as H1, encoding another hairpin can easily be inserted in the lentiviral vector downstream of the U6-shRNA. Using both U6 and H1 to express multiple hairpins has successfully been demonstrated by *Anderson et al.*, although it should be noted that shRNAs under control of the U6 promoter are expressed at higher levels compared to shRNAs under control of the H1 promoter (An et al. 2006; Anderson et al. 2007). Alternatively, multiple shRNAs may also be expressed as a multicistronic transcript from one promoter (ter Brake et al. 2008; Saayman et al. 2010).

Expression of LV-U6-shCD4-EF1-GOI-I-Puro^R in esMDM resulted in a significant reduction in HIV-1 infection, thereby confirming that the expression levels from the lentiviral vector are sufficiently high to study the effects of loss of protein function and demonstrates the principle of using a shRNA to study HIV-1 infection in esMDM. Other similar, suitable systems have been reported for knocking down other genes, illustrating the robustness of this approach (Anderson et al. 2007; Zhou et al. 2011; Walker et al. 2012). A higher inhibition of HIV-1 infection was achieved in this study using shCD4-esMDM, compared to other studies with esMDM expressing hairpins against other HIV-1 host factors such as the co-receptor CCR5, the nuclear integration facilitator Transportin 3 or transcription factor CDK2 (Anderson et al. 2007; Zhou et al. 2011; Jerebtsova et al. 2012). The multi-cycle p24 ELISA assay, measuring HIV-1 replication, is influenced by multiple confounding host and viral effects and is not an accurate assay to measure HIV-1 entry. This is illustrated by the fact that the single-cycle assay measuring HIV-1 integrants (luciferase assay) showed a 2-log higher inhibition of HIV-1 infection compared to the p24 ELISA assay. This is likely due to the fact that the p24 ELISA has a low sensitivity and dynamic range and is more prone to secondary effects. Nonetheless, the significant reduction of HIV-1 infection in CD4-knockdown esMDM was seen in all 4 different assays, providing strong evidence supporting the role of CD4 as the main receptor for HIV-1 infection of macrophages.

However, apart from facilitating HIV-1 entry, the role of CD4 on macrophages is unknown and few studies have addressed this, presumably due to difficulties in studying protein function in macrophages (Gibbings et al. 2009). The CD4-knockdown esMDM developed in this chapter provide an excellent model for studying this further, as for the first time, human macrophages have been produced with or without CD4 on an isogenic background. CD4-knockdown esMDM underwent normal differentiation and no cytotoxic or functional abnormalities were observed, indicating that CD4 does not have a crucial function in myelopoiesis. More in-depth functional analysis could be performed to find out whether CD4 has any other functional roles in esMDM apart from acting as a mere mechanical receptor for HIV-1 entry. CD4 could have a specific role in the macrophage immune response. For example, CD4 could mediate chemo-attractant activity, by binding IL-16 (Krautwald 1998; Qi et al. 2006; Yadav et al. 2010). If CD4 has a physiological function in macrophages, it could contribute to HIV-1 infection in these cells or HIV/AIDS pathogenesis. Better knowledge of CD4 as an HIV-1 receptor and/or as receptor for macrophage function, would allow more precise prevention and treatment of HIV-1 infection and pathogenesis. The cell lines developed in this chapter could be used to screen for a loss of function phenotypes using a range functional assays, such multiplex assays to detect cytokine and chemokine secretion or to detect activation of cell signaling molecules in control and shCD4 esMDM stimulated with potential CD4 ligands, such as IL-16 and opsonised pathogens, but also with recombinant gp120 or CD4 and CD4 antibodies. In addition to performing loss-of-function experiments, exogenous wild-type or mutant CD4, which are not affected by the shCD4 can be inserted, which would allow more extensive study of protein function, as demonstrated in Chapter 6.

Taken together, MDM derived from hESC represent a valuable, genetically-manipulable tool for investigating fundamental macrophage biology and HIV-macrophage interactions. A simple lentiviral dual expression vector, LV-U6-shRNA-EF1 α -GOI-IRES-P, allows effective knockdown and/or transgene expression in esMDM. The principle of using a

shRNA to study HIV-1 infection in esMDM was demonstrated by for the first time knocking down CD4 in esMDM leading to inhibition of successful HIV-1 entry. This reiterates the role of CD4 as the primary receptor of HIV-1 infection in macrophages. However, little is known about the role of sub-cellular distribution of CD4 in HIV-1 infection. This, as well as other HIV-macrophage interactions and macrophage biology, can now easily be investigated further with the use of stem cell technology and lentiviral vectors to achieve genetic modification of macrophages.

Chapter 5: Tethering of CD4 to the cell surface leads to increased HIV-1 entry but not to increased productive infection in esMDM

Introduction

Although CD4 is essential for HIV-1 entry into macrophages, T-cells and cell lines, its sub-cellular distribution (expression levels, location and recycling-rate) differs between each type of cell owing to the differential expression of LCK, which is only found in lymphoid cells (Perlmutter et al. 1988; Pelchen-Matthews et al. 1991; Pelchen-Matthews et al. 1998). In T-cells, LCK maintains the surface expression of CD4, whereas in macrophages, which do not express LCK, CD4 is constitutively recycled. It is currently not known whether the sub-cellular CD4 distribution in MDM contributes to the successful HIV-1 entry pathway in these cells, which is thought to be an endocytic-pathway similar to macropinocytosis that originates at lipid rafts (Marechal et al. 2001; Carter et al. 2009; Carter et al. 2011; Gobeil et al. 2012). In this chapter, the role of sub-cellular CD4 distribution in HIV-1 entry into macrophages is further investigated by expressing LCK heterologously in esMDM.

Previously, it has been shown that when LCK is introduced in myeloid cell lines, or when CD4 and LCK are co-introduced in non-lymphoid cell lines which normally do not express these proteins, LCK and CD4 associate, leading to inhibition of CD4 endocytosis, stabilised CD4 surface levels, and an increase in HIV-1 infection (Simpson et al. 1989; Pelchen-Matthews et al. 1992; Pelchen-Matthews et al. 1995; Pelchen-Matthews et al. 1998; Gray et al. 2005). In a simple model of infection, one would expect that, as has been found in the above studies on cell lines, increasing levels of CD4 would increase infection, considering that CD4 density on macrophages is highly correlated with infectivity (Dimitrov et al. 1999; Arrildt et al. 2012). However, myeloid cell lines may not

closely represent the HIV-1 entry pathway of macrophages, as described in Chapter 1.3.7.

For these reasons, conducting the LCK experiment in authentic MDM is desirable. However, it is challenging to introduce LCK directly into blood monocyte-derived MDM (bMDM), as bMDM are particularly refractory to genetic manipulation (Kaushik et al. 2009). By contrast, hESC-derived MDM (esMDM), which are comparable to bMDM and which replicate the HIV replication kinetics of primary MDM, offer a reliable system for genetic modification of human authentic MDM, as demonstrated in Chapter 3 and 4 (Karlsson et al. 2008; Cowley SA 2012). Therefore, I used this novel technology, developed in our laboratory, to express LCK in an authentic MDM.

I hypothesised that **successful** HIV-1 entry in MDM will be affected by redistribution of sub-cellular CD4. The null hypothesis — successful HIV-1 entry in MDM will *not* be affected by redistribution of sub-cellular CD4 — was tested by tethering CD4 to the surface with LCK. A panel of assays were used as readouts for HIV-1 infection, allowing dissection of the entry pathway. This included four single-cycle assays (“HIV virion fusion assay”, “qPCR of HIV-1 late reverse transcripts”, “2-LTR circles” and “HIV-1 integration (luciferase)” and a less informative multi-cycle assay (“quantification of p24 antigen by ELISA”). If tethering CD4 at the cell surface leads to an increase in infection as measured by at least all four single-cycle assays, it would provide evidence against the requirement for a particular sub-cellular CD4 distribution in HIV infection of MDM. However, if tethering CD4 at the cell surface does not lead to an overall increase in infection, it would imply that diverting CD4 away from its normal recycling route causes sequestering of HIV into an unproductive pathway.

5.1 Design and construction of lentiviral vectors encoding LCK_{WT}, LCK_{INACTIVE} or puromycin^R control

LCK is a Src family kinase important in T-cell activation and is regulated by the phosphorylation of specific tyrosine residues (Voronova et al. 1986; Acuto et al. 2008). Phosphorylation of LCK at the tyrosine (Y) at position 505 inhibits LCK activity, whereas phosphorylation of Y394 activates LCK (Amrein et al. 1988; Veillette et al. 1990). It is not known whether LCK can become activated when expressed in MDM, nor whether activated LCK can lead to any signaling events in MDM. In order to exclude the variable of possible LCK-induced signaling, a kinase-dead version of LCK ('LCK_{INACTIVE}') was also used, that can bind CD4 and tether it to the surface, but not transduce signals (Abraham et al. 1990; D'Oro et al. 1996). LCK_{INACTIVE} harbours a mutation at position 394 of tyrosine (Y) to phenylalanine (F).

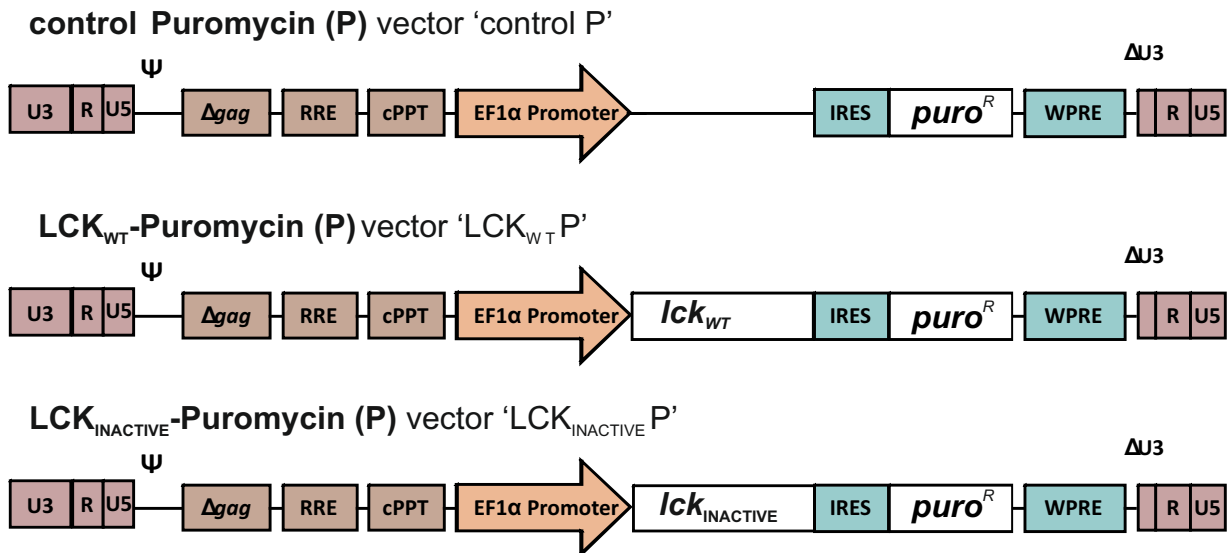
LCK_{WT} (LCK_{WT} P) or an inactive version of the protein, (LCK_{INACTIVE} P) were inserted into the vector pEF1-GOI-IRES-Puro^R so that their expression was under the control of the EF1 α promoter, followed by the IRES and puromycin resistance gene. Earlier work had shown that transgenes can successfully be expressed in esMDM using this design (see Chapter 3.9). The control vector (control P) lacks the LCK_{WT} or LCK_{INACTIVE} cistron. Figure 5.1A shows a schematic diagram and Figure 5.1B shows representative map of the vectors used in this chapter. The cloning strategy is described in Appendix IV.

Figure 5.1

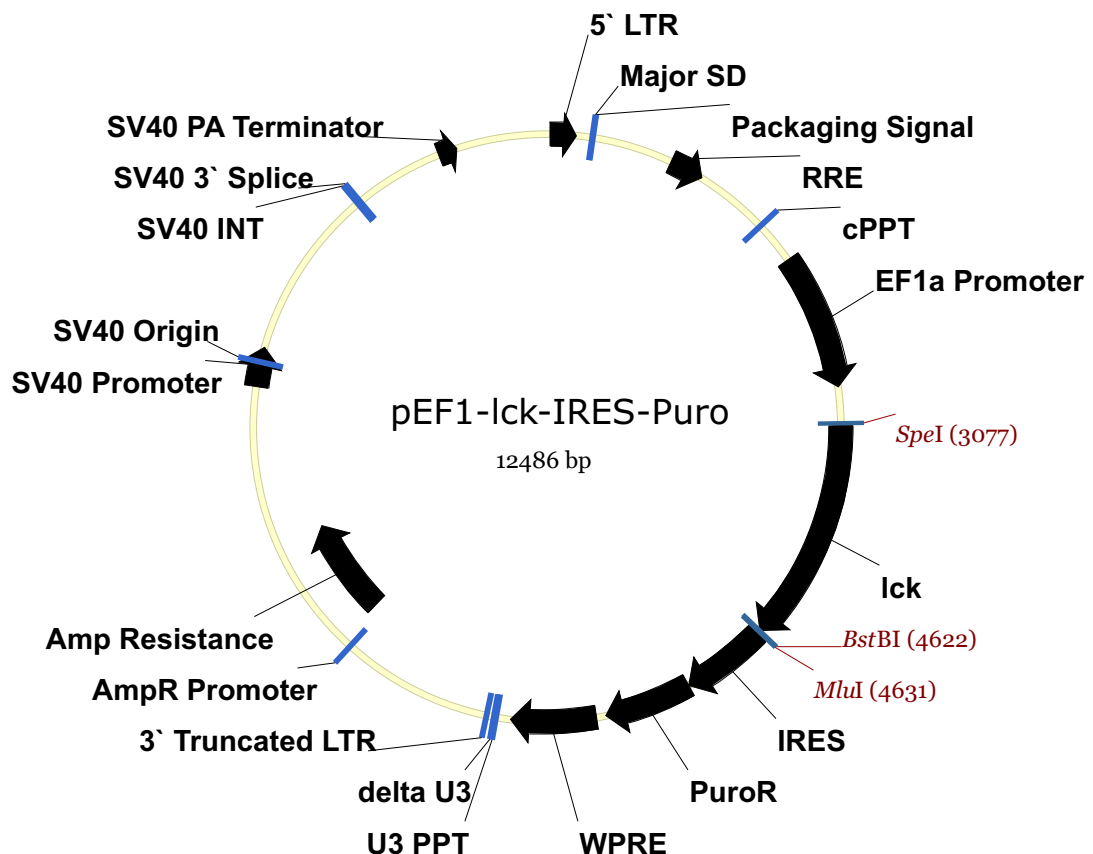
Figure 5.1. Design and construction of lentiviral vectors encoding LCK_{WT}, LCK_{INACTIVE} or puromycin^R control

A) Schematic diagrams of self-inactivating lentiviral constructs used to express *puromycin*^R ('control P'); wild-type *lck* and *puromycin*^R (LCK_{WT} P); or a kinase inactive form of *lck* and *puromycin*^R (LCK_{INACTIVE} P). B) Plasmid map for representative lentiviral vector, LCK_{WT} P.

A



B



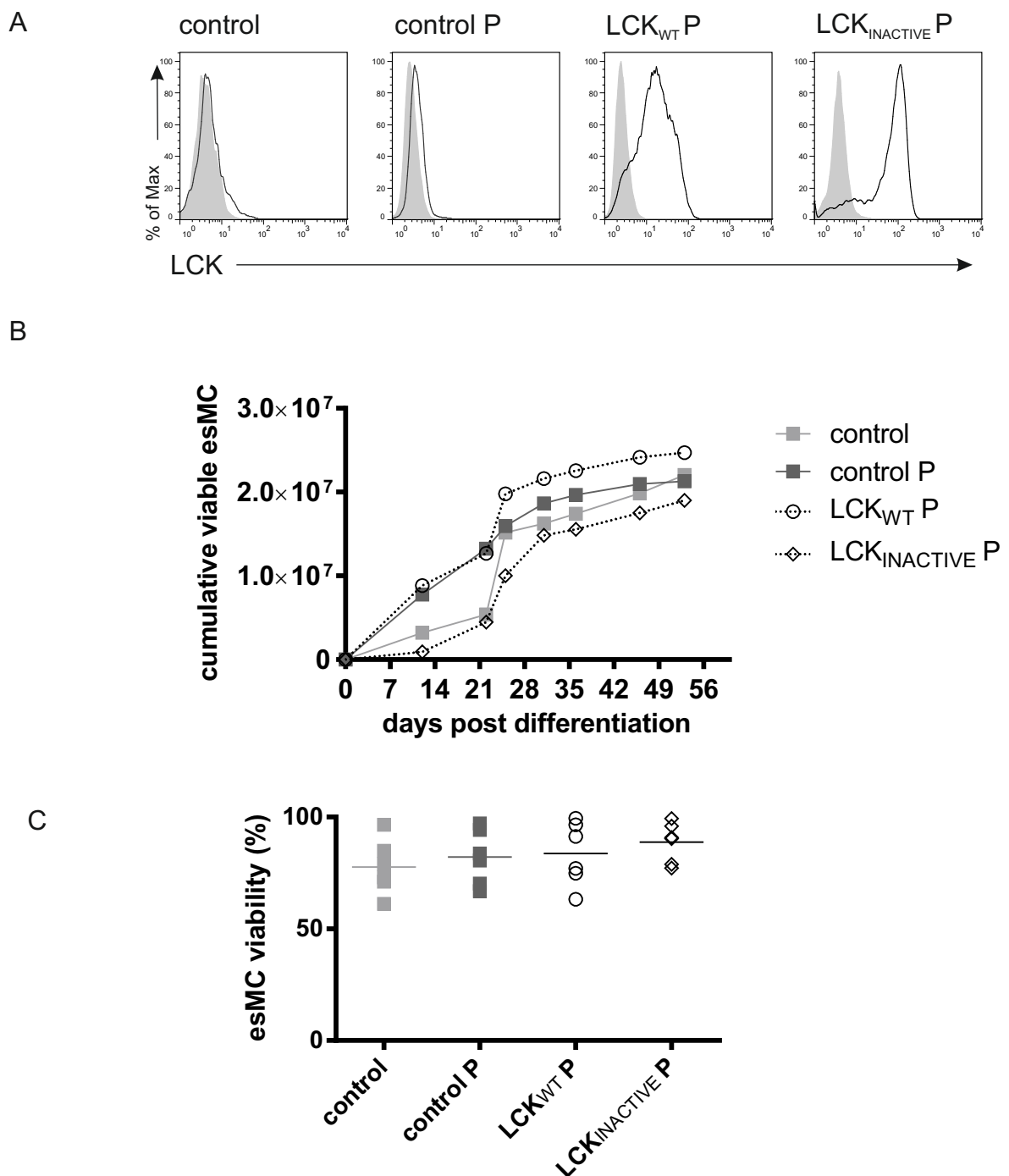
5.2 Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding LCK_{WT}, LCK_{INACTIVE} or puromycin^R control

hESC were transduced with lentiviral vectors encoding LCK_{WT}, LCK_{INACTIVE} or puromycin^R. Transduced hESC were continuously selected with 5 µg/mL puromycin (a concentration at which uninfected hESC died). The puromycin-selected and transduced hESC, as well as control untransduced hESC, were stained for total cellular LCK expression and analysed by flow cytometry. LCK was detected in hESC transduced with LCK_{WT} P, and LCK_{INACTIVE} P, but not in control P transduced and untransduced hESC (Figure 5.2A). Having confirmed successful transduction and selection of the hESC with LCK constructs, hESC were differentiated into functional monocytes (esMC). esMC were harvested roughly once a week over a period of 56 days. esMC were CD14⁺ selected to exclude any possible contamination of CD14⁻ esMC precursor cells present in the culture. Figure 5.2B shows the cumulative counts of viable esMC from >20 wells per condition. This shows that esMC were harvestable for several weeks from one differentiation culture. Figure 5.2C shows that the mean viability of CD14⁺ selected esMC, as determined by an AO-DAPI stain (Chemometec), was >83 % for all conditions.

Figure 5.2

Figure 5.2. Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding LCK_{WT}, LCK_{INACTIVE} or puromycin^R control.

A) hESC were transduced with lentiviral vectors encoding LCK_{WT}-IRES-Puromycin ('LCK_{WT} P') or LCK_{INACTIVE}-IRES-Puromycin ('LCK_{INACTIVE} P') or IRES-Puromycin control ('control P'). These and untreated control hESC ('control') were tested for total LCK expression measured by flow cytometry. Histograms represent total LCK staining (black line) compared to isotype control (gray). B) hESC were used to generate hESC-derived monocytes (esMC) using the Embryoid Body method. Non-adherent esMC were harvested from the supernatant of cultures and counted using a cell counter (Chemometec). The media were replaced for repeated esMC harvests over a period of 56 days. Data represent the cumulative number of viable esMC after CD14⁺ selection (milenyi) from >20 wells per condition. C) Viability of CD14⁺ selected esMC determined by an AO-DAPI stain (Chemometec).



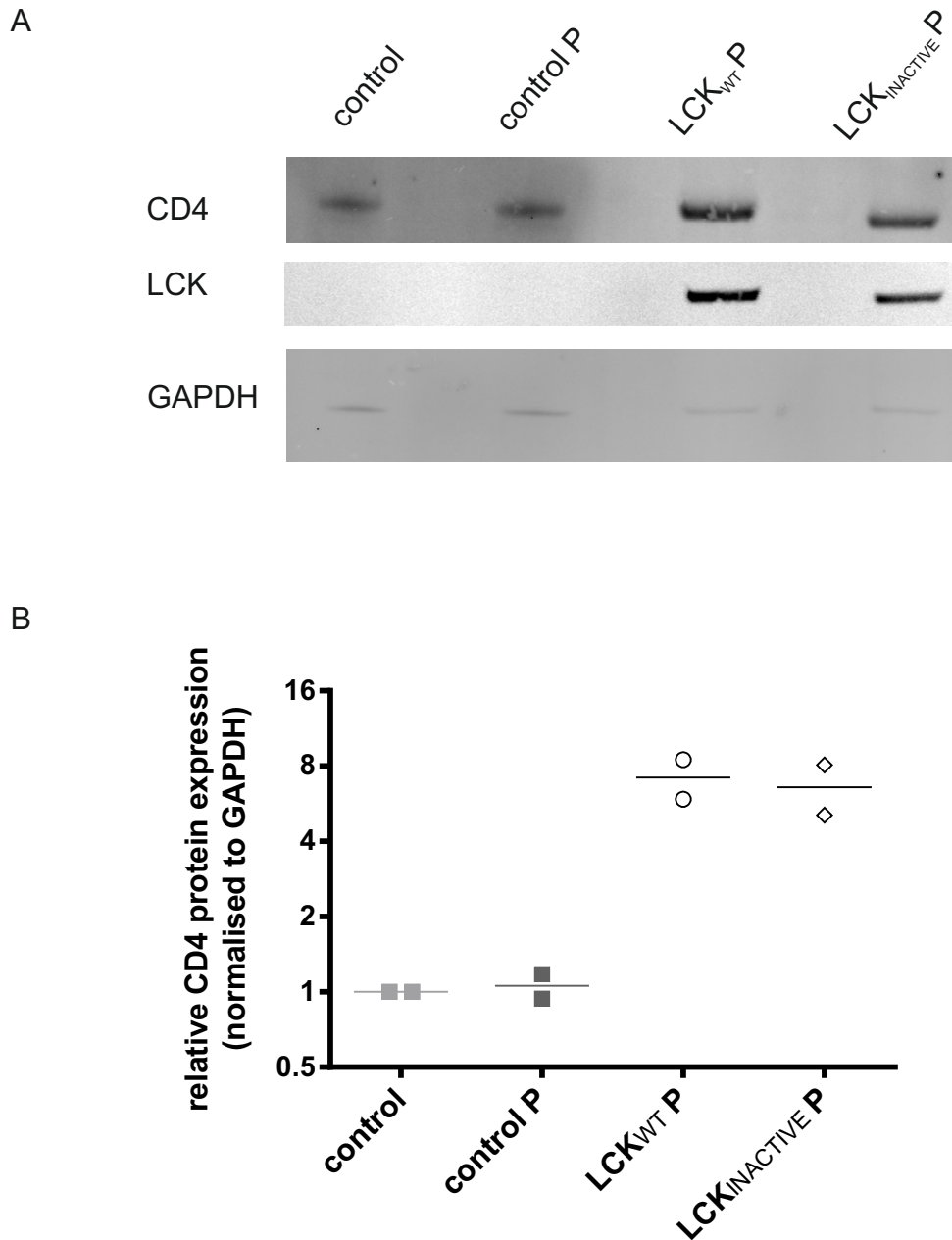
5.3 Higher levels of CD4 are found in esMDM expressing LCK

The pure population of esMC were further cultured in MDM medium supplemented with M-CSF for 4-7 days to direct the differentiation into esMDM. To find out whether esMDM derived from stem cells transduced with LCK_{WT} P or LCK_{INACTIVE} P maintained the expression of these transgenes throughout the differentiation process, esMDM lysates were analysed by western blotting using an anti-LCK antibody (Figure 5.3A). LCK was only detected in lysates from LCK_{WT} P and LCK_{INACTIVE} P esMDM and not in lysates from control and control P esMDM, whereas GAPDH (Figure 5.3A), used as a loading control, was detected in all conditions, thereby validating the constitutive transgene expression system. Having confirmed the expression of LCK in the differentiated MDM, CD4 expression was analysed (Figure 5.3A) and normalised to GAPDH expression. This shows that LCK⁺ esMDM have higher levels of CD4 expression compared to the control esMDM (Figure 5.3B), and this is consistent with previous reports by others using immortalized cell lines (Laguette et al. 2009).

Figure 5.3

Figure 5.3. Higher levels of Cd4 are found in esMDM expressing LCK.

A) To detect protein expression of LCK and CD4, control and transgenic esMDM lysates were analysed by western blotting using anti-LCK and anti-CD4 antibodies. GAPDH, a loading control was, detected using anti-GAPDH antibody. A) Representative blot is shown. B) Protein levels were measured with Odyssey software (Li-COR) and CD4 expression was normalised to GAPDH expression. Symbols represent normalised CD4 expression, relative to the esMDM control group, of two independent experiments.



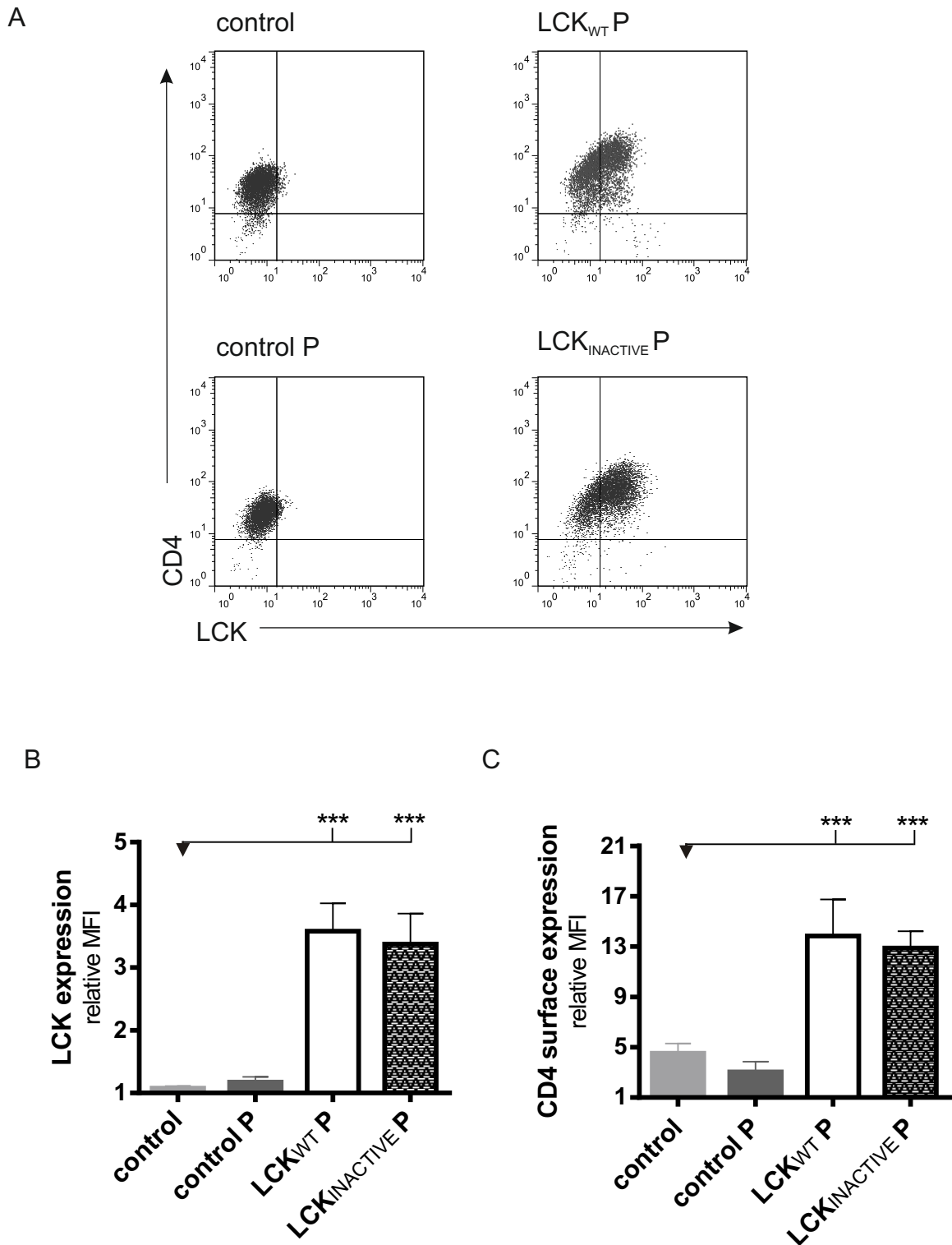
5.4 Higher levels of surface CD4 are found in esMDM expressing LCK

To determine the expression levels of CD4 at the cell surface (as opposed to total cellular CD4) in LCK⁺ esMDM, LCK and surface CD4 expression were measured using two-colour immunofluorescence flow cytometric analysis. LCK was detected in both 'LCK_{WT} P' and LCK_{INACTIVE} P esMDM, but not in control or control P esMDM (Figure 5.4B), as expected, and its expression was associated with significantly higher levels of surface CD4 expression (Figure 5.4A+C). This shows that by expressing LCK in esMDM, CD4 was tethered to the cell surface, as intended. These results are consistent with previous work that showed that when LCK is expressed in myeloid cell lines, CD4 surface levels are increased and CD4 endocytosis is inhibited (Pelchen-Matthews et al. 1998).

Figure 5.4

Figure 5.4. Higher levels of surface CD4 are found in esMDM expressing LCK.

Control and transgenic esMDM were tested for LCK and surface CD4 expression by flow cytometry. A) Representative two-colour immunofluorescence (dot plot) analysis of total LCK and surface CD4 expression. Gates were determined by using the two relevant isotype control antibodies. B) Quantification of LCK expression C) Quantification of CD4 expression. B+C) The bars reflect the ratio of the geometric mean fluorescence intensity (MFI) over the isotype control $\pm$ SEM of independent experiments (n=7). *** $P \leq 0.001$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



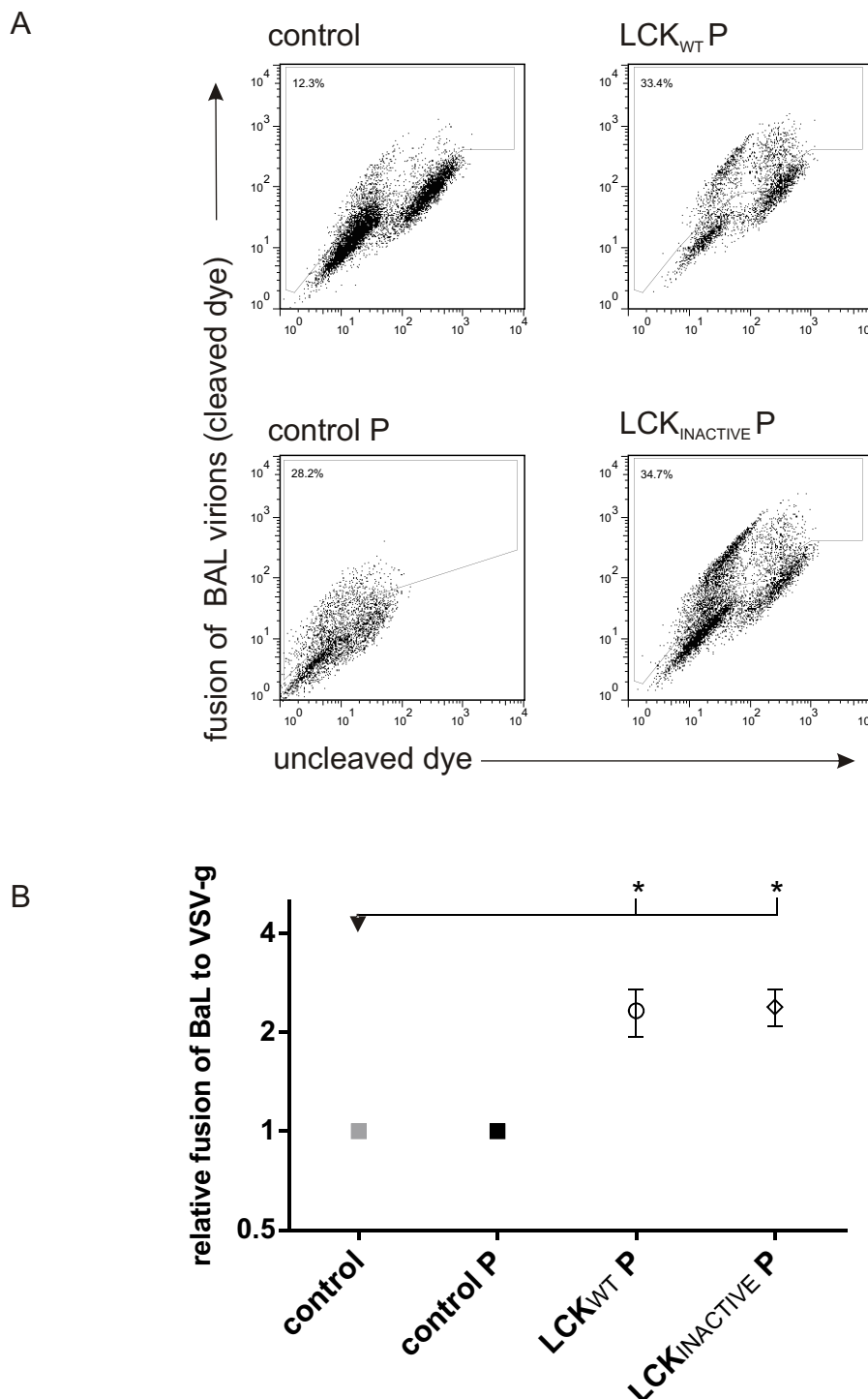
5.5 HIV-1 fusion is increased in esMDM with CD4 tethered to the cell surface

Having shown that LCK expression in authentic esMDM leads to elevated levels of CD4 at the surface, as a result of its ability to stabilise cell surface CD4 and reduce its endocytosis (Pelchen-Matthews et al. 1995), the impact this has on the different stages of HIV-1 infection was evaluated by a range of assays, as will be described here and in the following four sections of this chapter. Firstly, fusion of HIV-1 virions to the cells was measured using a fusion assay. BaL or VSV-G pseudotyped HIV-1 virions containing a β lactamase-Vpr (BlaM-Vpr) fusion protein were used to infect esMDM. esMDM were loaded with a fluorogenic substrate. Following virion fusion, BlaM gains access to the substrate in the cytosol and cleaves the substrate, changing its fluorescence emission, as detected by flow cytometry (Figure 5.5A shows a representative flow cytometry plot). The percentage of BaL-infected esMDM in which fusion had occurred was normalised to the equivalent percentage in VSV-G infected esMDM. VSV-G-pseudotyped virions would be expected to enter all cells with equal efficiency, independent of CD4 expression levels. Based on three independent experiments, compared to control esMDM, fusion was significantly greater in LCK_{WT} P (mean $\pm$ SEM: 2.3 ± 0.38 ; $P \leq 0.05$) and LCK_{INACTIVE} P (mean $\pm$ SEM: 2.4 ± 0.31 ; $P \leq 0.05$) expressing cells. This shows that tethering CD4 to the cell surface leads to increased HIV-1 fusion in esMDM.

