Demo Yemane Tesfaye

Characterization of Jurkat E6.1 T cell line as a potential model system for primary CD4+ T cells to study the role of TLR8 in HIV infection.

Master's thesis in Molecular Medicine

Trondheim, June 2016

Supervisor: Markus Haug, PhD, IKM/CEMIR

Co-supervisors: Hany Ibrahim, PhD candidate, IKM/CEMIR

Trude H. Flo, Professor, IKM/CEMIR

Norwegian University of Science and Technologies

Faculty of Medicine

Department of Cancer Research and Molecular Medicine

NTNU – Trondheim
Norwegian University of
Science and Technology

NTNU
Faculty of Medicine
Department of Cancer Research
and Molecular Medicine

ABSTRACT

HIV infects and destroys cells of the immune system such as macrophages, dendritic cells and CD4+ T helper cells. One route of HIV transmission is through cell-to-cell viral trans-infection where viral particles can be endocytosed by CD4+ T cells or other cell types and enter the endolysosmal degradation pathway. As other cells of the immune system, CD4+ T cells have been indicated to possess endosomal pattern recognition receptors (PRRs) such as Toll like receptors (TLRs), which might sense viral nucleic acids from these endocytosed HIV particles. Little is known today about the function of endosomal TLRs in CD4+ T cells. Preliminary findings in our group show that primary CD4+ T cells respond to endosomal TLR8 stimulation with inflammatory signaling. To further investigate the molecular mechanisms of TLR8 signaling in CD4+ T cells, it would be of great tool to have a suitable T cell line model system with defined TLR8 expression pattern. The human CD4+ T cell lymphoma cell line Jurkat E6.1 is a widely used T cell line to study molecular mechanisms in T cells.

The aims of this project were therefore to characterize the Jurkat E6.1 cell line for their suitability as a model to study TLR8 signaling in CD4+ T cells. These include, analysis of Jurkat cells for their endosomal TLR expression as well as their cytokine and activation marker response upon endosomal TLR and T cell receptor (TCR) stimulation. The results obtained with Jurkat cells should then be compared to findings in primary CD4+ T cells. In addition, we aimed to develop Jurkat cell lines with defined TLR8 expression pattern by knocking-down or overexpressing TLR8 gene, with which the specific role of endosomal TLR8 in HIV infection of CD4+ T cells could be further investigated.

We found that primary CD4+ T cells expressed TLR7, 8 and 9, while Jurkat cells expressed the genes for TLR7 and 9 but lacked TLR8 at both mRNA and protein level. The majority of Jurkat cells expressed the T cell co-receptor CD3 and the co-receptor for HIV entry CXCR4, while CD4+ expression was lower and greatly fluctuated.

Similar to primary CD4+ T cells, Jurkat cells upregulated the activation markers CD69, CD40L and CD25 upon stimulation with T cell activators. However, in contrast to primary CD4+ T cells, the cytokine response profile of Jurkat cells was found to be very limited and mainly restricted to the secretion of IL-2, IL-8 and TNF. Jurkat cells did not respond to stimulation with TLR7, 8 and 9 ligands by means of cytokine production or activation marker upregulation.

As Jurkat cells were found to lack TLR8, lentiviral transduction system and nucleofection methods were utilized to induce TLR8 expression. We found both methods to induce TLR8

mRNA expression but TLR8 protein was not detected. Hence, we tested the possibility that undetected TLR8 protein might exert TLR8 function in the engineered Jurkat cells. However, stimulation with TLR8 ligand neither induced cytokine production nor triggered nuclear translocation of the transcription factor NF-κB both on the nucleofected and transduced Jurkat cells.

In conclusion, we found that Jurkat cells do express endosomal TLR7 and TLR9 but lack TLR8. Successful expression of functional TLR8 protein in Jurkat cells was not achieved after repeated trials using two approaches. Use of alternative protocols and expression vectors may potentially lead to successful expression of functional TLR8 protein in Jurkat cells in future experiments. However, since it appeared difficult to introduce functional TLR8 into Jurkat cell and functional responses were not apparent for TLR7 and TLR9 stimulation, it might be considered to rather continue working with other T cell lines or directly with human primary CD4+ T cells. Primary CD4+ T cells are more difficult to modify than cell lines, but studies have succeeded and protocols are available. Knockdown of TLR8 in primary CD4+ T cells could then directly proof the importance of TLR8 signaling in CD4+ T cells and a possible role for TLR8 in CD4+ T cells during HIV infection.

ACKNOWLEDGMENT

I would first like to thank my supervisor Dr. Markus Haug for your all rounded support. Your willingness to give your time so generously is much appreciated. You have provided me with constructive suggestions and useful critiques during the planning and development of this thesis write-up. I have learned a lot from you and it was fun working with you as well. I remain indebted to your kindness. The professional lessons learned from you will always stay with me.

I would also like to offer my special thanks to Hany Ibrahim for bearing with me during my naïve experiences in the lab. Your contribution in formulating this project plan was also profound. You took your time to see me learn new things every day: Thank you. My gratitude also goes to Professor Trude H. Flo for the opportunity to work in your lab. You suggested valuable ideas, followed up and took an interest in this project. I appreciate everything.

To Dr. Zekarias Ginbot, you have been so candid in helping me with Lentivirus preparation and transduction experiments, you have also been a great moral support, thank you so much. Advice given by Dr. Jane A Awuh, has been a great help when performing lentiviral experiments. To former student Ida Schrøder for the TLR8 expression plasmid. To Claire Louet for the help with SYBRgreen PCR analysis and for your genuine advises and lessons on PCR. I want to thank Dr. Lobke Gieman as well for the provision of primers for SYBRgreen PCR.

I wish to extend my depth of gratitude to all the members of our lab for your valuable trainings in the lab and for your moral support and guidance. To my colleagues, for rocking in the same boat with me, you guys made this year really amazing.

Finally, to my loving and supporting family throughout my study stay here. I can't even begin to think how I would manage to pursue my education without your support. Words cannot express how fortunate I am to have you as family, you believed in me and pushed me forward. To Aster for all your love, patience and bringing life to us.

Trondheim, June 2016

Demo Yemane Tesfaye

CEMIR
Centre of Molecular Inflammation Research

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AP1	Activation protein 1
APC	Antigen presenting cell
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CCR5	C-C chemokine receptor type 5
CD	Cluster of Differentiation
CpG	5'-Cytosine-phosphate-Guanine-3'
Ct	Cycle threshold
CXCR4	C-X-C chemokine receptor type 4
DAG	Diacylglycerol
DAMP	Damage associated molecular pattern
DC(m/p)	myeloid/plasmacytoid Dendritic Cells
DMEM	Dulbeccos's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
ECACC	The European Collection of Cell Cultures
ECD	Ectodomain
ELISA	Enzyme Linked Immuno Sorben Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FOXP3	Forkhead box P3
GFP	Green fluorescent proten
gp120	Envelope glycoprotein 120
gp41	Glycoprotein 41
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IFN	Interferon

IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate (IP3)
IRAK	interleukin-1 receptor-associated kinase
IRF	Interferon Regulatory Factors
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilo Daltons
LASER	Light amplification by stimulated emission of radiation
LDS	Lithium dodecyl sulfate
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPK	Mitogen activated protein kinase
MHC/pMHC	Major histocompatibility complex/ peptide-MHC complex
MIP-1α	Macrophage inflammatory protein-1alpha
MyD88	Myeloid differentiation primary response protein 88
NF-κB	Nuclear Factor κ light-chain enhancer of activated B cells
NK cells	Natural killer cells
ODN	Oligodeoxynucleotides
ORN	Oligoribonucleotide
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFA	paraformaldehyde
PHA	Phytohemagglutinin
PI	Propidium iodide
PI3K	Posphoinsitide 3-kinase
PIP2	Phosphatidylinositol 4,5 bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Porbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate

PTEN	Phosphatase and tensin homolog
pU/pLA	Poly-uridine complexed with poly-L-arginine
qPCR	Real-time reverse transcription polymerase chain reaction
RNA/ss/ds	Single stranded/double stranded ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
Rq	Relative quantification
SDS	Sodium dodecyl sulfate
SDF-1	Stromal derived factor-1
TAB	TAK1- binding protein
TAK	TGF-beta activated kinase
TBS-T	Tris Buffered Saline-with Tween-20
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th1/2	T helper 1/T helper 2 cells
TIR	Toll/interleukin-1 Receptor
TIRAP	Toll-Interleukin 1 Receptor (TIR) domain containing Adaptor Protein
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
UNK93B1	Unc-93 homolog B1

1 INTRODUCTION

1.1 Toll Like Receptors

Toll like receptors (TLRs) are a group of germline encoded pattern recognition receptors (PRRs) that are specialized in recognizing different pathogen derived components called Pathogen Associated Molecular Patterns (PAMP) and also cell damage or stress byproducts of self-cells called Damage Associated Molecular Patterns (DAMP) (Janeway Jr & Medzhitov, 2002; Piccinini & Midwood, 2010). They were first described as functional genes that encode for a transmembrane protein involved in the process of dorso-ventral axis formation in fly embryos (Hashimoto et al, 1988; Nüsslein-Volhard et al, 1984). Later, Lemaitre et al (1996) elucidated that the Toll signaling pathway also has a role in Drosophila immune system by showing that flies harboring a mutant Toll protein are unable to resist aspergillus fungal infection.

The role of TLRs in the mammalian immune system was first established when it was shown that mice with mutated intracellular TLR4 domain are hyper-susceptible to Gram-negative bacterial infection (Poltorak et al, 1998). In this study, they also indicated that TLR4 is the receptor for bacterial lipopolysaccharide (LPS). To date twelve TLRs in mice and ten in human have been characterized, TLR1 to 10 in humans, and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice: the homolog of TLR10 in mice is non-functional (pseudogene) while the human genome does not contain TLR 11, 12 and 13 (Kawai & Akira, 2009).