Figure 5.5

Figure 5.5. HIV-1 fusion is increased in esMDM with CD4 tethered to the cell surface.

Viral fusion was detected by the fluorescence shift associated with cleavage of the BLaM substrate after 24h of infection of esMDM with NL4.3 BaL (BlaM-Vpr) or NL4.3 VSV-g (BlaM-Vpr), as described in material and methods. A) Representative plots of control and transgenic esMDM infected with NL4.3 BaL (BlaM-Vpr). Gates were set based on the fluorescent signal of uninfected samples loaded with the BlaM substrate. B) The percentage of fusion of BaL pseudotyped virions was normalised to the percentage fusion of VSV-g pseudotyped virions. Symbols represent the normalised mean fusion, relative to the esMDM control group $\pm$ SEM of independent experiments. Control-, LCK_{WT} P and LCK_{INACTIVE} P (n=3) and control P esMDM (n=1). **P $\leq$ 0.01; ***P $\leq$ 0.001 One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



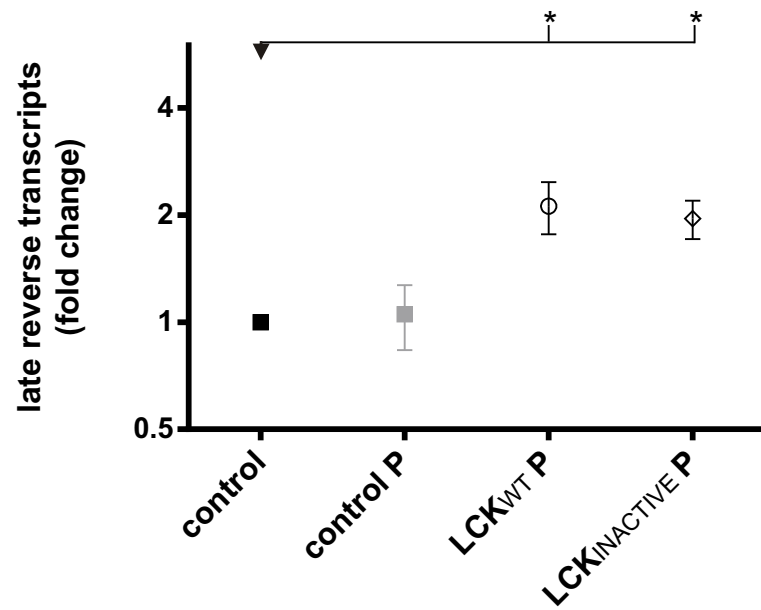
5.6 HIV-1 reverse transcription is increased in esMDM with CD4 tethered to the cell surface

Post fusion, an essential step in the HIV-1 life cycle is reverse transcription: the conversion of vRNA into DNA. To determine whether this stage was affected by surface CD4 stabilisation, reverse transcription was measured in esMDM-infected cells. To quantify HIV-1 reverse transcription, the number of copies of HIV-1 late reverse transcription products by qPCR after spinoculation and 28 h of infection were measured, using β actin primers to normalise to the number of cells present. Based on three separate experiments, 2.1X and 1.9X more copies of late reverse transcript per cell were detected in esMDM expressing LCK_{WT} P or LCK_{INACTIVE} P compared to control esMDM (Figure 5.6). This shows that, in addition to increased fusion, tethering CD4 to the cell surface leads to increased HIV-1 reverse transcription as well.

Figure 5.6

Figure 5.6. HIV-1 reverse transcription is increased in esMDM with CD4 tethered to the cell surface.

Reverse transcription of HIV-1 in esMDM was measured by detecting late (pol) products by qPCR after 30h of infection. Symbols represent the relative mean number of copies of HIV-1 DNA $\pm$ SEM of independent experiments ($n=3$), normalised to the number of cells using a β -actin control. * $P \leq 0.05$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



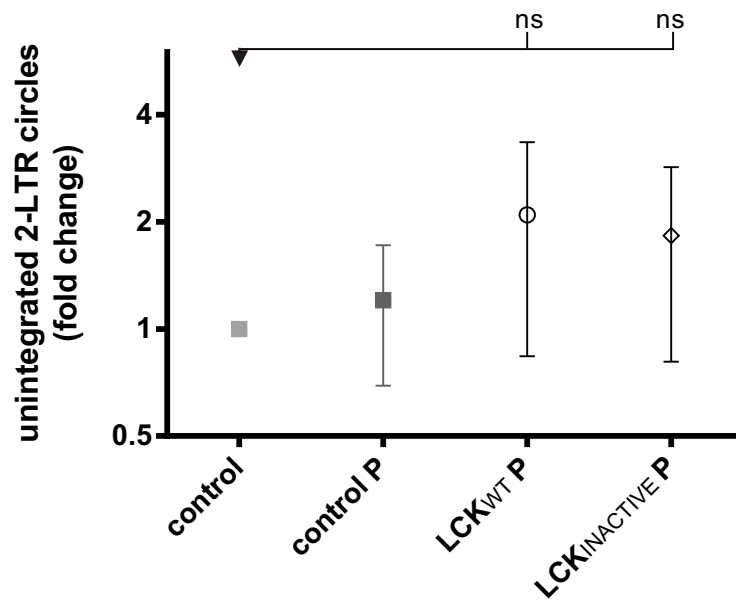
5.7 2-LTR circles are not significantly increased in esMDM with CD4 tethered to the cell surface

To investigate the efficiency of stages of viral infection following the early events of viral fusion and cDNA synthesis, the presence of a different DNA viral form, namely unintegrated 2-LTRs circles, was quantified. HIV-1 2-LTR circles are formed in the cell nucleus from HIV-1 cDNA that fails to integrate. Their concentration is directly proportional to the amount of viral DNA imported into the nucleus, and consequently is used as a useful marker for nuclear import (Bukrinsky et al. 1993; Mandal et al. 2009). The relative levels of 2-LTR circles by qPCR after spinoculation and 28h of infection was measured, using β actin primers as an internal control. With three separate experiments, levels of unintegrated 2-LTR circles in esMDM expressing LCK_{WT} P (mean $\pm$ SEM: 2.0 ± 1.14) or LCK_{INACTIVE} P (mean $\pm$ SEM: 1.8 ± 1.2) were not significantly different compared to control cells (Figure 5.7). The results suggest that when CD4 is tethered to the cell surface in esMDM, HIV-1 fusion and reverse transcription are enhanced, but this does not result in an increase in viruses following a successful route into to the nucleus.

Figure 5.7

Figure 5.7. 2-LTR circles are not significantly increased in esMDM with CD4 tethered to the cell surface.

As a marker for nuclear import of viral DNA in esMDM, unintegrated 2-LTR DNA circles were measured by qPCR after 30h of infection. Symbols represent the mean number of copies of HIV-1 2-LTR DNA circles, relative to the esMDM control group $\pm$ SEM of independent experiments. Control, control P and LCK_{WT} P (n=4) and LCK_{INACTIVE} (n=3). ns: non-significant (P>0.05). One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



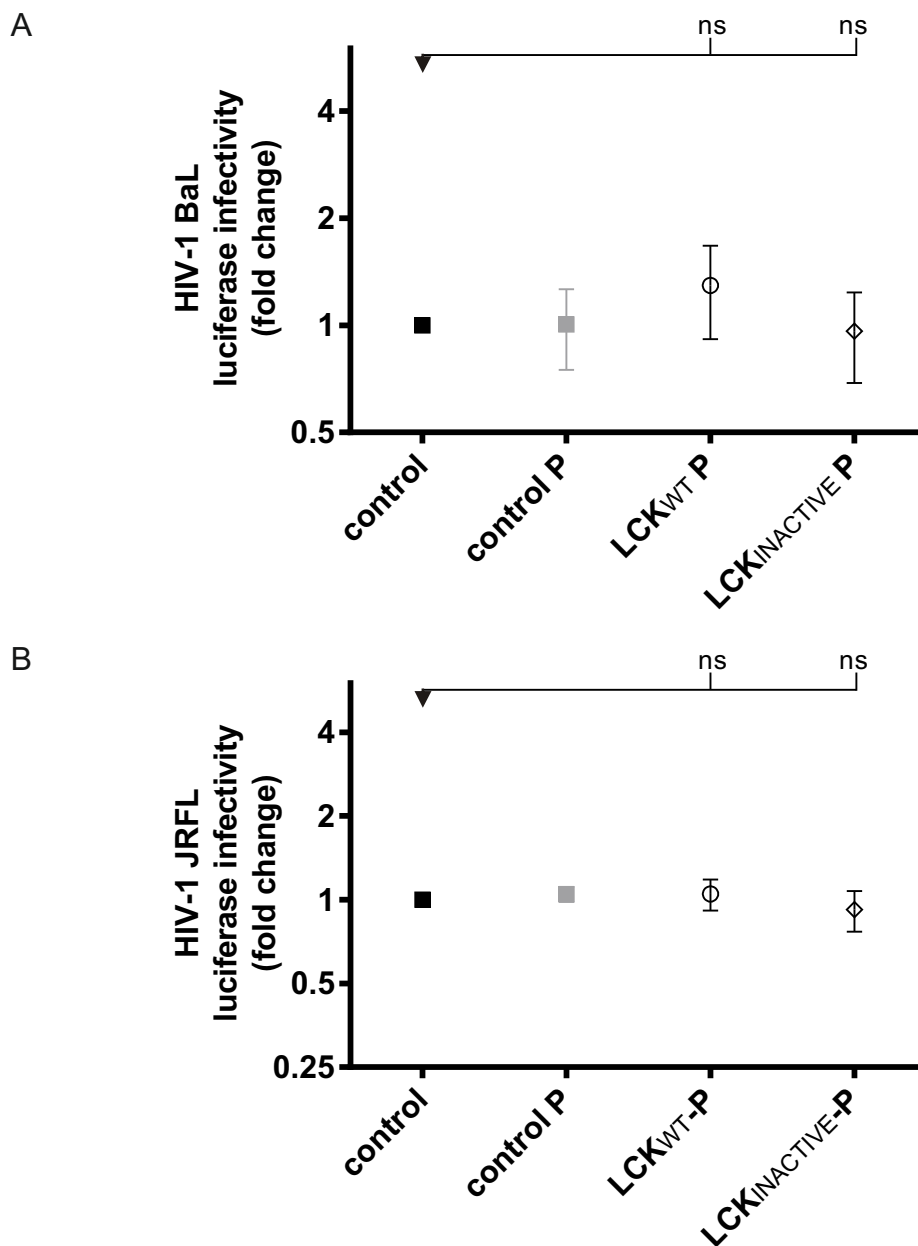
5.8 Reporter expression from HIV-1 integrants is not significantly increased in esMDM with CD4 tethered to the cell surface

Once the vRNA has been reverse transcribed into a strand of cDNA and has been imported into the nucleus, the viral cDNA can be integrated into the host cell DNA. Only once the viral DNA (provirus) is transcribed, can new viruses be produced. Therefore, a reporter assay from HIV-1 integrants was used as measure of a single round of productive infection. This assay uses a replication-defective pseudotyped HIV-1 virus that has the gene for luciferase inserted in place of the *nef* gene, and can therefore function as a reporter gene for infection. esMDM were infected with JRFL-NL4.3, BaL-NL4.3 or VSV-G-NL4.3, for 3 days before cells were lysed and luciferase expression was measured. The luciferase activity of esMDM infected with JRFL-NL4.3 and BaL-NL4.3 was normalised to esMDM infected with VSV-G-NL4.3. There was no significant difference in detection of luciferase protein produced in lysates from esMDM expressing LCK_{WT} P or esMDM LCK_{INACTIVE} P compared to control esMDM, that had been infected with either BaL-NL4.3 (Figure 5.8A; mean $\pm$ SEM LCK_{WT} P: 1.3 ± 0.35 ; mean $\pm$ SEM LCK_{INACTIVE} P: 0.9 ± 0.36) or JRFL-NL4.3 (Figure 5.8B; mean $\pm$ SEM LCK_{WT} P: 1.0 ± 0.15 ; mean $\pm$ SEM LCK_{INACTIVE} P: 0.9 ± 0.15). The results are in accordance with the previous result, suggesting that when CD4 is tethered to the cell surface in esMDM, HIV-1 fusion and reverse transcription are enhanced, but this does not result in an increase in viruses following a successful route into to the nucleus.

Figure 5.8

Figure 5.8. Reporter expression from HIV-1 integrants is not significantly increased in esMDM with CD4 tethered to the cell surface.

HIV-1 integration was measured by detecting luciferase activity in esMDM three days after transduction with HIV-1 NL4.3.Luc.R-E- virus pseudotyped with JRFL, BaL or VSV-g envelope. Luciferase expression was quantified using Promega One-Glo luciferase substrate and a luminometer. A) BaL NL4.3.Luc.R-E- transduced cells were normalised to VSV-g NL4.3.Luc.R-E- transduced cells. Symbols represent the mean luciferase detection relative to the esMDM control group, $\pm$ SEM of independent experiments (control, n=8; control P, n=3; LCK_{WT} P, n=8; LCK_{INACTIVE} P, n=7). B) JRFL NL4.3.Luc.R-E- transduced cells were normalised to VSV-g NL4.3.Luc.R-E- transduced cells. Symbols represent the mean luciferase detection relative to the esMDM control group $\pm$ SEM of independent experiments (n=3). ns: non-significant (P>0.05). One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM control group.



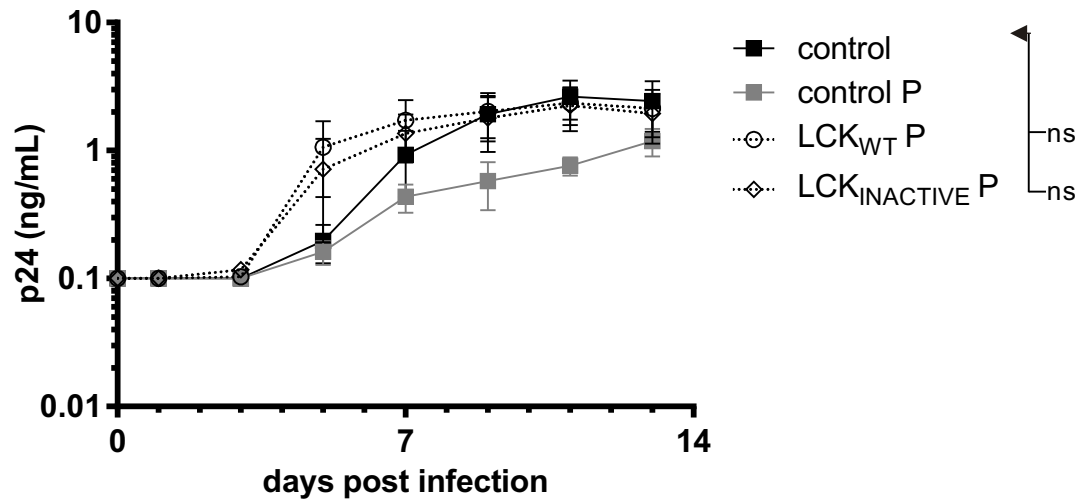
5.9 Multiple rounds of HIV-1 replication are not significantly increased in esMDM with CD4 tethered to the cell surface.

The above assays measured events during a single cycle of HIV-1 infection. Once new viruses are produced and bud out of the infected cell, the cycle repeats itself. A multi-cycle assay was also performed. It is important to note here that HIV-1 replication in LCK⁺ MDM over multiple generations may change the phenotype of these cells, and this might complicate the interpretation of the results. HIV-1 replication kinetics in LCK⁺ and control esMDM were measured by culturing HIV-1 BaL infected esMDM for 2 weeks. Samples of the supernatant were taken at intervals and assayed for viral p24 protein levels by ELISA. Infection with HIV-1 BaL resulted in similar levels of virus being produced by LCK_{WT} P or LCK_{INACTIVE} P esMDM compared to control esMDM. Although this assay probably does not give as accurate reflection of successful infection as the previous experiments, owing to amplification effects, the results are in accordance with the previous results. That is, when CD4 is tethered to the cell surface in esMDM, more HIV-1 virions fuse and reverse transcribe, but this does not result in an increase in viruses following a successful route into to the nucleus. Accordingly, 2-LTR circle formation, reporter expression from HIV-1 integrants and HIV-1 replication are not increased.

Figure 5.9

Figure 5.9. Multiple rounds of HIV-1 replication are not significantly increased in esMDM with CD4 tethered to the cell surface.

HIV-1 replication in esMDM was measured by p24 ELISA. esMDM were infected with HIV-1 BaL. Samples of the supernatant were taken at regular intervals and assayed for p24 levels by ELISA. Symbols represent the mean p24 concentration at different time points $\pm$ SEM of independent experiments, esMDM control; LCK_{WT} P; LCK_{INACTIVE} P (n=3) and control P (n=2). ns: non-significant ($P>0.05$). Repeated measure one-way ANOVA followed by Holm-Sidak test or multiple t-test for different time points versus the esMDM control group.



5.10 Summary and discussion

Exogenous LCK was expressed for the first time in a hESC-derived macrophage. This led to tethering of CD4 to the cell surface, and increased CD4 expression. Challenge of these cells with HIV-1 did not affect productive HIV-1 infection efficiency (determined by reporter expression from DNA integrants and a replication assay). Therefore, the null hypothesis is accepted: **successful** HIV-1 entry in MDM is not affected by redistribution of sub-cellular CD4. This is in contrast to results found by others in cell lines, and confirms that entry in MDM occurs through a distinct pathway. Interestingly, despite the fact that productive infection was unaffected, redistribution of sub-cellular CD4 did affect early steps of HIV-1 entry in MDM. CD4 surface tethering led to increased fusion and reverse transcription, but this did not result in an increase in viruses following a successful route into the nucleus. This implies that diverting CD4 away from its normal recycling route causes sequestering of HIV-1 into an unproductive pathway.

The findings confirmed that hESC-derived MDM offer a successful genetic modification system allowing constitutive expression of LCK in human authentic MDM to manipulate sub-cellular CD4 distribution. In addition to its mechanical function to stabilise CD4 at the cell surface and prevent CD4 endocytosis, LCK may contribute to HIV-1 signaling pathways (Pelchen-Matthews et al. 1992; Briant et al. 1998; Foti et al. 2002). To account for this variable, a kinase-dead version of LCK (LCK_{INACTIVE}) was expressed in addition to LCK_{WT}. Both versions of LCK had a similar effect on CD4 distribution and HIV-1 infection in all assays. This indicates that wild-type LCK likely did not interfere in any relevant signaling pathways in MDM and predominantly functioned mechanically, to tether CD4 at the cell surface. However, LCK may have affected CD4 dimerization. CD4 dimerization has been observed in many cell types, including myeloid cell lines and Langerhans cells, but has not been studied in bMDM or esMDM (Lynch et al. 1999; Lynch et al. 2003;

Fournier et al. 2010). It would be valuable to study CD4 dimerization in the context of HIV-1 and LCK expression, considering dimerization could reduce CD4 localization to DRM and LCK may prevent CD4 dimerization, as CD4 dimerization occurs at the same two cysteine residues at positions 420/422 that are required for the CD4-LCK association (Fournier et al. 2010). Irrespective of CD4 dimerization, expressing either LCK_{WT} or LCK_{INACTIVE} in MDM resulted in increased CD4 surface levels (Figure 5.4). Also, total expression levels (Figure 5.3) were increased, suggesting that tethering CD4 at the surface reduced the rates of CD4 degradation. This is as expected, considering LCK, which binds two cysteine residues at positions 420/422 in CD4, masks the di-leucine motif in CD4 at position L413/L414, that would otherwise interact with the clathrin adaptor complex AP2, which would lead to CD4 recycling to lysosomes (Turner et al. 1990; Pitcher et al. 1999; Bentham et al. 2003; Laguette et al. 2009). So, LCK reduces clathrin-mediated CD4 internalization and the resultant proteolytic turnover of CD4.

The increased expression of CD4 levels led to an increase of HIV-1 fusion. This is as expected, unless saturating CD4 levels are achieved, which is unlikely given that CD4 levels are very low in MDM compared to for example T-cells (Lee et al. 1999). Despite the fact that M-tropic isolates generally evolve to infect cells with low levels of CD4 and CCR5, several studies using cell lines expressing varying concentrations of CD4 and/or co-receptors have shown that infection of cells expressing high concentrations of CD4 leads to increased infection, which may also depend on CCR5 concentration (Kabat et al. 1994; Kozak et al. 1997; Bannert et al. 2000; Peters et al. 2004; Gray et al. 2005; Platt et al. 2005; Arrildt et al. 2012). These studies suggest that faster HIV-1 fusion kinetics occur with increased CD4-co-receptor complexes, whereby the requirements for CD4 or CCR5 is decreased when the other is present at higher concentrations (Platt et al. 1998; Lapham et al. 1999; Platt et al. 2005). As such, the increased HIV-1 fusion observed in CD4^{high} LCK⁺ MDM likely occurred more quickly and therefore closer to or at the plasma membrane, whereas in MDM with normal levels of CD4, HIV-1 fusion is thought to occur

predominantly after macropinocytosis-like uptake, inside the cytosol (Pelchen-Matthews et al. 1998; Marechal et al. 2001; Carter et al. 2011; Gobeil et al. 2012). Low levels of CD4, as normally found on the surface of MDM, would result in low fusion kinetics and lead to the accumulation of unfused virions at the cell surface, as such favouring virion uptake through endocytosis over plasma membrane fusion (Permanyer et al. 2010).

Interestingly, despite the higher levels of CD4 and enhancement of HIV-1 fusion in LCK⁺ MDM, the diversion of CD4 away from its normal recycling route sequestered HIV-1 into an **unproductive** pathway, likely proximal/at the surface membrane. This result reiterates the importance of the macropinocytosis-like **productive** entry route in MDM, because if HIV infection in MDM was by surface fusion, enhancing plasma membrane fusion by tethering CD4 to the cell surface through LCK expression would have resulted in increased infection efficiency during all stages of the HIV-1 entry pathway. If expressing LCK would re-direct all virions to fuse at the plasma membrane, one would expect a reduction of productive infection. However, although the LCK-CD4 interaction prevents clathrin-mediated endocytosis of CD4, it is not expected to reduce the rate of CD4 undergoing macropinocytosis-like internalisation, as this is a non-specific uptake process (Mercer et al. 2010). As such, HIV-1 could still enter through the macropinocytosis-like entry pathway and lead to productive infection. Nonetheless, the increase in fusion and reverse transcription in LCK⁺ MDM, resulted from high CD4 surface levels and increased fusion, most likely closer by, or at the plasma membrane. The results are summarized in Figure 5.10 and suggest the following model of HIV-1 infection in MDM: HIV-1 can enter both by plasma membrane fusion or fusion after endocytosis, but the latter pathway is more productive and favourable in MDM. The type of endocytic route may be different in different cell types. In the case of MDM, the productive endocytic route is probably a raft dependent macropinocytosis-like pathway, as described by *Carter et al.* (Carter et al. 2009; Carter et al. 2011). The role of CD4 raft localization in HIV-1 entry in MDM is unclear. LCK can associate with rafts due to myristoylation at glycine at position 2 at the

N-terminus and palmitoylation of at least one of the two cysteine residues at position 3 and 5 (Rodgers et al. 1994; Kwong et al. 1995; Filipp et al. 2003). LCK can form a complex with CD4 inside rafts, which in T-cells may be dependent on the cell activation state (Nyakeriga et al. 2009). To clarify the importance of CD4 raft localization on the successful HIV-1 entry pathway in MDM, it would be valuable to manipulate the CD4 raft localization in the absence of LCK. The latter was achieved in Chapter 6.

The location of fusion events that leads to successful HIV-1 entry in cell lines and primary T-cells may be different from that in MDM. For example, by analysing the presence of the HIV-1 p24 capsid protein in cytosolic and membrane-bound fractions enriched in endosomes 1 h post infection, *Schaeffer et al.* showed that in Jurkat T cells ~40 % of fusion events occur at the plasma membrane and ~60 % in the cytosol, whereas in HeLa-CD4 cells, 85 to 90 % of total viral entry is mediated through endocytosis (Schaeffer et al. 2001). However, in contrast to MDM, endocytic entry in T-cells and cell lines is thought to be predominantly an unproductive pathway that leads to HIV-1 being secreted or inactivated in acidified endosomes and degraded in lysosomes (Bomsel 1997; Marechal et al. 1998; Fredericksen et al. 2002; Vidricaire et al. 2003; Schaeffer et al. 2004). Several studies in cell lines show that expression of CD4 mutants that are impaired of endocytosis do not results in decreased infection, implying that productive infection does not occur after endocytosis in these cells (Bedinger et al. 1988; Maddon et al. 1988; Corbeil et al. 1996). Moreover, inhibiting CD4 endocytosis and increasing plasma membrane fusion using hypertonic medium, enhances productive infection in HeLa-CD4 cell (Pelchen-Matthews et al. 1995). In a similar approach as used in this chapter, LCK⁺ HeLa cells (expressing a poorly endocytosed CD4 construct) resulted in increased successful HIV-1 entry compared to LCK⁻ HeLa cells (expressing wild-type CD4 or a poorly endocytosed CD4 construct) (Pelchen-Matthews et al. 1995). This is in contrast with what was found in LCK⁺ MDM, highlighting the fact that HIV-1 does not behave the same in different cell-types. Although many studies indicate that in cell lines and T-cells productive infection

occurs predominantly at the cell surface, there are also reports arguing that some of the virions entering through endocytosis in these cell types may lead to productive infection, yet the efficiency, type of endocytic pathway and molecules involved may vary (Stein et al. 1987; Daেকে et al. 2005; de la Vega et al. 2011). The contribution of fusion after endocytosis may be determined by cell type-specific differences including, CD4-co-receptor complex levels, fusion kinetics, cell morphology, restriction factors, cytoskeleton and signaling pathways, which may also explain why fusion at or proximal to the plasma membrane may be unproductive in MDM.

The relatively large size of MDM compared to T-cells, could offer a simple, hypothetical explanation for a reduction in productive infection when fusion is re-directed from fusion-after-endocytosis to plasma membrane fusion in MDM. MDM have a diameter of about 21 μM with a relatively large cytoplasm, whereas lymphoid cell are about half the size with a relatively small cytoplasm, so virions would have to travel a relatively long way to reach the nucleus in MDM (Geha et al. 1973; Krombach et al. 1997). For example, entry through endocytic vesicles is important for viruses infecting neurons, in order to migrate along long axons to reach the cell body (Mercer et al. 2010). As endosomes mature, changes in the vesicle microenvironment may inform viruses about their location within the cell and may facilitate correct timing of fusion (Marsh et al. 1989; Mercer et al. 2010). Furthermore, hijacking the endocytic machinery to migrate to the nucleus could allow escape from some innate immune response and cellular restriction factors. For example, HIV-1 is recognized by Pattern Recognition Receptors (PRR), such as the cytosolic RNA helicases retinoic acid-inducible gene I (RIG-I) (Berg et al. 2012). However, endocytosis of HIV-1 may also activate the innate immune response, for example Toll-Like Receptors can be activated by viral nucleic acids that have been endosomally delivered (Beignon et al. 2005; Lepelley et al. 2011). Moreover, HIV-1 activates interferon-stimulated genes, without inducing any interferons through PPRs in MDM by inhibiting nuclear translocation of interferon-regulated factor (IRF3) (Nasr et al. 2012). Nonetheless, irrespective of its

fusion location, HIV-1 has developed effective strategies to evade immune responses. For example, the HIV-1 protease can inhibit IRF-3 phosphorylation, as such decreasing expression of interferon (IFN) and IFN-stimulated genes (Solis et al. 2011). Moreover, the innate immune response is inhibited by the exonuclease TREX1, which binds to excess HIV-1 DNA that would otherwise activate interferon expression (Yan et al. 2010). So, although using an endocytic entry route may not necessarily be more beneficial to evade an immune response, it nonetheless may enhance HIV-1 infection in large cell types such as MDM that have multiple pathogen detection mechanisms, by facilitating transport of virions to the nuclear membrane. Movements through the cytoplasm are guided by the cytoskeleton, along microtubules and actin filaments (Rogers et al. 2000). Differences in actin dynamics between cell types affect their susceptibility to HIV-1 infection and may explain why plasma membrane fusion is not a productive entry path in MDM (Wang et al. 2012).

The cytoskeleton has been shown to be important in HIV-1 entry and may function as post-entry barrier as well as a necessary cofactor for productive infection, as recently reviewed by (Spear et al. 2012). In order to enter cells, viruses must pass through a cross-linked actin network present at the plasma membrane, which is also known as the actin barrier (Salbreux et al. 2012). Endocytosis naturally overcomes this barrier and can function as a productive entry route, providing that the endocytic pathway hijacked by HIV-1 allows the virus to fuse after uptake in order to escape virus degradation in endosomal compartments (Stolp et al. 2011; Mooren et al. 2012). Viruses that fuse at the plasma membrane must actively overcome the actin-barrier, which is achieved by activation of cellular signaling pathways. In HeLa cells, actin remodelling is achieved through a function of its Nef protein, which is not important for fusion after endocytosis of VSV-G pseudotyped HIV-1 (Campbell et al. 2004). In resting T cells, cortical actin rearrangements are achieved by a signaling cascade induced by gp120 binding to CD4-CXCR4-complex or CXCR4, involving actin regulators such as cofilin and LIMK (Yoder et

al. 2008; Vorster et al. 2011). The importance of plasma membrane fusion and these signaling pathways in resting T-cells is highlighted by the finding that viral DNA synthesis is decreased when virions are delivered directly to the cytosol without engaging the CD4-co-receptor complex using VSV-G-mediated entry (Yu et al. 2009). In active T-cells, cofilin is constitutively active to facilitate constant actin remodelling, allowing HIV-1 to efficiently overcome the actin barrier (Spina et al. 1997; Yoder et al. 2008). In MDM, it is unclear whether HIV-1 can induce signaling pathways to induce remodelling of the actin cortex. Actin remodelling and LIMK activation have been implicated in HIV-1 entry in MDM, but have not been described in detail and may function predominantly during the endocytic pathway, rather than during fusion at the plasma membrane (Carter et al. 2011; Vorster et al. 2011). The results presented in this chapter showed that fusion and reverse transcription, but not 2-LTR circle formation were increased in LCK⁺ MDM, suggesting that when HIV-1 fusion is re-directed to the plasma membrane, virions cannot penetrate the actin barrier in MDM to establish productive infection. In this scenario, according to the suggested model, reverse transcription complexes are sequestered in the actin cortex and do not succeed to uncoat nor to form a pre-integration complexes at the-nuclear pore. In cell lines and T-cells, it has been demonstrated that the large subunit of the reverse transcriptase interacts with actin and that blocking actin polymerization with inhibitors of actin remodelling inhibits viral DNA synthesis (Hottiger et al. 1995; Yoder et al. 2008; Yu et al. 2009). The results presented in this chapter suggest that also in MDM, the actin cortex can function as an anchor for reverse transcription (Bukrinskaya et al. 1998; Iyengar et al. 1998; Wu et al. 2009; Yu et al. 2009). Unlike other cell types however, HIV-1 is unable to hijack the cytoskeleton of MDM to transport the reverse transcription complexes from the plasma membrane to the nuclear pore, where HIV uncoating and nuclear import is thought to take place (Arhel et al. 2007).

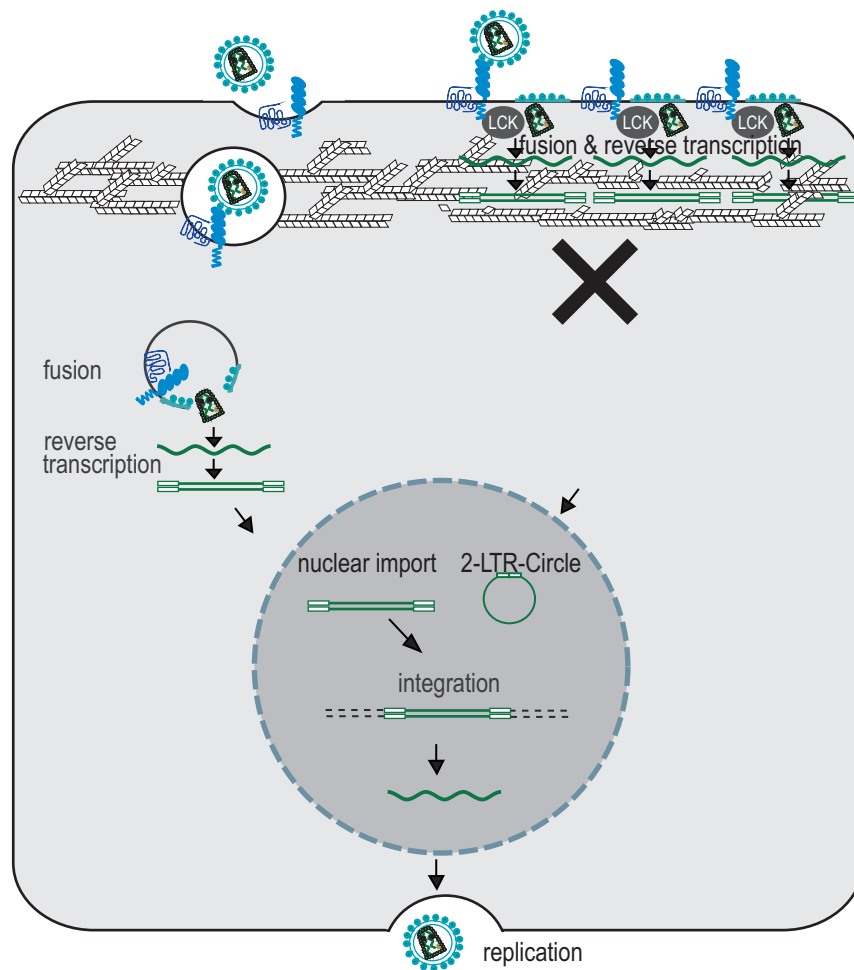
Taken together, as illustrated in Figure 5.10, the model proposed, is that the cortical actin barrier is overcome by fusion after endocytosis in MDM, but when fusion is redirected

close to, or at the plasma membrane, virions are unable to induce cortical actin remodelling to overcome this barrier. Currently it is unclear whether this is due to differences between the actin cortex of MDM and T-cells or whether MDM lack adequate chemokine co-receptor signaling pathways. Future studies addressing the involvement of actin remodelling and signaling pathways upon HIV-1 entry in MDM would be valuable. Considering the actin-cytoskeleton is differentially involved in many different cellular processes, it would be important to differentiate between both the location of fusion, as well as the different stages of entry (e.g. endocytosis, fusion, reverse transcription, nuclear migration, uncoating). The results presented in this Chapter highlight the importance for HIV-1 to use the macropinocytosis-like entry pathway in MDM, as opposed to the plasma membrane pathway, to achieve productive infection, and to do so, requires MDM-specific sub-cellular CD4 distribution.

Figure 5.10

Figure 5.10. Tethering CD4 at the cell surface leads to increased HIV entry but not in productive infection in esMDM.