Generally, TLRs share similar structural features: They are type I transmembrane proteins with the N-terminus outside of membrane and the C-terminus towards the cytoplasm. The N-terminal region contains a Leucine-Rich Repeat (LRR) ectodomain (ECD) followed by a single transmembrane region and an intracellular C-terminal domain which is homologous to the intracellular domain of IL-1 receptor, and is known as Toll/interleukin-1 Receptor homology (TIR) domain (Moresco et al, 2011). TLRs differ on the number of tandem repeats of LRR contained in their ECD that ranges from 19 to 25 tandem LRR motifs. A single LRR module comprises 24-29 amino acids with a general consensus motif of XLXXLXLXX (L=leucine residue, X= any amino acid). LRR domains also contain other conserved hydrophobic residues which are important in ligand interaction. They are placed at distinct intervals and have consensus sequence of XØXXØXXXXFXXLX (Ø=hydrophobic residue, F=phenylalanine residue) (Akira et al, 2006). Structurally a LRR module is made up of a β-strand followed by a variable loop connected to α-helical segments (Ohto et al, 2014). In the three dimensional

folding, multiple consecutive LRRs form a solenoid structure. The β-strands are placed parallel to each other forming a β-sheet structure and are more closely packed than the remaining part of LRR. This forces the solenoid to bend with the β-sheet on the concave surface while the loops and α-helices placed on the convex side. This gives the ECD a characteristic horse shoe shape (Fig. 1.1) (Botos et al, 2011).

The intracellular TIR domain of TLRs is also known to contain three highly conserved regions. These regions are known as box 1, 2 and 3 and are stretched over a total of approximately 200 amino acid residues. They are reported to be of importance for signaling, with box 1 and box 2 being more essential for signaling than box 3 (Watters et al, 2007).

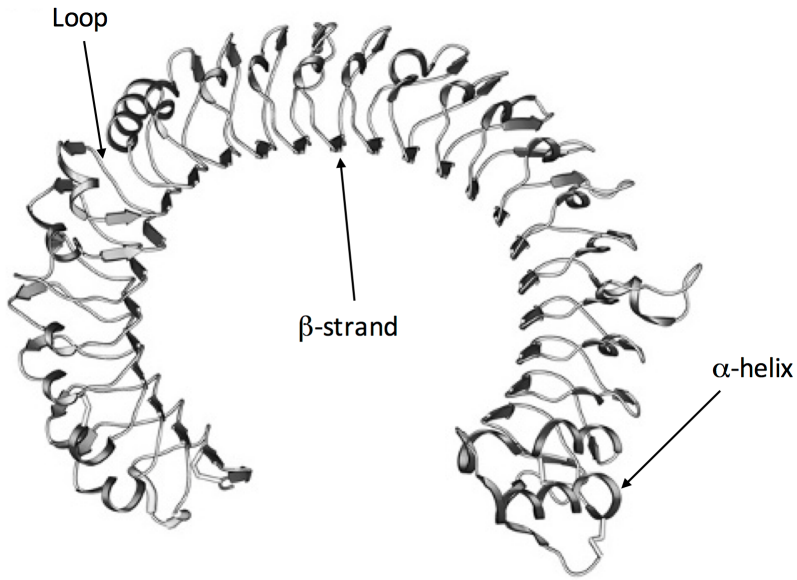


Figure 1.1: Schematics of Leucine Rich Repeats at the ectodomain of TLRs.
LRRs viewed from the side of the solenoid of TLR3 ectodomain made of 25 LRRs of different lengths. Continuous β-sheet forms the concave side while the convex side shows helical structures as well as stretches of short β-strand. Figure modified from Bella et al (2008)

Downstream signaling of TLRs is only possible when two TLR monomers dimerize. Dimerization of the receptors is usually induced by ligand recognition (Takeda & Akira, 2015). This recognition in turn triggers inflammatory and antiviral responses, facilitate innate immune cells activation and maturity which in turn shapes and directs the adaptive immune response (Kawai & Akira, 2006).

TLRs differ in their cellular localization (cell surface vs endosomal), ligand specificity, downstream signaling pathways, and target gene they induce. Cell surface localized TLRs include TLR1, 2, 4, 5, and 6 and sense a range of microbial components. For instance, TLR4

recognizes gram negative bacteria (LPS), while TLR2 in conjunction with TLR1 or TLR6 recognizes molecular patterns of triacyllipopeptide or diacylipopeptide of gram positive bacteria respectively (Kawai & Akira, 2006). A list of ligands recognized by cell surface expressed TLRs is shown in Table 1.

Table 1:Microbial components recognized by cell surface TLRs.
Table modified from Akira et al (2006)

TLR	**Microbial component**	**Origin of Species**
TLR1/2	Triacyl lipopeptide (Pam_3CSK_4)	Bacteria and mycobacteria
TLR2	Peptidoglycan	Gram-positive bacteria
	Porins	*Neisseria*
	Lipoarabinomannan	Mycobateria
	Phospholipmannan	*Candida albicans*
	Glycosylphosphatidylinositol-mucin	*Trypanosoma*
	Hemagglutinin protein	Measles virus
	Not Determined	HSV1, HCMV
TLR6/2	Diacyl lipopeptides (Pam_2CSK_4)	*Mycoplasma*
	Lipoteichoic acid	Group B *Streptococcus*
	Zymosan	*Saccharomyces cerevisiae*
TLR2 and TLR4	Glucuronoxylomannan	*Cryptococcus neoformans*
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Mannan	*Candida albicans*
	Glycoinositolphospholipids	*Trypanosoma*
	Viral envelope proteins	RSV, MMTV
	Heat-shock protein 60, 70	Host DAMP
	Fibrinogen	Host DAMP
TLR5	Flagellin	Flagellated bacteria

DAMP: Damage associated Molecular Pattern; MMTV: mouse mammary tumor virus; RSV: respiratory syncytial virus; HSV1: herpes simplex virus 1; HCMV: human cytomegalovirus

1.2 Endosomal Toll Like Receptors 7, 8 and 9

By engaging TLR signaling, cells have the ability to discriminate between self and non-self-nucleic acids. In the endosomal TLRs, this is to some extent provided by different modifications between cellular nucleic acids and nucleic acids that originate from pathogens such as viruses or bacteria. TLR9, for instance, recognizes un-methylated CpG containing DNA motifs which characteristically are found only in non-vertebrate genetic material like bacterial and viral DNA. Compartmentalization of endosomal TLRs also helps in discriminating self-derived nucleic acids, since self-derived nucleic acids are normally not found in the endosome. Here, the endosome can be viewed as a compartment through which endocytosed pathogens transit and thereby their nucleic acids can be detected by TLRs (Krieg & Vollmer, 2007).

The TLRs localized in the endosome include; TLR3, TLR7, TLR8 and 9. They have their N-terminal ECDs with multiple LRRs facing towards the lumen of the endosome, unlike the cell surface TLRs that have extracellular ECDs (Pandey et al, 2015). Moreover, TLR7, 8 & 9 have a higher molecular weight due to a longer ECD compared to the surface expressed TLRs (Chuang & Ulevitch, 2000).

Endosomal TLRs are stored in endoplasmic reticulum (ER) and have a controlled and guided exit from the ER to the endosomes via binding to an ER-resident membrane protein called UNC93B1 (Unc-93 homolog B1) (Itoh et al, 2011; Kim et al, 2008). These endosomal TLRs are then processed in endolysosomal compartments by resident proteases that result in the formation of functional receptors (Ewald et al, 2011; Ishii et al, 2014; Lee & Barton, 2014).

Endosomal TLRs signaling can be divided on the basis of adaptor protein requirement for downstream signaling. TLR7,8 and 9 use MyD88 (myeloid differentiation primary response protein 88) as an adaptor while TLR3 uses TRIF (TIR-domain-containing adapter-inducing interferon-β) (Kawai & Akira, 2007). Moreover, a study involved in generating molecular tree of all vertebrate TLRs using GeneBank database has shown that TLR7, 8 and 9 split from same branch and hence belong to a sub-family of hTLRs, while TLR3 has its own early bifurcating branch (Roach et al, 2005). Furthermore, TLR7 and TLR8 are phylogenetically closely related TLRs and are both located on chromosome Xp22 while TLR9 is located on chromosome 3p23.1 (Fig. 1.2) (Chuang & Ulevitch, 2000).

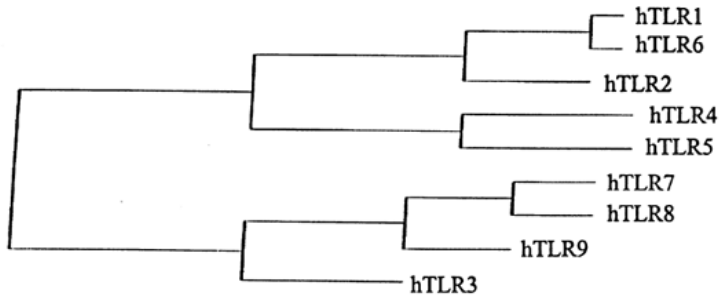


Figure 1.2: Phylogenetic tree of the hTLR members .
Phylogenetic tree derived from alignment of protein sequences of hTLRs from GeneBank. Figure from Chuang and Ulevitch (2000).

The gene for TLR3, is mapped to chromosome 4q35 and is known to be expressed mainly on innate immune cells, with the exception of neutrophils and plasmacytoid dendritic cells (pDCs) (Akira et al, 2001; Thompson et al, 2011). High amounts of human TLR8 mRNA expression is found in monocytes, monocyte-derived dendritic cells (mDCs) and macrophages, (Zarember & Godowski, 2002). On the other hand, TLR 7 and 9 are strongly expressed in pDCs and human B cells (Hornung et al, 2002).

Generally, the endosomal TLRs recognize and get activated by nucleic acid materials within an acidified endolysosomal compartment. TLR3 is activated by double-stranded (ds) RNA, TLR7 and TLR8 by single-stranded (ss) RNA, while TLR9 is activated by unmethylated CpG motifs within ssDNA (Krieg & Vollmer, 2007). Apart from these natural ligands for TLR7,8 & 9, a number of synthetic agonists also exist that can trigger signaling from these receptors. For instance, synthetic oligoribonucleotides derived from the U5 region of human immunodeficiency virus-1 RNA (called ssRNA40) which is rich in guanosine and uridine mimics ssRNA and can induce strong NF-kB response upon stimulation of human TLR 8 (Heil et al, 2004). Similarly, synthetic oligodeoxynucleotides containing CpG-motifs (CpG-ODN) have been used to stimulate TLR9 response (Lingnau et al, 2002).

A recent study on the crystal structure of human TLR8 has shown two distinct sites on TLR8 that recognize two degradation products of ssRNA, rather than the whole fragment of ssRNA (Tanji et al, 2015). This two fragments are; a single uridine residue recognized at dimerization interface of the two TLR8 monomers as well as a short stretch of oligonucleotids recognized on the concave surface of the TLR8's ECD (Tanji et al, 2015).

Other synthetic immune response modifiers known to activate TLR7 or TLR8 are small tricyclic organic molecules called imidazoquinolines. Initially, Hemmi et al (2002) showed that the imidazoquinoline compounds imiquimod and R-848, activate macrophages from mice via

a TLR7-MyD88-dependent signaling pathway resulting in induction of the transcription factor Nuclear Factor κ light-chain enhancer of activated B cells (NF-κB) and secretion of pro-inflammatory cytokines such as Tumor necrosis factor-α (TNF-α). Here, macrophages from TLR7-deficient mice did not respond to stimulation by R-848 (Hemmi et al, 2002). Later R848 was shown to stimulate the induction of NF-κB in HEK293 cells transiently transfected with human TLR8 (hTLR8) and failed to induce similar response in HEK293 cells transiently transfected with mouse TLR8 (Jurk et al, 2002). This indicates that TLR8 in mice might be non-functional. This is in accordance with the above study that reported macrophages from TLR7-deficient mice did not respond to R-848 stimulation even at higher concentration even if they were expressing the mice TLR8 (Hemmi et al, 2002).

The presence of selective imidazoquinoline agonists towards TLR7 or TLR8 greatly improved the study of the specific roles of the two receptors. Gorden et al (2005) identified 3M-001 as TLR7 selective ligand and 3M-002 (CL075) as TLR8 selective ligand. They showed that the TLR7 agonists induce IFN-α more efficiently than TLR8 agonists from pDCs. On the other hand, TLR8 agonists were more effective in inducing pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-12, IL-6, IL-8 and MIP-1α from mDCs (Gorden et al, 2005). Ever since, there have been a number of studies about the role of TLR7 and/or TLR8 using selective immune response modulators.

Table 2: Agonists for TLR7, TLR8 and TLR9

TLR	Agonist	Source	Reference
TLR7/8	ssRNA	Virus	Heil et al (2004)
	R848	Synthetic	(Hemmi et al, 2002).
	CL097	Synthetic	(Smits et al, 2008)
TLR7 selective	CL264	Synthetic	(Dominguez-Villar et al, 2015)
	R-837 (Imiquimod)	Synthetic	(Smits et al, 2008)
	Loxoribin	Synthetic	(Dominguez-Villar et al, 2015)
	Gardiquimoid	Synthetic	(Dominguez-Villar et al, 2015)
TLR8 selective	CL075 (3M-002)	Synthetic	(Gorden et al, 2005)
	ssRNA40	Synthetic	Heil et al (2004)
	TLR-506 (VTX-2337)	Synthetic	(Lu et al, 2012)
TLR9	Unmethylated CpG-DNA	mycrobs	(Krieg & Vollmer, 2007)
	CpG-ODN	Synthetic	(Lingnau et al, 2002)

CpG-ODN: 5'-Cytosine-phosphate-Guanine-3'-oligodeoxynucleotides, DNA: Deoxyribonucleic acid, ssRNA: single stranded Ribonucleic acid, TLR: Toll like receptor

1.3 Toll Like Receptors 7, 8 and 9 signaling

The initial phase of TLR-ligand binding is marked by dimerization of the cytosolic TIR domains of the TLRs, which then interact with TIR domains of cytosolic adaptors. Some TLRs, such as TLR8, already have a preformed dimerized ECD. However, this initial dimer in TLR8 is not able to initiate downstream signaling since the TIR domains are wide apart (≈53Å). Upon ligand recognition, the two cytoplasmic domains are brought to close proximity (≈30Å), which results in dimerization of TIR domains and commencement of signaling (Tanji et al, 2013).

Endosomal TLR7,8 & 9 utilize MyD88 as adapter for downstream signaling, while TLR3 signals through the adaptor TRIF. Furthermore, the endosomal TLR 7, 8 and 9 depend completely on MyD88 to relay signaling, whereas, cell surface TLRs use the shuttling adaptor TIRAP (Toll-Interleukin 1 Receptor (TIR) domain containing Adaptor Protein) to mediate recruitment of MyD88 (Blasius & Beutler, 2010; Troutman et al, 2012). Once MyD88 is recruited, it stimulates the recruitment of interleukin-1 receptor-associated kinase 4, 1 and 2 (IRAK4, IRAK1 and IRAK2) and forms homotypic interaction via the death domain found both on MyD88 and the IRAKs. Phosphorylation of these IRAKs results in their dissociation from MyD88. They then associate with and activate the E3 ubiquitin ligase TRAF6 (TNF receptor associated factor 6) (Kawai & Akira, 2007). TRAF6 in combination with E2 ubiquitin ligases catalyzes the synthesis of Lys-63 linked polyubiqutin chain on itself and on free ubiquitin. Polyubiquitinated TRAF6 in turn ubiquitinates and activates TGF-beta activated kinase 1 (TAK1), a member of the MAPkinase kinase kinase (MAPKKK) family, that is associated with TAK1- binding proteins 1, 2 &3 (TAB1, TAB2 & TAB3). TAK1 activates the IκB kinase (IKK) protein complex, IKKα, IKKβ and IKKγ (also known as NEMO) complex, by posphorylating IKKβ. The IKK protein complex then phosphorylate IκBs (inhibitor of NF-κB) that is found associated with and inhibiting NFκB. Phosphorylation of IκBs leads to its degradation via targeted ubiquitin-mediated proteasome degradation thereby freeing NF-κB to translocate into the nucleus and initiates transcription of pro-inflammatory genes (Fig. 1. 3) (Blasius & Beutler, 2010; Kawai & Akira, 2006; Troutman et al, 2012).

TAK1 phosphorylation can also lead to the activation of MAPK that initiates alternative downstream cascade that ultimately activates the transcription factor AP1. The TLR-MyD88 pathway is also shown to activate the Interferon Regulatory Factors (IRFs) - 3,5 and/or 7 via association of TRAF6 to IRF (Bergstrom et al, 2015; Kawai & Akira, 2007). In general, activation of the endosomal TLR7, TLR8 and TLR9 leads to the activation of IRFs, NF-κB and

AP-1 transcription factors that leads to the induction of pro-inflammatory cytokines and type 1 interferons (Blasius & Beutler, 2010).

However, the cytokine profile produced and the signaling cascade triggered upon TLR7,8 and 9 mediated sensing of PAMP depends on the cell type involved. For instance, pDCs are known to be the major producers of type I IFN in response to TLR7/8/9 agonists (Kader et al, 2013) while mDCs, tend to secret more pro-inflammatory cytokines (Hemont et al, 2013). Furthermore, induction of type I IFN response by TLR9-ligand recognition is mediated via activation of IRF7 in pDC while this response is mediated by IRF-1, and not IRF3/7, in mDCs and macrophages (Colonna, 2007).Therefore, the particular signaling pathway and the specific mechanism of signal cascading triggered upon TLR mediated sensing of ligands may differ from cell type to cell type.