HIV-1 infection in MDM can occur at the plasma membrane or inside the cell after endocytosis. Fusion after endocytosis is more productive in MDM. This is illustrated by expressing LCK in MDM, which tethers CD4 to the cell surface and allows more virions to fuse at the cell surface (right). Although this leads to increased fusion and reverse transcription, this does not lead to an increase in productive infection. This is likely due to the barrier formed by cortical actin. In contrast, by entering via an endocytic route, virions can overcome the actin barrier and fuse after endocytosis.



Chapter 6: CD4 localization to Detergent Resistant Membrane is required for successful HIV-1 entry in esMDM

Introduction

HIV-1 entry in macrophages (MDM) is dependent on intact Detergent Resistant Membranes (DRM), which are extracts of cells that are believed to represent the composition of lipid rafts (Carter et al. 2009). Lipid rafts were discussed in more detail the introduction (Chapter 1.3.5). The HIV-1 receptors CD4 and CCR5 associate within these plasma membrane microdomains in MDM (Carter et al. 2009). The role of lipid rafts in authentic MDM has only been studied using pharmacological agents, which may have multiple (off-target) or non-specific effects (Carter et al. 2009). Here, the role of CD4 localization to DRM in HIV-1 entry into authentic MDM was further refined in a highly specific and controlled manner using a genetic approach.

The endocytic entry pathway used by HIV-1 to enter MDM has some unique features, but shares many cellular and signaling components with macropinocytosis, and requires cholesterol and, as mentioned above, intact DRM (Marechal et al. 2001; Carter et al. 2009; Carter et al. 2011). Localization of HIV-1 to DRM in MDM can be visualized using fluorescent Cholera toxin B subunit binding to the raft glycolipid GM1 (Carter et al. 2009). Cholesterol disruption of membranes using four different pharmacological agents all significantly inhibit successful HIV-1 entry in MDM (Carter et al. 2009). Raft- and cholesterol-dependent endocytic routes have also been shown to be important for HIV-1 entry into other cell types, such as brain microvascular endothelium and placental trophoblasts, respectively (Liu et al. 2002; Vidricaire et al. 2007). HIV-1 transcytosis through the mucosal epithelium is dependent on lipid raft associated proteins (Alfsen et al. 2001). Other pathogens, such as the bacteria *Brucella* and *Afipia felis*, enter MDM by raft-dependent macropinocytosis (Watarai et al. 2002; Schneider et al. 2007). Several pathogens, including the respiratory bacterium *Legionella pneumophila*, the urobacterium

FimH⁺ *Escherichia coli*, the parasite *Toxoplasma gondii*, the virus SV40 and cholera toxin B secreted by the bacterium *Vibrio cholera*, associate with lipid rafts before taken up by the MDM autophagy machinery (Baorto et al. 1997; Mordue et al. 1999; Norkin et al. 2002; Fujinaga et al. 2003; Amer et al. 2005). Finally, a range of viral infections in different cell types can use macropinocytosis, or similar endocytic routes, to infect a range of cell types (Mercer et al. 2009). Taken all together, raft-dependent macropinocytosis-like pathways are frequently hijacked by pathogens to enter a cell, as is the case for HIV-1 entry into MDM (Carter et al. 2009; Carter et al. 2011).

In addition to HIV-1 itself, CCR5 (the coreceptor typically used by M-tropic viruses) localizes exclusively in DRM in MDM and CD4 predominantly localizes in DRM (Carter et al. 2009). The surface expression levels of CD4 and CCR5 are decreased by 4 different pharmacological agents which disrupt cholesterol, the organising component of lipid raft microdomains (Carter et al. 2009). Similar to other cell types, electron micrographs revealed that CD4 and CCR5 localize on the outer membranes of microvilli and blebs in MDM, forming homogeneous microclusters (Singer et al. 2001). Proteomic analysis showed that CD4 associates with DRM associated proteins in MDM (Raposo et al. 2011). However, unlike in other cell types, CD4 DRM localization has not yet been demonstrated using a genetic approach in MDM. DRM exclusion can be achieved by mutating two regions in the cytoplasmic tail of CD4 that have been shown to be important for the DRM localization of CD4. The first region is composed of two cysteines at position 394 and 397 that are palmitoylated in wild-type CD4, but not in CD4 in which these Cysteines are substituted with Serines (Crise et al. 1992; Popik et al. 2004). This mutant has been found to reduce DRM-localization in a range of cell types, including primary T-cells and cell lines, as determined biochemically using Triton X-100 solubilisation and by confocal microscopy (Crise et al. 1992; Fragoso et al. 2003; Percherancier et al. 2003; Balamuth et al. 2004). Nonetheless, palmitoylation may not be absolutely necessary for CD4 DRM localization, considering *Popik et al.* reported that irrespective of CD4 palmitoylation a

second region in the cytoplasmic tail of CD4, a short sequence of positively charged residues (RHRRR), may control CD4 partitioning to DRM (Popik et al. 2004). Substitution of these residues with Alanine residues excludes CD4 from DRM, as determined by Triton X-100 solubilisation and by confocal microscopy using a B-cell line (Popik et al. 2004).

The exact role of DRM localization of CD4 in HIV-1 infection of MDM remains unclear. Cholesterol depletion reduces HIV-1 infection in MDM, likely by reducing CD4 and CCR5 DRM association and abundance, thereby reducing HIV-1 fusion after endocytosis. However, there could be other factors that lead to reduced HIV-1 infection upon cholesterol depletion, such as inhibition of the endocytic HIV-1 uptake pathway in MDM, as has been reported in brain microvascular endothelia (Liu et al. 2002). To clarify the role of CD4 localization to DRM microdomains during HIV-1 infection of MDM, I hypothesised that successful HIV-1 entry in MDM will be affected by excluding CD4 from DRM. The hypothesis was tested by expressing CD4 cytoplasmic tail mutants in authentic MDM. In order to test this hypothesis, I also reasoned that the lentiviral shRNA expression system that allows knockdown of endogenous CD4 in esMDM (as described in Chapter 4) could be further developed to also allow knock-in of exogenous wild-type or mutant CD4 in esMDM, which is not affected by the shCD4.

In this Chapter, a novel version of wild-type and mutant CD4 were designed and inserted in the lentiviral expression system, LV-U6-shCD4-EF1-GOI-I-Puro^R. Expression from the lentiviral vectors were first tested in a cell line. Next, hESC were transduced with these second-generation lentiviral based vectors and differentiated into esMDM. Integration of the constructs in esMDM was confirmed using qPCR. CD4 knockdown and knock-in were tested by measuring mRNA and protein expression levels. Further, the phenotype of the CD4 cytoplasmic tail mutants was analysed, using a flow cytometric assay of detergent resistance. After assessing the novel lentiviral knockdown knock-in expression system in esMDM, the hypothesis that successful HIV-1 entry in MDM will be affected by excluding

CD4 from DRM was tested. A panel of assays were used as readouts for HIV-1 infection, allowing dissection of the entry pathway. This included three single-cycle assays (“HIV virion fusion assay”, “qPCR of HIV-1 late reverse transcripts” and “HIV-1 integration (luciferase)” and a less informative multi-cycle assay (“quantification of p24 antigen by ELISA”). If CD4 exclusion from DRM leads to a decrease in infection as measured by at least the first three single-cycle assays, it would provide direct evidence for the requirement for CD4 DRM localization in HIV infection of MDM.

6.1 Design and construction of lentiviral vectors encoding CD4 DRM localization mutants

Previously, as described in Chapter 3, efficient CD4-knockdown was achieved in esMDM, using LV-U6-shCD4-EF1-GOI-I-Puro^R. CD4-knockdown esMDM inhibited successful HIV-1 entry efficiency. Here, the same lentiviral expression system was used to not only knockdown endogenous CD4, but also simultaneous knock-in shCD4-resistant wild-type or mutant CD4, collectively referred to as LV-U6-shCD4-EF1-CD4-I-Puro^R. To differentiate between endogenous CD4 and CD4 expression from this lentiviral vector, chimeric versions of CD4 were designed using a combination of rat and human CD4 domains. Although the overall protein sequence overlap is only 54 %, human and rat CD4 are homologous proteins and share a similar structure: an extracellular region of 4 immunoglobulin domains, a transmembrane (TM) domain and a cytoplasmic tail (Clark et al. 1987). As such, one would expect the wild-type chimera to localize and function similar to endogenous human CD4. Human and rat CD4 consist of a signaling peptide of 25 amino acids in humans (position -25-0) or 27 amino acids in rat (position -27-0) and a chain of 433 amino acids in human or 430 amino acids in rat. A comparison of the rat and human CD4 protein sequences are described in the table and alignment below.

	Human CD4		Rat CD4	
	length	position	length	position
Signal peptide	25	-25-0	27	-27-0
Domain 1 (D1)	100	1-100	100	0-100
Domain 2 (D2)	78	101-178	79	101-179
Domain 3 (D3)	114	179- 292	110	180-289
Domain 4 (D4)	57	293- 349	58	290-347
Transmembrane (TM)	22	350- 393	23	348-390
Tail	40	394- 433	40	391-430

Alignment of human and rat CD4 protein sequences using standard one-letter amino acid symbols as presented by *Clark et al.*, underlining identical residues and marking potential N-glycosylation sites (asterisks) (Clark et al. 1987).

```

<-----SIGNAL PEPTIDE----->
HUMAN  MNRGVPPFRHLL--LVLQLALLPAATQGNKVVLGKKGDTVELTCTASQKKSIQ
RAT    MCRGFSFRHLLPLLLQLSKLLVVTQGKTVVLGKEGSAELPCESTSRRSAS
      -27                               -1.1

PHWKNSNQIKILGNQGSFLT*KGPSKLNDRADSRRLWDQGNFPLI*IKNLKIEDSDTYIC
FAWKSSDQKTILGYKNKLLIKGSL*ELYSRFD*SRKNAW*ERGSPFLI*INKLRMEDSQTYVC
      50

EVEDQKEEVQLLVFGLTANS*DTHLLQGQSLTLTLESPP-GSSPSVQCRSPRGKNIQGGK
ELENKKEEVELWYFRVT*FNPGRLLQGQSLTLILD*SNPKVSDPPIE*CKHKSSNIVKDSK
      100

TLSVSQLELQDSGTWTCTVLQ*NQKKVEFKIDIVVLA*FQKASSIVYKKEGEQVEFSFPLA
AFSTHS*RIQDSGIW*NCVT*TLNQKKHS*FDMKLSV*LGFAST*SITAYK*EGESA*EFSFPLN
      150                               200

FTVEKLTGSGELWQAERASS*SKSWITFDLKNKEVSVKRV*TQDPKLQMGKKLPLHLTLP
LGEESLQG--ELRWKA*EKAPSSQSWIT*FSLKNQKVSVQKST*SNPKFQLSETLPLTLQIP
      250

      *                               *
QALPQYAGSGNLTAL*EAKTGKLHQEVN*LVVMRATQLQKN-LTCEVWGPTSPKLMLSLK
QVSLQFAGSGNLT*LTLD--RGILYQEVN*LVVMKV*TQPD*SN*TLTCEVWGPTSPKMRLILK
      300

LENKEAKVSKREKAVVVLNPEAGMWQCLLSDSGQVLL*ESNIKVLPTWSTPVQPMAL-IV
QENQEARS*RQEKV*IQQVQAP*EAGVWQCLLS*EGEEVKMD*SKI*QVLSK--GLNQT*MF*LAVV
      350                               *

MEMBRANE REGION-><-----CYTOPLASMIC DOMAIN----->
LGGVAGLLLF*IGLGIFFCVRCR*HRRRQAERMSQIKRLLSEKKT*CQC*PHRFQKTCSP*I
LGSAP*SFLVFTGL*CLFCVRCR*HQQRQAERMSQIKRLLSEKKT*CQC*SHRMQKSHNLI
      400

```

The chimeric CD4 consists of domain 1+2, as well the cytoplasmic tail of human CD4, but domain 3+4 and TM region of rat CD4. Domains 3+4 of rat CD4 were used, as these can easily be recognized and distinguished from endogenous CD4 with a specific anti-CD4 rat antibody (clone OX68)(Brown et al. 1994). *Brown et al.* showed that domains 3+4 of rat CD4 (starting at the Serine residue corresponding to position 182 in human CD4) can be used to engineer other chimeric leucocyte cell surface proteins, although these were engineered without a TM domain and expressed as soluble proteins (Brown et al. 1994). Because CD4 cell surface expression was desired, the TM and cytoplasmic tail of CD4 were also included in the novel design of the human-rat chimera. The TM of rat CD4 was used as there was a clear homology between rat and human CD4 after the TM region. Both CD4 homologs contain Phenylalanine and Cysteine residues, corresponding to position 393 and 394 of human CD4, respectively. These residues are located at the start

of the cytoplasmic tail, offering a good transition area. The remaining regions of the chimera (domain 1+2, as well as the cytoplasmic tail) consist of human CD4. This is especially important for Domain 1 and the cytoplasmic tail. Residues in domain 1 of human CD4 are required for the interaction with gp120 and HIV-1 infection (Jameson et al. 1988; Peterson et al. 1988; Arthos et al. 1989; Brodsky et al. 1990). Rat CD4 does not function as a receptor for HIV-1, but the gp120 binding site that mediates HIV-1 infection can be constructed in rat CD4 by replacing residues 33-62 of domain 1 in rat CD4 with the corresponding residues of human CD4 (Schockmel et al. 1992; Simon et al. 1993). The cytoplasmic tail of human CD4 was used to specifically manipulate the sub-cellular distribution of CD4. The tail contains two regions that are implicated in localization of CD4 to DRM: the palmitoylation region ³⁹⁴CVRC³⁹⁷ ('P') and raft localization region ³⁹⁸RHRRR⁴²² ('R') (Crise et al. 1992; Percherancier et al. 2003; Popik et al. 2004). Three chimeric CD4-DRM mutants were designed, containing mutations in one or both of these regions: ³⁹⁴SVRS³⁹⁷ (CD4_P) and/or ³⁹⁸AAAA⁴²² (CD4_R). The chimeric CD4 sequences were designed and codon-optimised using a multiparameter optimization tool and DNA2.0 GeneDesigner software. Codon-optimisation may improve expression, as codon usage is species-specific and several DNA- or mRNA-based sequence may modulate mRNA stability levels, gene transcription and translation (Plotkin et al. 2004; Welch et al. 2009; Fath et al. 2011). The transgene was flanked with XmaI and SpeI restriction sites to facilitate sub-cloning. The wild-type (CD4_{WT}) and mutant (CD4_P, CD4_R, or CD4_{P-R}) CD4 sequences were synthesized by GeneArt (Life Technologies) and delivered in a simple Amp^R plasmid (pMA-RQ). The chimeric CD4 sequences were sub-cloned into the 'GOI' site of the knockdown system described in Chapter 4 (LV-pU6-shCD4-EF1-GOI-I-Puro^R), creating a lentiviral knockdown knock-in expression system (LV-U6-shCD4-EF1-CD4-I-Puro^R, see Appendix V for further details). The U6 promoter drives the expression of the shCD4 and is responsible for the knockdown of endogenous CD4. Protection of chimeric CD4 mRNA from degradation by the shCD4 is ensured for two reasons. Firstly, the target sequence of

shCD4 resides in domain 3 of human CD4, which is replaced by rat domain 3 in the chimera. Secondly, the chimeric CD4 sequence was codon-optimized, further changing the DNA sequence of the rat Domain 3 in chimeric CD4. The EF1 α promoter drives the expression of chimeric CD4 and is responsible for the knock-in of CD4. So, by inserting chimeric CD4 into the shRNA knockdown expression system, the system was further developed to also allow knock-in of exogenous wild-type or mutant CD4 in esMDM. By using a chimeric version, CD4 is not affected by the shCD4 and endogenous and transgenic CD4 expression can be distinguished. Selection of a 100% pure cell population expressing chimeric CD4 is ensured, as chimeric CD4 is co-ordinately expressed with puromycin^R as they are transcribed bi-cistronically from the same mRNA transcript (Adam et al. 1991).

Figure 6.1A shows schematic diagrams of the resulting lentiviral vectors and Figure 6.1B shows a representative vector map. The expected phenotype (presence or absence of endogenous human CD4 and wild-type or mutant transgenic CD4) in cells expressing this lentiviral vector is indicated by the symbols shown in Figure 6.1C. The amino acid sequence of the cytoplasmic domain and mutated regions are shown in Figure 6.1D, adapted from (Crise et al. 1992).

The results of experiments with control, shCNTRL and shCD4 cells are not shown in this Chapter as the results have already been presented in Chapter 4. All experiments presented in Chapter 4 were performed in parallel with the corresponding experiments presented in this Chapter. Only in Figures 6.4-6.7, in which the expression levels of CD4 are analysed, the results from the shCNTRL group are shown again, to allow easy comparison of endogenous and chimeric CD4 expression. Where appropriate, there is a reference to the corresponding Figure in Chapter 4.

Figure 6.1

Figure 6.1. Design and construction of lentiviral vectors encoding chimeric CD4 DRM localisation mutants.

A) Schematic diagrams of self-inactivating lentiviral constructs used to knockdown endogenous CD4 and express puromycin^R and wild-type CD4 (CD4_{WT}) or CD4 mutants that can not localise to Detergent Resistant Membranes (DRM). B) Plasmid map for representative lentiviral vector, CD4_{WT}.

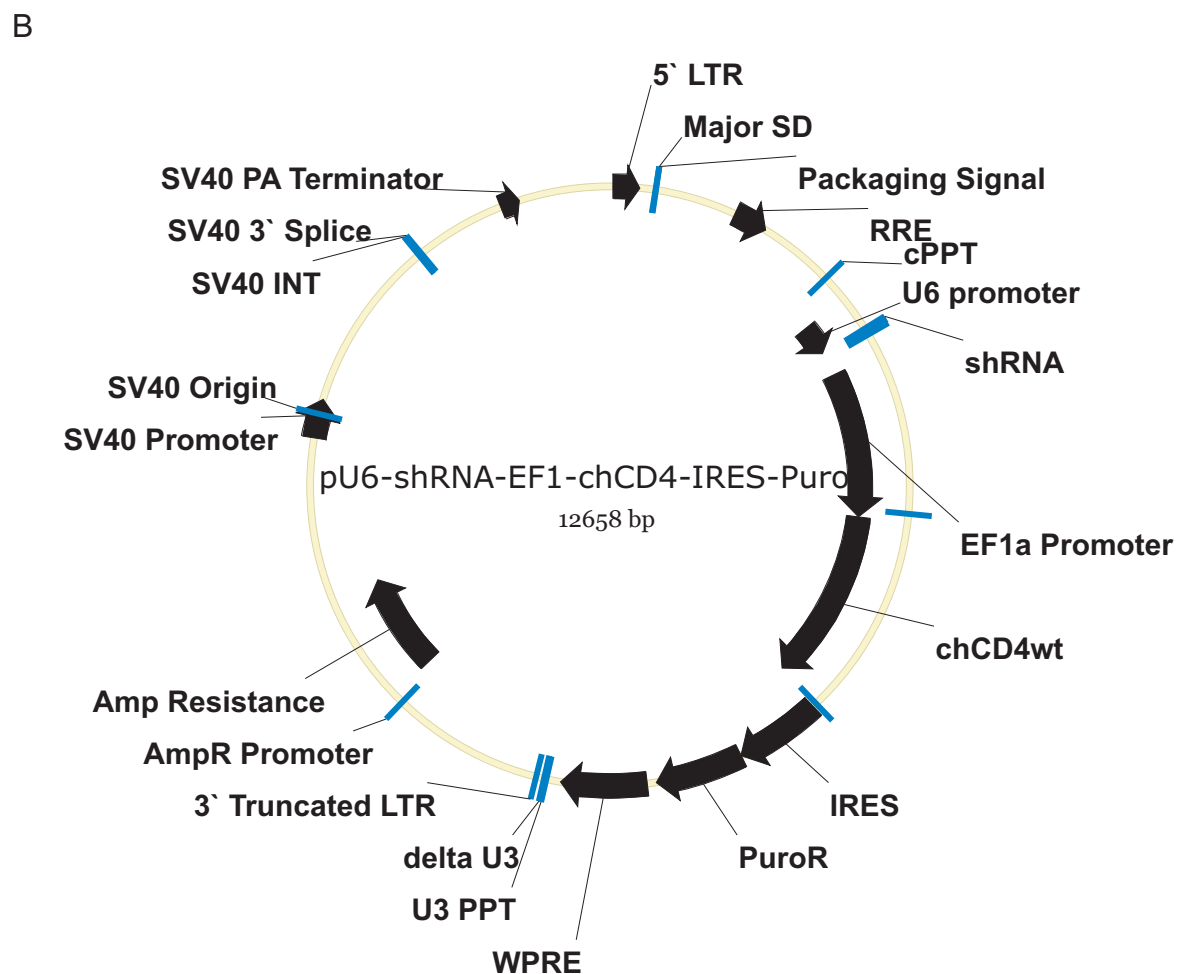
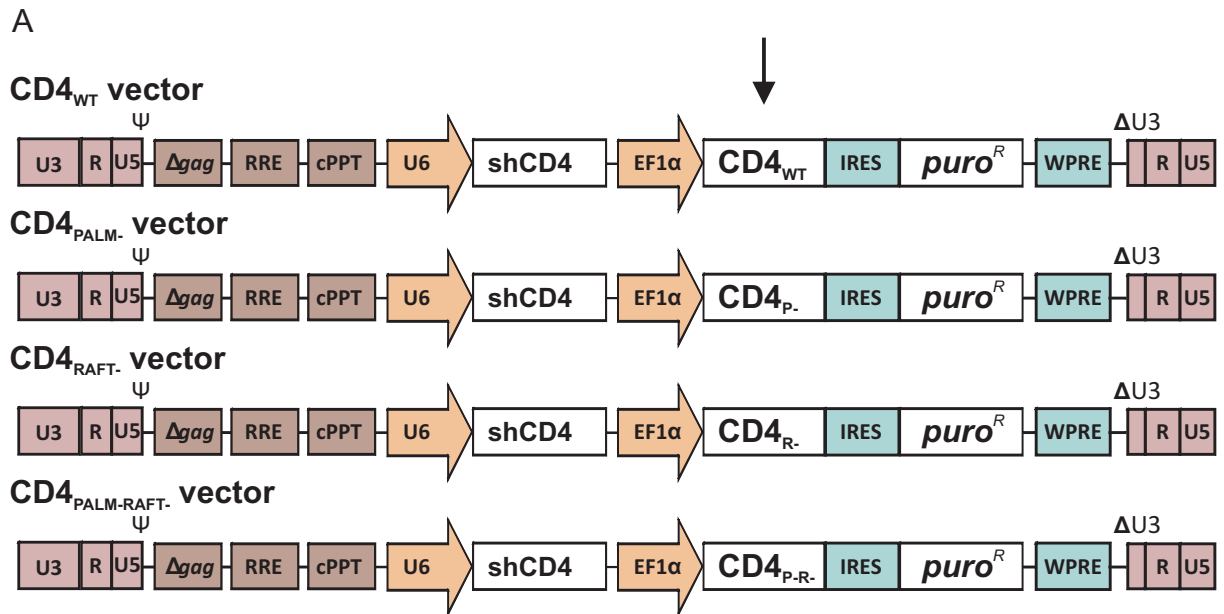
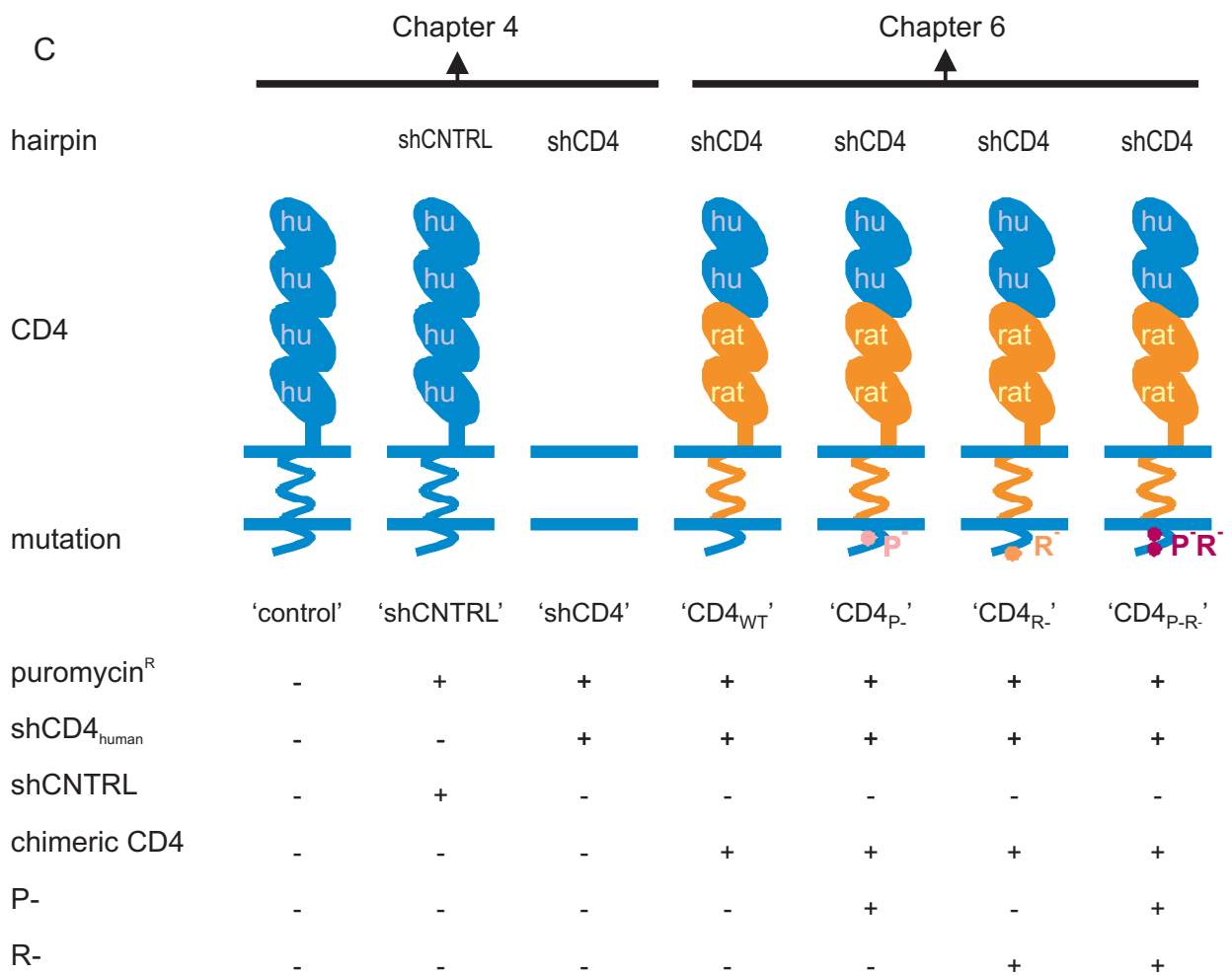


Figure 6.1

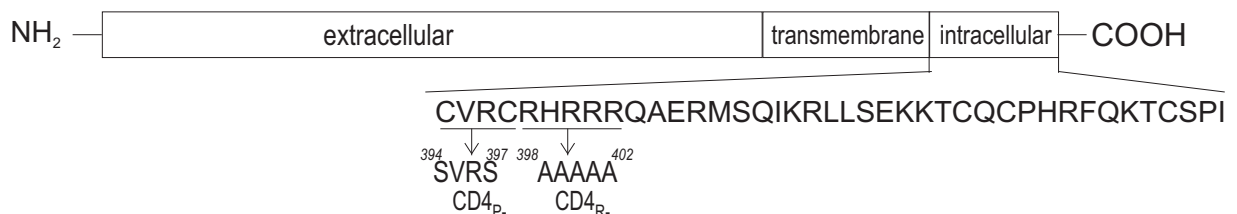
Figure 6.1. (continued) Design and construction of lentiviral vectors encoding CD4 DRM localisation mutants.

C) Schematic diagram and table of lentiviral vectors encoding an shRNA alone, or an shRNA together with wild-type or mutant CD4. The shCD4 and shCNTRL vectors were validated and tested in esMDM, as described in Chapter 4. The shCD4 targets endogenous human CD4 (depicted in blue), but not the transgenic wild-type/mutant CD4, as the latter is a chimera of human and rat CD4 (depicted in orange/blue). It contains domain 1+2 and the cytoplasmic tail of human CD4, but domain 3+4 and transmembrane domain of rat CD4. The chimeric CD4 versions contained one or two mutated regions of the cytoplasmic domain, namely 'CD4_P' and/or 'CD4_R'. D) Amino acid sequence of cytoplasmic tail of CD4 showing location of mutants.



P-refers to chimeric CD4 containing substitutions in its palmitoylation domain: ³⁹⁴CVRC³⁹⁷ → ³⁹⁴SVRS³⁹⁷
R-refers to chimeric CD4 containing substitutions in its raft association domain: ³⁹⁸RHRRR⁴²² → ³⁹⁸AAAAA⁴⁰²

D



6.2 Lentiviral vectors allow effective knock down of endogenous human CD4 and expression of chimeric CD4 in TZM-bl cells.

To validate the design and expression of chimeric CD4, the novel lentiviral knockdown knock-in expression system was first tested in a cell line. TZM-bl cells were transduced with the lentiviral knockdown vectors expressing shCNTRL or shCD4 alone (also used in Chapter 4), or the lentiviral knockdown knock-in vectors expressing both shCD4 and wild-type CD4 (CD4_{WT}) or chimeric CD4 DRM localization mutants (CD4_{P-}, CD4_{R-}, or CD4_{P-R-}). A pure population of TZM-bl cells containing the lentiviral vector were selected using Puromycin (5 µg/mL). To determine the expression levels of human and chimeric CD4 at the cell surface, the puromycin-selected as well as control untransduced cells, were stained for surface CD4 expression using 3 different CD4 antibodies and analysed by flow cytometry. Two-colour immunofluorescence flow cytometric analysis with mAb clone OKT4, which recognises endogenous CD4 (domain 3 of human CD4) and mAb clone OX68, which recognises chimeric CD4 (domain 3+4 of rat CD4), showed that high endogenous CD4 levels were detected in control cells and cells transduced with shCNTRL, but CD4 levels in cells transduced with lentiviruses encoding shCD4 (shCD4, CD4_{WT}, CD4_{P-}, CD4_{R-}, or CD4_{P-R-}) were reduced by 98%-99%. (Fig 6.2A and 6.2D). This shows that a hairpin targeting CD4, expressed under the control of the U6 promoter, can effectively knock down endogenous CD4 to background levels and this is not affected by the expression of chimeric CD4 on the same lentiviral vector.

In addition to knocking down endogenous CD4, chimeric CD4 was simultaneously expressed in cells transduced with lentiviruses encoding CD4_{WT}, CD4_{P-}, CD4_{R-}, or CD4_{P-R-}, as detected by mAb clone OX68 (Fig 6.2A and 6.2E). To compare the expression levels of endogenous CD4 and chimeric CD4, cells were stained with mAb clone 11830, recognising Domain 1+2 of CD4 (Figure 6.2B and 6.2F). Chimeric CD4 surface

expression levels were at least as high as endogenous CD4 levels on controls cells, confirming that the structural similarities between human and rat CD4 allow the design of a chimeric CD4 version that can successfully expressed at the cell surface. These experiments also show that the three CD4 antibodies (Clone OKT4, OX68 and 11830) provide a useful tool to monitor the expression of endogenous and chimeric CD4. Representation of the binding site of the three antibodies on human and chimeric CD4 is depicted in Figure 6.2C. Although clone OX68 specifically recognises chimeric CD4, clone 11830 is a more sensitive antibody and allows comparison of endogenous and chimeric CD4 expression levels once endogenous CD4 knockdown has been confirmed with clone OKT-4.

Figure 6.2

Figure 6.2. Lentiviral vectors allow effective knock down of endogenous human CD4 and expression of chimeric CD4 in TZM-bl cells.

To validate the design of the lentiviral constructs, control TZM-bl cells or TZM-bl cells transduced with lentiviral vectors encoding shCNTRL or shCD4 alone (top row), or shCD4 and wild-type (WT) or mutant versions of chimeric CD4 (P-, R-, or P-R-) (bottom row). Cells were stained with anti-CD4 mAb clone OKT4, which recognises endogenous CD4 (domain 3 of human CD4) and anti-CD4 mAb clone OX68, which recognises chimeric CD4 (domain 3+4 of rat CD4) or with anti-CD4 mAb clone 11830, which recognises both endogenous human CD4 and chimeric CD4. Cells were analysed by flow cytometry. A) Two-colour immunofluorescence (dot plot), showing the mean fluorescence intensities of mAb clones OKT4 and OX68, plotted on the Y-axis and X-axis, respectively. Gates were determined by using the two relevant isotype control antibodies. B) Representative histogram showing CD4 surface staining with mAb clone 11830 (red/brown line) compared to the matched isotype control (grey line). C) Representation of the binding site of the three antibodies on human and chimeric CD4.