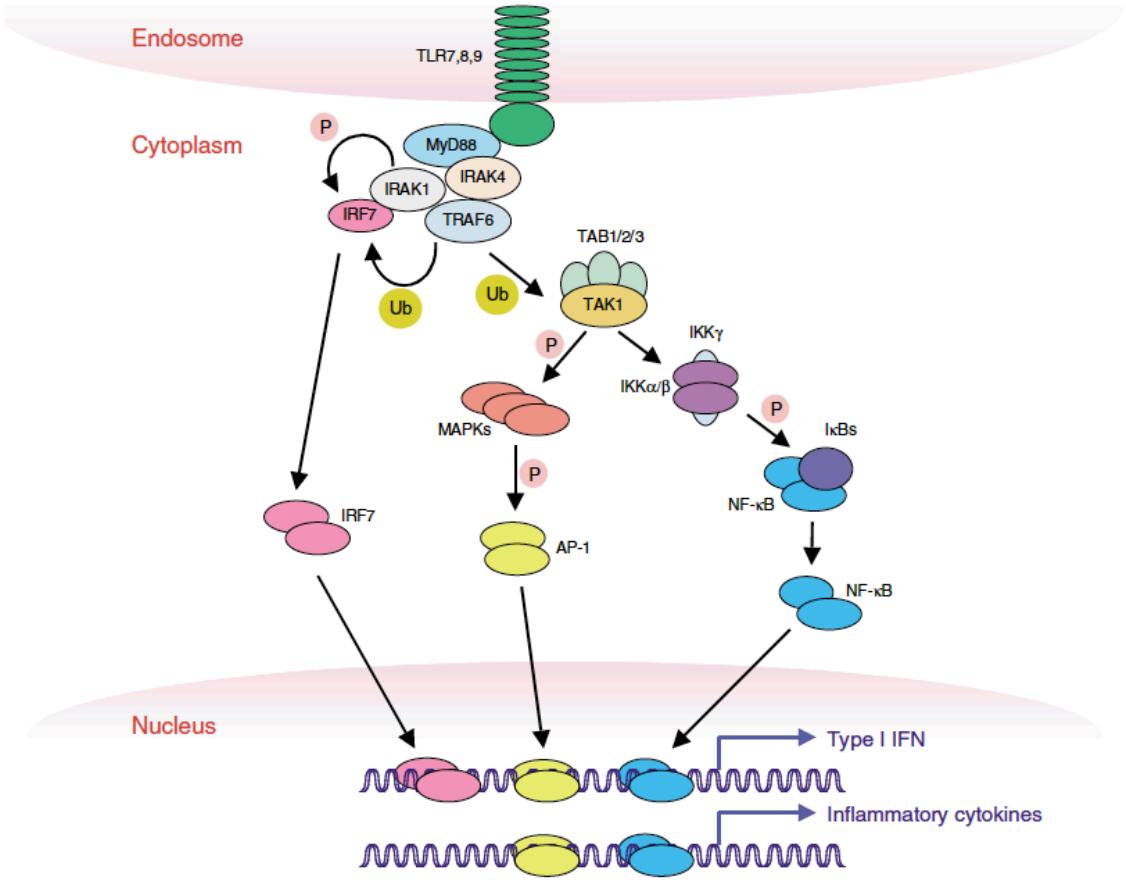


Figure 1.3: Schematics of TLR7,8 and 9 signaling.
TLR7,8 and9 use MyD88 as an adaptor to shuttle signaling downstream that culminate in activation of NF-kB, AP-q and IRF transcription factures and results in the induction of Type I interferons and pro-inflammatory cytokines. Figure adopted from Kawai and Akira (2006)

1.4 Toll Like Receptors in T Cells: Implications in the context of HIV trans-infection

The role and signaling pathway of TLRs seem to be well described in APCs. TLRs as well as other PRRs in APCs are involved in the early sensing of invading microorganisms. Recognition of PAMP by the TLRs activates different downstream signals that results in activation and maturation of the APCs which in turn results in stimulating and directing of the adaptive immune cells such as T and B cells (Kulkarni et al, 2011).

The expression profile of TLRs in adaptive immune cell subsets is indicated but still not well defined. Moreover, few studies have been published regarding the role of TLR signaling in T cell functions (Caron et al, 2005; Dominguez-Villar et al, 2015; Gelman et al, 2004). However, the function and significance of TLR signaling in adaptive immune cells in general and CD4+ T cells in particular has not yet been fully explored.

1.4.1 Overview of CD4+ T cells

T cells, together with B cells, are the main effector cell types of the adaptive immune response. Both B and T cells are cells of lymphoid lineage and their progenitors originate from hematopoietic stem cells in the bone marrow. T cell progenitors move to the thymus and mature to naïve T cells. CD4+ T and CD8+ T cells make up the majority of T lymphocytes in the peripheral blood. CD4+ T cells are also called T helper cells and are the central orchestrators of adaptive immune system and enhancers of innate immune cell responses (Klein et al, 2009). Other minor CD4+ expressing lineage generated in the medulla of thymus is the forkhead box p3 (FOXP3) expressing natural regulatory T cells (nTreg cells) which has CD4+CD25+ marker (Takahama, 2006).

Once mature, naïve CD4+ T cells leave the thymus and circulate in the secondary lymphoid organs such as the spleen, lymph nodes and mucosal associated lymphoid tissues where they constantly scan for peptide complexed with Major Histocompatibility Complex class II (pMHC II) on professional antigen presenting cells (APCs), mainly dendritic cells (DCs). The initial step for activation and differentiation of naïve CD4+ cell is triggered upon recognition of cognate pMHC II complex via their T cell receptor (TCR) and CD4 as co-receptor (Jenkins et al, 2001). Three different signals are required for full activation of CD4+ T cells and polarization into CD4+ T effector cell subsets. DCs presenting antigenic peptides on MHC II to CD4+ T cells in the lymph node are capable of providing all three signals. Signal one is acquired from the binding of TCR to pMHC II on cell surface of APC. Signal 2 is from the

interaction of the co-stimulatory receptor CD28 found on CD4+ T cells with CD80 (B7.1) and CD86 (B7.2) expressed on APCs. Signal 1 and signal 2 activate the cell and up regulate IL-2 receptor expression, which is the receptor for cell proliferating cytokine IL-2. The third signal is obtained from cytokines in the surrounding of the activated T cell that induce polarization and clonal expansion of the stimulated CD4+ T cell (Fig. 1.4).

The cytokines that act as 3rd signal for CD4+ T cells polarization are mainly produced by the professional APCs such as dendritic cells, and by the activated CD4+ T cells creating a positive feedback loop. Depending on the type of the third signal, CD4+ T cells are polarized into Th1, Th2, Th17, Tfh and iTregs effector cell subsets. Il-12 and IFN-γ are, for example, Th1 polarizing cytokines while IL-4 is the main Th2 polarizing cytokine (Luckheeram et al, 2012). Induced Tregs (iTregs) are derived from polarization of CD4+ T cells by TGF-β and IL-10 (Kapsenberg, 2003).

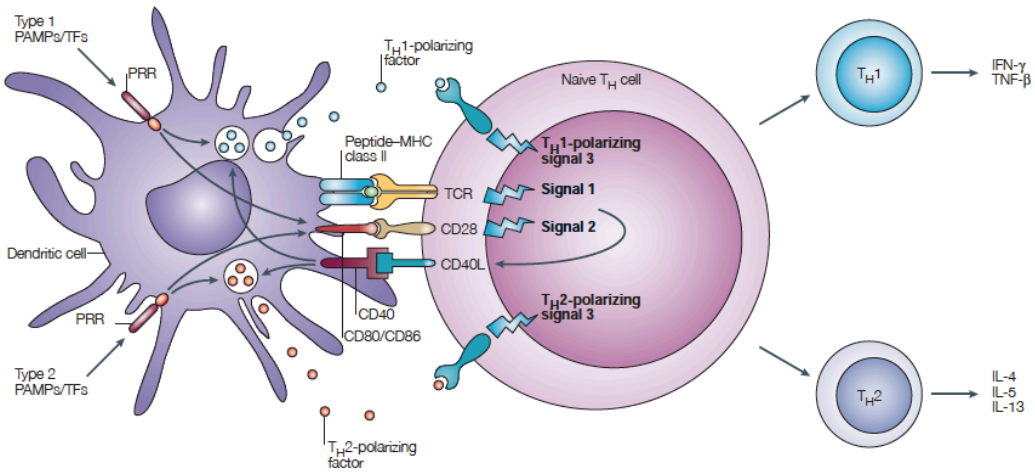


Figure 1.4: T cell stimulation and polarization.
Three signals are required for activation of naïve CD4+ T cells. Signal one is from the interaction of pMHC complex interaction with TCR. Signal two is the co-stimulatory signal derived from the interaction of CD28 on the T cell with CD80/CD86 on the APC. Signal 1 & 2 increases the proliferation of T cells. Depending on the nature of third signal the T cells polarize into subsets with different effector response. Figure from Kapsenberg (2003)

1.4.2 CD4+ T cells and HIV infection

CD4+ T cells are the principal targets of the human immune deficiency virus (HIV). This single stranded RNA (ssRNA) virus needs a receptor complex consisting of CD4 and chemokine receptor, either CXCR4 or CCR5, for entrance into target cells. Furthermore, it has been reported that activated and proliferating CD4+ T cells are more permissive for productive HIV infection than resting CD4+ T cells (Pan et al, 2013; Stevenson, 2003).

HIV enters target cells through the fusion of viral membrane to target cell's plasma membrane. The initial interaction of the viral envelope glycoprotein gp120 with CD4 receptor and CXCR4 or CCR5 co-receptor on target cells triggers membrane fusion (Melikyan, 2014). This results in conformational change in gp120 and allows unfolding and insertion of gp41 subunit's N terminus to target cell membrane. Refolding of gp41 then results in the fusion of the viral membrane and injection of viral core into target cell (Wilen et al, 2012).

Though free HIV viral particles released from infected cells can diffuse and infect new target cells, spread of the virus among adjacent cells via cell-to-cell transmission is efficient and rapid (Jolly et al, 2004). Moreover, transmission of HIV particles between infected and target cells through nanotubes connecting the two cells has also been described (Sowinski et al, 2008). Cell-to-cell transferred HIV viral particle may go through one of three different pathways: It can either be secreted back, endocytosed by the target cell where it is degraded in the endolysosome or fuse with vesicular membranes to inject the viral core into the cytoplasm and result in infection of the target cell (Blanco et al, 2004). In the case of a virus that enters infectious viral cycle, its genetic material in the capsid is uncoated, reverse transcribed and transported to the nucleus where it integrates into the host cell genome. The provirus will be transcribed and translated in the cytoplasm, and finally form new viral components, that assemble into a nucleocapsid core containing the RNA genome. Finally, the newly assembled virus is released by budding from the cellular membrane where it acquires its envelope (Guerrero et al, 2015).

1.4.3 Expression pattern and roles of endosomal TLRs in CD4+ T cells

Although, most that is known about TLR signaling and function originated from studies in APCs, studies have also indicated that the adaptive immune cells, such as T cells, also express most of the TLRs. However, review on TLR expression pattern in human and mouse T cells described considerable variation in the mRNA expression profile of TLRs in different studies (Rahman et al, 2009).