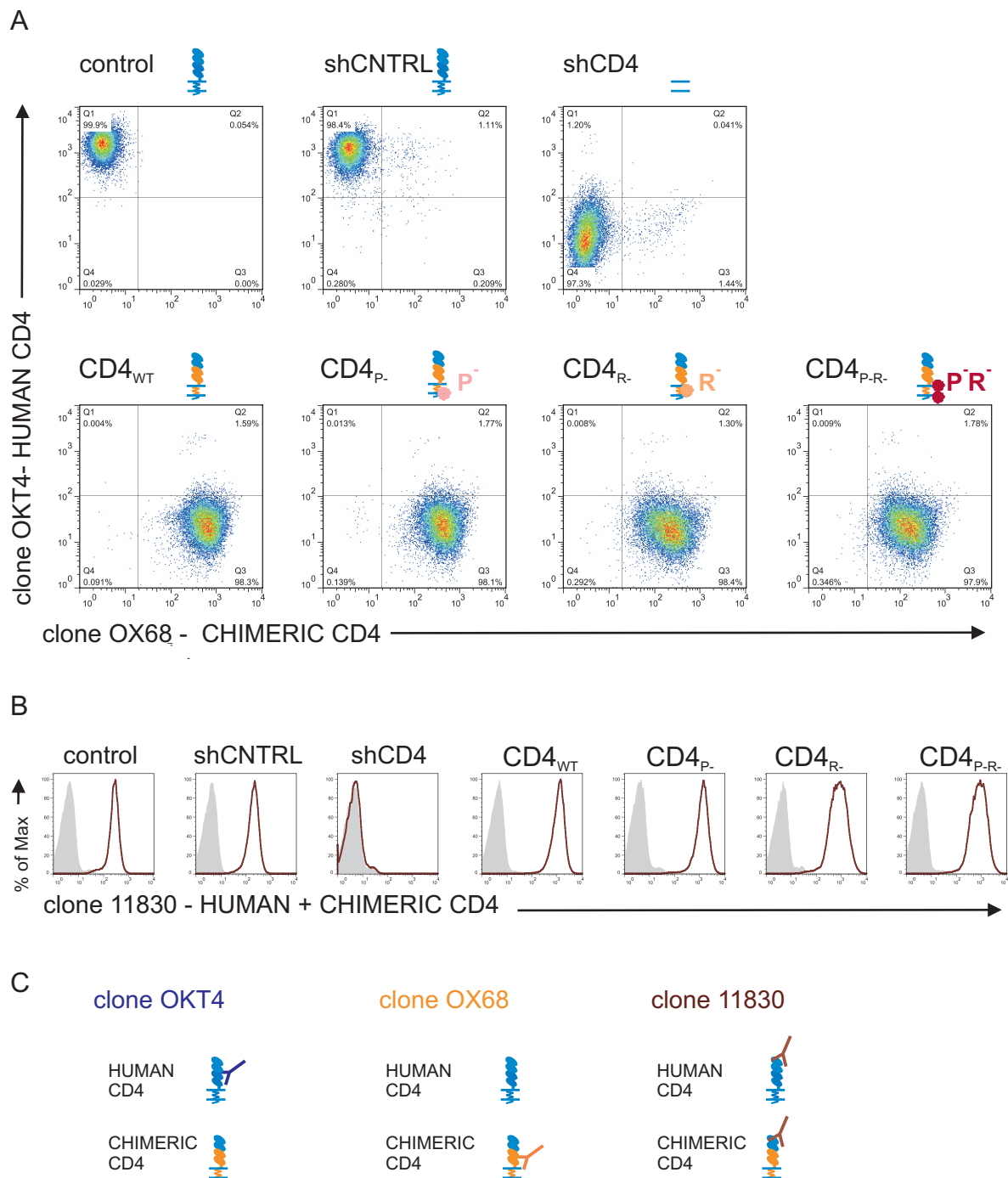
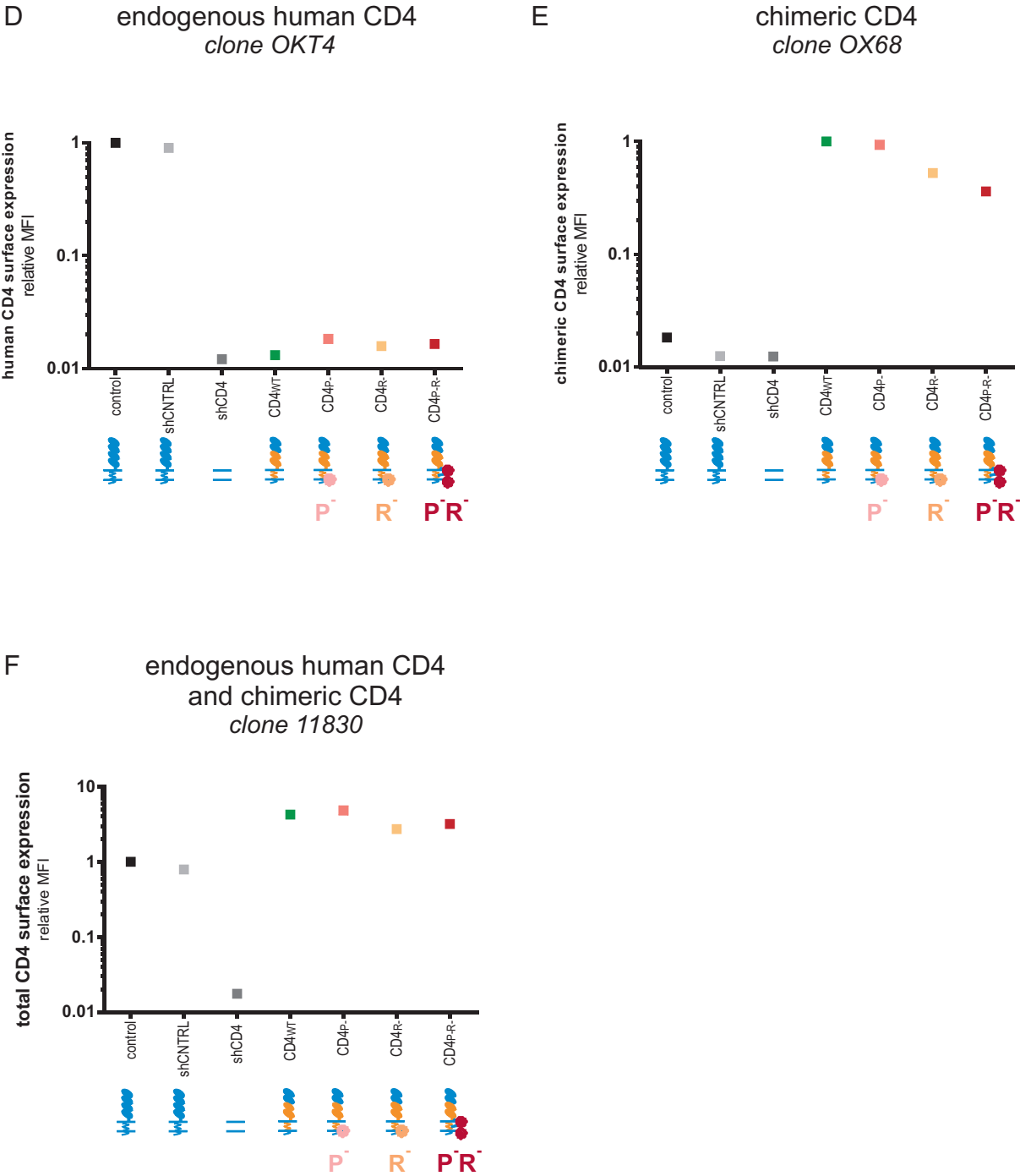


Figure 6.2. (continued) Lentiviral vectors allow effective knock down of endogenous human CD4 and expression of chimeric CD4 in T2M-bl cells.

Quantification of D) endogenous human CD4 expression relative to the control group, using mAb OKT4, E) chimeric CD4 expression relative to the chimeric CD4 wild-type group, using mAb OX68, F) endogenous human CD4 and chimeric CD4 expression, relative to the esMDM control group, using mAb 11830. D-F) The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control.



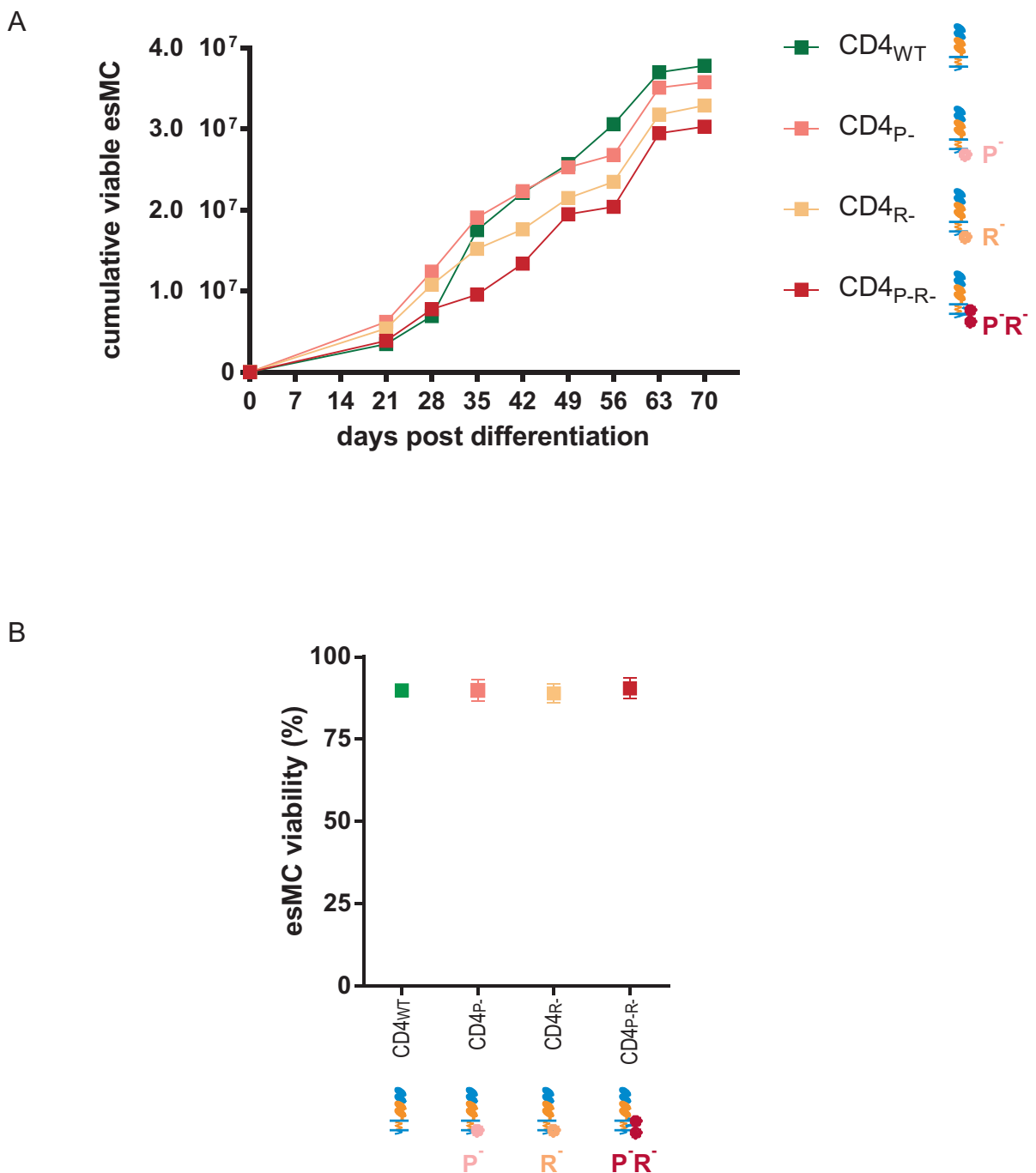
6.3 Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding CD4-DRM mutants

hESC were transduced with lentiviral vectors expressing shCNTRL or shCD4 alone, or with lentiviral vectors expressing both shCD4 and wild-type chimeric CD4 (CD4_{WT}) or DRM localization mutant versions of chimeric CD4 (CD4_{P-}, CD4_{R-}, or CD4_{P-R-}). Control hESC and transduced hESC were differentiated into functional monocytes (esMC). Transduced hESC were kept under continuous puromycin (5 µg/mL) selection (a concentration at which untransduced cells died). esMC were harvested weekly over a period of 70 days. esMC were CD14⁺ selected (Miltenyi Biotec, 130-050-201) to exclude any possible contamination of CD14⁻ esMC precursor cells present in the culture. Figure 6.3A shows the cumulative counts of viable esMC from >20 wells per condition for CD4_{WT}, CD4_{P-}, CD4_{R-}, and CD4_{P-R-} esMC. Figure 6.3C shows that the mean viability of CD14⁺ selected esMC, as determined by an AO-DAPI stain (Chemometec), was >75 % for all conditions. Results for control, shCNTRL and shCD4 esMC are shown in Figure 4.3. Normal differentiation and production of viable monocytes shows that the hairpin or CD4 mutants have any apparent toxic effects. The puromycin-resistance shows that the esMC contain the lentiviral vector.

Figure 6.3

Figure 6.3. Viable transgenic monocytes can be produced from hESC transduced with lentiviral vectors encoding CD4 DRM mutants.

A) hESC were infected with lentiviral vectors encoding shCD4 to knockdown endogenous CD4 and introduce wild-type (WT) or mutant versions of chimeric CD4 (P-, R-, or P-R-) that can not localise to Detergent Resistant Membranes (DRM). Also, hESC were infected with control lentiviral vectors encoding shCD4 alone or a control hairpin (shCNTRL) (data shown in Chapter 4). hESC were used to generate hESC-derived monocytes (esMC) using the Embryoid Body method. Non-adherent esMC were harvested from the supernatant of cultures and counted using a cell counter (Chemometec). The media were replaced for repeated esMC harvests over a period of 70 days. Data represent the cumulative number of viable esMC after CD14⁺ selection (Miltenyi) from >20 wells per condition. B) Viability of CD14⁺ selected esMC determined by an AO-DAPI stain (Chemometec).



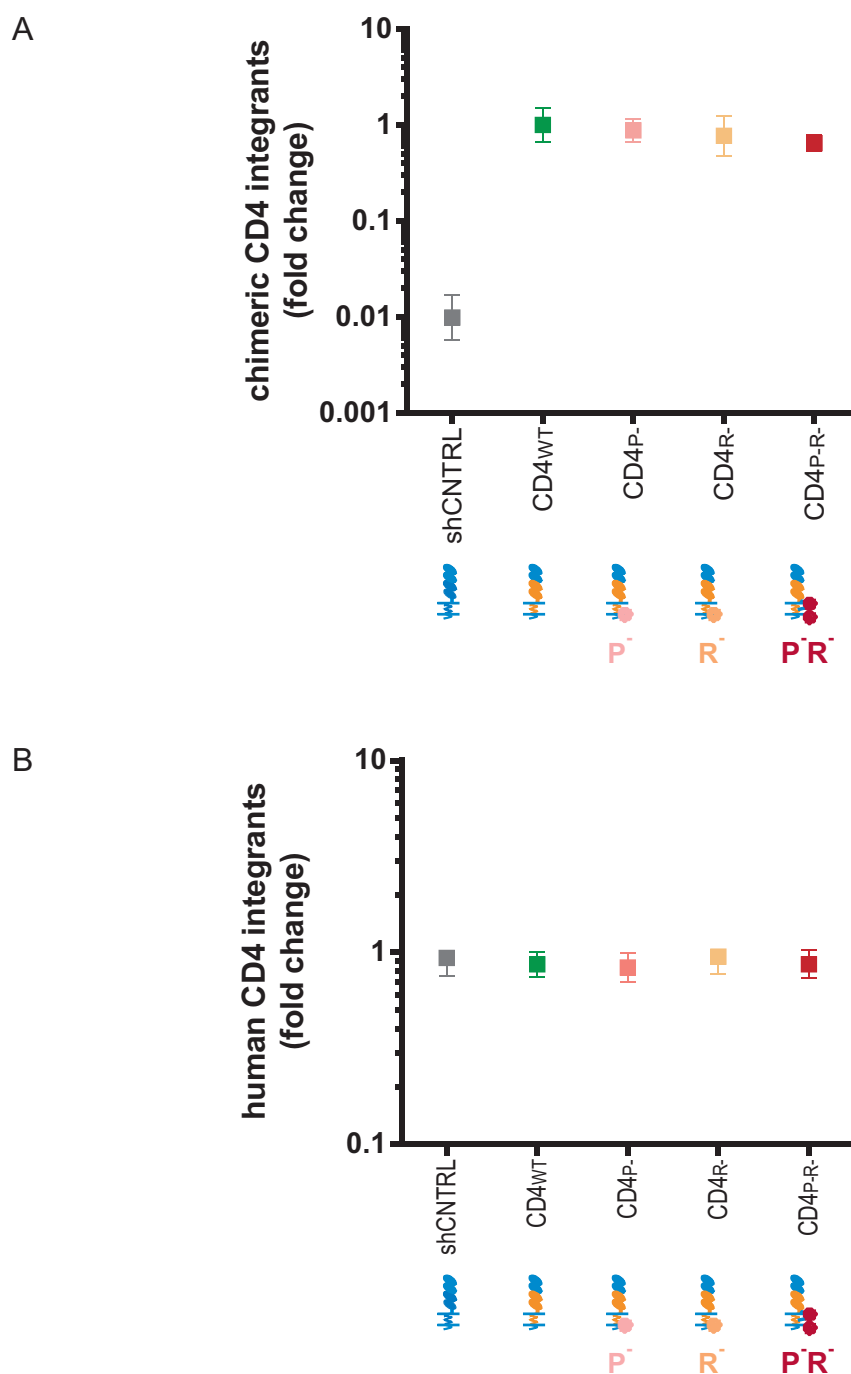
6.4 Similar levels of the integrated lentiviral vectors are found in CD4-DRM mutant esMDM.

The pure population of viable esMC were further cultured in MDM medium supplemented with M-CSF for 4-7 days to direct the differentiation into esMDM. To find out whether esMDM derived from hESC transduced with CD4_{WT}, CD4_{P-}, CD4_{R-}, and CD4_{P-R-} contained the integrated lentiviral vector in their genome, DNA was isolated from these and shCNTRL esMDM cells and analysed by qPCR using primers specific for chimeric CD4 (Figure 6.4A). Similar levels of the integrated chimeric CD4 sequences were found in CD4_{WT}, CD4_{P-}, CD4_{R-}, and CD4_{P-R-} esMDM. Expression levels were 2-logs above non-specific background levels, as inferred from the shCNTRL esMDM group which do not contain any chimeric CD4 sequences. As a negative control, esMDM were also analysed by qPCR using primers specific for endogenous human CD4. Genomic human CD4 integrants were expressed at similar levels in all conditions (Figure 6.4B + Figure 4.3B). These results show that hESC were successfully transduced with LV-U6-shCD4-EF1-CD4-I-Puro^R and esMDM maintained the lentiviral sequences in their genome throughout differentiation

Figure 6.4

Figure 6.4. Similar levels of the integrated lentiviral vectors are found in CD4-DRM mutant esMDM.

A) To detect integration of lentiviral vectors, DNA was isolated from MDM derived from hESC infected with lentiviral vectors expressing shCD4 and chimeric CD4 ($CD4_{WT}$, $CD4_{P-}$, $CD4_{R-}$, $CD4_{P-R-}$) or from hESC infected with lentiviral vectors expressing only a control hairpin (shCNTRL) and analysed by qPCR using specific primers. B) To detect genomic human CD4, DNA was isolated from shCNTRL/ $CD4_{WT}$ / $CD4_{P-}$ / $CD4_{R-}$ / $CD4_{P-R-}$ esMDM and analysed by qPCR using specific primers. A+B) Symbols represent the mean number of copies $\pm$ SEM from technical replicates (n=3) using pooled DNA from three independent experiments, of (A) chimeric CD4 relative to the $CD4_{WT}$ group or (B) human CD4 relative to the control group (shown in Figure 4.4B).



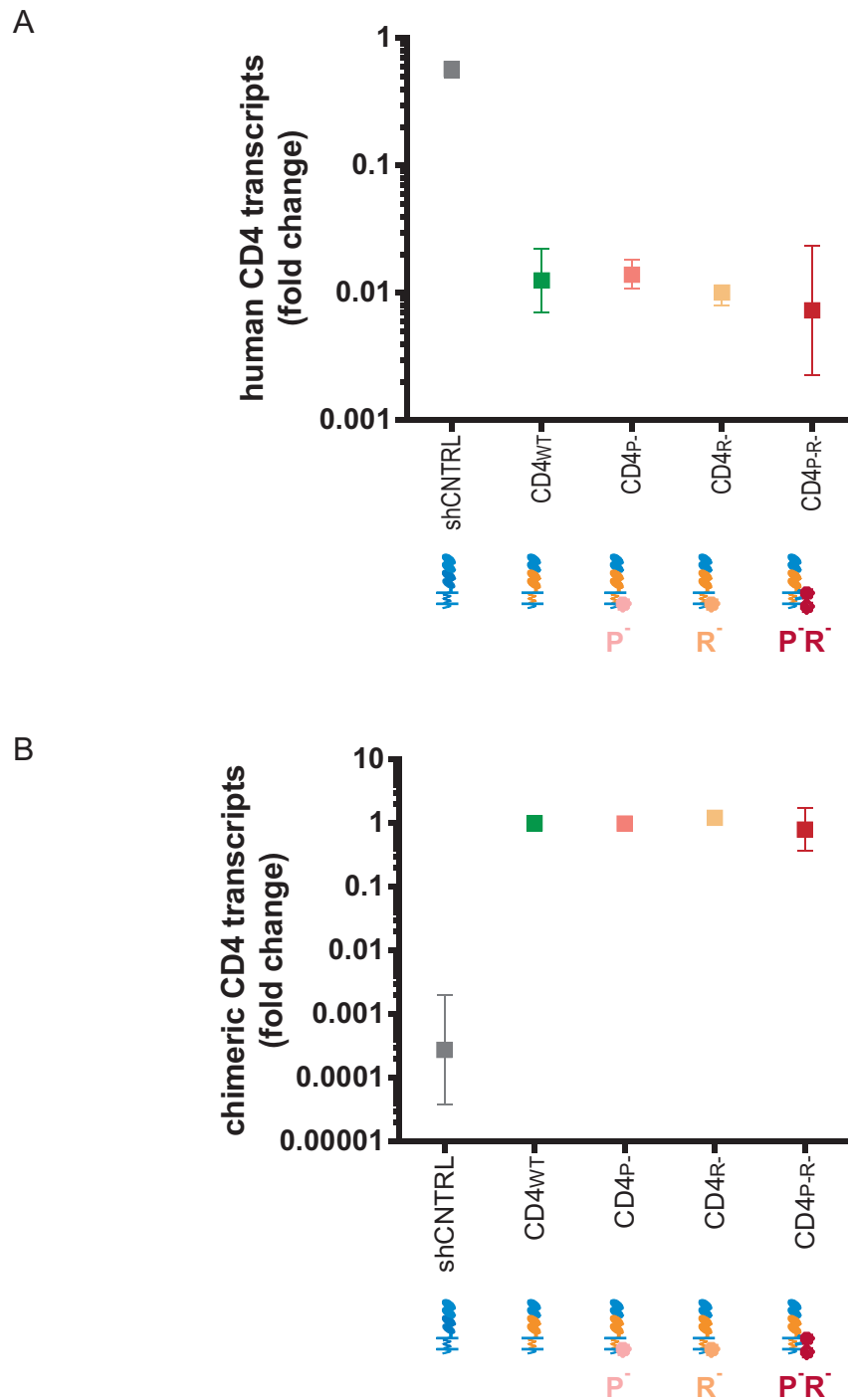
6.5 Negligible levels of endogenous human CD4 mRNA and uniform levels of chimeric CD4 mRNA are expressed by CD4-DRM mutant esMDM.

To find out whether esMDM derived from transduced hESC maintained expression from the integrated lentiviral vectors throughout the differentiation process, RNA was isolated from control and transgenic esMDM and analysed by RT-qPCR using primers specific for human and chimeric CD4 mRNA. Endogenous human CD4 transcripts were reduced by a factor of a 100 in esMDM expressing LV-U6-shCD4-EF1-CD4-I-Puro^R (Figure 6.5A) compared to control esMDM (Figure 4.4A). This knockdown was as effective in shCD4 esMDM, which express the same vector without chimeric CD4 (Figure 4.4A). Chimeric CD4 transcripts were expressed at similar levels in CD4_{WT}, CD4_{P-}, CD4_{R-}, and CD4_{P-R-} esMDM (Figure 6.5C). Expression levels were more than 3-logs above non-specific background levels as inferred from the shCNTRL esMDM group which do not contain any chimeric CD4 sequences. The results confirm that a CD4 hairpin expressed by the U6 promoter can effectively knockdown endogenous CD4 transcripts in esMDM and this is not affected by simultaneous knock-in of chimeric CD4. This shows that LV-U6-shRNA-EF1-GOI-I-Puro^R is a suitable lentiviral expression system that allows knockdown mediated by an shRNA, as well as knock-in of a Gene Of Interest in authentic MDM.

Figure 6.5

Figure 6.5. Negligible levels of endogenous human CD4 mRNA and uniform levels of chimeric CD4 mRNA are expressed by CD4-DRM mutant esMDM.

To detect human and chimeric CD4 transcripts, RNA was isolated from transgenic esMDM and analysed by RT-qPCR using specific primers. Symbols represent the relative mean number of copies of (A) human endogenous CD4 mRNA relative to the control group (shown in Figure 4.4A) and (B) transgenic chimeric CD4 mRNA relative to the CD4_{WT} group $\pm$ SEM of technical replicates (n=3) using pooled RNA from three independent experiments.



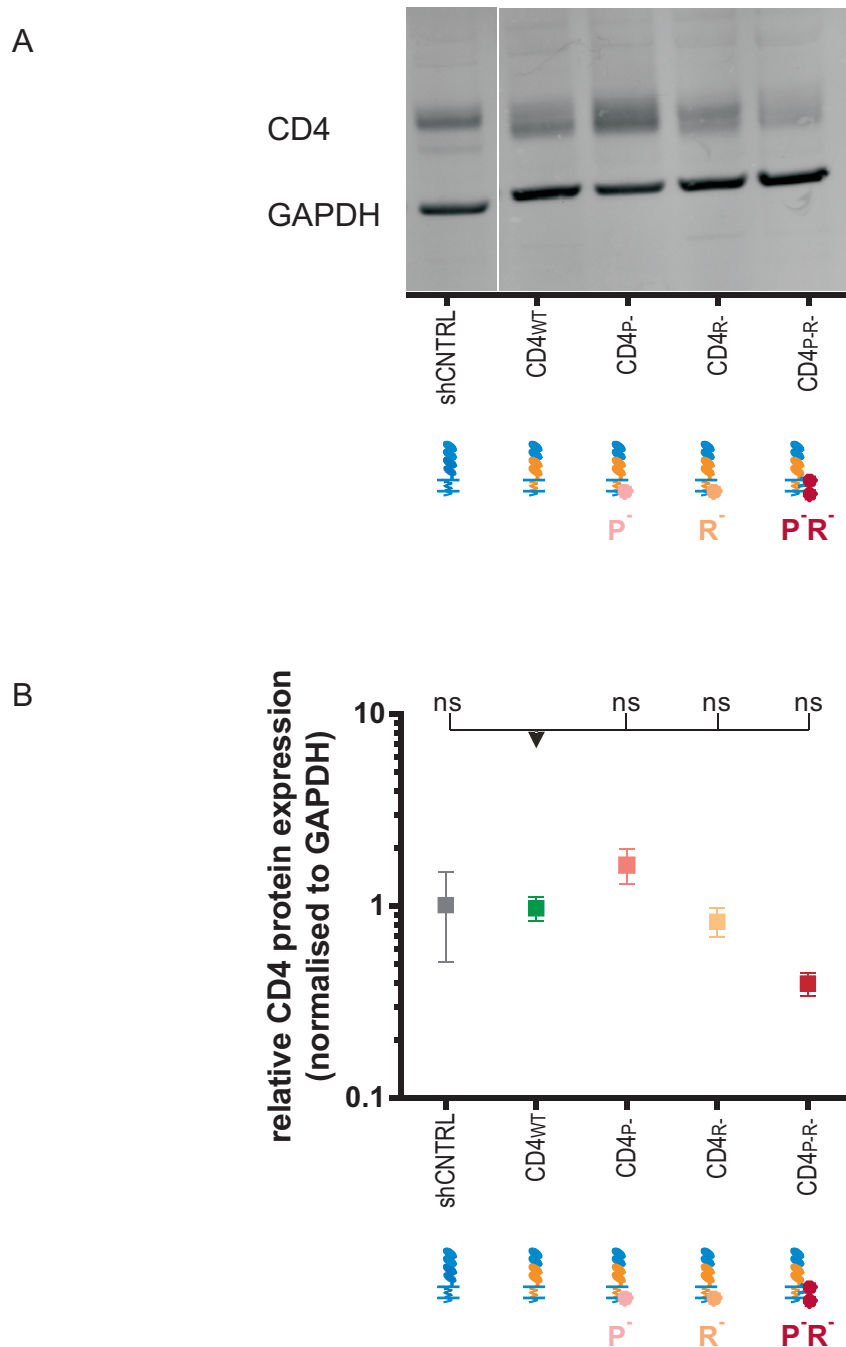
6.6 Chimeric CD4 protein is expressed by CD4-DRM mutant esMDM.

To confirm whether the chimeric CD4 transcripts were translated and expressed as proteins, esMDM lysates were analysed by western blotting using an anti-CD4 antibody (Figure 6.6A). CD4 expression was normalised to GAPDH expression and, when compared to levels of endogenous CD4 (shCNTRL, mean $\pm$ SEM: 1.0 ± 0.29), similar levels of total CD4 protein were detected in lysates from esMDM expressing CD4_{WT} (mean $\pm$ SEM: 1.0 ± 0.14 ; ns), CD4_{P.} (mean $\pm$ SEM: 1.6 ± 0.33 ; ns), CD4_{R.} (mean $\pm$ SEM: 0.8 ± 0.14 ; ns), and CD4_{P-R.} (mean $\pm$ SEM: 0.4 ± 0.05 ; ns). Together with the qRT-PCR results showing endogenous CD4 mRNA reduction and transcription of knocked-in chimeric CD4, the western blot results validate the lentiviral expression system and show that knocked-in versions of CD4 are expressed in authentic esMDM.

Figure 6.6

Figure 6.6. Chimeric CD4 protein is expressed by CD4-DRM mutant esMDM.

To detect protein expression of CD4, transgenic esMDM lysates were analysed by western blotting using anti-CD4 antibody. GAPDH, a loading control, was detected using anti-GAPDH antibody. A) Representative blot is shown. shCNTRL esMDM were run on the same blot, but with other experimental conditions in between (shown in Figure 4.5). B) Protein levels were measured with Odyssey software (Li-COR) and CD4 expression was normalised to GAPDH expression. Symbols represent normalised CD4 expression, relative to the esMDM control group (shown in Figure 4.5), of three independent experiments $\pm$ SEM (n=3). ns: non-significant ($P>0.05$). One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM CD4_{WT} group.



6.7 Effective knockdown of endogenous CD4 and comparable surface levels of chimeric CD4 are found in CD4-DRM mutant esMDM.

To describe the protein expression levels in more detail, and distinguish between endogenous knocked-down CD4 and chimeric knocked-in CD4, surface CD4 expression levels (as opposed to total cellular CD4) were measured using flow cytometric analysis and three different clones of anti-CD4 antibodies (Figure 6.7A+B). The relative ratios of the geometric mean fluorescence intensity (MFI) over the isotype control were quantified (Figure 6.7C-E).

Clone 11830 is a relatively sensitive antibody that binds domain 1+2 of human CD4. These domains are present in both endogenous and chimeric CD4 and as such detection with clone 11830 allows good comparison of overall CD4 surface expression levels of control and transgenic esMDM. As effective knockdown has previously been demonstrated (Figure 6.5), CD4 detected on CD4_{WT}, CD4_{P-}, CD4_{R-} and CD4_{P-R-} esMDM will predominantly be knocked-in CD4. Compared to CD4_{WT}, similar levels of surface CD4 were detected in CD4_{P-}, CD4_{R-}, and CD4_{P-R-} esMDM. As CD4 surface expression levels are similar in wild-type and mutant versions of chimeric CD4, any functional changes in CD4 mutant esMDM, such as changes in HIV-1 infectivity, can be attributed to the exclusion of CD4 from DRM, rather than changes in expression levels. Chimeric CD4 expression levels were similar to endogenous CD4 expression levels, so chimeric CD4 should provide a good, representative model to study endogenous human CD4 (Figure 6.7A, left panel and Figure 6.7E).

Although clone OX68 is less sensitive, it allows detection of knocked-in CD4 as it binds domain 3+4 of rat CD4. These domains are present in chimeric CD4, but not in endogenous human CD4. Accordingly, only background levels of chimeric CD4 were

detected in shCNTRL esMDM, whereas, in agreement with the previous results, similar levels of surface CD4 expression were detected in CD4_{WT} and mutant CD4 esMDM.

Previous qRT-PCR results showed that the shCD4 reduced endogenous CD4 transcripts a 100-fold. Knockdown of endogenous CD4 was confirmed using clone OKT-4, which binds to domain 3+4 of human CD4. These domains are not present in chimeric CD4. Compared to shCNTRL esMDM, endogenous CD4 levels were significantly reduced in esMDM expressing chimeric CD4 (Figure 6.7A right panel and Figure 6.7C).

To further illustrate how CD4 knockdown and knock-in occurs simultaneously, cells were analysed by two-colour immunofluorescence using clone OKT4 (endogenous CD4) and clone OX68 (chimeric CD4) (Figure 6.7B).

These results confirm that endogenous CD4 is knocked-down and that comparable surface levels of chimeric CD4 are expressed in CD4_{WT}, CD4_{P-}, CD4_{R-}, or CD4_{P-R-} esMDM. The uniform expression levels between wild-type and mutant CD4 provide a good basis for studying the effects of the DRM localization mutations on HIV-1 infection. Chimeric CD4 expression levels were comparable to human CD4 expression levels in control cells.

Figure 6.7

Figure 6.7. Effective knock down of endogenous CD4 and comparable surface levels of chimeric CD4 is found in CD4-DRM mutant esMDM.

esMDM were tested for surface CD4 expression by flow cytometry using three different clones of anti-CD4 antibodies. A) Representative histogram showing CD4 surface staining with mAb clone OKT4 (blue line, left panel, recognises endogenous human CD4 only); or with mAb clone OX-68 (orange line, middle panel, recognises chimeric CD4 only); or with mAb clone 11830 (red/brown line, right panel, recognises both endogenous human and chimeric Cd4). All lines are depicted compared to the matched isotype control (gray). Representation of the binding site of the three anti-bodies on human and chimeric CD4 are shown at the bottom. B) Representative two-colour immunofluorescence (dot plot) analysis of human CD4 expression (using mAb clone OKT4 and chimeric CD4 (using mAb clone OX68). Gates were determined by using the two relevant isotype control antibodies.