Two studies on C57/BL6 mice showed that CD4+CD45RN^{high} T cells express mRNA for TLR 1, 2, 3, 6, 7, 8 but low or undetectable expression for TLR3, 5 and 9. Both the studies utilized FACS sorting for purification of CD4+ T cells (Caramalho et al, 2003; Tomita et al, 2008). While a quantitative real-time PCR analysis on negatively isolated (untouched) T cells from human PBMC revealed mRNA expression for TLR1,2,3,5,6,7,8 and 9 (Hornung et al, 2002). In this study, TLR8 mRNA-expression is low on T cells and NK cells, while it is moderately expressed on monocytes. Furthermore TLR8 seems to be absent on pDCs and B cells (Hornung

et al, 2002). Another similar study with purified subpopulation of T cell reported that human CD4+ T cells express TLRs 1 to 10 (Zarember & Godowski, 2002).

Contrary to the above findings, there are some studies that reported absence of TLR8 in CD4+ T cells. A study done on negatively isolated CD4+ T cells followed by FACS sorting reported that expression of TLR6, 8 and 10 mRNA was weak or undetectable in CD4+ T cells (Caron et al, 2005). Another report for the absence of TLR8 mRNA expression on CD4+ T cells came from Mansson et al (2006) on a study done on T cells isolated from tonsil. Here, it was also shown that the level of mRNA expression for TLR3 and 7 was very low while TLR6 was undetectable (Mansson et al, 2006).

Accounts for the protein level expression of TLR8 in CD4+ T cells are lacking. However, reports about the expression of TLR2, 3, 4, 5, and 9 proteins on CD4+ T cells analyzed using flow cytometry technique have published (Crellin et al, 2005; Fukata et al, 2008; Komai-Koma et al, 2004).

The controversies in expression of TLR8 on CD4+ T cells might partly stem from technical and method variation. qPCR is a powerful, accurate and very sensitive method for the detection of mRNA level expression of genes. However, of note here is that mRNA levels may not correspond with protein expression and most of the studies done to characterize TLRs in T cells so far are based on mRNA level detection by PCR. Furthermore, there is a lack of consensus on how to interpret qPCR experiments since the relative quantification in each study is made from different reference genes or cells (Bustin et al, 2009). This makes comparison of findings from different studies more challenging. Moreover, contamination of purified T cells by APCs may significantly influence PCR output. In light of such issues protein level TLR examination seems an attractive alternative for PCR data. However, lack of robust and reliable antibody reagent hampers determination of TLR protein levels in T cells by use of western blotting or flow cytometer (Rahman et al, 2009). Therefore, induction of signaling pathways by TLR-ligands appears to be a more reliable method to assess and characterize TLRs on T cells than detection of TLRs themselves.

A few studies have assessed for the immunological role and responses of TLR8 in CD4+ T cells to further understand the link between endosomal TLRs and adaptive immune cells during viral infections. For instance, higher expression of mRNA for TLR 7, 8 & 9 in hepatitis C virus infected patients' T cells were reported compared to healthy control. However, the exact T cell population (CD4+, CD8+ or other sub population) were not discriminated in this study (Dolganiuc et al, 2006). The first report about the effect of R848 (TLR7/8 ligand) on CD4+ T

cells came from Caron et al (2005). This study showed an innate immune role for T cells by demonstrating that R848 up regulates the production of IFN-γ and IL-8 and increases proliferation of human CD4+ T cells. Here, the memory CD4+ T (described by $CD45RO^+$) cells were found to be more sensitive to R848 stimulation than naïve CD4+ T cells ($CD45RA^+$). Further discrimination showed that the effector memory T cells (known by lack of chemokine receptor 7; $CCR7^-$) were more sensitive than the central ($CCR7^+$) memory T cells (Caron et al, 2005). Similar effector cytokine response and cell proliferation was found when CD4+ CD45RO memory T cells were stimulated by TLR2 ligand Pam_3CSK_4 and the TLR5 ligand flagellin (Caron et al, 2005; Komai-Koma et al, 2004).

CD28 mediated signaling in CD4+ T cell, *i.e.* signal two, complement the TCR induced signal to drive effector functions such as cytokine production and cell proliferation. TLR9 ligand (CpG) has been shown to provide the costimulatory role in the absence of anti-CD28 to enhance IL-2 production on CD4+ T cells isolated from B6 mice (Rahman et al, 2009). Furthermore, T cell proliferation was induced when Pam_3CSK_4, flagellin and R848 were used as a co-stimulant for anti-CD3, anti-CD2 or IL-2 (Caron et al, 2005; Komai-Koma et al, 2004). However, such synergism was not always consistent in driving effector cytokine production. For instance when used alone without anti-CD3, R848 as well as Pam_3CSK_4 and flagellin failed to induce IL-2 and IL-10 production (Caron et al, 2005). To the contrary IL-8 induction in this study was increased when R848 was used alone and inhibited when R848 was used as a co-stimulant to anti-CD3(Caron et al, 2005).

Although, both TLR7 and 8 have same natural ligand, (ssRNA fragments) and both respond to the synthetic ligand R848, their effector functions as well as their signaling pathways are distinctively different. The intricacy of the matter gets even more complicated when considering different cell types from an organism and same cell types among different organisms induce different downstream immune response profile in response to TLR7 and 8 stimulations. For instance, HIV ssRNA in human mDCs has been shown to activate NF kB through the TLR8 - endosomal MyD88 mediated pathway and not via TLR7 (Gringhuis et al, 2010). Another study reported ssRNA from influenza virus triggered large amount of IFN-α via TLR7 – MyD88 pathway in pDCs from C57BL/6 mice (Diebold et al, 2004). A study reported that murine TLR7 in DCs recognizes GU rich ssRNA derived from HIV and induce IFN-α and TNF-α, while human TLR8 transfected HEK293 responds to GU and U rich ssRNA and increases NF-κB induction (Heil et al, 2004).

The regulatory T cells (Tregs) are subtypes of CD4+ T cells with a distinct role of suppressing immune responses which is essential in prevention of autoimmunity. Tregs are reported to have TLR8 and induction of TLR8 signaling in this cells was shown to inhibit their suppressive activity, hence permitting heightened immune responses indicated by enhanced naïve CD4+ T cells proliferation (Peng et al, 2005).

A surprising role for the endosomal TLR7 in human CD4+ T cells infected with HIV was reported in a recent study. TLR7 stimulation was found to negatively regulate both the activation and cytokine production by the CD4+ T cells deriving them into unresponsiveness and a complete state of anergy, which was claimed to enhance HIV replication (Dominguez-Villar et al, 2015).

1.4.4 Endosomal TLRs in CD4+ T cells in the context of HIV

A study indicated that the majority of HIV viral particles, which are transferred into CD4+ target cells, end up in the endo-lysosomal compartment (Fredericksen et al, 2002). Furthermore, it was shown that preventing the acidification of the endolysosome prevents viral particle degradation and hence enhanced efficiency of HIV virus infectivity to the host cell (Fredericksen et al, 2002). This phenomenon of HIV viral particles degradation and containment in the acidic endolysosome sparks a multitude of questions in the perspective of HIV derived nucleic acids detection by TLRs present in the endosome of CD4+ T cells. Endosomal TLRs are activated by acidic environment of endolysosome and sense fragmented viral or bacterial nucleic acids.

It has been reported that HIV derived nucleic acids-endosomal TLRs interactions mediate activation of innate cells, such as pDCs, monocytes and macrophages (Beignon et al, 2005; Chattergoon et al, 2014). Human TLR8 and mouse TLR7 have been reported to recognize HIV viral ssRNA fragments in APCs such as DCs and macrophages. The response triggered by this recognition induces release of IFN-α and the pro-inflammatory cytokines TNF-α, IL-6 and IL-12 (Heil et al, 2004). Guo et al (2014) also reported that HIV infection in human monocytes induce IL-1β production via TLR8 dependent pathway in concert with activation of caspase-1 through NLRP3 inflammasome. Meanwhile TLR8 stimulation has been implicated with activation of HIV from latently infected myeloid-monocytic U1 cell line (Schlaepfer & Speck, 2011).

Overall endosomal TLR signaling in viral infections have both direct and indirect modulation of T cell responses (Fig. 1.5). The indirect role which is mediated by APCs is well established

where recognition of pathogen nucleic acids by the TLRs in APCs drives their maturation and induction of cytokine production that creates all the necessary milieu (Signal 1- 3) for activation and polarization of naïve T cells into the different subtypes (Th1, Th2) (Akira et al, 2001). Whereas the direct role of TLRs in CD4+ T cells involves recognition of viral nucleic acid directly by endosomal TLRs expressed in CD4+ T that in turn drives the stimulation and cytokine secretion of the cells. However, information regarding the direct roles of these endosomal TLRs in modulating effector functions of CD4+ T cells in the context of viral infections is scarce.

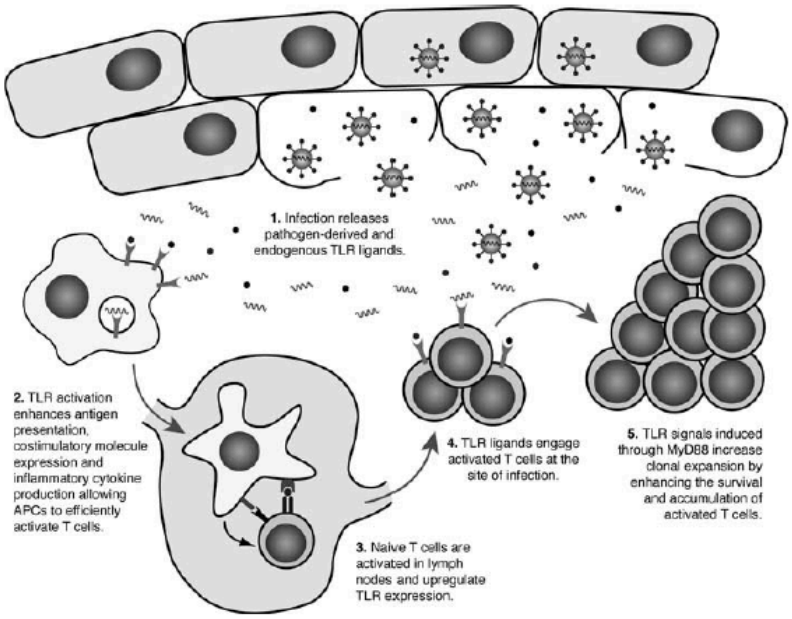


Figure 1.5: Direct and indirect regulation of T cells by TLRs.
Indirect role of TLRs on T cells is mediated by APCs. APCs present pMHC complex derived from pathogens and and at the same time secrete cytokines that direct the type of T cell immune response triggered. However, T cells may also directly interact with PAMPs via their TLRs such as in the case of HIV derived ssRNA molecules recognized in the endosomes of CD4+ T cells. Figure from (Rahman et al, 2009)

In a recent study, TLR7 stimulation was found to negatively regulate both the activation and cytokine production by CD4+ T cells infected by HIV deriving them into unresponsive state known as anergy (Dominguez-Villar et al, 2015). Immiquimoid (TLR7 ligand) stopped proliferation of CD4+ T cells which was induced activation with anti-CD3 and the co-receptor anti-CD28. Moreover, production of cytokines such as IFN-γ, IL-2, IL-4, IL-17 were also diminished as well as the expression of CD4+ T cells surface activation markers such as CD25, CD69 and CD137 inhibited. They further elucidated that impaired responses in productive HIV infected CD4+ T cells were reduced when TLR7 is knocked-down, by showing significantly more IL-2 and IFN-γ secretion in TLR7 knockdown CD4+ T cells compared to their non-

targeting shRNA transduced counterparts (Dominguez-Villar et al, 2015). On the other hand, stimulation of the CD4+ T cells by TLR8 ligand (ssRNA40 and CL075) increased secretion of IFN-γ and inhibited IL-4 secretion (Dominguez-Villar et al, 2015). However, the implication of HIV viral particle containment inside the endo-lysosome of non-productively infected CD4+ T cells was not addressed by the above study and the role of TLR8 in the context of HIV infection was not studies. Therefore, studies focusing on endosomal contained viral particles, rather than productively infectious virus, would open opportunities to understand the exact roles of the endosomal TLRs such as TLR 7, 8 & 9 in the context of HIV derived genetic material in the endosome of CD4+ T cells. Currently, our group is working to address those issues, with an emphasis on the role of TLR8 (ongoing experiments by M. Haug and H. Ibrahim). In these investigations we found that stimulation of the human primary CD4+ T cells with synthetic TLR8 ligands alone can induce production of pro-inflammatory cytokines even in the absence of additional TCR activation. Among other cytokines, we found that TLR8-stimulation of TCR-unactivated CD4+ T cells induced production of the innate immune cytokine IL-6, whereas no such response was observed when unactivated CD4+ T cells were stimulated with TLR 7 or TLR 9 ligands (Fig 1.6).

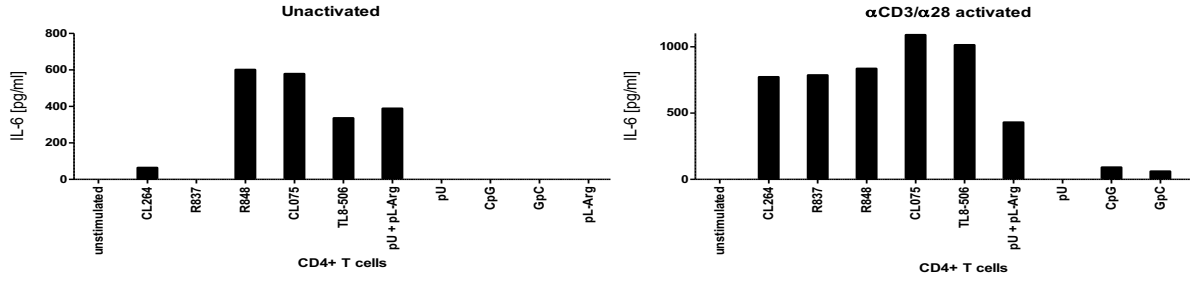


Figure 1.6: TLR8 mediated IL-6 production of human primary CD4+ T cells.
Primary CD4+ T cells unactivated or activated with anti-CD3/antiCD28 were stimulated with TLR7 ligands (CL264, R837), TLR7/8 ligand (R848), TLR8 ligand (CL75, TLR8-506, pU/pLA), and TLR9 ligand (CpG). (unpublished data in our group, experiment done by M. Haug/ H. Ibrahim)

1.5 Jurkat E6.1 T cells as model for CD4+ T cell functions

Some of the disadvantages in working with primary human CD4+ T cells are that primary CD4+ T cells are a heterogeneous cell population, have to be freshly isolated from blood donation for each experiment, issues of donor variability in immune responses studies and that it is difficult to overexpress or knock-down genes of interest in primary cells. Working with a good cell model overcomes some of this difficulties, since it is usually easier, faster and more reproducible in comparison with primary cells. Furthermore, overexpression or knockdown of proteins is relatively easier to achieve in cell lines. In these regards, Jurkat cells have been utilized extensively to model primary CD4+ T cells in the study for T cell signaling systems.

Jurkat E6.1 Cell line is a CD4+ T-cell leukemia cell line that was established from the peripheral blood of a 14 year old boy with acute T cell leukemia (Schneider et al, 1977). The initial Jurkat cell line which was investigated for IL-2 production upon PHA stimulation were found to be heavily contaminated with mycoplasma. Jurkat E6-1 clone (referred to as Jurkat cell hereafter) was the product after the process of curing of the cell line from this infection (Abraham & Weiss, 2004). It expresses the T cell antigen receptor (TCR) and the invariant signaling protein CD3, which are the defining marker for the T cell lineage (Smith-Garvin et al, 2009).

Substantial amount of information about T cell signaling is known via *in vitro* investigation of Jurkat cells. These includes the discovery that TCR ligation triggers intracellular Ca^{2+} mobilization, identification and use of TCR/CD3 specific antibodies as stimuli for IL-2 production, the observation that TCR stimulation triggers protein tyrosine phosphorylation and its subsequent role in TCR signaling, identification of ITAMs as signal-transducing motifs in the CD3 cytoplasmic domain, identification of PLC-γ1 as a TCR-linked signaling enzyme, characterization of ZAP70, identification of stimulatory pathways that skew T helper cell activation towards either to Th1 or the Th2 like response and so on (reviewed in Abraham and Weiss (2004); (Smeets et al, 2012).

Jurkat cells show low expression of a variety of chemokine receptors, including CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 (Maki et al, 2002). They also express CXCR4 that can bind to its natural ligand stromal-derived factor (SDF-1), and also is a known co-receptor for HIV envelope glycoprotein binding (Hesselgesser et al, 1998). Jurkat cell line is susceptible to infection by HIV and allow viral replication and is therefore the most common lymphocytic cell line used to study HIV. This is in part due to convenience for transfection of Jurkat cells with HIV proviral DNA such as *tat* (transactivator) gene and acquire

the unique ability to support replication of slow replicating HIV isolated from asymptomatic individual (Cervantes-Acosta et al, 2001; Korneyeva et al, 1993). Furthermore, Jurkat cell has been used to study human mRNA targets needed for HIV replication by use of short interfering RNAs (siRNA), or shRNA, (short hairpinRNA) targeting and knocking down a range of human mRNA needed for virus replication (Yeung et al, 2009).

However, usage of Jurkat cells for the investigation of T cell functions should also be treated with caution, as with any other leukemic cell lines. For instance, it was shown that Jurkat cells were defective in the expression of PTEN (phosphatase and tensin homologue), a tumor suppressor that regulates level of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) negatively by dephosphorylating it. This will result in the constitutive activation of phosphatidylinositol 3-kinase (PI3K) signaling pathway (Astoul et al, 2001; Shan et al, 2000).

Furthermore, long term culture of Jurkat cell has been linked with significant alteration in DNA fingerprinting profile resulting in heterogeneous sub-clones differing in up to eight STR (short tandem repeat) loci from parental culture (Parson et al, 2005).

Despite the upper mentioned drawbacks, Jurkat cell line remains a trusted model system; since the basic aspects of Ag-TCR signaling defined in Jurkat cells are similar in primary CD4+ T cells physiology (Zhang & Samelson, 2000). However, much hasn't been done on Jurkat cells regarding investigations of TLRs using the cell line as a model system. Caron et al (2005) have reported that PCR analysis on Jurkat cells revealed that they do not encode for TLR6, 8 & 10. A study aiming to investigate the regulation of TLR5 in T cells using Jurkat cell line as a model showed that stimulation of the cells with flagellin (TLR5 agonist) resulted in heightened IL-8 secretion. In this study, Jurkat stimulated with TLR5 agonist in the absence of serum showed an increased IL-8 secretion compared to Jurkat maintained in serum supplemented media. This inhibition of IL-8 secretion to TLR5 stimulation was attributed to the serum-borne bioactive lipid called sphingosine-1-phosphate (S1P) (Sharma et al, 2013). The TLR5 agonist flagellin was also found to be able to co-stimulate Jurkat cells treated with plate bound anti-CD3 and drive increased secretion of IL-2 (Ye & Gan, 2007). Such an increase in IL-2 secretion was not found when the cells were stimulated with TLR2 (Pam_3CSK_4) and TLR4 (LPS) ligands and they further described that mRNA expression was not detectable for TLR2 and TLR4 (Ye & Gan, 2007). This is contrary to (Caron et al, 2005) report that Jurkat cell line was shown to express mRNA for TLR1, 2 ,3,4,5,7 and 9. Another report for the role of TLR5 response on Jurkat came from Akhade and Qadri (2015) where they showed flagellin induces IL-8 secretion on Jurkat. But the secretion of IL-8 was minimal or absent when the cells were pre-activated

with plate coated anti-CD3 together with soluble anti-CD28.This is surprising result since Ye and Gan (2007) reported flagelin could only drive IL-2 production when used as a co-stimulant with plate-coated anti-CD3.