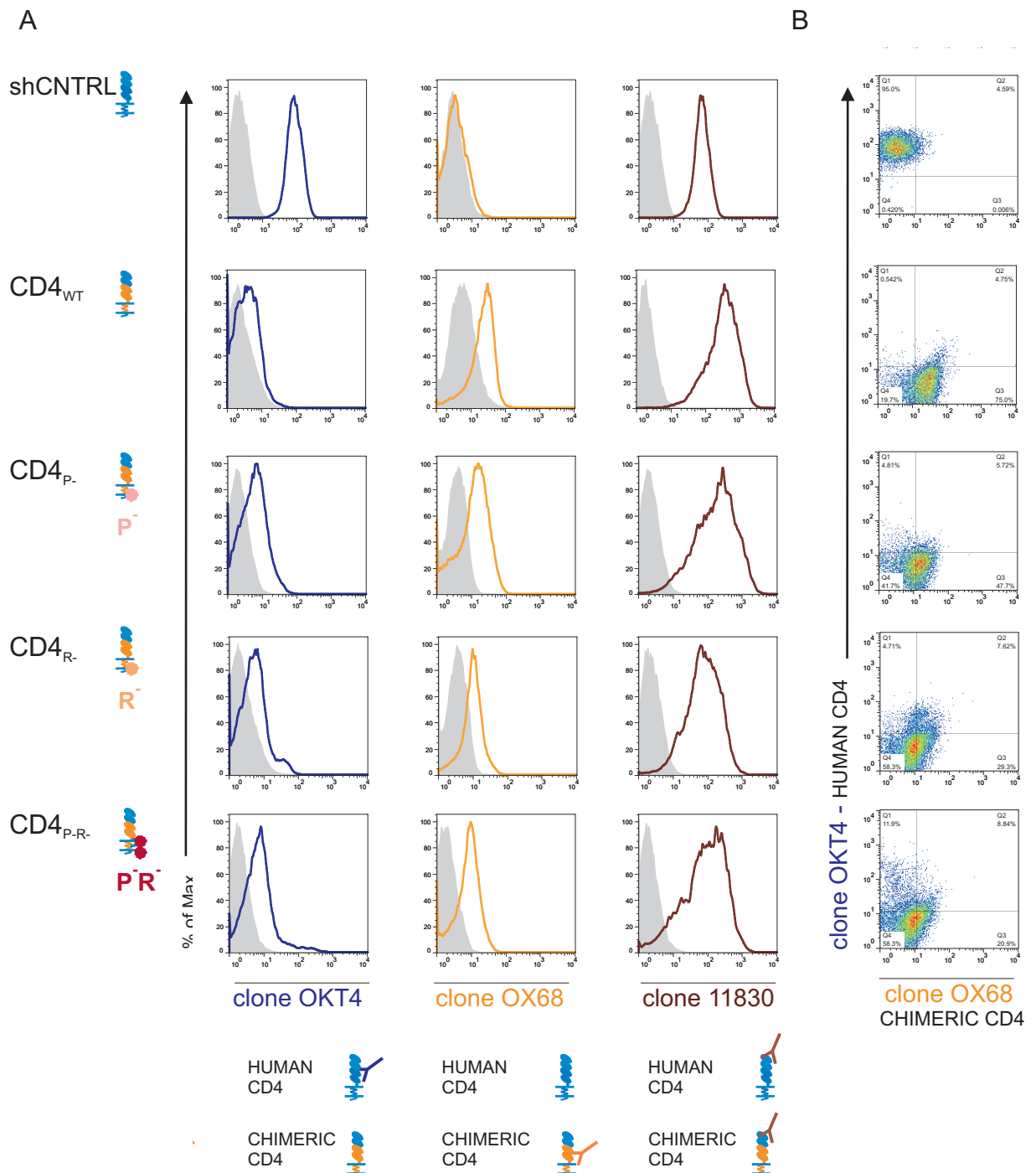
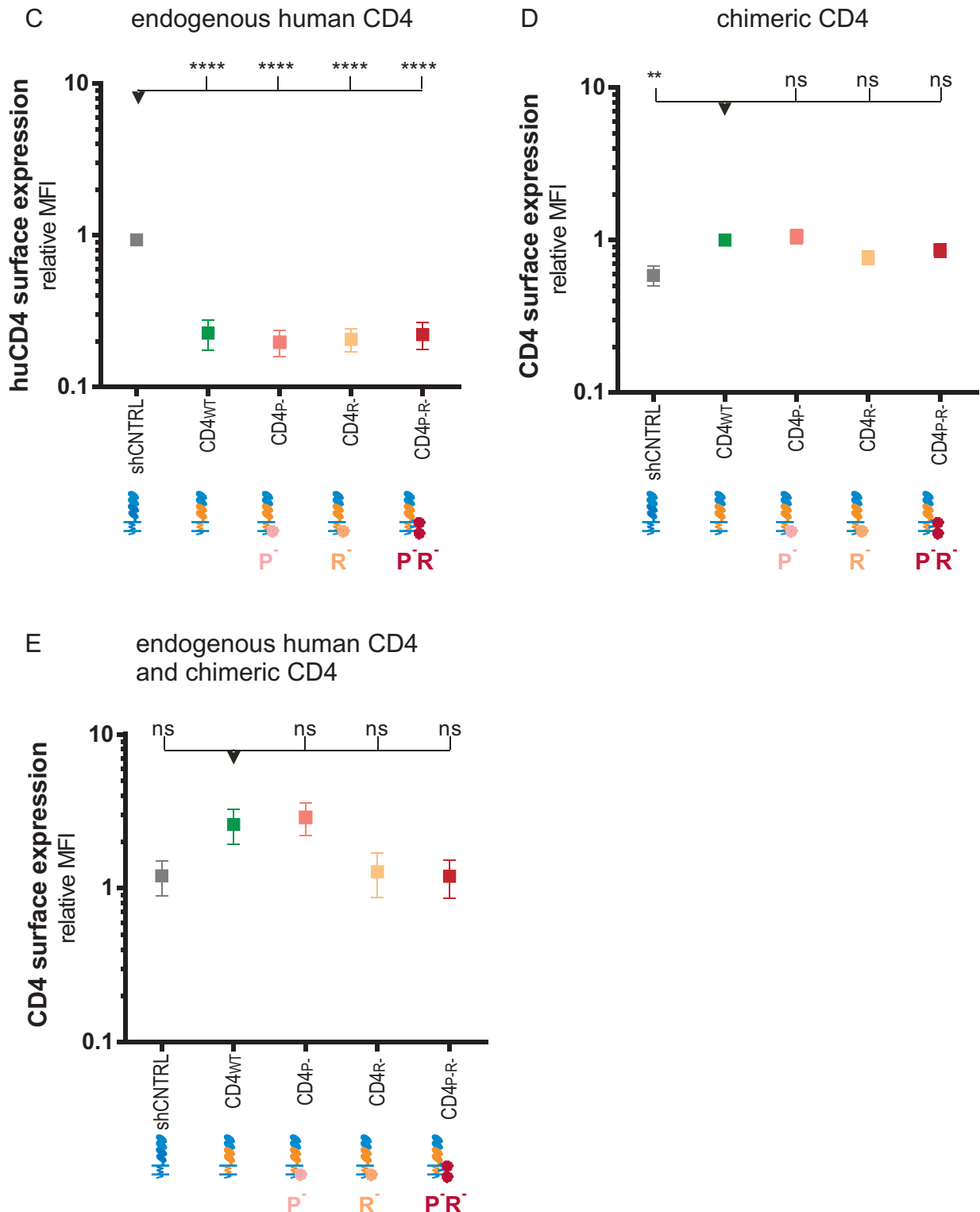


Figure 6.7

Figure 6.7. (continued) Effective knock down of endogenous CD4 and comparable surface levels of chimeric CD4 are found in CD4-DRM mutant esMDM.

Quantification of (C) endogenous human CD4 expression relative to the esMDM control group (shown in Figure 4.6), using mAb OKT4 (n=4), (D) chimeric CD4 relative to the esMDM CD4_{WT} group, using mAb OX68 (n=6), (E) endogenous human CD4 and chimeric CD4 expression relative to the esMDM control group (shown in Figure 4.6), using mAb 11830 (n=7). C-E The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control $\pm$ SEM. ns: non-significant ($P > 0.05$); ** $P \leq 0.01$; **** $P \leq 0.0001$, one-way ANOVA followed by Holm-Sidak's multiple comparison test.



6.8 Testing of a Flow Cytometric screening test for Detergent Resistant surface antigens in esMDM

Having thoroughly tested CD4 knockdown and knock-in, the phenotype of the CD4 DRM localization mutants was analysed using a rapid, reliable, and reproducible flow cytometric assay of differential detergent resistance (FCDR) that has been developed by *Gombos et al.* (Gombos et al. 2004). Since it was first described in 2004, it has been validated in a range of cell types, including primary monocytes, T-cells, B-cells and various cell lines (Bacso et al. 2004; Gombos et al. 2004; Calzolari et al. 2006; Wolf et al. 2006; Wolf et al. 2007; Kiss et al. 2008). Although the FCDR is highly reproducible, the assay had never been used with macrophages. Therefore, the method was first validated in esMDM.

Compared to other biochemical approaches to study lipid rafts, the FCDR assay is a rapid, easy procedure, with lower risk of artefacts and requires fewer cells (Gombos et al. 2004; Kiss et al. 2008). In the FCDR assay, cells are first labelled with fluorescent antibodies and treated with low concentrations of cold Triton X-100 (TX-100) and immediately analysed by flow cytometry. Using low concentrations of detergent (<0.5 % TX-100) for a short period, results in a discontinuous plasma membrane and a slightly decreased cell volume, but does not affect cellular integrity, so that cells still can be analysed by flow cytometry. This is in contrast to using a higher dose of TX-100 (~1 %), which usually results in significant loss of cellular integrity. After detergent treatment, detection of a surface protein that do not localize to DRM is gradually lost, as it solubilise. In contrast, detection of a surface protein that localizes in rafts is unaffected by detergent treatment, as it does not solubilise. Using this assay, the extent of detergent resistance (FCDR index) can be estimated. The value is close to 0, if a surface protein is highly soluble after detergent treatment (i.e. does not localize to DRM) and the value is close to 1 if the surface protein is not soluble after detergent treatment (i.e. localizes to DRM).

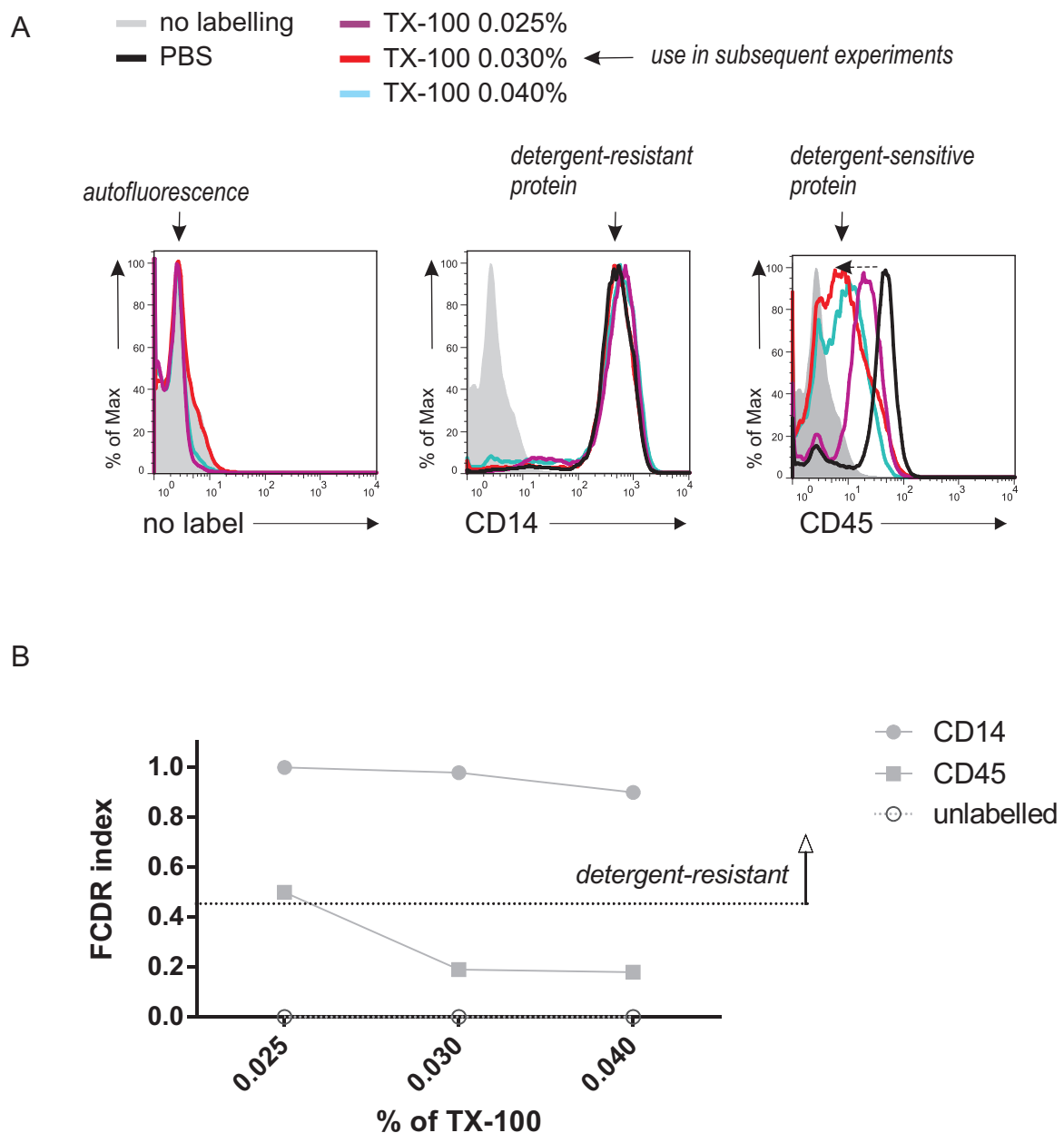
Proteins with a FCDR index higher than 0.45 are thought to be detergent resistant and raft-associated (Gombos et al. 2004; Wolf et al. 2006).

The optimal concentration of TX-100 that solubilises proteins that localize outside detergent resistant membranes (such as the non-raft marker CD45), but does not affect detergent resistant proteins (such as the raft-marker CD14) in esMDM was determined. Treatment of esMDM with 0.025 %, 0.030 %, 0.040 % TX-100 for 4 min did not affect the expression of the raft marker CD14, but reduced the expression of the non-raft marker CD45 (Figure 6.8A). 4 min PBS treatment (negative control) did not affect expression of CD14 or CD45. 0.030 % TX-100 was the lowest concentration that reduced the FCDR index of CD45 below the 0.45 cut-off, whereas the FCDR index of CD14 remained high (Figure 6.8B), as such this concentration was used in subsequent experiments. The results demonstrated that the FCDR assay is suitable, easy method for studying the raft localization of proteins in MDM.

Figure 6.8

Figure 6.8. Testing of a Flow Cytometric screening test for Detergent Resistant surface antigens in esMDM.

To determine the optimal concentration of Triton X-100 (TX-100) that solubilises proteins that localise outside detergent resistant membranes (such as CD45), but does not affect detergent resistant proteins (such as CD14) in esMDM, esMDM were labelled with anti-CD14 and anti-CD45 or left unlabelled and treated with PBS or 0.025%, 0.030%, 0.040% cold TX-100 for 4 min. A) Fluorescence histograms of unlabelled (shaded grey) or anti-CD14 and anti-CD45 labeled esMDM after 4min treatment with PBS (black line) or with 0.025% (purple line); 0.030% (red line); 0.040% (blue line) cold TX-100. B) Flow Cytometric Detergent Resistance (FCDR) index of unlabelled/autofluorescence (open circles), CD14 (solid squares) and CD45 (solid circles) on esMDM, plotted against the detergent concentrations (0.025%, 0.030%, 0.040%).



6.9 CD4 localization to DRM is reduced in CD4-DRM mutants compared to wild-type CD4 in esMDM

The previous results validated the FCDR assay in esMDM and here this method was used to characterize the detergent resistance of wild-type CD4 and the DRM localization mutants.

First, the experimental conditions were confirmed once again. 4-min mild detergent treatment (0.03 % TX-100) was sufficient to resolve raft sensitive (CD45) and resistant (CD14) proteins in esMDM (Figure 6.9A+C). Next, the detergent resistance of endogenous CD4 and transgenic wild-type/mutant CD4 was determined. esMDM were stained with anti-CD4 (clone 11830) and treated for 4 min with PBS or 0.030 % TX-100 (Figure 6.9B). As summarised in Figure 6.9D, Both endogenous wild-type CD4 (shCNTRL FCDR index: 0.50) and chimeric wild-type CD4 (CD4_{WT} FCDR index: 0.49) were resistant to detergent treatment, whereas the raft mutant (CD4_R FCDR index: 0.35) and double mutant (CD4_{P-R} FCDR index: 0.29) were sensitive to detergent treatment in macrophages. The palmitoylation mutant (CD4_P FCDR index: 0.45) had marginally reduced sensitivity to detergent compared to wild-type CD4.

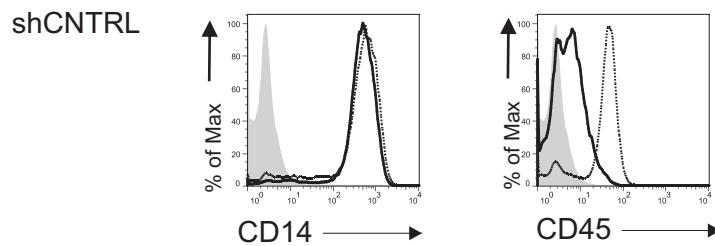
These results suggest that wild-type CD4 localizes to raft microdomains in esMDM and that the localization mutants CD4_R, CD4_{P-R}, and to a lesser extent CD4_P localize outside raft microdomains in esMDM.

Figure 6.9

Figure 6.9. CD4 localisation to DRM is reduced in CD4-DRM mutant esMDM compared to wild-type CD4.

A) To confirm the experimental conditions, control esMDM were labelled with anti-CD14 and anti-CD45 or left unlabeled and treated with PBS (black dotted line) or 0.030% cold TX-100 (black solid line) for 4 min. B) To determine the detergent resistance of endogenous CD4 and transgenic wild-type or mutant CD4, control and transgenic esMDM were left unlabelled (shaded grey) or labelled with anti-CD4 (clone 11830) and treated for 4 min with PBS (dotted red/brown line) or with 0.030% TX-100 (solid red/brown line) for 4 min. C+D) Fluorescence histograms of detergent resistance are shown.

A



B

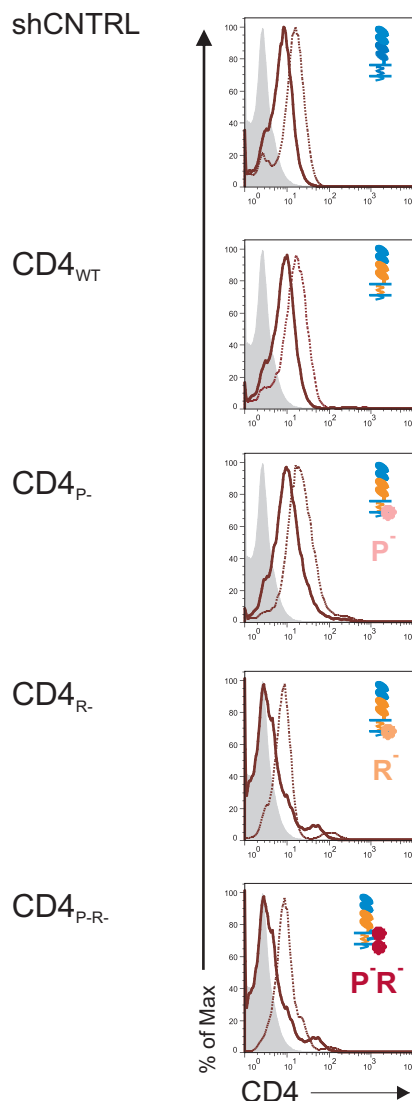
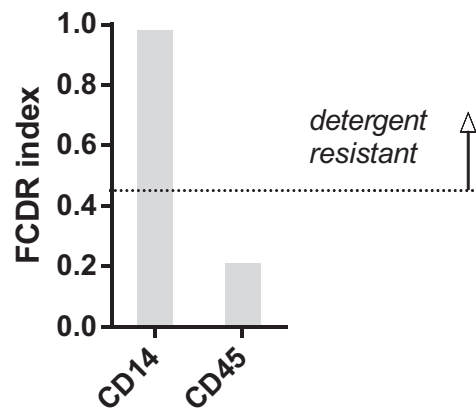


Figure 6.9

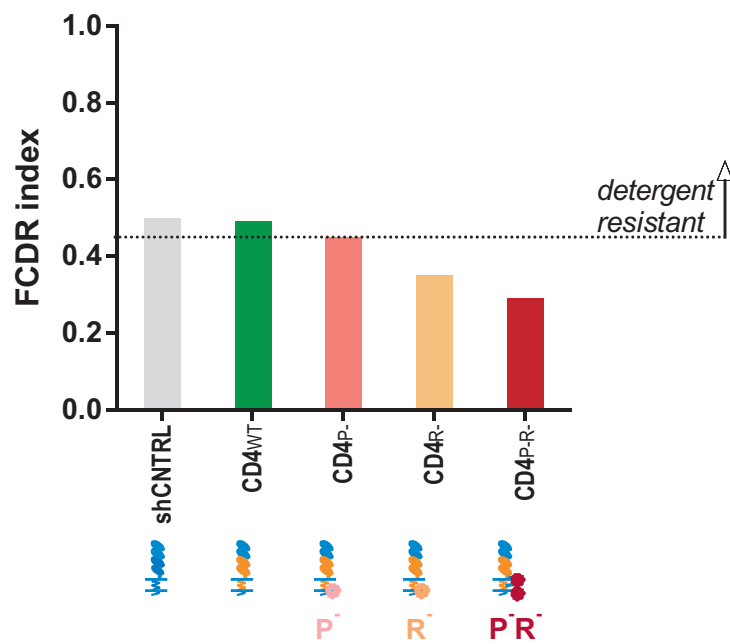
Figure 6.9. (continued) CD4 localisation to DRM is reduced in CD4-DRM mutant esMDM compared to wild-type CD4.

Flow Cytometric Detergent Resistance (FCDR) index of C) CD14 and CD45 on control (shCNTRL) esMDM and D) endogenous/chimeric mutant CD4 on esMDM expressing endogenous CD4 (shCNTRL) or expressing chimeric wild-type (CD4_{WT}) or mutant (CD4_P, CD4_R, CD4_{P-R}) CD4.

C



D



6.10 HIV-1 fusion is reduced in CD4-DRM mutant esMDM

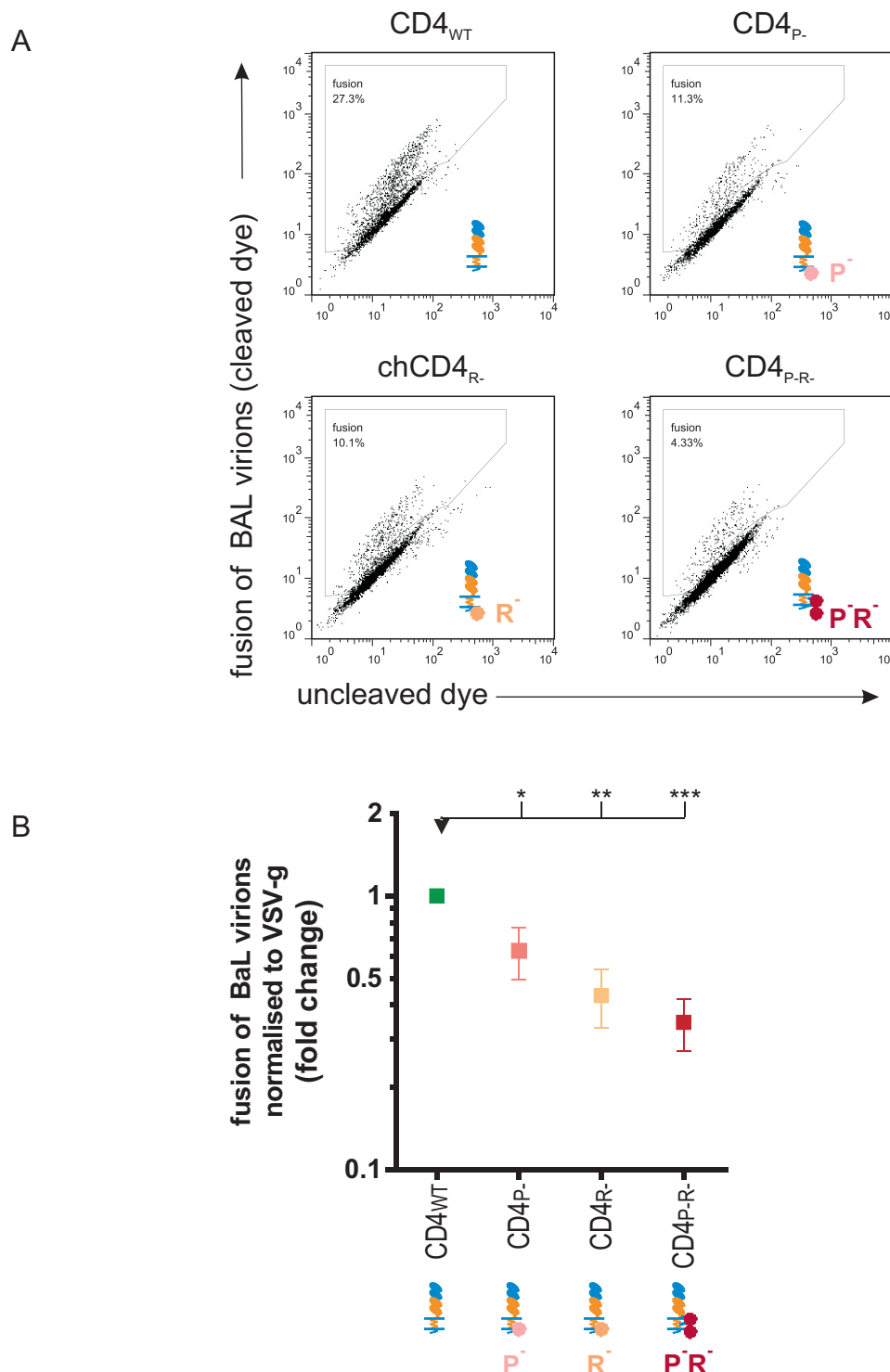
The previous results showed that esMDM were successfully genetically modified with the use of a lentiviral vector knocking down endogenous CD4 and knocking in chimeric CD4, as well expressing a puromycin resistance gene, to allow selection of a cell population that expressed the lentiviral construct. The phenotype of CD4_{WT} esMDM compared to control esMDM was validated and the mutant phenotype of the CD4 DRM localization mutants was confirmed. In the last part of this chapter, the impact of these CD4 mutants on the different stages of HIV-1 infection was evaluated by a range of assays and compared to CD4_{WT} esMDM. The results of control, shCNTRL and shCD4 esMDM from the same HIV-1 experiments were shown in Chapter 4.

Firstly, fusion of HIV-1 virions to the cells was measured using a fusion assay (Figure 6.10A shows a representative flow cytometry plot). It is important to note that HIV-1 fusion in CD4_{WT} esMDM (mean set to 1) was similar to esMDM expressing endogenous human CD4 (shCNTRL, shown in Figure 4.7; mean $\pm$ SEM: 0.9 ± 0.16 , ns). This demonstrates that chimeric CD4 can act as a 'functional' HIV receptor. However, fusion was significantly lower in all three CD4 DRM localization mutants: CD4_P esMDM (mean $\pm$ SEM: 0.6 ± 0.13 ; $P \leq 0.05$), CD4_R esMDM (mean $\pm$ SEM: 0.4 ± 0.10 ; $P \leq 0.01$) and CD4_{P-R} esMDM (mean $\pm$ SEM: 0.3 ± 0.07 ; $P \leq 0.001$) (Figure 6.10B). These results show that CD4 exclusion from DRM leads to reduced HIV-1 fusion in esMDM and suggests that CD4 localization to lipid rafts is required for HIV-1 entry.

Figure 6.10

Figure 6.10. HIV-1 fusion is reduced in CD4-DRM mutant esMDM.

Viral fusion was detected by the fluorescence shift associated with cleavage of the BL_{AM} substrate after 24h of infection of esMDM with NL4.3 BaL (BL_{AM}-Vpr) or NL4.3 VSV-g (BL_{AM}-Vpr), as described in material and methods. A) Representative FACS[®] plots of control and transgenic esMDM infected with NL4.3 BaL (BL_{AM}-Vpr). Gates were set based on the fluorescent signal of uninfected samples loaded with the BL_{AM} substrate. B) The percentage of fusion of BaL pseudotyped virions was normalised to the percentage fusion of VSV-g pseudotyped virions. Symbols represent the normalised mean fusion relative to the esMDM CD4_{WT} group of four independent experiments $\pm$ SEM (n=4). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM CD4_{WT} group.



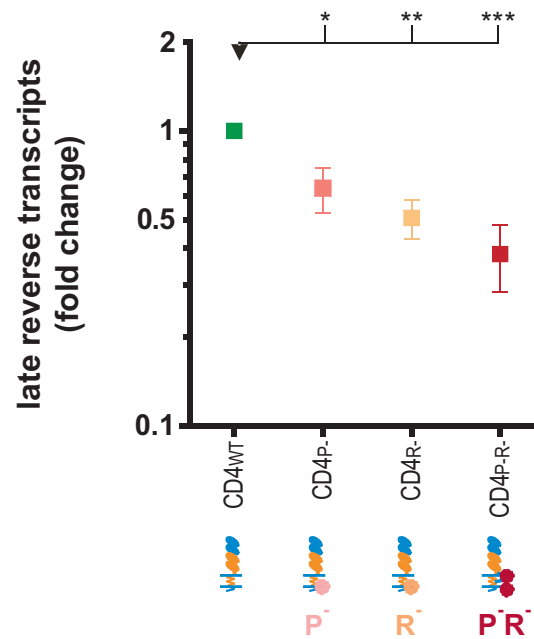
6.11 HIV-1 reverse transcription is reduced in CD4-DRM mutant esMDM

Post fusion, an essential step in the HIV-1 life cycle is reverse transcription: the conversion of vRNA into DNA. To determine whether this stage was affected by preventing CD4 DRM localization, reverse transcription was measured in transgenic esMDM-infected cells. To quantify HIV-1 reverse transcription, the number of copies of HIV-1 late reverse transcription products by qPCR after spinoculation and 28h of infection were measured, using β -actin primers to normalise to the number of cells present. Compared to CD4_{WT} esMDM (mean set to 1), based on four separate experiments, fewer copies of late reverse transcript per cell were detected in all three CD4 DRM localization mutants: CD4_{P-} esMDM (mean $\pm$ SEM: 0.6 ± 0.11 ; $P \leq 0.05$), CD4_{R-} esMDM (mean $\pm$ SEM: 0.5 ± 0.08 ; $P \leq 0.01$) and CD4_{P-R-} esMDM (mean $\pm$ SEM: 0.4 ± 0.10 ; $P \leq 0.001$) (Figure 6.11B). This shows that, in addition to reduced HIV-1 fusion, excluding CD4 localization from DRM results in reduced HIV-1 reverse transcription and suggests that CD4 localization to lipid rafts is required for HIV-1 entry.

Figure 6.11

Figure 6.11. HIV-1 reverse transcription is reduced in CD4-DRM mutant esMDM.

Reverse transcription of HIV-1 in esMDM was measured by detecting late (pol) products by qPCR after 30h of infection. Symbols represent the relative mean number of copies of HIV-1 DNA of four independent experiments $\pm$ SEM (n=4), normalised to the number of cells using a β -actin control. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the CD4_{WT} group.



6.12 Reporter expression from HIV-1 integrants is reduced in CD4-DRM mutant esMDM

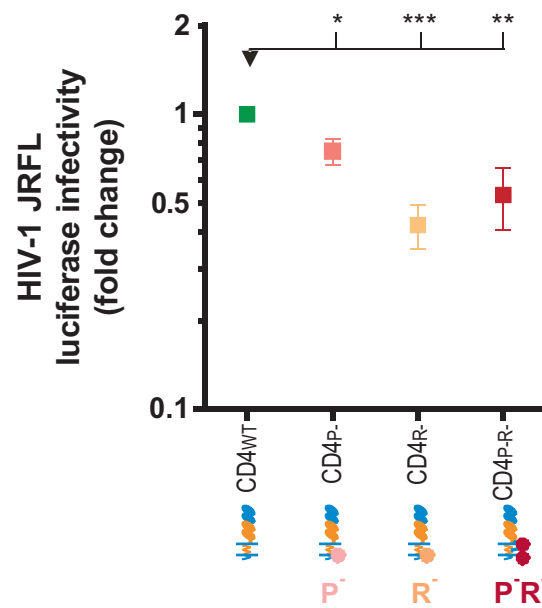
Once the vRNA has been reverse transcribed into a strand of cDNA and has been imported into the nucleus, the viral cDNA can be integrated into the host cell DNA. Only once the viral DNA (provirus) is transcribed, can new viruses be produced. Therefore, a reporter assay from HIV-1 integrants was used as measure of a single round of productive infection. This assay uses a replication-defective pseudotyped HIV-1 that has the gene for luciferase inserted in place of the *nef* gene, and can therefore function as a reporter gene for infection. esMDM were infected with JRFL-NL4.3 or VSV-G-NL4.3, for 3 days before cells were lysed and luciferase expression was measured. The luciferase activity of esMDM infected with JRFL-NL4.3 was normalised to esMDM infected with VSV-G-NL4.3. Compared to CD4_{WT} esMDM (mean set to 1), detection of luciferase protein produced in lysates from esMDM expressing the CD4 DRM localization mutants was significantly reduced (CD4_P. mean $\pm$ SEM: 0.8 ± 0.08 ; $P \leq 0.05$; CD4_R. mean $\pm$ SEM: 0.4 ± 0.07 ; $P \leq 0.001$; CD4_{P-R}. mean $\pm$ SEM: 0.5 ± 0.13 ; $P \leq 0.01$) (Figure 6.12).

These results are in accordance with the previous result, showing that when CD4 is excluded from DRM in esMDM, HIV-1 fusion, reverse transcription and productive infection are reduced. These results suggest that CD4 localization to lipid rafts is required for successful HIV-1 entry.

Figure 6.12

Figure 6.12. Reporter expression from HIV-1 integrants is reduced in CD4-DRM mutant esMDM.

HIV-1 integration was measured by detecting luciferase activity in esMDM three days after transduction with HIV-1 NL4.3.Luc.R-E- virus pseudotyped with JRFL or VSV-g envelope. Luciferase expression was quantified using Promega One-Glo luciferase substrate and a luminometer. JRFL NL4.3.Luc.R-E- transduced cells were normalised to VSV-g NL4.3.Luc.R-E- transduced cells. Symbols represent the mean luciferase detection relative to the esMDM CD4_{WT} group of six independent experiment, $\pm$ SEM (n=6). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. One-way ANOVA followed by Holm-Sidak's multiple comparison test versus the esMDM CD4_{WT} group.



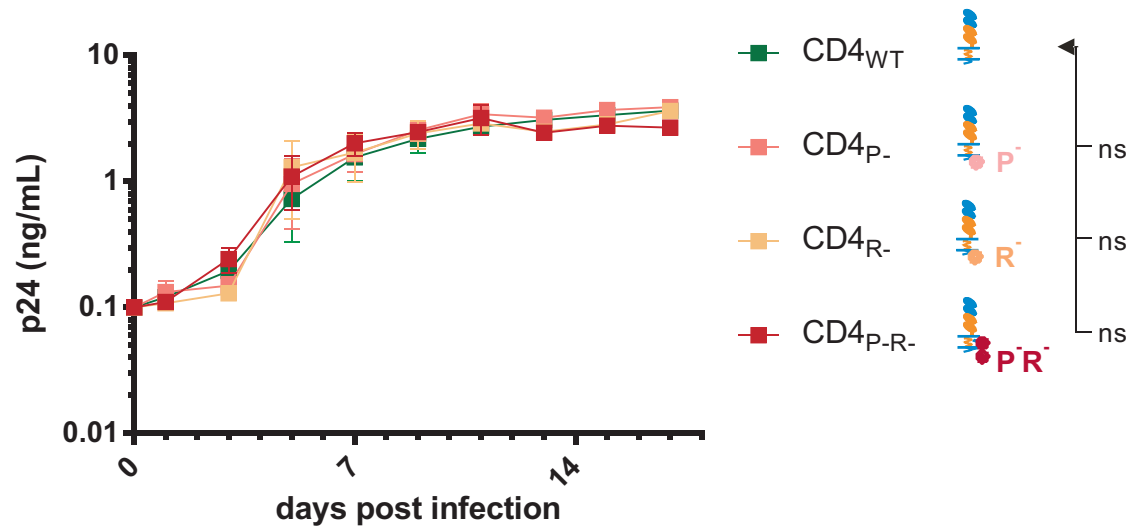
6.13 Multiple rounds of HIV-1 replication are not significantly reduced in CD4-DRM mutant esMDM

The above assays measured events during a single cycle of HIV-1 infection. Once new viruses are produced and bud from the infected cell, the cycle repeats itself. A multi-cycle assay was, therefore, also performed even though HIV-1 replication in MDM over multiple generations may change the phenotype of these cells, and this might complicate the interpretation of the results. HIV-1 replication kinetics were measured by culturing HIV-1 BaL infected esMDM for 11-17 days. Samples of the supernatant were taken at intervals and assayed for viral p24 protein levels by ELISA. Compared to CD4_{WT} esMDM, infection with HIV-1 BaL resulted in similar levels of virus being produced by esMDM expressing CD4 DRM localization mutants. Although, this assay does not give as accurate reflection of successful infection as the previous experiments, owing to amplification effects of various confounding factors, the results show that when CD4 is excluded from DRM in esMDM, HIV-1 replication is not affected, whereas a single round of HIV-1 infection (fusion, reverse transcription and productive infection) is reduced. These results suggest that CD4 localization to lipid rafts is required for successful HIV-1 entry.

Figure 6.13

Figure 6.13. Multiple rounds of HIV-1 replication are not significantly reduced in CD4-DRM mutant esMDM.

HIV-1 replication in esMDM was measured by p24 ELISA. esMDM were infected with HIV-1 BaL. Samples of the supernatant were taken at regular intervals and assayed for p24 levels by ELISA. Symbols represent the mean p24 concentration at different time points $\pm$ SEM of independent experiments. $n=3$ for day 1-11; $n=2$ for day 13; and $n=1$ for day 15-17. ns: non-significant ($P>0.05$). Repeated measure one-way ANOVA followed by Holm-Sidak test.



6.14 Summary and Discussion

Endogenous CD4 was knocked down and three mutant versions of CD4 were knocked in for the first time in authentic macrophages. The CD4 mutants were expressed at the cell surface, but excluded from DRM. A set of HIV-1 assays produced evidence in favour of the hypothesis that successful HIV-1 entry in MDM is affected (reduced) by excluding CD4 from DRM.

To achieve lentiviral expression in MDM, which are inherently refractory to genetic modification, hESC, which are easily genetically manipulable, were transduced and differentiated into authentic MDM. An advanced lentiviral-based dual expression system, as described in Chapter 4, was further modified to not only allow knockdown of endogenous CD4, but also knock-in of novel versions of wild-type or mutant CD4 that were shCD4 resistant (LV-U6-shCD4-EF1-CD4-I-Puro^R). Endogenous CD4 was effectively knocked-down to background levels in esMDM expressing LV-U6-shCD4-EF1-CD4-I-Puro^R. The knockdown efficiency was similar in esMDM expression shCD4 alone (as presented in Chapter 4), as such confirming that the hairpin expression from the U6 promoter is not affected by transgenes expression from the downstream EF1 α promoter. This system, using two separate promoters for simultaneous expression of a hairpin and transgenes, is more effective than a system that uses one promoter to co-transcribe an shRNA-miR and the GOI, as previously developed in our laboratory (Cowley SA 2012). Chimeric versions of CD4 were designed using a combination of rat and human CD4. Chimeric CD4 versions were resistant to the shCD4 and easily distinguished from endogenous CD4 using an anti-CD4 rat antibody clone OX68 (Brown et al. 1994). Chimeric CD4 mediated HIV-1 infection, as it contained domain 1 of human CD4, which encompasses the residues that are required for the interaction with gp120 and HIV-1 infection (Jameson et al. 1988; Peterson et al. 1988; Arthos et al. 1989; Brodsky et al.

1990). Cell surface expression and raft localization of chimeric CD4 was similar to that of human CD4, considering chimeric CD4 contained the cytoplasmic tail of human CD4, containing the sub-cellular localization signals, although there was a trend towards a lower surface expression of the CD4 raft localization mutant, indicating that when CD4 is excluded from DRM, more CD4 becomes available for down-modulation endocytosis through clathrin-coated pits, which indeed occurs outside lipid rafts. An inducible system would have allowed control of expression levels, the use of the EF1 α to express codon-optimized chimeric CD4 resulted in expression levels similar to that of endogenous CD4 in esMDM. Accordingly, HIV-1 entry in esMDM mediated by wild-type chimeric CD4 was similar to infection mediated by endogenous human CD4.

The utility of this novel lentiviral expression to knockdown, as well as, knock in CD4, was demonstrated by creating three DRM localization mutant versions of chimeric CD4, containing mutations in its palmitoylation region ³⁹⁴CVRC³⁹⁷ ('P') and/or raft localization region ³⁹⁸RHRRR⁴⁰² ('R'). mRNA and protein expression levels were similar between wild-type CD4 and the CD4 DRM localization mutants, which is in agreement with previous reports. In cell lines, it has been shown that CD4_P is excluded from DRM without affecting its biosynthesis and cell surface expression, and that CD4_P has transport kinetics identical to that of wild-type CD4 (Crise et al. 1992; Percherancier et al. 2003). CD4_R is also excluded from DRM in cell lines, without affecting CD4 palmitoylation or total expression levels (Popik et al. 2004). The double mutant, CD4_{P-R} has not been described in the literature before.

In order to assess the localization of CD4 to DRM, a Flow Cytometric assay for Detergent Resistance was performed. This assay, first described by *Gombos et al.* is highly reproducible and overcomes problems associated with the classic biochemical technique that applies high concentrations of TX-100 and density gradient centrifugation, to study DRM associated proteins (Gombos et al. 2004). For example, raft proteins that are

associated with the cytoskeleton may not float after detergent extraction; or DRM localization of proteins that are only weakly associated with rafts may be disrupted by the high concentrations of TX-100; there is no distinction between surface and total expression of DRM proteins using the classic assay (Simons et al. 2000; Schuck et al. 2003; Le Roy et al. 2005). FCDR analysis of DRM localization of the raft marker CD14 and non-raft marker CD45 confirmed that the FCDR assay is rapid as well as reliable, and demonstrated for the first time that the assay is suitable to study the detergent resistance of proteins in MDM. Although the FCDR index represents a spectrum of varying degrees of DRM associations, proteins with a FCDR index higher than 0.45 are considered to be detergent-resistant and thought to localize to raft micro-domains; conversely, proteins with a FCDR index below 0.45 are considered to be excluded from DRM (Gombos et al. 2004; Wolf et al. 2006). For example, *Gombos et al.* showed that the raft-marker G_{M1} -ganglioside has a FCDR index of 0.49 (Gombos et al. 2004). Endogenous human CD4 and chimeric wild-type CD4 had an FCDR index of 0.50 and 0.49, respectively, confirming that CD4 is associated with DRM in MDM. This is consistent with findings using the classic DRM biochemical approach in MDM, and similar to CD4 DRM localization in monocytes (Wolf et al. 2007; Carter et al. 2009). In contrast, the raft mutant (CD4_R, FCDR index: 0.35) and double mutant (CD4_{P-R}, FCDR index: 0.29) were sensitive to detergent treatment in macrophages. The palmitoylation mutant (CD4_P, FCDR index: 0.45) had marginally reduced sensitivity to detergent compared to wild-type CD4. This shows for the first time in MDM, that raft localization region, and to a lesser extent the palmitoylation region, are involved with the DRM localization of CD4 and this is consistent with findings in other cell types (Crise et al. 1992; Fragoso et al. 2003; Percherancier et al. 2003; Balamuth et al. 2004; Popik et al. 2004; Baker et al. 2007).

The raft localization region (³⁹⁸RHRRR⁴⁰²) has been suggested to be the dominant signal for raft targeting of CD4 in a B-cell line, but has not been studied further in other cell types (Popik et al. 2004). Here, esMDM expressing CD4_R had a lower FCDR index compared

to CD4_P, which may suggest that in esMDM too, ³⁹⁸RHRRR⁴⁰² is the dominant DRM signal. Like the ³⁹⁸RHRRR⁴⁰² motif in CD4, Arginine residues and polybasic motifs are responsible for DRM localization in other proteins, such as CD8 (Pang et al. 2007; Hrdinka et al. 2012). Such basic motifs may stabilise proteins in DRM through an electrostatic interaction with a negatively charged phosphate group of sphingomyelin and/or sialic acids of gangliosides found in DRM (Fantini 2003). Palmitoylation increases the affinity of integral membrane proteins to localize to DRM and may be necessary for DRM localization of some proteins, but protein acylation is generally not sufficient on its own to signal raft association (Melkonian et al. 1999; Levental et al. 2010). Palmitoylation is the only lipid modification that can be reversible and although this has not specifically been reported for CD4, one could hypothesize that this may partially control sub-cellular localization of CD4 (Resh 1999). Depalmitoylation can occur throughout the cell and redirects proteins to the Golgi where proteins become palmitoylated again (Rocks et al. 2010). Further studies exploring the molecular mechanism involved in DRM localization of CD4 and palmitoylation are lacking.

In T-cells, CD4 can associate with DRM, however, unlike in MDM which do not express LCK, CD4 DRM localization may depend also on LCK association and the cell activation state (Zlatkine et al. 1997; Balamuth et al. 2001; Filipp et al. 2003; Fragoso et al. 2003). In primary resting T cells, the majority of LCK and CD4 are generally thought to localize outside DRM, although some reports suggest that CD4 is constitutively localized to DRM, possibly together with LCK in smaller raft sub-micro-domains (Millan et al. 1999; Alonso et al. 2001; Kozak et al. 2002; Popik et al. 2002; Filipp et al. 2003; Percherancier et al. 2003). Interaction between the TCR and MHC complex on antigen-presenting cells results in an 'Immunological Synapse' which is a specialized contact surface that is formed between the T cell and the APC, where proteins aggregate (Grakoui et al. 1999; Holdorf et al. 2002). The Immunological Synapse is enriched in DRM components, and TCR signaling depends on the integrity of DRM (Dykstra et al. 2003). Upon antigen T-cell

stimulation, pre-existing smaller raft sub-micro-domains may come together and CD4 and LCK form a complex in DRM together with other TCR signaling components to form the Immunological Synapse (Balamuth et al. 2001; Filipp et al. 2003; Levental et al. 2010). LCK can associate with DRM due to myristoylation of its Glycine at position 2 at the N-terminus and palmitoylation of at least one of the two Cysteine residues at position 3 and 5 (Rodgers et al. 1994; Kwong et al. 1995; Filipp et al. 2003). However, the relative distribution of CD4/LCK in DRM or non-DRM membranes in T-cells remains unclear and the underlying mechanisms of lipidation are a topic of debate. Nevertheless, the importance of CD4 and LCK DRM localization during T-cell activation is generally accepted (Del Real et al. 2002; Filipp et al. 2003; Popik et al. 2004).

The effect of CD4 exclusion from DRM on HIV-1 infection in MDM was studied. Excluding CD4 from DRM in macrophages expressing CD4_{P-}, CD4_{R-} or CD4_{P-R-}, in all cases led to a decrease in infection as observed using three different HIV-1 assays. This effect was more pronounced in the DRM excluded CD4_{R-} compared to the partially DRM excluded mutant CD4_{P-}. This provides direct evidence for the requirement for CD4 DRM localization in HIV infection of MDM. Unlike a single cycle of HIV-1 infection however, HIV-1 replication, as measured by the multi-cycle p24 ELISA assay, was not affected by CD4 exclusion of DRM. This assay is a relatively crude measure of HIV-1 entry, and multiple confounding host and viral effects contribute to HIV-1 replication, so it does not differentiate small differences in one specific step of the infection cycle. Irrespective of the consequence on HIV-1 replication however, the single-cycle assays used here demonstrate that CD4 localization to DRM is required for HIV-1 entry. The results in this chapter are in agreement with previous results that applied a pharmaceutical approach to demonstrate the requirement of intact DRM for HIV-1 infection of MDM and supports Carter *et al.*'s suggestion that cholesterol depletion reduces CD4 DRM localization and as such HIV-1 infection (Carter et al. 2009).

The genetic approach used in this chapter, avoided the problems associated with the pharmacological approach. For example, the reduced HIV-1 infection observed after cholesterol depletion by *Carter et al.*, may have occurred due to other effects of drug treatment, such as reduced CD4 levels, or inhibition of the endocytic HIV-1 entry pathway in MDM (*Carter et al.* 2009). Also, cholesterol depletion may affect HIV-1 fusion irrespective of its DRM location, considering cholesterol is present everywhere on the plasma membrane, albeit enriched in lipid rafts (*Simons et al.* 1997; *Ilangumaran et al.* 1998). Cholesterol is likely involved in the fusion process (*Salzwedel et al.* 1999; *Haque et al.* 2001; *Epand* 2006). Gp41, which mediates HIV-1 fusion after gp120-CD4 binding, has a Cholesterol Recognition/interaction Amino acid Consensus (CRAC) domain (the sequence LWYIK) that specifically recognizes the cholesterol in DRM and promotes membrane fusion (*Li et al.* 1998; *Salzwedel et al.* 1999). So, although cholesterol-disrupting drugs can result in pleiotropic effects, their use demonstrates the importance of cholesterol during HIV-1 entry in MDM and highlights that cholesterol may affect different HIV-1 entry processes (*Carter et al.* 2009). Cholesterol has been implicated in regulating viral entry into many other cell types (*Manes et al.* 2000; *Liao et al.* 2001; *Percherancier et al.* 2003; *Carter et al.* 2009). For example, depletion of cell plasma membrane cholesterol also inhibits HIV-1 infection in T-cells, however, unlike in MDM, it is unclear whether in T-cells depletion of cell plasma membrane cholesterol also affects CD4 DRM distribution (*Percherancier et al.* 2003; *Carter et al.* 2009).

Although cholesterol plays a role during HIV-1 infection in many cell types, the importance of CD4 localization to DRM during HIV-1 entry in non-myeloid cells is controversial. The CD4 mutants used in this Chapter were expressed for the first time in authentic MDM, but have been expressed previously in other cell types. Unlike in MDM, these mutants did not affect HIV-1 entry in cell lines. Several studies expressed CD4_p in a T-cell or B-cell line or HeLa cells and showed that CD4 palmitoylation is not required for HIV entry in these cell lines (*Crise et al.* 1992; *Percherancier et al.* 2003; *Popik et al.* 2004). Also, a B-cell

line expressing CD4_R supports HIV-1 entry (Popik et al. 2004). This suggests that the CD4 DRM localization may not be important for HIV-1 entry into cell lines. However, another non-DRM CD4 mutant with no CD4 cytoplasmic tail, generated by replacing CD4 transmembrane and cytoplasmic domains with the transmembrane and a very short cytoplasmic tail from the non-raft low density lipoprotein (LDL) receptor, resulted in a diminished capacity for HIV entry in cell lines (Del Real et al. 2002). It remains controversial whether in T-cells or cell lines, CCR5 and CXCR4 can localize to DRM too and whether CD4, CCR5 and CXCR4 DRM localization is important for HIV-1 entry (Manes et al. 2000; Kozak et al. 2002; Nguyen et al. 2002; Percherancier et al. 2003). The conflicting results may be due to differences in methods and cell types used, but also highlight the importance of studying HIV-1 infection in a physiologically relevant cell type and emphasizes that HIV-1 likely enters MDM through a cell type specific entry route, as recently described by *Carter et al.* (Carter et al. 2011). The results in this chapter confirm that DRM, and specifically CD4 localization to DRM, is required for successful HIV-1 entry into MDM (Marechal et al. 2001; Carter et al. 2009; Carter et al. 2011).

The finding that CD4 DRM localization is important for HIV-1 entry in MDM complements the existing raft-dependent endocytic entry model for successful HIV-1 entry in MDM (Marechal et al. 2001; Carter et al. 2009; Carter et al. 2011). Like many non-clathrin dependent entry routes, the productive HIV-1 endocytic entry route into MDM is DRM-dependent (Carter et al. 2009; Carter et al. 2011). To allow fusion after endocytosis, the presence of CD4 and the co-receptor CCR5 in the endocytic HIV-1 uptake vesicles are necessary, although they are most likely not required for viral attachment at the surface, considering cholesterol depletion did not affect viral attachment, but did reduce CD4 and CCR5 surface levels (Carter et al. 2009). Lipid rafts are rich in components that are recognized by HIV-1. The envelope protein is palmitoylated on two cysteine residues and contains a common sphingolipid-binding motif (Yang et al. 1995; Rousso et al. 2000; Mahfoud et al. 2002). Gp120 interacts with various sphingolipids that are enriched in lipid

rafts, including ceramides, gangliosides and sphingomyelin (Harouse et al. 1991; Hammache et al. 1998; Puri et al. 1998; Hammache et al. 1999; Van Mau et al. 2000; Mahfoud et al. 2002). Glycosphingolipids can mediate the clustering of rafts and organisation of a membrane fusion complex containing the HIV-1 envelope as well as CD4 and CCR5 (Hug et al. 2000; Manes et al. 2000; Rawat et al. 2005). Further studies would have to be performed to confirm the involvement the above mentioned envelope-lipid interactions in macrophages. In addition to lipids, HIV-1 attachment can attach to components at the cell surface in MDM, such as syndecans, which have been implicated in various raft-dependent endocytic routes (Fuki et al. 2000; Saphire et al. 2001; Tkachenko et al. 2002; Wilsie et al. 2006).

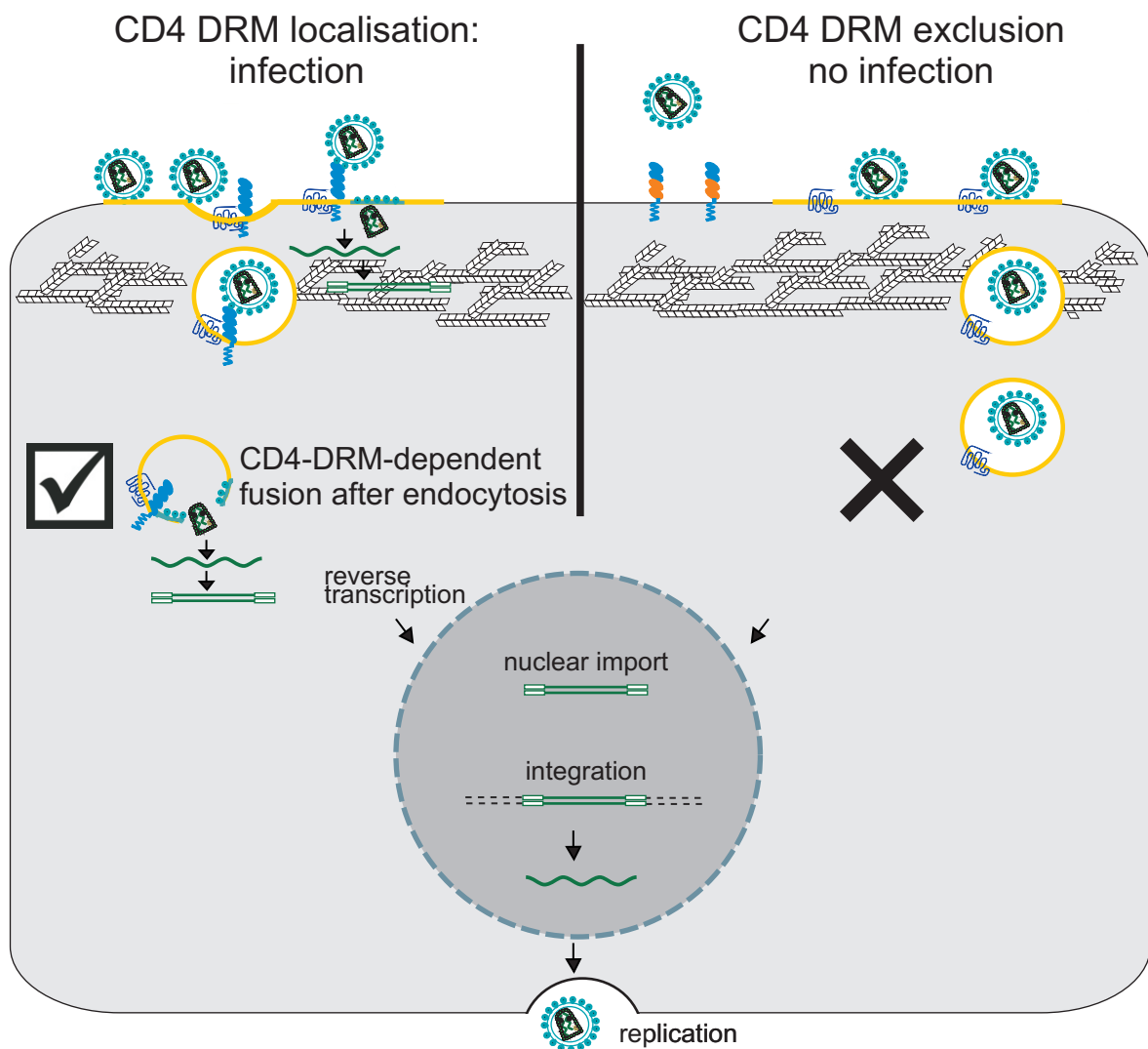
The result suggest the following model of HIV-1 infection in MDM, as illustrated in Figure 6.14: although raft-dependent uptake pathway is unspecific, CD4 and CCR5 presence in raft microdomains are required to allow fusion after endocytosis. When CD4 is localized outside DRM, HIV-1 may still be taken up through macropinocytosis-like entry route, but no fusion can occur. The requirement for CD4 raft localization during HIV-1 entry in MDM was demonstrated in this chapter. The requirement for CCR5 raft localization is most likely obligatory, considering all CCR5 molecules in MDM localize to lipid rafts (Carter et al. 2009). Accordingly, electron micrographs revealed that CD4 and CCR5 localize on the outer membranes of cell protrusions in MDM, forming homogeneous microclusters (Singer et al. 2001). From this follows, that when CD4 is localized outside DRM, surface fusion can also not occur, because no CCR5 would be available outside raft domains to associate with CD4 and HIV-1 to allow fusion. A more simple interpretation of the results presented in this chapter would be that CD4 has to be proximal to CCR5, independent of its DRM location, however, previous publications have shown that intact lipid rafts are required for successful HIV-1 entry (Carter et al. 2009).

Although the productive uptake pathway originates in DRM, HIV-1 may also be taken up via DRM-independent endocytic routes in MDM, such as clathrin-mediated endocytosis. However, this is a non-productive entry route, associated with rapid HIV-1 degradation (Carter et al. 2011; Gobeil et al. 2012). Accordingly, the clathrin assembly inhibitor chlorpromazine does not affect HIV-1 infection in MDM and neither Pak-1 nor Rac, both required for the HIV-1 endocytic-entry route in MDM, are thought to be involved in clathrin-dependent endocytosis (Wang et al. 1993; Grassart et al. 2008; Carter et al. 2011). HIV-1 entry through clathrin-mediated endocytosis is likely to be unproductive

Figure 6.14

Figure 6.14. CD4 localisation to DRM required for productive HIV-1 infection.

Model illustrating the requirement of CD4 DRM localisation for productive HIV-1 infection (left side). The proposed model suggests that productive infection follows a raft (shown in yellow) dependent, unspecific uptake pathway and requires the presence of both CD4 and CCR5 in rafts. HIV-1 surface fusion may occur, but is unproductive as the actin-cortex forms a barrier. This barrier is overcome by endocytosis. However, when CD4 localises outside DRM (right side), no fusion can occur, neither at the surface or after raft-dependent uptake, likely due to the absence of CCR5 outside lipid rafts.



considering the raft-associated co-receptor CCR5 is presumably absent from the non-DRM clathrin coated pits. Although CCR5 agonists can induce clathrin-mediated endocytosis of CCR5, all CCR5 molecules in MDM localize to DRM microdomains (Signoret et al. 2005; Carter et al. 2009). The C-terminal domain of CCR5 contains a basic domain followed by palmitoylated cysteine residues that anchor CCR5 to plasma membrane rafts (Venkatesan et al. 2001; Venkatesan et al. 2003). A truncated CCR5 mutant 'KRFX' that lacks the palmitoylation motif, is excluded from DRM and is endocytosed by a clathrin-dependent pathway (Venkatesan et al. 2001; Venkatesan et al. 2003). Expression of such a non-DRM CCR5 mutant in MDM would shed light on the role of CCR5 DRM localization in the productive and non-productive entry pathways of HIV-1 in MDM. Alternatively, the role of clathrin-dependent endocytosis could in HIV-1 entry in MDM could be studied directly using a genetic approach, by knocking-down components of the clathrin-dependent endocytosis pathway (McMahon et al. 2011).

To summarize, the importance of CD4 rafts localization in HIV-1 entry was studied for the first time in MDM. A lentiviral expression system was designed that achieves knockdown of endogenous CD4, as well as, knock-in of shCD4-resistant wild-type or mutant CD4 in MDM. Whereas expression of wild-type CD4 restored the infectability of MDM with HIV-1, expression of non-raft CD4 mutants resulted in reduced HIV-1 entry. These results demonstrate that successful HIV-1 entry in MDM relies on a CD4- and raft-dependent entry route.

Chapter 7: Summary and final discussion

7.1 Summary of results

The general hypothesis of this thesis was that sub-cellular CD4 distribution determines the HIV-1 entry pathway of macrophages. To this end a method for producing large numbers of genetically modifiable, functional macrophages was developed. The hematopoietic differentiation of hESC could be induced based on the identification of CD34+CD38- and CD34+CD90+ HSC populations and CD45+CD14+ and CD33+ myeloid populations in the differentiating cultures. The optimized protocol allowed the production 1×10^7 viable esMC after 5 weeks of differentiation and monocyte production continued for up to 1 year. Monocytes were CD14+CD16+CD163+, similar to bMC and could be differentiated into functional macrophages, despite low MHCII expression levels. esMDM were capable of phagocytosis, could be classically activated and were phenotypically similar to bMDM, expressing myeloid markers as well as the HIV-1 receptors CD4 and CCR5. esMDM were permissive to HIV-1 infection and allowed HIV-1 replication.

Transduction of human embryonic stem cells with a second-generation SIN lentiviral vector and differentiating these genetically modified hESC into esMDM, allowed the continuous production of a pure population of genetically modified esMDM. This was achieved using a lentiviral vector that expressed RFP under the control of the EF1 promoter. This then enabled the use of genetic methods to manipulate the sub-cellular distribution of CD4 and study HIV-1 infection in esMDM. The usefulness of this system was demonstrated by developing an advanced lentiviral-based dual expression vector, which not only has the option to express a transgene, but also allows the expression of a short hairpin targeting CD4 (or another target) under the control of the U6 promoter. In esMDM expressing this vector, CD4 transcripts were reduced by a factor of 100 and CD4

protein was reduced to background levels compared to control esMDM. HIV-1 infection in CD4-knockdown esMDM was significantly reduced, as measured by a fusion-, reverse transcription-, integration-, and replication assay. This provides strong direct evidence that CD4 is necessary as the main receptor for HIV-1 infection of MDM.

hESC-derived MDM as genetic modification system was used to address the general hypothesis specifically by constitutively expressing LCK esMDM to manipulate sub-cellular CD4 distribution. In LCK expressing esMDM, CD4 was tethered to the cell surface and the early steps of the HIV-1 entry pathway were affected (as fusion and reverse transcription were increased), but successful HIV-1 entry efficiency (determined by reporter expression from DNA integrants and a replication assay) was not increased. This implies that diverting CD4 away from its normal recycling route causes sequestering of HIV-1 into a pathway that is unproductive in esMDM, presumably due to the actin cortex barrier that cannot be overcome by virions that fuse close to the surface in esMDM. This result highlights the importance for HIV-1 to use an endocytic entry pathway in MDM, as opposed to a plasma membrane pathway, to achieve productive infection, and to do so, requires MDM-specific sub-cellular CD4 distribution.

As endocytic entry pathways for HIV-1 have previously been reported to be raft-dependent, the requirement of CD4 DRM localization for HIV-1 entry in esMDM was tested using the advanced lentiviral-based dual expression system (Liu et al. 2002; Vidricaire et al. 2007; Carter et al. 2009). Endogenous CD4 was knocked down and three mutant versions of CD4 were knocked in (a technical advance in authentic macrophages). The CD4 mutants were expressed at the cell surface, but excluded from DRM (measured by the FCDR assay) and HIV-1 fusion-, reverse-transcription- and integration assays produced evidence in favour of the hypothesis that successful HIV-1 entry in MDM is affected (reduced) when CD4 is excluded from DRM.

7.2 Model

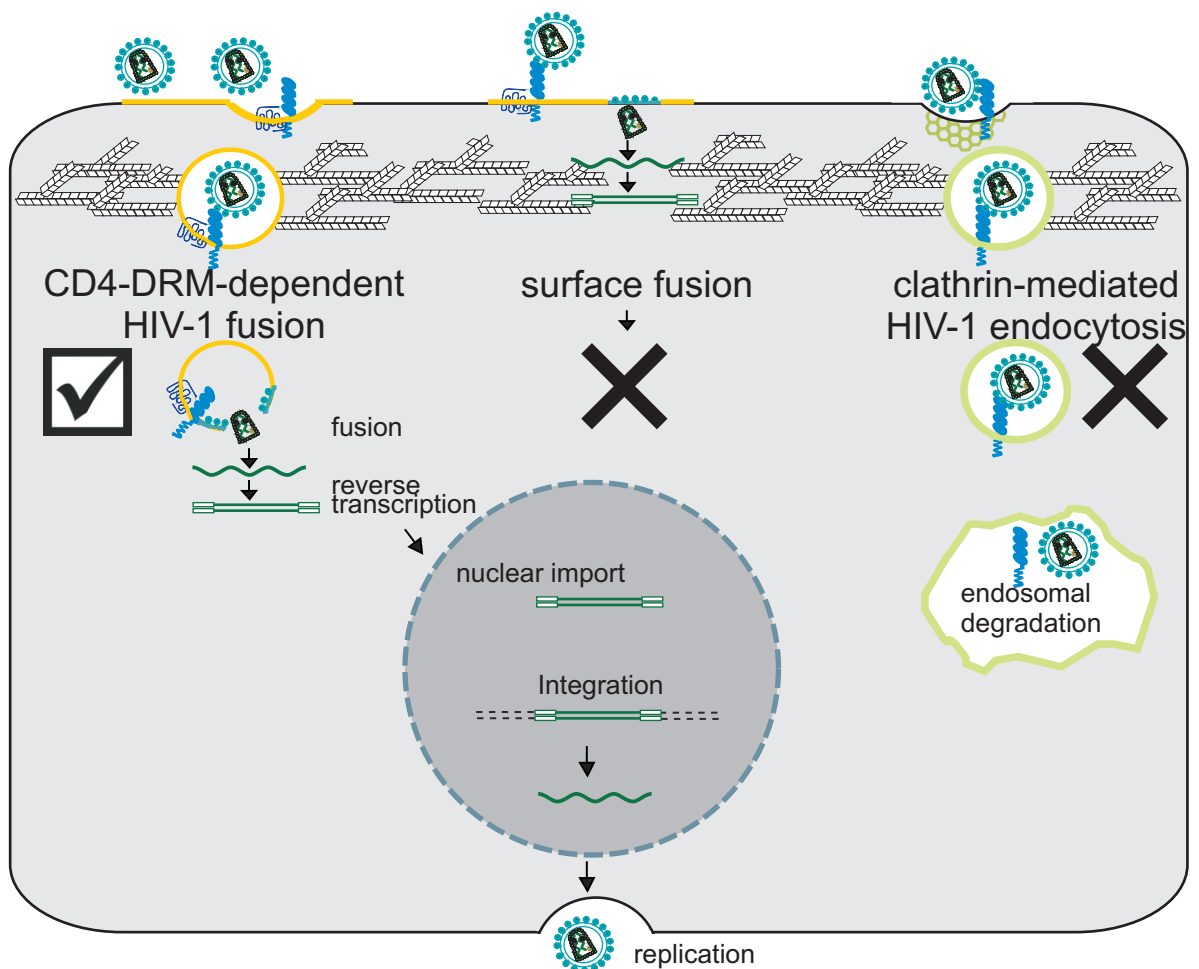
From the results described, based on studies using genetically modified esMDM and a panel of assays as readouts for sequential stages of HIV-1 infection, an entry model for HIV-1 infection in macrophages is proposed, as further described below and illustrated in Figure 7.1.

It is proposed that HIV-1 is taken up non-specifically into MDM by a raft-dependent pathway that resembles macropinocytosis (Marechal et al. 2001; Carter et al. 2009; Carter et al. 2011). Importantly, the localization of CD4 and presumably CCR5 to lipid rafts is required for **successful** HIV-1 entry, demonstrated using CD4-DRM mutants. Alternatively, HIV-1 can enter MDM through **unsuccessful** routes, demonstrated by tethering CD4 to the cell surface using LCK expression. The results suggested that in MDM, virions may fuse at the plasma membrane, but are unable to pass the actin cortex to establish productive infection. This highlights the importance of the endocytic route in MDM, which allows HIV-1 to overcome this actin barrier before fusing with the host membrane (Stolp et al. 2011; Mooren et al. 2012). Virions may also overcome the actin barrier through clathrin-mediated endocytosis, but are unable to fuse from clathrin-coated vesicles, presumably due to the absence of CCR5 in these vesicles. Virions taken up through clathrin-mediated endocytosis continue to be degraded through the endosomal/lysosomal pathway (Stolp et al. 2011; Gobeil et al. 2012; Mooren et al. 2012). It is plausible that some virions that enter through the productive raft-dependent endocytic HIV-1 entry route may also fail to fuse before they become degraded in lysosomes. Conversely, it is plausible that some virions do succeed in fusing from clathrin-coated vesicles or establish productive infection after surface fusion, albeit less efficiently compared to the raft-dependent endocytic entry route. As such, the ability of HIV-1 to establish productive infection associated with a certain entry pathway could be represented as a spectrum. This model of HIV-1 entry implies that productive infection

Figure 7.1

Figure 7.1. Model of HIV-1 entry in MDM.

Proposed model of the HIV-1 entry pathways in MDM. Virions can be taken up unspecifically via raft or clathrin-mediated endocytosis. However, only after raft-dependent endocytosis, virions can fuse and productively infect MDM, as CCR5 predominantly associates with lipid raft microdomains and clathrin-mediated endocytosis occurs outside lipid rafts. As such, CD4 localisation to DRM is a requirement for HIV-1 fusion. Virions taken up via clathrin-mediated endocytosis are not able to fuse and will be degraded in endosomes. Alternatively, virions can fuse at the cell surface, but unlike endosomes, cannot overcome the actin-barrier.



depends on the balance between virus fusion kinetics (influenced by (co-)receptor expression levels and raft localization) and the endolysosomal degradation rates, as well as the actin barrier (Carter et al. 2011; Gobeil et al. 2012). Low levels of surface CD4, as normally found on the surface of MDM, would result in low fusion kinetics. This would lead to the accumulation of unfused virions at the cell surface and increased endocytic uptake. Therefore, it has been suggested that low surface CD4 levels favour virion uptake through endocytosis over unproductive plasma membrane fusion (Permanyer et al. 2010). Because CD4 and CCR5 localize to raft micro-domains, the raft-dependent endocytic uptake pathway allows for productive fusion after endocytosis, whereas clathrin-mediated endocytosis leads to viral degradation. The model also illustrates the paradoxical role of actin. Although the actin cytoskeleton facilitates the formation of HIV-1 endocytic uptake vesicles, actin also forms a barrier to virions fused at the cell surface (Spear et al. 2012). This model is in agreement with HIV-1 entry models proposed by *Permanyer et al.* and *Gobeil et al.* and demonstrates the diverse routes in which HIV-1 can facilitate its cellular entry and how the successful route may be cell type specific (Permanyer et al. 2010; Gobeil et al. 2012).

7.3 Implications and future challenges

To strengthen the model of HIV-1 entry in MDM and confirm that productive HIV-1 uptake is via raft-mediated endocytosis, one could express both a non-raft CCR5 mutant and/or a non-raft CD4 mutant in esMDM. When studying the raft association of proteins using detergent resistance, which operationally define lipid rafts, further studies ought to be performed to confirm that DRM represent lipid rafts (Simons et al. 2000)(Prof. Tony Magee, personal communication). HIV-1 entry in esMDM expressing a non-raft CCR5 mutant and/or a non-raft CD4 mutant could be further studied using the panel of assays as readouts for sequential stages of HIV-1 infection, as used in this thesis, but also include a HIV-1 binding experiment, as described by *Carter et al.* in the (Carter et al. 2009). Moreover, it would be valuable to show the location of HIV-1 fusion over time, which can be achieved using two different methods. The first method, as described by *Miyauchi et al.*, uses the Vpr-BlaM fusion assay to measure HIV-1 fusion after adding membrane-impermeant inhibitors of fusion (to specifically inhibit plasma membrane fusion) or after lowering the temperature (to inhibit both plasma membrane fusion and fusion after endocytosis) at different time-points (Miyauchi et al. 2009). To deduce the location of fusion, the rate of virus escape from both inhibitors can be compared. The second method visualizes the location of fusion using single virus imaging, as developed by *Markosyan et al.* (Markosyan et al. 2005). Viral envelopes are labelled with a fluorescent lipid dye (DiD) and virus content is visualized using fluorescently tagged Gag (Gag-GFP). Irrespective of the location of fusion, fusion will lead to the loss of the virus content marker, due to its virtually infinite dilution in the cytosol. In contrast, the viral envelope marker will only be lost when virions fuse at the plasma membrane, as the marker will dilute within the plasma membrane, but not in the membrane of an endosome, that is not continuous with the plasma membrane (Miyauchi et al. 2009).