All in all, reports that utilize Jurkat as model to investigate the role of TLRs is quite scarce, and the studies done so far are more focused on the bacterial flagellin associated responses in Jurkat (Akhade & Qadri, 2015; Sharma et al, 2013; Sharma et al, 2016; Ye & Gan, 2007). Even if lack of TLR8 mRNA expression has been indicated in Jurkat cell (Caron et al, 2005), the same uncertainty about TLR8 expression pattern in CD4+ T cell exist. Hence, investigation of TLR8 expression in both primary CD4+ T cells and Jurkat cells remain crucial. Furthermore, it is of paramount importance to have a model system to understand the molecular mechanisms of viral nucleic acid sensing by TLR8 and characterization of innate immune signaling in the adaptive immune CD4+ T cell. Therefore, the expression of TLR8 in both primary CD4+ T cells and Jurkat cells should be more carefully assessed. These assessments together with functional assays of endosomal TLR induced immune responses help in determining if Jurkat cells can be considered a potential model system for the study of TLR8 mediated signaling in CD4+ cells and to understand the mechanisms behind sensing of endocytosed HIV particles by endosomal TLRs in CD4+ T cells.

2 OBJECTIVES

2.1 General Objective

CD4+ T cells are main targets of HIV infection. One of the routes for HIV infection is via cell-to-cell viral trans-infection, in which case the viral particles are endocytosed by CD4+ T cells. We hypothesize that nucleic acids from HIV particles entering the endosomes of CD4+ T cells might be sensed by endosomal TLRs. Such a sensing of nucleic acids from HIV by endosomal TLRs might induce activation and cytokine production and thus modulate CD4+ T cell mediated immune responses.

Working with a good cell model usually provides faster and more reproducible results. Overexpression or knockdown of a protein of interest is also relatively easier to achieve in cell lines. However, even if cell lines are useful in the study of molecular signaling systems of primary cells, they do not function identical to primary cells. A good characterization of a cell model and comparison to the features of primary cell is therefore important. Jurkat E6.1 is a leukemic CD4+ T cell line that has been widely used to study mechanisms in T cell functions and signaling. The overall aim of this project was to characterize the Jurkat E6.1 T cell line as a potential model system to study endosomal TLR (TLR7, 8 and 9) signaling and if findings made with the Jurkat T cell line show comparable results to studies with primary CD4+ T cells.

2.2 Specific Objectives

- Endosomal TLR expression in Jurkat E6.1 cells and primary CD4+ T cells
  - Determine the expression level of endosomal TLR7, 8 and 9 in Jurkat E6.1 cells, with an emphasis on TLR8: Compare the findings with primary CD4+ T cells
- Response of Jurkat E6.1 T cells to stimulation with endosomal TLR and TCR stimulation
  - Analyze the activation marker expression and cytokine production in Jurkat E6.1 cells in response to TLR7, 8 and 9 ligands as well as T cell receptor stimulation: Compare the findings with primary CD4+ T cell
- Generate TLR8 expressing or TLR8 negative Jurkat cell line
  - overexpress/knockdown the TLR8 gene in Jurkat E6.1 cell: depending on TLR8 expression status of wild type Jurkat E6.1 cell line.
  - Determine the effect of TLR8 mediated signaling in T cells by comparing between TLR8 overexpressing and TLR8 negative Jurkat cell lines.

3 METHODS

3.1 Cell culture and maintenance

3.1.1 Jurkat E6.1 T cells

The Human Jurkat T cell line (clone JE6.1) was obtained from The European Collection of Authenticated Cell Cultures (ECACC, UK, Cat No: 88042803) and cultured in Roswell Park Memorial Institute media (RPMI 1640, Sigma Aldrich, USA) supplemented with 10% Fetal Calf Serum (FCS), 2mM L-glutamine, 1% HEPES (Gibco™, life technologies) and 1% penicillin/streptomycin antibiotic. The cells were seeded in T75 culture flasks at a concentration of 1 x 10^5 cells/ml at 37°C in 5% CO_2 and split every 3 - 4 days. Splitting was always done after counting the cell concentration. The cells were counted and their viability checked using 0.4% Trypan Blue dye exclusion assay by; mixing 10µl cell suspension and 10µl of Trypan Blue then pipetting into disposable Countess™ chamber slide and analyzing in Countess™ Automated Cell Counter (Invitrogen). Live cells possess intact cell membrane that excludes the trypan blue dye, whereas dead cells do not and the Countess™ uses image analysis to automate cell counting and viability.

Stock cells were maintained by freezing down in liquid nitrogen at -196°C. For freezing down, Jurkat cells were resuspended at a concentration of $6x10^6$ cells/ml in freezing media and 1 ml aliquoted into cryogenic vial. The freezing media contained a 10% cryoprotectant Dimethyl Sulphoxide (DMSO) (Sigma® Life Science, USA) and an additional 10% FCS supplementation to the Jurkat media described above. To maintain cooling rate of 1°C/min, each vial was then placed in Nunc Cooler (Mr. Frosty, a plastic holder with propanol fluid-filled base coolant that insulates the container and gives a cooling rate of about 1°C/min in the ampules). The Nunc Cooler was then placed in -80°C for 24 hours and then transferred to liquid nitrogen (≈-196°C) for prolonged storage.

Resuscitation of frozen cells for culturing was done by quick thawing of vials in 37° water bath, followed by resuspending cells in 10 ml Jurkat media (described above) and centrifugation at 1,500 rpm for 5 minutes. Supernatant was then decanted and the cells were seeded in a T75 flask containing 20 ml media.

3.1.2 Other cell lines used in the project

U373 cells transfected with CD14 and TLR8, used as positive control for TLR8 analysis in western blot, were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FCS, 2mM L-gluthamine and the antibiotics Geneticin® (G418 sulfate) (select for CD14), Puromycin (select for TLR8) and Gentamycin as anti-contaminant and were incubated at 37°C in 8% CO_2.

HEK 293T cells maintained in DMEM supplemented with 10% FCS and 2mM L-glutamine were used as transfection packaging cells for the production of lentiviral particles for transduction of the Jurkat cells. HEK293T cells were incubated at 37°C in 5%CO_2 atmosphere in a T75 flask in the presence of 12ml of the complete media. Splitting of the adherent cells (U373 and HEK293T cells) was made after pipetting out the media and washing the cells gently with PBS. 3ml Trypsin-EDTA was then added to the culture flask followed by 3-minute incubation at 47°C. The effect of Trypsin was then neutralized by adding a complete medium in a 1:1 ratio. Appropriate volume of cells (containing roughly 10 x10^5 cells/ml) was then passaged to a new T-75 flask and complete growth medium added to a final volume of 12 ml. All experiments were done on cells passaged for less than 20 times.

3.1.3 Isolation of primary CD4+ T cells.

Untouched (negative) and positive isolated CD4+ T cells were purified from PBMC (Peripheral Blood Mononuclear Cells) according to the manufacturer's instructions using MACS human CD4+ T cell isolation kit and MACS human CD4 MicroBeads respectively (Miltenyi Biotec, Germany). During the negative isolation, the CD4+ T cells are isolated by targeting all the other non CD4+ T cells in PBMC by use of cocktail of biotin-conjugated monoclonal antibodies to magnetic beads. The non-target cells are then depleted by use of magnetic column (MACS® Colum) that retain all the non-target cells and lets through the purified CD4+ T cells. On the other hand, during positive isolation the CD4+ cells are magnetically labeled with CD4 MicroBeads and are retained in the magnetic column while all the other non-target cells run through. Then the retained purified CD4+ T cells are eluted by removing the column from the magnetic field (Miltenyi: human CD4+ isolation kit, and CD4 MicroBeads kit data sheet).

3.2 Cell stimulation

Cell surface marker expression and cytokine production of Jurkat E6.1 cells were assessed after cells have been stimulated by; plate bound anti-CD3 (1μg/mL) with soluble anti-CD28 (1μg/mL) antibodies, 1μg/mL of Phytohemagglutinin (PHA) or 100ng/ml of Phorbol12-Myristate 13-Acetate (PMA) + 1μg/mL Ionomycine. Generally, all of this stimulants can activate CD4+ T cells without the presence of antigen, unlike *in vivo* stimulation of T cells that requires signal one derived by a specific antigen presented in association with MHC. Anti-CD3 with anti-CD28 provides a more physiological response by crosslinking of TCR with antibody to the invariant signaling protein CD3 (anti-CD3) and crosslinking of the co-receptor CD28 with anti-CD28 antibody that initiates downstream signaling. PHA is a lectin that can bind to glycosylated proteins on cell surface resulting in crosslinking (agglutinating) of multiple cell surface glycoproteins thereby initiating cell activation and proliferation (Tiefenthaler & Hunig, 1989). Downstream signaling of activated T cells involves hydrolysis of phosphatidyl-inosytol 4,5-bisphoshpate (PIP_2) that produces inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 increases intracellular calcium (Ca^{++}) concentration and DAG activates protein kinase C (PKC)(Jenkins et al, 2001). PMA is DAG analogue that can directly activate PKC while the calcium ionophore ionomycin increases cytosolic Ca^{++} by increasing extracellular Ca^{++} influx, thus these two (PMA and Ionomycin), synergize to induce direct intracellular T cell activation (Chopra et al, 1987).