To confirm further experimentally the proposed model and investigate the role of clathrin-mediated endocytosis, CD4 mutants that cannot bind to clathrin adaptor complexes could be expressed. Such mutants exclude CD4 from clathrin coated vesicles and abrogate the ability of CD4 to undergo endocytosis (Pitcher et al. 1999). This could be achieved by using the advance lentiviral dual expression vector (as developed in Chapter 3 + 6) to knockdown endogenous CD4 and express chimeric CD4 with a mutations in the dileucine sequence (L413 and L414) and neighbouring serine residues (S408 and S415) in the cytoplasmic tail or express tailless CD4 (Pitcher et al. 1999). One would expect an increase of CD4 surface expression and HIV-1 fusion, but not an increase in HIV-1 productive infection, as observed in LCK+ MDM. Alternatively, the role of clathrin-dependent endocytosis in HIV-1 entry in MDM could be studied by knocking-down components of the clathrin-dependent endocytosis pathway or by expressing a dominant negative inhibitor of Eps15 which interacts with the clathrin adaptor AP2 and inhibits clathrin-mediated endocytosis (Daecke et al. 2005; Kennedy et al. 2009; McMahon et al. 2011). However, as these proteins may be critical or toxic to normal cell function and haematopoiesis, the lentiviral expression system would have to be further developed into an inducible expression system instead of a constitutive expression system.

An inducible lentiviral expression system would also be useful to address CD4 expression levels and fusion kinetics, as it would allow control over expression levels of a GOI, in this case wild-type or mutant CD4 and/or CCR5 (Zhou et al. 2007; Johnston et al. 2009). CD4 surface expression levels are lower on MDM compared to other cell types. This may contribute to the distinct HIV-1 entry pathway in MDM, as low CD4 expression levels favour low fusion kinetics and accumulation of virions at the cell surface, followed by endocytic uptake, as described by *Permyer et al.* (Permyer et al. 2010). In chapter 5, it was shown that HIV-1 infection of MDM expressing high CD4 surface levels is less productive, likely due to high fusion kinetics leading to fusion at the plasma membrane, as opposed to fusion after endocytosis. Interestingly, M-tropic isolates generally evolve to

infect cells with low levels of CD4 and CCR5 (Arrildt et al. 2012). Irrespective of CD4 expression level and location of fusion, the formation of a tri-molecular Env-CD4-CCR5 complex is an essential step in the HIV-1 fusion process (Lapham et al. 1996; Wu et al. 1996; Doms 2000). However, the threshold number and the stoichiometry of Env-CD4-CCR5 complexes necessary for successful HIV-1 entry in MDM are lacking. Co-precipitation studies suggest that CD4 is constitutively associated with CCR5 in MDM (Lapham et al. 1999). It would also be valuable to study CD4 receptor clustering in the HIV-1 entry pathway of MDM, for example using FRET microscopy studies. In other cell types, a sufficient number of CD4 and co-receptor molecules cluster in rafts upon HIV-1 binding to achieve timely fusion (Iyengar et al. 1998; Kuhmann et al. 2000; Manes et al. 2000; Viard et al. 2002; Jimenez-Baranda et al. 2007). It is currently unclear whether receptor clustering occurs on the surface of MDM and in HIV-1 uptake vesicles in MDM and how this affects entry. In addition to receptor clustering, signaling and actin rearrangements upon HIV-1 entry in MDM require further investigation. CD4 and co-receptor clustering in T-cell lines is dependent on Rho/filamin A-mediated actin rearrangements and has been shown to occur in rafts and also may involve clustering of rafts (Manes et al. 2000; Popik et al. 2002; Jimenez-Baranda et al. 2007). Rho, Cdc42 and Rac, are members of the Rho-family GTPases, which are involved in the CCR5 signaling pathway, which can induce changes in the actin cytoskeleton (Rossi et al. 2000; Vicente-Manzanares et al. 2002; Eitzen 2003; Pontow et al. 2004). The Rho-family GTPases can bind filamin A, an actin filament-crosslinking protein that connects the membrane, as well as proteins, to actin (Ohta et al. 1999). Drug studies in MDM suggest that RhoA/filamin A-mediated actin-dependent clustering of HIV-1 receptors have no effect on HIV-1 entry in MDM although a dynamic actin cytoskeleton is required for successful HIV-1 entry in MDM, considering actin-disrupting drugs decrease HIV-1 infection in MDM, but do not affect CD4 and CCR5 surface expression levels (Carter et al. 2011). Interestingly however, the Rac GTPase or its effector kinase Pak1, are required for

successful HIV-1 entry in MDM and as such could be involved in clustering of receptors in MDM (Carter et al. 2011). However, Rac-induced actin rearrangements may also be important for HIV-1 fusion, as demonstrated in cell lines (Pontow et al. 2004; Harmon et al. 2008). It would be interesting to explore the role of signaling molecules such as the Rho GTPases in MDM HIV-1 entry. This can be achieved employing a genetic approach as described in Chapter 3, using mutant or dominant negative versions of signaling proteins, as is currently being undertaken in the James lab. Furthermore, to investigate the role of Filamin-A and CD4 in receptor clustering and HIV-1 uptake/fusion in MDM, one could use the lentiviral expression system described in Chapter 4+6, to express a truncated/mutated CD4 molecule as developed by *Jimenez-Baranda et al.*, that cannot interact with Filamin-A (Jimenez-Baranda et al. 2007). However, if the Env-CD4-CCR5 complex does initiate a signaling pathway, it may have effects on multiple aspects of the entry pathway (e.g. receptor-clustering, endosome formation, actin cortex rearrangements, and fusion) and it would be a challenge to differentiate between these.

7.4 Therapeutic implications

The lentiviral system used in this thesis to knockdown CD4 can also be extended to other applications, such as myeloid-specific knockdown and gene therapy, as well as patient-specific research or the identification of other host proteins involved in HIV-1 infection. Firstly, if CD4 is found not to have a physiological role in myeloid cells other than as a receptor for HIV-1, then there is a potential therapeutic opportunity to knockdown CD4 specifically in the myeloid lineage to render these cells HIV-resistant in patients. The lentiviral vector can in principle be modified to use a myeloid specific promoter to knockdown CD4 in MDM, without affecting CD4 expression in other cells, such as T-cells, which would offer unique potential for stem cell-based anti-HIV myeloid cell-targeted gene therapies, to protect MDM from infection. This would reduce viral spread and progression to AIDS and enable MDM to maintain immune system homeostasis (Verani et al. 2005;

Martinez et al. 2008; Kuroda 2010). Myeloid-specificity is important, as unlike in MDM where CD4 has no known functions, CD4 has critical biological functions in T-cells. The U6 promoter (used to express a hairpin in the lentiviral system in this thesis), is not a myeloid specific promoter and is ubiquitously expressed, like most Pol III promoters, although cell-specific Pol III promoters do exist (Sandy et al. 2005; Oler et al. 2010). To achieve myeloid-specific expression, one could use an RNA-miR, instead of shRNA, driven by a myeloid specific Pol II promoter such as MRP8 or miR223 (Cullere et al. 2008). The miR223 promoter has been shown to allow transgene expression in esMDM without antibiotic selection and as such would be a suitable promoter for myeloid-specific gene therapy, although not 100% of cells express the GOI is achieved (Hänseler et al, *in progress*). Knockdown of CD4 in myeloid cells could be used in combination with other therapeutic strategies to target or prevent HIV-1 infection in a range of cell types, although further studies would be required to achieve myeloid-specific shCD4 expression; to further investigate the functional consequences of knocking down CD4; and to address *in vivo* safety as well as efficacy. The concept of using lentivirus-based RNAi in HIV-1 gene therapy has already been explored *in vivo* (MacGregor 2001; Humeau et al. 2004; Kumar et al. 2008; DiGiusto et al. 2010). For example, in a phase I trial, a lentiviral encoded anti-HIV-1 shRNA was used to treat HIV-infected patients suffering from AIDS lymphoma (DiGiusto et al. 2010). Secondly, the lentiviral vector system and stem cell technology can be extended to investigate MDM biology and host-pathogen interactions against known patient and control genetic backgrounds. This could be achieved using hiPSC obtained from patients to obtain genetically modified hiPSC-derived MDM. This is regularly achieved in our laboratory and produces hiPSC-derived MDM sharing similar functional features with esMDM and bMDM (Jiang et al. 2012). Thirdly, using RNAi to characterize the cellular pathways and signaling components that allow successful HIV-1 entry into primary cells, could identify new potential targets for novel antiviral agents

7.5 Conclusion

This thesis showed that hESC technology can be used to produce large numbers of genetically modifiable, functional macrophages to study HIV-1 infection in macrophages. This technology was successfully used to manipulate sub-cellular CD4 distribution in macrophages and, using a panel HIV-1 assays, allowed dissection of the HIV-1 entry pathway. Findings in this thesis indicate that CD4 DRM-localization enhances the productive entry of HIV-1 entry into macrophages. The findings further suggest that the productive entry pathway of HIV-1 in macrophages occurs via fusion after a raft-dependent endocytic uptake pathway.

Appendix I: Further modifications of esMDM differentiation protocol

Defined, serum- and feeder-free versions of esMDM differentiation protocol

In order to minimize variability for the differentiation of esMDM, a serum- and feeder-free version of the improved protocol was developed, as described below and in Figure AI.1A. This protocol also has the option to avoid the use of undefined tissue culture products, making it a fully chemically defined, serum- and feeder-free protocol. The defined steps are highlighted in blue in Figure AI.1A.

In the original protocols, hESC were cultured on a layer of mouse feeder cells. Adhesion to extracellular matrix and soluble factors produced by feeder cells are important for cell survival, growth and self-renewal *in vitro* (Thomson et al. 1998; Xu et al. 2001). However, hESC can also be grown successfully using feeder-free systems, such as on MatrigelTM coated tissue culture plates or on SynthemaxTM plates. Nonetheless, the MatrigelTM culture system is not free of animal products, nor is it defined, considering MatrigelTM is a gelatinous protein mixture obtained from mouse tumor cells and may be variable between batches (Hughes et al. 2010). In contrast, SynthemaxTM plates are feeder-free and defined, as the plates are coated with synthetic extracellular matrix-derived cell adhesion-promoting peptides (Baranov et al. 2012). In order to achieve a serum- and feeder-free protocol, hESC were grown on MatrigelTM coated tissue culture plates or on SynthemaxTM plates and cultured in the defined, serum-free mTeSRTM-1 medium.

EB formation was also further defined. In the original protocol, mechanical dissociation was used to generate EBs of varied size, whereas the defined protocol used enzymatic dissociation and centrifugal forced-aggregation to seed a pre-defined number of hESC into a round-bottomed Low-Attachment 96-well, or an AggreWellTM to create uniform EBs of a defined size and shape. Centrifugation allows hESC to concentrate at the bottom of the well, promoting cell-cell contact and cell aggregation (Kurosawa 2007). Such spin-EBs

are reproducible and easy to use (Ungrin et al. 2008). Spin-EBs are morphologically organized and symmetrical, ensuring maximal control over the micro-environment and reflects the tissue-level organization of *in vivo* development (Ungrin et al. 2008). The use of 96-well plates allowed the isolation of a single EB per well (i.e. a useful format for medium-scale screening assays), whereas AggreWell™ plates allowed large scale production of EBs, generating 300 EB per AggreWell™. In order to induce haematopoiesis, soluble growth factors and cytokines may be required during EB formation, depending on the type of differentiation scheme that is employed (Tian et al. 2004). The defined EBs were formed in mTeSR™-1 medium and required the addition of three growth factors: bone morphogenetic protein 4 (BMP-4), vascular endothelial growth factor (VEGF) and stem cell factor (SCF). mTeSR™-1 medium is defined, but not xeno-free (free of components that are not from human origin). Alternatively, TeSR™2 can be used during EB formation, which is a more recently developed xeno-free version of mTeSR™1. Next, to induce monocytopoiesis, EBs were plated in the defined medium, X-VIVO™ 15, and supplemented with IL-3 and M-CSF. This step was similar across all versions of the improved protocol. EBs adhered and produced esMC that could be harvested routinely from the supernatant of the differentiation cultures. To allow the differentiation into esMDM, harvested esMC could be cultured in RPMI supplemented with serum, or, in order to follow defined, serum-free differentiation at all stages of the process, in X-VIVO™ 15. In both cases, the culture medium was supplement with M-CSF, for 5-7 days.

Figure AI.1

Figure AI.1. Defined, serum- and feeder-free versions of esMDM differentiation protocol.

The optimised esMDM differentiation method can also be performed in a serum- and feeder-free, as well as, defined (highlighted in blue) manner. A) Diagram, using light microscopy images of hESC (10x), EB (2x), differentiating EBs (20x) and MDM (10x), outlining the protocols. In short, hESC can be cultured serum- and feeder-free (hESC 'MatrigelTM') or serum- and feeder-free and defined (hESC 'SynthemaxTM'). Defined EB formation can be achieved by spinning, using a 96-well plate to isolate a single EB (image taken 21 days after spinning) or AggreWellTM (image taken 1 day after spinning). Spin EBs require the addition of three growth factors: BMP-4, VEGF and SCF (+++). Myeloid differentiation is achieved using defined products as described in Chapter 3.1. esMDM can be produced serum-free and defined by culturing esMC in X-VIVOTM15 + M-CSF.

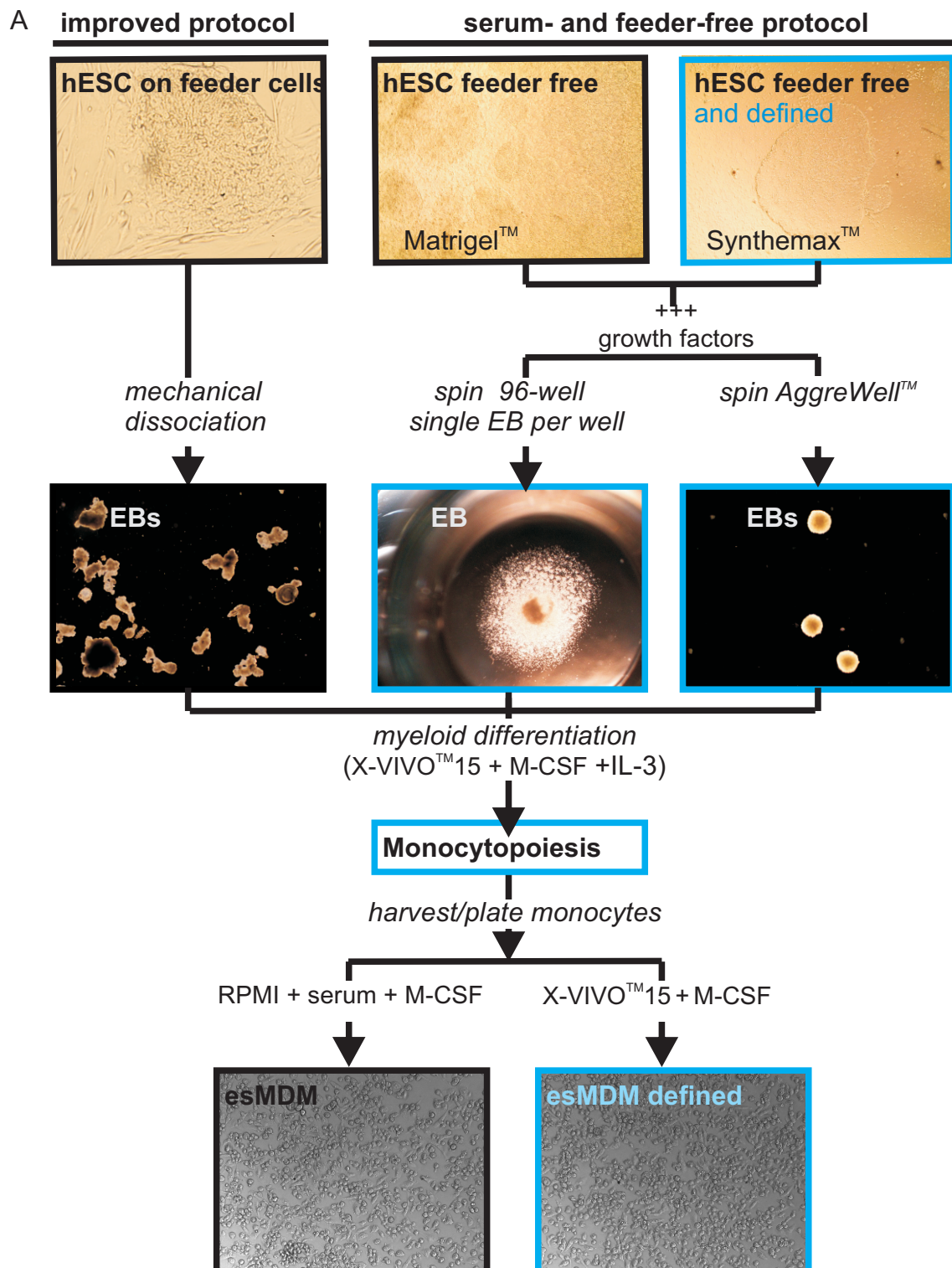


Figure AI.1

Figure AI.1. (continued) Defined, serum- and feeder-free versions of esMDM differentiation protocol.

B+C) To test the requirement of growth factors during EB formation using the defined and feeder free protocol, hESC were grown on Matrigel™ and transferred to a 96-well plate to generate EBs by spinning without growth factors (-) or with the four growth factors BMP-4, VEGF, SCF and GM-CSF (++++). esMC were produced according to protocol 'X'. D+E) To test the requirement of GM-CSF, hESC were grown on Matrigel™ and transferred to an AggreWell™ with three (+++) or four (++++). esMC were produced according to protocol X. esMC-X were counted and viability was assessed using a cell counter (Chemometec). The media were replaced for repeated esMC-X harvests over a period of 7-10 weeks. B+D) Data represent the cumulative number of viable esMC-X from 6-wells per condition. C+E) Viability of esMC-X.(n=5, C; n=4, E).

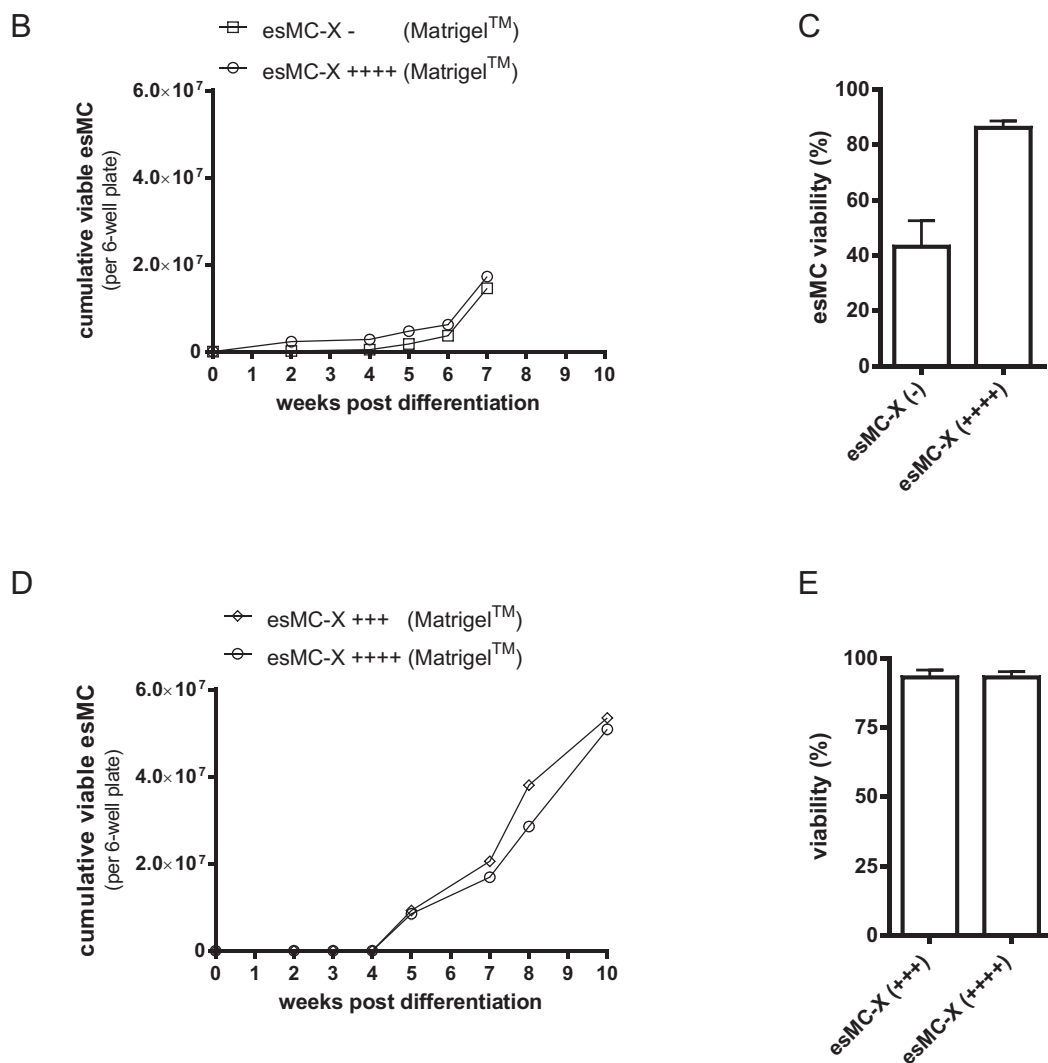
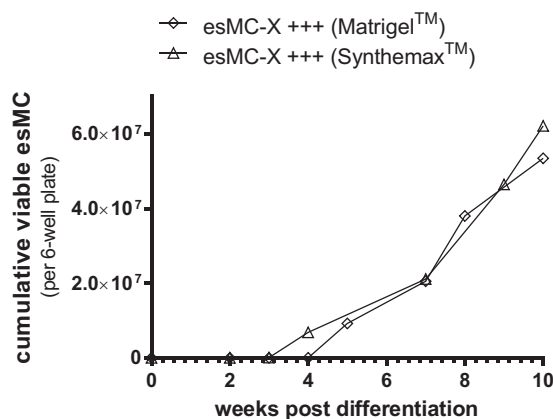


Figure AI.1

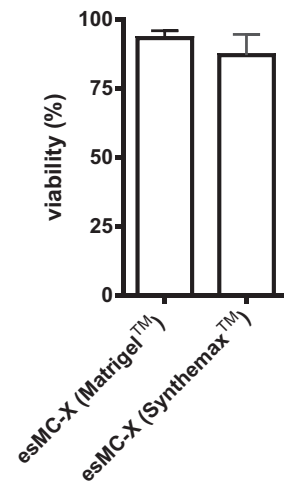
Figure AI.1. (continued) Defined, serum- and feeder-free versions of esMDM differentiation protocol.

F) Non-adherent esMC-X were harvested from the supernatant of cultures that had been derived according to the defined, feeder free protocol using hESC grown on Matrigel™ or Synthemax™ and EBs were generated using AggreWell™ (+ BMP-4 + VEGF + SCF). esMC-X were counted using a cell counter (Chemometec). The media were replaced for repeated esMC harvests over a period of 10 weeks. Data represent the cumulative number of viable esMC from 6 wells per condition. G) Viability of esMC-X (Matrigel™, n=4; Synthemax™, n=3). H) esMC-X (Matrigel™ and Synthemax™) were further differentiated into esMDM and surface expression of CD14, CD16, CD163 and CD86 were measured by flow cytometry. Histograms represent surface staining (black line) compared to isotype control (shaded gray).

F



G



H

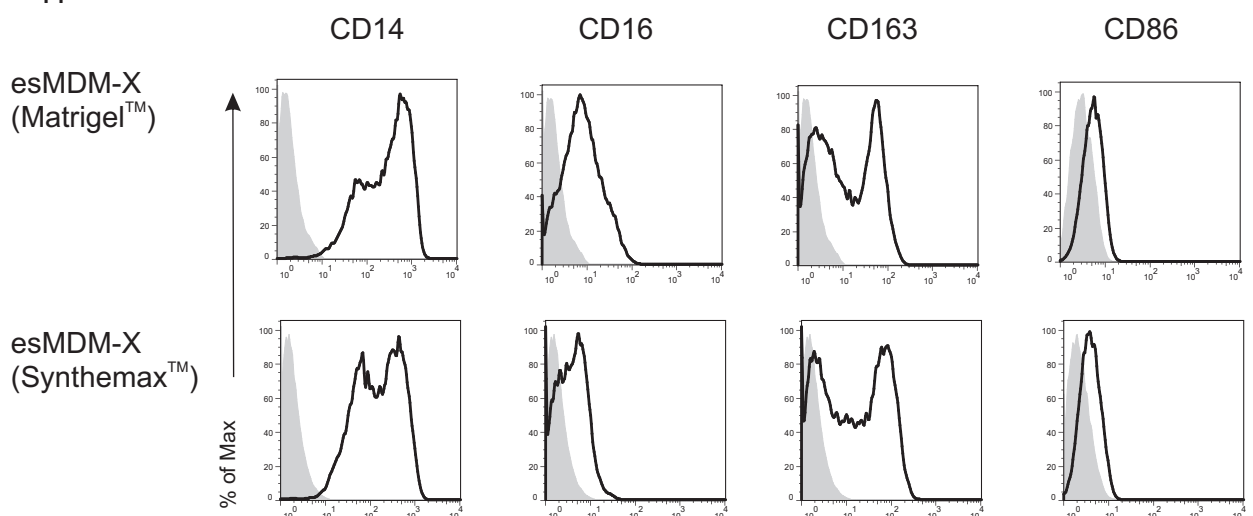
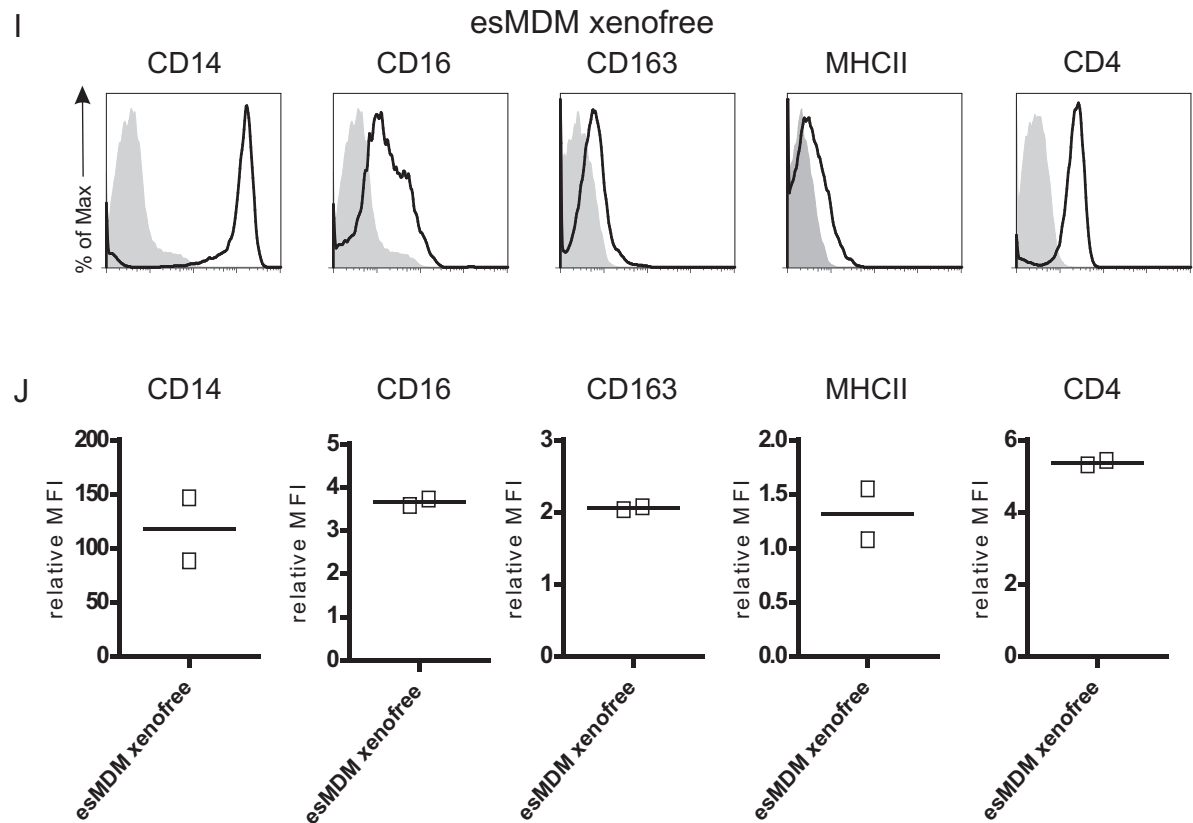


Figure AI.1

Figure AI.1. (continued) Defined, serum- and feeder-free versions of esMDM differentiation protocol.

I+J) esMDM were generated xenofree by culturing esMC-X (derived using hESC grown on Matrigel™ coated plates and spin-EB's on AggreWell™) for 7 days in X-VIVO™15 supplemented with M-CSF. Surface expression of CD14, CD16, CD163, MHCII and CD4 were measured by flow cytometry. Histograms represent surface staining (black line) compared to isotype control (shaded gray). J) The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control of independent experiments.



To test the requirement of growth factors during EB formation using the serum- and feeder-free protocol, hESC were grown on Matrigel™ and transferred to a 96-well plate to generate EBs by spinning without growth factors (-) or with the four growth factors, BMP-4, VEGF, SCF and GM-CSF (++++). esMC were produced according to the improved protocol X. BMP-4, VEGF, SCF and GM-CSF were selected as they have been used in several published protocols during EB formation for the derivation of haematopoietic cells (Slukvin et al. 2006; Tseng et al. 2009; Xu et al. 2012). BMP-4 is a critical signaling molecule required for mesoderm commitment during early embryogenesis and strongly promotes hematopoietic differentiation (Chadwick et al. 2003; Goldman et al. 2009). VEGF is a growth factor involved in regulating haematopoiesis, as well as angiogenesis, although likely through separate mechanisms (Gerber et al. 2003). VEGF may control hematopoietic stem cell survival and repopulation (Gerber et al. 2003). SCF is a key hematopoietic cytokine and may influence the migration of hematopoietic cells (Broudy 1997). GM-CSF is a major regulator of myeloid lineage populations. esMC, produced from EBs, formed with or without growth factors, were harvested over a period of 7 weeks. esMC were counted and viability was assessed (Figure AI.1B+C). esMC derived from EBs grown without growth factors were less viable (mean $\pm$ SEM: 43.2 ± 9.42) compared to esMC derived from EBs grown with the four growth factors (mean viability $\pm$ SEM: 86.2 ± 2.5). This shows that the addition of haematopoietic growth factors during spin-EB formation is required for the production of viable, defined esMC. Unlike the three growth factors SCF, VEGF and BMP-4, which regulate early human haematopoiesis, the fourth growth factor, GM-CSF, is a later acting hematopoietic growth factor (Metcalf 1985; Donahue et al. 1988; Xu et al. 2012). As such, the requirement of GM-CSF during EB formation may be redundant and therefore was tested. hESC were grown on Matrigel™ and transferred to an AggreWell™ with three growth factors (+++) or with four growth factors (++++). esMC were produced according to the improved protocol X. esMC yields and viability were similar, irrespective of the presence of GM-CSF during EB formation

(Figure AI.1D+E). This shows that the presence of GM-CSF during spin-EB formation is redundant. The addition of growth factors were not required, nor enhanced monocyte production or viability, when forming EBs using mechanical dissociation in the haematopoietic differentiation protocol (Figure AI.1 and data not shown). Unlike spin-EBs, mechanically dissociated EBs, presumably, spontaneously differentiate sufficiently well to mesoderm and hematopoietic progenitors.

Next, it was tested whether esMC produced in a defined manner, using either hESC cultured on MatrigelTM coated plates or on SynthemaxTM plates, could differentiate into esMDM. EBs were formed using the spin-EB method (using AggreWellsTM) and monocytopoiesis was induced according to the improved protocol X. Using either the serum- and feeder-free (MatrigelTM) or defined, serum- and feeder-free (SynthemaxTM) differentiation method, $\sim 1 \times 10^7$ esMC were collected after 5 weeks of monocytopoiesis (Figure AI.1F). High levels of esMC production continued for the duration of the experiment similar to that of esMC derived from hESC which were grown on feeder cells (Figure 3.3). The (defined) serum- and feeder-free esMC were viable (>87 %, Figure AI.G). To confirm that these esMC could further differentiate into esMDM, esMC were differentiated according to the protocol presented in chapter 3, in serum-containing RPMI, supplemented with M-CSF. Expression of surface markers on esMDM was assessed. CD14, CD16, CD163 and CD86 were expressed, although CD14 was present at variable, yet high levels (Figure AI.1H).

To define the last stage of the protocol, avoiding the use of serum-containing media, serum- and feeder-free esMC (derived using hESC grown on MatrigelTM coated plates and spin-EBs on AggreWellTM) were grown for 7 days in X-VIVOTM 15, instead of RPMI + serum, and supplemented with M-CSF. esMC adhered similar to bMC and expressed CD14, CD16, CD163. MHCII and CD4 (Figure AI.1 I+J). All esMDM expressed CD163, suggesting that esMDM were phenotypically more similar to M-type MDM.

Taking these results together, the improved protocol for the differentiation of MDM from hESC, not only increases esMC yield and production kinetics, but also minimizes variability by further defining the differentiation process, avoiding the use of feeder-cells, animal-derived, and serum-containing products. The protocol could easily be further adjusted to a xeno-free protocol by using TeSR™-2 medium instead of mTeSR™-1 medium, which would be useful if wanting to generate 'good manufacturing practice'-grade myeloid cells for cell therapy.

esDC can be differentiated from same myeloid precursor as esMDM

Monocyte-derived DC and MDM share a common myeloid precursor and exposing blood-derived monocytes to GM-CSF and IL-4 induces the differentiation of MC into immature DC (imDC) (Sallusto et al. 1994; Fogg et al. 2006; Lehtonen et al. 2007; Geissmann et al. 2008; Auffray et al. 2009; Geissmann et al. 2010). imDC can be matured with various stimuli, for example with GM-CSF, IFN- γ , IL1- β , TNF- α , and PGE2, to generate mature bDC (Lehtonen et al. 2007; Tseng et al. 2009). Similar to MDM, DC are specialized antigen-presenting cells. imDC can be highly phagocytic, whereas mDC, have increased cytokine production (Banchereau et al. 1998; Mellman et al. 2001).

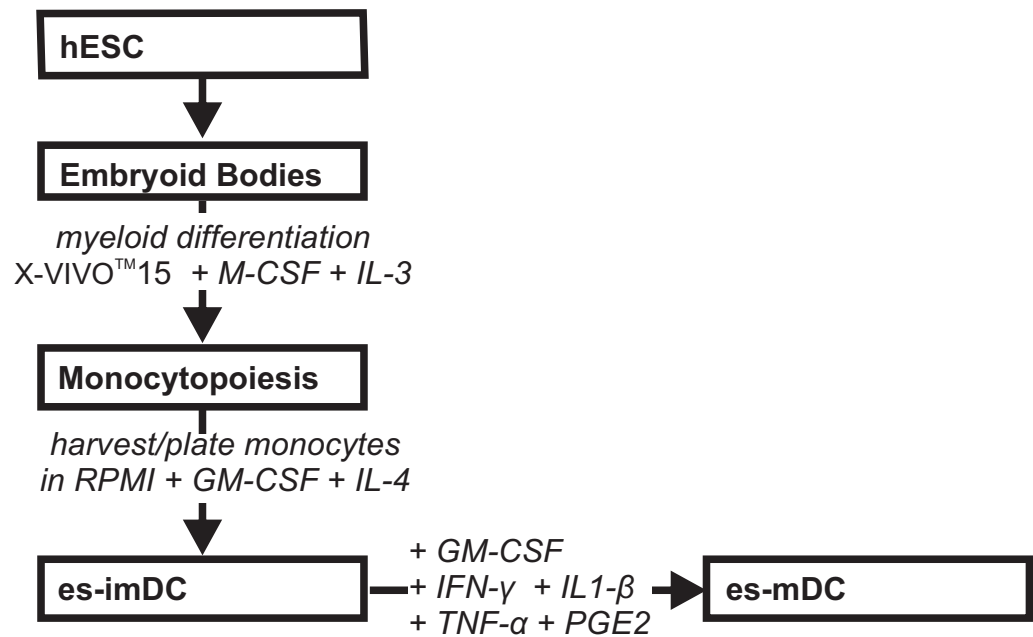
Unlike bMC, esMC differentiated according to the original protocol containing serum (protocol v1), are impaired from differentiating into DC (Dr. Karlsson, thesis 2008). It was tested whether esMC generated using the improved protocol X could differentiate into hESC-derived imDC (es-imDC), as outlined in Figure AI.2A and described next. esMC were supplemented with GM-CSF and IL-4, instead of M-CSF, for four days. To further induce the differentiation of es-imDC into hESC-derived mDC (es-mDC), es-imDC were cultured for an additional 3 days with a maturation cocktail (GM-CSF, IFN- γ , IL1- β , TNF- α , and PGE2).

Figure AI.2

Figure AI.2. esDC can be differentiated from same myeloid precursor as esMDM.

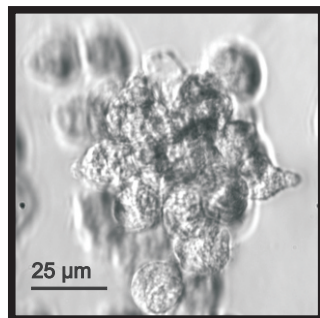
A) Schematic diagram illustrating the modification of the improved protocol 'X' to derive immature dendritic cells (es-imDC) and mature dendritic cells (es-mDC) from the esMC-X precursor cells. B) Light microscopy images of esDC and blood derived dendritic cells (bDC), 40x magnification.

A

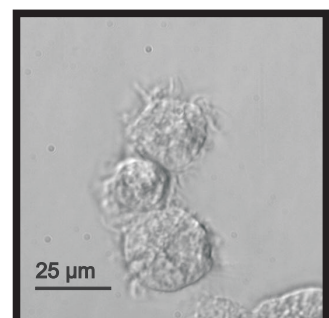


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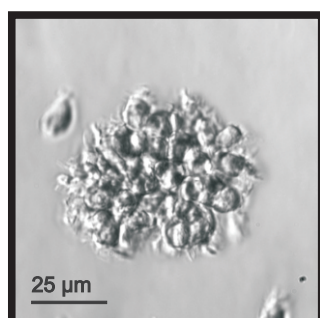
es-imDC



es-mDC



b-imDC



b-mDC

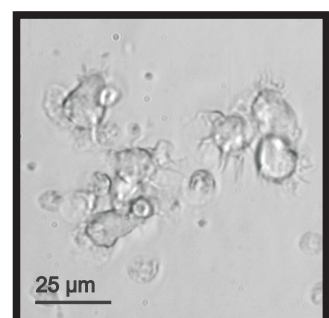
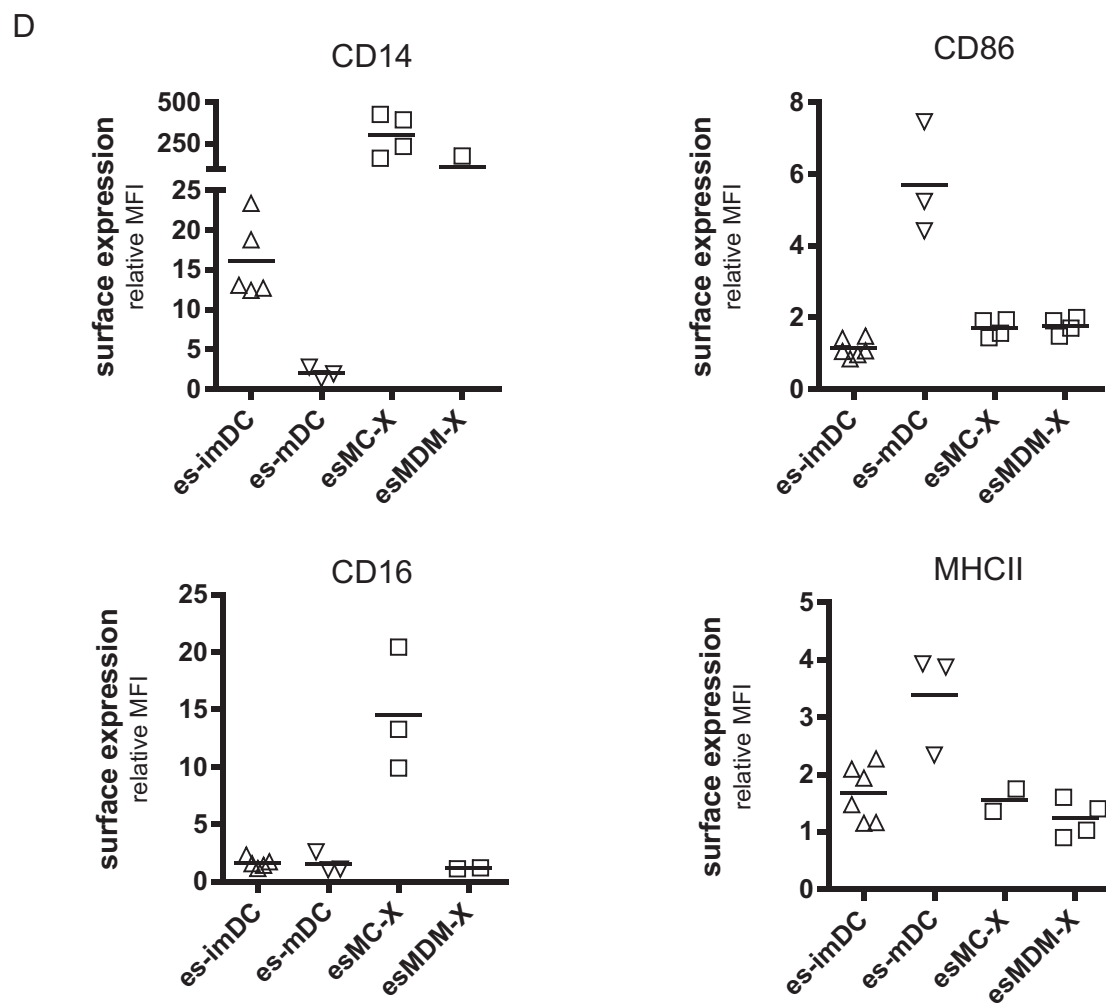
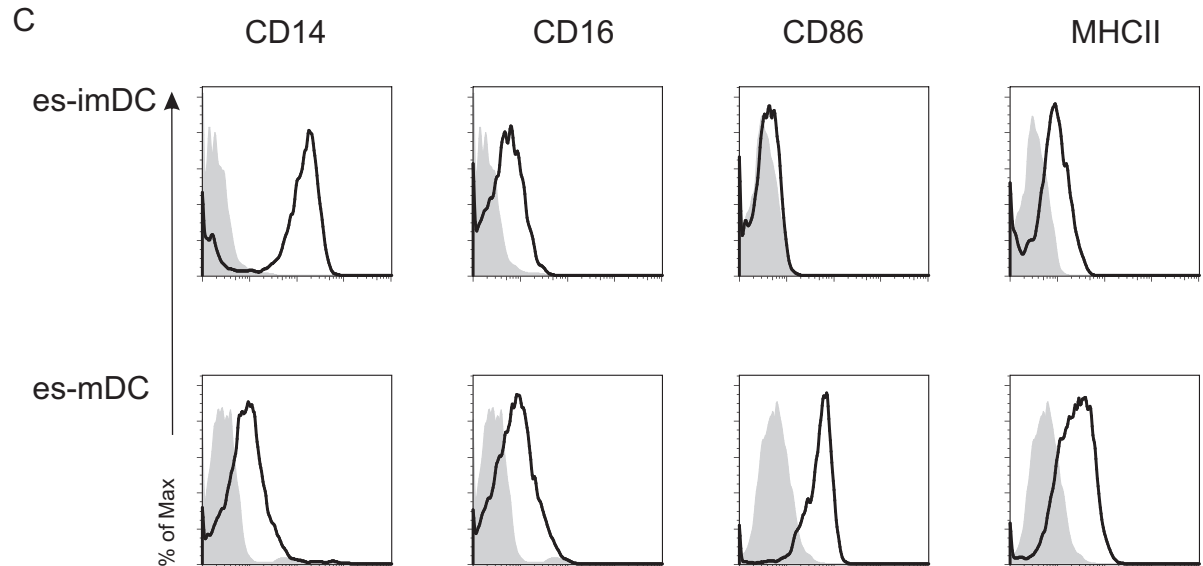


Figure AI.2

Figure AI.2 (continued) esDC can be differentiated from same myeloid precursor as esMDM.

C) Surface expression of CD14, CD16, CD86 and MHCII was measured by flow cytometry. Histograms represent surface staining (black line) compared to isotype control (shaded gray). D) The symbols reflect the relative ratio of the geometric mean fluorescence intensity (MFI) over the isotype control of independent experiments.



Es-imDC (Figure AI.2B, top left) were homogenous, round, non-adherent cells, forming tight clusters, similar to blood-derived imDC's (b-imDC, Figure AI.2B, bottom left), but distinct from adherent bMDM (Figure 3.5A). Es-imDC were morphologically similar to imDC described by other researchers (Pickl et al. 1996; Tseng et al. 2009; Silk et al. 2011). Es-mDC (Figure AI.2B, top right) formed fine cytoplasmic protrusions, typical of mDC development, and similar to blood-derived mDC (b-mDC, Figure AI.2B, bottom right) and similar to imDC's as published by other researchers (Dauer et al. 2003; Lehtonen et al. 2007; Tseng et al. 2009). The phenotype of the es-imDC and es-mDC were analysed. As expected, CD14 was down-regulated upon and CD86 was up-regulated es-mDC maturation (Figure AI.2C) (Tseng et al. 2009; Mohib et al. 2012). This receptor profile is distinct from the esMC and esMDM (Figure AI.2D). Furthermore, MHCII was expressed at higher levels, and CD16 at lower levels, on es-DC compared to esMC.

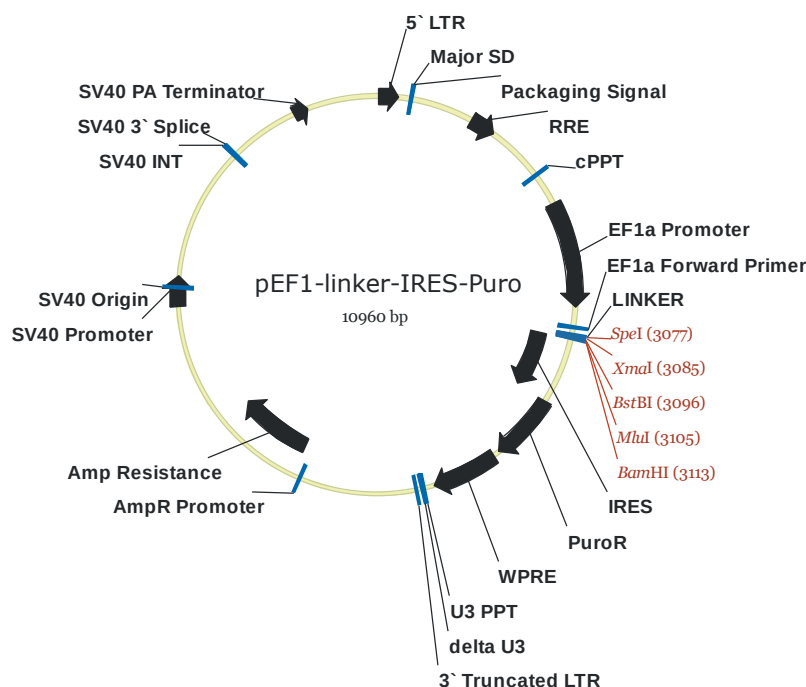
Although functional characterization was not conducted in this study, morphological and phenotypical analyses suggest that esMC differentiated according to the improved protocol X have the potential to differentiate into DC. Moreover, this protocol for DC differentiation is easy to use and less complex than many other published protocols. The ability of esMC to differentiate into DC widens the application of the protocol and also offers a model to directly study DC functions, compared to MDM.

Appendix II: Construction of pEF1 α -GOI-IRES-Puromycin^R/Neomycin^R lentiviral cloning vectors

Two second-generation, lentiviral SIN vectors (pGIN: pEF1 α -GFP-IRES-Neomycin^R and GIP: pEF1 α -GFP-IRES-Puromycin^R), were modified to insert a linker instead of GFP, driven by the EF1 α promoter, so that other Genes Of Interest (GOI) could easily be inserted into the transfer plasmids. pGIN and pGIP were obtained from Prof. William James' lab and are based on the SIN lentiviral vector design of *Zufferey et al.* (pHR'SIN plasmid) (Zufferey et al. 1998). The plasmids were restricted with SpeI and BamHI-HF, in order to insert a linker instead of the GFP. The linker contains the following restriction sites: SpeI—XmaI—BstBI—MluI—BamHI. The sequence 'GCTA' was added at the start, to make a stable 5' end, and added at the end and in between the restriction sites, to separate them. The sense sequence of the linker: 5→3:GCTAACTAGTTACCCGGGGCTATT CGAAGCTAACGCGTTAGGATCCGCTA

The sense and anti-sense oligonucleotides were produced by Sigma and 3 μ L of each oligonucleotide (20pmol/ μ L) and 6 μ L of water were heated at 50°C for 5min to disrupt internal annealing within each oligonucleotide and then gradually cooled to anneal each oligonucleotide, by turning off the waterbath. After several hours the annealed linker was stored at 4°C. Next, 12 μ L linker, 3 μ g pGIN or pGIP were digested with 1 μ L SpeI, 1 μ L BamHI-HF, 4 μ L Buffer 4 (New England Biolabs) in a 40 μ L reaction for 2h at 37°C and run on a 1% Agarose gel, cut out. The linearized pGIN and pGIP were purified and eluted into 30 μ L (QIAquick Gel Extraction Kit) and the linker was purified and eluted into 100 μ L (QIAquick Nucleotide Removal Kit). 5 μ L of the linker were ligated into 3 μ L of the linearized pGIP or pGIN using 2 μ L T4 ligase and 10 μ L 2x rapid buffer (10min RT). Midiprep quality plasmid preparations were made and the correct sequences of plasmids were confirmed. The plasmid map can be found on the CD-ROM under the following file path: 'H:\WSJLAB\BONNIE\Cloning\Thesis CD-ROM\LCK SEQUENCE AND CONSTRUCT INFO\plasmdis' The resulting plasmids were named pEF1-linker-IRES-Puromycin^R (or pEF1-GOI-IRES-

Puromycin^R or “control P”). and pEF1-linker-IRES-Neomycin^R (or pEF1-GOI-IRES-Neomycin^R). Abbreviation for IRES (I), Puromycin^R (P^R or Puro^R) and Neomycin (N^R or Neo^R) may be used. The plasmid map of pEF1-linker-IRES-Puromycin^R is shown below.



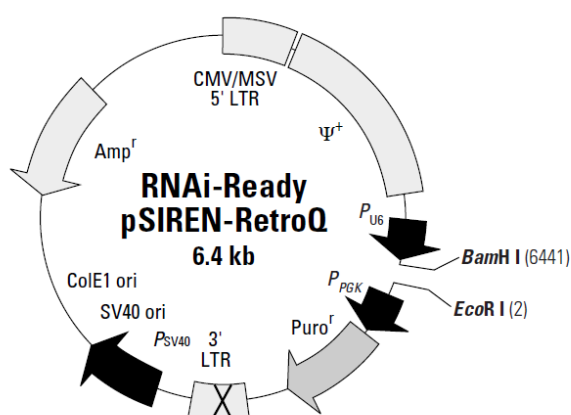
Cloning RFP into pEF1α-GOI-IRES-Puromycin^R

DNA sequence were obtained from the plasmid pEF1α-RFP-IRES-Neomycin^R (previously made in the laboratory, by cloning RFP from the pCMV_{minimal}-SIN-RFP:shRNA-UBC-rtTA3-I-P-WPRE, V2THS_37234, Open Biosystems into the above described pEF1-GOI-IRES-Neomycin^R). pEF1α-RFP-IRES-Neomycin^R was digested with BamHI and SpeI and cloned into the pEF1-linker-IRES-Puromycin^R, also digested with BamHI and SpeI. The digested vectors were separated on an agarose gel (QIAquick Gel Extraction Kit) and the RFP sequence and the digested pEF1-linker-IRES-Puromycin^R backbone, were extracted from the gel (QIAquick Gel Extraction Kit). The pEF1-linker-IRES-Puromycin^R backbone and RFP fragments were ligated and transformed into competent bacteria. Plasmid preparations were made and successful cloning was confirmed with an analytical restriction digest.

The selected target regions are not within the 5' and 3' untranslated regions (UTRs) nor regions near the start codon, in order for the RISC complex to bind without being hampered by regulatory and UTR-binding proteins; or translation initiation complexes. The selected sequences have a GC content of approximately 45%. Moreover, the sequences have at least 3 A or T residues in positions 15–19 of the sense sequence, which is associated with increased knockdown activity. (Anti-)sense sequences that had 3 or more T residues next to each other were avoided, as this can potentially cause premature termination the shRNA transcript. The 19-base oligonucleotides were selected to have little secondary structure, which otherwise would interfere with annealing. The selected sequences are specific for CD4 and show no significant homology to other genes. Target sequence #1, was based on a pre-existing miR-shRNA target sequence from Open Biosystems, that had been previously validated in cell lines (Cowley SA 2012) and was optimized for shRNA design. Target sequence #2 was based on a siRNA used by *Novina et al.* and was optimized for shRNA design (Novina et al. 2002). Target sequences #3 was identical to the siRNA sequence used by *García-Expósito et al.*, and compatible with an optimal shRNA design (García-Expósito et al. 2011). The control shRNA target sequence was adapted from *Kamiya et al.* (Kamiya et al. 2011). The designed oligonucleotides (bottom and top strands) were produced by Sigma and were annealed by taking 15µL of each (100µM, final concentration of double stranded oligonucleotides would become 50µM), and heating as follows: 95 °C (30 sec to disrupt internal hairpin of each oligonucleotide), and gradually cooled as follows to anneal the bottom and top strand: 72 °C (2 min), 37 °C (2 min), 25 °C (2 min), and was stored at -20 °C. The double stranded oligonucleotide was ligated into the linearized (BamHI and EcoRI restriction) RNAi-Ready pSIREN (#631526, Clontech, plasmid map shown below), using 10x rapid ligase buffer and quick T4 DNA ligase (promega) for 10min at RT, according to manufacturer's protocol.

RNAi-Ready pSIREN-RetroQ Vector Information

PT3737-5
Catalog No. 631526



Plasmid preparations were made and successful cloning was confirmed with an analytical restriction using XhoI. The sequences of plasmids containing the insert were confirmed using the following primers (Geneservice order 404460401):

Name	Sequence (5'→3')
U6_seqF	ACGGTAAGCATATGATAGTC
MSCVrev	CAGCGGGGCTGCTAAAGCGCATGC

The raw sequence data files and more information on pSIREN-RetroQ can be found on the CD-ROM under the following file path: 'shRNA SEQUENCE AND CONSTRUCT INFO\LV SIREN U6-shRNA'. Lentiviruses were produced as described in material and method and used to transduce TZM-bl cells to test the ability of the shRNAs to knockdown CD4 (data shown in Chapter 3).

Cloning the U6 shRNA sequences into a constitutive, Puromycin resistant vector to create pU6-shRNA-EF1-linker-IRES-Puromycin

The sequence of the U6 promoter and shCD4 #3 (which gave the best knockdown), as well as the U6 promoter and shCNTRL, were amplified and inserted into a plasmid encoding, pEF1-linker-IRES-Puromycin^R (see Appendix I for details about this vector) in front of the EF1 α promoter using the EcoRI restriction site. This plasmid was chosen, as a similar approach had previously been shown to allow successful transgene expression in esMDM (Cowley SA 2012) and would allow insertion of other genes at the linker site, to achieve simultaneous knockdown (under the control of the U6 promoter) and expression of another transgene (under the control of the EF1 α promoter). An EcoRI site (AATTC) was already present at the 3' of the shRNA sequence in the pSIREN vector, but another EcoRI site had to be inserted at the 5' of the U6 promoter and primers were designed accordingly (see below). The forward primer (U6f EcoRI) contains 4 base pairs (GCTA) to create space for the enzyme to be able to sufficiently bind and cut.

Name	Sequence (5'→3')
EcoRI_U6_F	GCTAGAATTCGGGCAGGAAGAGGGC
shRNA_R	CCTACCCGGTAGAATTCCTCGAG

The PCR was performed using Phusion DNA polymerase (Finnzymes/Biolabs). In a 20 μ L reaction 2 μ L of template (midiprep quality); 2x Phusion High-Fidelity mastermix; 0.5 μ M forward primer F1 and 0.5 μ M reverse primer were added. The PCR programme used was as follows: 98 °C (30 sec), followed by 35 cycles of 98 °C (10 sec), 62 °C (15 sec), 72 °C (10 sec), and a final step of 72 °C (10 min). The amplified products were purified (QIAquick PCR Purification Kit) and eluted in 30 μ L. The purified, amplified fragments and pEF1-linker-IRES-Puromycin^R (5 μ g) were digested with EcoRI (2 μ L) in a 50 μ L reaction and run on a 1% Agarose gel, cut out, extracted, purified and eluted into 30 μ L (QIAquick

Gel Extraction Kit). The purified, linearized pEF1-linker-IRES-Puromycin^R (8µL or ~1µg) was treated with 1µL Alkaline Phosphatase and 1µL buffer (FastAP™, Fermentas) at 37 °C (10 min) and 75 °C (5 min) to prevent re-ligation. Next, the amplified sequences (5 µL) were ligated into the linearized pEF1-linker-IRES-Puromycin^R (3 µL) and the ligation products were transformed into competent bacteria and several plasmid preparations were made. Plasmid preparations were screened for the correct direction of the inserts, by performing an analytical restriction (using 0.5µL XhoI (which cuts next to the shRNA as well as in the backbone at position 3178), 2µL buffer, 16.5µL water and 1µL of plasmid preparations (miniprep quality) and running the products on a gel. The correct sequences of plasmids containing the inserts in the correct direction were confirmed using the following primers (Geneservice order 404991801 and 405044301):

Name	Sequence (5'→3')
U6_seqF	ACGGTAAGCATATGATAGTC
U6_seqR	GACTATCATATGCTTACCGT
EF1a_seq_F	TCAAGCCTCAGACAGTGGTTC
IRES_seq_R	CCTCACATTGCCAAAAGACG

The raw sequence data files and analysis of sequences can be found on the CD-ROM under the following file path: 'shRNA SEQUENCE AND CONSTRUCT INFO\LV U6-shRNA-EF1-linker-IRES-PURO'. The U6-shRNA was similarly inserted into a plasmid expressing EF1α, but RFP instead of the linker (data not shown). Sequence results are included on the CD-ROM for future reference and use. Lentiviruses were produced as described in material and method and used to transduce TZM-bl cells to confirm the ability of the shRNAs to knockdown CD4, after sub-cloning the U6 promoter and shRNA into a different backbone. Data can be found on the CD-ROM: 'shRNA SEQUENCE AND CONSTRUCT INFO\LV U6-shRNA-EF1-linker-IRES-PURO\TZM-bl flow cytometry analysis of constructs'

Appendix IV: LCK cloning and sequence

Sequencing original LCK constructs

The plasmid used successfully in Chapter 5 evolved after sub-cloning the inserts into various other plasmids (as described below), which due to a variety of reasons were not suitable for the experiments in Chapter 5. The human Lck inserts originated from plasmids expressing Lck wt, a kinase inactive Lck (Y394F, TAC→TTC) which were kindly provided by Dr. Nika (Sir William Dunn School of Pathology). The original sequences were confirmed using the following primers (Geneservice order 2094441):

Name	Sequence (5'→3')
Seq f1	TCCTTCCTCATCCGGGAGAG
Seq r2	CGTTGTCCAGATTACGGATC
Seq f3	ACATGGAGAATGGGAGTCTAG

The raw sequence data files can be found on the CD-ROM under the following file path:

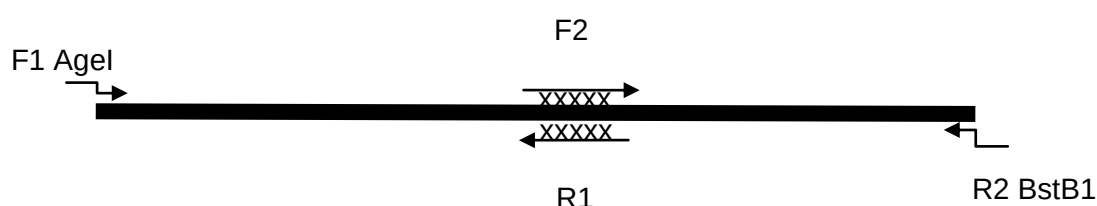
'\LCK SEQUENCE AND CONSTRUCT INFO\original plasmids raw sequence files (order 2094441)'

The Lck WT sequence corresponds to sequence found on the NCBI (NM_001042771.1) and can be found on the CD-ROM under the following file path: \LCK SEQUENCE AND CONSTRUCT INFO\lck wt. The sequence results confirmed the correct mutation of Lck Y394F (TAC→TTC) and the correct mutation of the Lck Y505F (TAC→TTC). An alignment of the above mentioned sequences can be found on the CD ROM under the following file path: LCK SEQUENCE AND CONSTRUCT INFO\original plasmids raw sequence files (order 2094441)\alignment

Amplification of Lck sequences

The three Lck sequences (WT, Y394F and Y505F) were amplified from the original construct using a two-step PCR. During the PCR amplification, five silent mutations were

introduced into these three Lck sequences in order to make them resistant to silencing by a Lck hairpin (shLck, V2THS_37234, Open Biosystems, sense: GGAATTATATTCATCGTGA). This shLck targets 19 nucleotides in the kinase domain of the Lck transcript and can be used to silence endogenously expressed Lck, if present. To avoid recognition of these Lck sequences by Primer R1 and Primer F2 were designed by binding the first 20nt of the primer binding site with full complementary, followed by the missense mutations and another 10nt of the correct complement sequence. In order to continue the cloning of the Lck amplicons, restriction sites were added to the F1 and R2 primers. In both cases 4 base pairs (GCTA) were added at the end to create allow sufficient space for the enzyme to be able to bind and cut. Because the F1 primer binds the start of the ORF, the Kozak sequence (ACC) was added, to allow recognition by the ribosome as the translational start site.



Name	Sequence (5'→3')
F1 Age	GCTAACCGGTACCATGGGCTGTGGCTGCAGCTC
R1*	CAGCCCGAAGGTCACGGTGGATGTAGTTTCGCTCTTCAATGAATGCCATGCCTT
F2*	TTACAACCGTCGGGCTTCCAGTGCCACCTACATCAAAGCGAGAAGTTACTTA
R2 BstBI	TAGCTTCGAATCAAGGCTGAGGCTG
*contains 5 silent mutations (underscored), so that the heterologous lck is resistant to the short-hairpin LCK.	

In the first step, the first ~1120bp the Lck sequence (similar for WT, Y394F and Y505F) was amplified from the provided lck wt construct by PCR using a forward primer ('F1') which included the AgeI restriction site and is complementary to the first 20nt of WT Lck and a reverse primer that included the 5 non-sense mutations ('R1'). The final 480bp

(1050-1530) of the wild-type and mutant Lck sequences were amplified from the provided constructs in a separate PCR reaction. In this case the forward primer ('F2') contained the 5 non-sense mutations and the reverse primer ('R2') contained the BstBI restriction site. The PCR was performed using Phusion DNA polymerase (Finnzymes/Biolabs). In a 20µL reaction 250ng template; 5x Phusion buffer; 10mM dNTPs, 0.5µM forward primer and 0.5µM reverse primer were added. The PCR programme used was as follows: 94 °C (2 min), followed by 20 cycles of 94 °C (10 sec), 58 °C (15 sec), 72 °C (20 sec), and a final step of 72 °C (10 min). The amplified products were run on a 1% Agarose gel, cut out, extracted and purified (QIAquick Gel Extraction Kit).

The full Lck sequence and its mutants, including the two restriction sites and 5 silent mutations, were obtained by adding the F1 and R1 product to a second PCR reaction with the F2 and R2 product. In a 50µL reaction, 3µL of each product was added to 5x Phusion buffer; 10mM dNTPs. The first PCR programme used was as follows: 94 °C (2 min), followed by 8 cycles of 94 °C (15 sec), 58 °C (15 sec), 72 °C (20 sec). Next, 0.5µM forward primer F1 and 0.5µM reverse primer R2 were added and the following PCR programme was used: 94 °C (2 min), followed by 30 cycles of 94 °C (10 sec), 58 °C (15 sec), 72 °C (20 sec), and a final step of 72 °C (10 min). The amplified products were run on a 1% Agarose gel, cut out, extracted and purified (QIAquick Gel Extraction Kit).

Cloning the Lck PCR products into a Doxycycline inducible shRNA vector

The amplified Lck fragment were digested with AgeI and BstBI and ligated into a lentiviral Dox inducible shRNA vector (pCMV_{minimal}-SIN-RFP:shRNA-UBC-rtTA3-I-P-WPRE, V2THS_37234, Open Biosystems), which had been digested with AgeI and ClaI (thereby removing the RFP gene). The ligation products were transformed into competent bacteria and plasmid preparations were made and verified by sequencing using primers 'Seq f1', 'Seq r2' and 'Seq f3' (see table above, Geneservice order 2098180). The raw sequence

data files can be found on the CD-ROM under the following file path: 'LCK SEQUENCE AND CONSTRUCT INFO\LV Dox inducible shLck vectors (order 2098180)

Lentiviruses encoding doxycycline (Dox) inducible LCK were produced. The expression system was tested successfully in cell lines, but expression of the transgenes was lost in esMDM (data not shown), which may be related to silencing of the promoter (CMV) or impairments with the Dox induction. Therefore, the vector was simplified by excluding the inducible promoter and the short hairpin. Instead, a bicistronic mRNA containing the gene of interest (lck), IRES and a gene for Neomycin resistance, driven by the EF1 α constitutive promoter was created as described below.

Cloning the Lck sequences into a constitutive, Neomycin resistant vector

The Lck sequences were amplified from the lentiviral Dox inducible shRNA vectors and the AgeI and BstBI restriction sites were added at the 5' and 3' end, respectively. The PCR was performed using Phusion DNA polymerase (Finnzymes/Biolabs). In a 20 μ L reaction 2 μ L of template (midprep quality); 2x Phusion High-Fidelity mastermix; 0.5 μ M forward primer F1 and 0.5 μ M reverse primer were added. The PCR programme used was as follows: 98 °C (30 sec), followed by 30 cycles of 98 °C (10 sec), 62 °C (15 sec), 72 °C (10 sec), and a final step of 72 °C (10 min). The amplified products were run on a 1% Agarose gel, cut out, extracted and purified (QIAquick Gel Extraction Kit). Next, the products were digested with AgeI-HF and ClaI (65°C O/N), and the pEF1 α -linker-IRES-Neomycin^R (as described above) was digested with XmaI and BstBI. Both were run on a 1% Agarose gel, cut out, extracted and purified (QIAquick Gel Extraction Kit). The Lck inserts were ligated into the linearized pEF1 α -linker-IRES-Neomycin^R and transformed into competent bacteria. Plasmid preparations were produced and sequenced; the expected sequences were obtained using the primers 'Seq f1', 'Seq r2' and 'Seq f3' (see table above, Geneservice order 2133372 and 2134397). The raw sequence data files can be found on the CD-ROM under the following file path: 'LCK SEQUENCE AND CONSTRUCT

INFOLV lck-IRES-Neo (order 2133372). Lentiviruses were produced as described in material and methods and were tested in cell lines, stem cells and esMDM. Despite culturing cells with G418, no cell population could be selected with 100% transgene expression (data not shown). As previous results had shown that Puromycin selection does allow 100% transgene expression, the vector was re-designed to exchange the Neomycin resistance with Puromycin resistance.

Cloning the Lck sequences into a constitutive, Puromycin resistant vector

The plasmids encoding pEF1 α -lck_{WT}-IRES-Neomycin^R, pEF1 α -lck_{INACTIVE}-IRES-Neomycin^R, as well as a plasmid encoding, pEF1-linker-IRES-Puromycin^R (described above), were digested with SpeI and MluI. The digested vectors were separated on an agarose gel (QIAquick Gel Extraction Kit) and the Lck sequences and the digested pEF1-linker-IRES-Puromycin^R backbone, were extracted from the gel (QIAquick Gel Extraction Kit). The digested plasmid vector and Lck fragments were ligated and transformed into competent bacteria. Plasmid preparations were produced and sequenced; the expected sequences were obtained (Geneservice order 402827901) using the primers 'Seq f1', 'Seq r2' (see table above) and 'EF1a_fwd' and 'Seq f4' (see table below).

Name	Sequence (5'→3')
Seq f4	AGCACGCCTCATTGAGGACAACGAGT
EF1a_fwd	TCAAGCCTCAGACAGTGGTTC

The raw sequence data files can be found on the CD-ROM under the following file path: 'LCK SEQUENCE AND CONSTRUCT INFOLV lck-IRES-Puro (order 402827901)' Lentiviruses were produced as described in material and methods and were tested successfully in stem cells and esMDM (see chapter 5).

Appendix V: Chimeric CD4 cloning and sequences

The chimeric wild-type and mutant CD4 sequences, as described in Chapter 6, were designed and codon-optimized using DNA2.0 GeneDesigner software. The chimeric CD4 sequence was flanked with *SpeI* (ACTAGT) and *XmaI* (CCCGGG) restriction sites at the start and end of the chimeric CD4 gene, respectively, to facilitate sub-cloning of chimeric CD4 into pU6-shRNA-EF1-linker-IRES-Puromycin^R, which contains the *SpeI* and *XmaI* restriction sites in the linker. The designed fragments were produced by GeneArt (Invitrogen) and provided in an Ampicillin^R vector pMA-RQ (see the attached CD-ROM: 'CHIMERA DESIGN\pMA-RQ-chCD4'). The wild-type (WT) and 3 mutant CD4 sequences were sub-cloned into the 'GOI' site of pU6-shCD4-EF1-GOI-I-Puro^R. The pMA-RQ vectors containing chimeric CD4 and the pU6-shRNA-EF1-linker-IRES-Puromycin vectors containing shCNTRL and shCD4 were digested with *SpeI* and *XmaI* (in a 40µL reaction, using 5µg of DNA; 1µL of each restriction enzyme and .5 µL BSA; 4µL of buffer (New England Biolands) and water, for 2h 37°C) and run on a 1% Agarose gel, cut out, extracted, purified and eluted into 50 µL (QIAquick Gel Extraction Kit). 5µL of the chimeric CD4 inserts were ligated into 3µL of the linearized pU6-shRNA-EF1-linker-IRES-Puromycin^R using 2µL T4 ligase and 10µL 2x rapid buffer (10min RT) and run on a 1% Agarose gel, cut out, extracted, purified and eluted into 50 µL (QIAquick Gel Extraction Kit). The ligation products were transformed into competent bacteria and plasmid preparations were made. Analytical restrictions (using 1µL *XmaI*; 1µL *SpeI*; 0.5µL BSA, 1µL buffer, 2.5µL plasmid preparations (miniprep quality) and 4µL water) were performed for 1h at 37°C and run on a 1% Agarose gel to confirm correct cloning. Midiprep quality plasmid preparations were made and the correct sequences of plasmids were confirmed (Geneservice order 405685401) using the following primers: U6_seqF, IRES_seq_R and EF1a_seq_F (primer sequence shown above). The raw sequence data files can be found on the CD-ROM under the following file path: 'pU6-shRNA-EF1-chCD4-IRES-Puromycin

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