During anti-CD3/anti-CD28 stimulation of the cells, first 5μg/mL solution of anti-CD3 in Dulbecco's Phosphate Buffer Saline (DPBS) was prepared and 200uL of the antibody in DPBS solution was dispensed in polystyrene microplate surface and incubated at 37°C for 1hour to immobilize the ant-CD3 antibodies. Cells to be stimulated were added to the wells after DPBS solution was removed and the wells were rinsed two times with 800 μL of DPBS. Then 1μg/mL of anti-CD28 was added to the wells.

Furthermore, responses of the cells for TLR 7 ligands (CL264, 5 μg/mL or R837, 5 μg/mL), TLR8 ligand CL75 (5 μg/mL), and TLR9 ligand (CpG, 10μM,) were also investigated. The TLR8 ligand Oligoribonucleotide (ORN) polyU complexed with the polycationic polypeptide poly-L-arginine (pU/pLA) in the presence of buffered Opti-MEM® media at a ratio of 1:1:3 was also used to stimulate Jurkat cell (in our experiment 5μl of 1ug/μl pU + 5μl of 1μg/μl pLA + 15 μl of Opti-MEM® media was added into a 1ml of 5 x 10^5 Jurkat cells in a 24 well plate)

3.3 Flow cytometry

Phenotypic characterization of Jurkat E6.1 cells was assayed using flow cytometry technique. Flow cytometry is a technique of measuring properties of cells as they flow in fluid suspension across an illuminated light path. The basic principle of flow cytometry lies on the fact that passage of single suspension cells in hydrodynamically-focused stream of fluid in front of a laser can be detected, counted and sorted.

Cell components can be labeled with fluorochrome either directly or bound to an antibody that bind to cell component. Up on excitation by beam of laser, the flurochrome absorbs light energy from the laser and then releases some of the energy in the form of vibration and heat dissipation and the remaining as photon. This results in re-emission of absorbed photon at a lower energy, *i.e.* with a longer wavelength than the source (Fig. 3.1). Moreover, multiple parameters on a single cell can be analyzed simultaneously by use of different fluorochromes with different spectral characteristics that are tagged on range of antibodies targeting different markers on a cell.

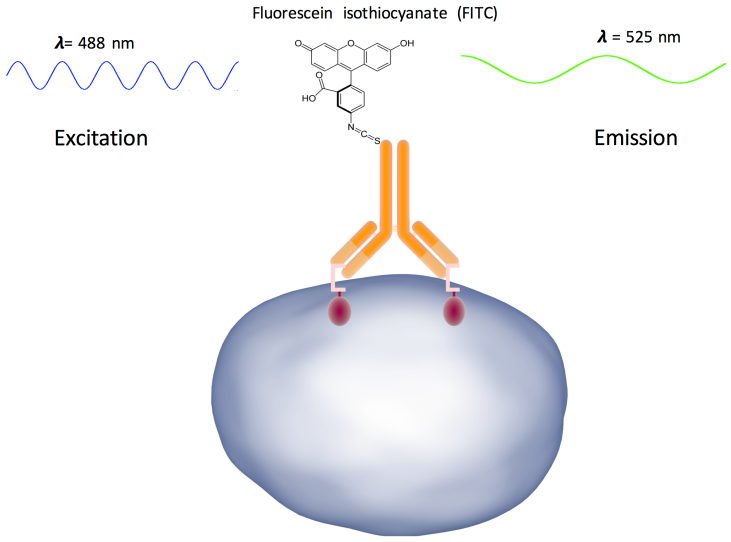


Figure 3.1: Schematic representation of fluorochrome excitation. Fluorescein ispthiocyanate (FITC) fluorochrome attached to an antibody that in turn is directed to specific cell surface protein. Illumination source excites FITC at 488nm and FITC in turn has emission peak at 525nm that is detected. Figure created by author.

In this project, cells were acquired for the characterization of cell surface markers and activation molecules using BD LSR II flow cytometer (**BD** Biosciences, San Jose, CA, USA) with FACSDiva 6.1 software (BD Biosciences), and then the data were analyzed with FlowJo 10.1 (TreeStar Inc).

The BD LSR II used has been fitted with 4 LASER (Light Amplification by Stimulated Emission of Radiation) sources; blue (488nm), red (640 nm), yellow-green (561nm) and violet (405nm) lasers. For each laser source there is in turn 3 or more user configurable detection channels, thus with a potential ability of detecting more that 12 colors simultaneously. The blue laser has 4 detection channels, the red 3, yellow-green 5 and the violet 5 detection channels. These detectors in turn collect and convert the fluorescence signals into electronic signals.

Sample preparation involved platting of cells at a concentration of 1 x 10^5 cells/ml in 24 wells plate under different experimental conditions and time points (section 3.2). The cells were then harvested and centrifuged at 1,500 rpm for 5 minutes and the media discarded. The pellet was resuspended in 2ml PBS supplemented with 2% FCS (Flow washing buffer) and centrifuged at 1500 rpm for 5 minutes and decant the flow wash buffer. Cell pellets were then stained with antibodies against surface markers of interest for 15 minutes on ice in dark. Unbound antibodies were washed using flow wash buffer and the cells were ready for acquisition using the BD LSRII.

The cell markers, either as constitutively expressed or stimulated, investigated in this project include CD3, CD4, CXCR4, CCR5, CD25, CD40L, CD69, HLADR (MHC II). The fluorochrome conjugated anti-human monoclonal antibodies used against these markers include: CD3-FITC, CD4-BV711, CD25-BV510, CD69-PE, CD40L-BV605 and HLADR-BV785.

3.4 Western immunoblotting for TLR7, 8 and 9 expression

TLR7, 8 and 9 protein expression was assessed by western blotting from cell lysates. Here proteins are denatured and reduced with lithium dodecyl sulfate (LDS) in presence of dithiothreitol (DTT) and are separated according to their molecular weight by electrophoresing them on separating gel (10 % NuPage Novex Bis-Tris, Life technologies), then blotted on to nitrocellulose paper where specific antibodies for detection were added to target proteins.

Cells untreated, stimulated with 1μg/μl of PHA with or without TLR7, 8 and 9 ligands were seeded at 1x10^6/ml into 24 well plate. After 24-hour incubation cell pellet was collected after centrifugation at 1000 rpm for 7 minutes and washed with 500ul PBS. Cells were lysed using urea lysis buffer (appendix: 5) in ice with intermittent vortexing every 5 minutes for 15minutes. Supernatant cell lysate was collected into Ependorf tube after the centrifugation of the cells treated with lysis buffer at 10,000 rpm for 20 min at 4 °C.

Bio-Rad colorimetric protein assay technique was used to determine the total protein concentration of the cell lysates. The assay was based on color change in response to various concentrations of protein. Here the Bio-Rad protein assay dye reagent (BIORAD Laboratories, Hercules, CA) diluted 1:5 in in ddH_2O was used as the colorimetric reagent for protein concentration determination. Protein lysate (1 µl) was dispensed to 1ml of the diluted Bio-Rad solution and incubated at room temperature in dark for 10 minutes. The absorbance in the samples was measured at 595 nm. Protein concentration was calculated by multiplying the absorbance value by 19 (a factor derived from standard curve developed from serial measurement of BSA at different concentrations.)

All samples were brought to a final concentration of 50 µg total protein in 20 µl final volume in a fresh Ependorf tube (For samples with high protein concentration lysis buffer solution was used to equilibrate them at 50µg in 20µl). 10 µl of NuPage LDS sample buffer 4X containing 0.1M DDT (Invitrogen, USA) was added to the samples and was heated for 10 minutes at 70°C using a heat block. Hence, LDS, DTT, and heat are responsible for the actual denaturation of the sample. 30µl of the solution (the sample with the sample buffer) was loaded to 10 % NuPage Novex Bis-Tris Gel immersed in 1X NuPAGE MOPS SDS Running buffer. Each run was accompanied by parallel running of the standard ladders SeeBlue standard marker (5µl) (Life technologies, USA) and 1 µl of MagicMark XP Western Protein Standard (Life technologies, USA). The ladders were later used for the determination of the molecular weight of the protein of interest.

Electrophoresis running conditions were set at 100V for the first 30 minutes followed by 150V for 90 minutes. The separated protein fragments on the gel electrophoresis are then blotted onto nitrocellulose membrane using Invitrogen's iBlot dry blotting system by applying 20V horizontally for 9 minutes. The blotted nitrocellulose membrane is wetted by immersing it in Tris Buffered Saline-with Tween-20 (TBS-T) for a minute. Before addition of specific antibodies to proteins of interest, the whole surface of the membrane was blocked to prevent nonspecific binding of antibodies and creating background noise. Blocking was achieved by incubation of the membrane for 1 hour under agitation in 5 % bovine serum albumin (BSA) in TBS-T. Excess blocking buffer was then rinsed of by washing the membrane by TBS-T for 5. Rabbit monoclonal antibody against TLR 8 (Cell signaling Technologies, USA, Ref. No. 11886) diluted 1:1000 with 5% BSA in TBS-T was used as a primary antibody. The membrane was incubated with the primary antibody overnight at 4°C under agitation, then excess unbound primary antibody was washed three times, for 5 minutes each, with TBS-T under agitation. The

membrane was then incubated with Horseradish Peroxidase (HRP)-conjugated swine-anti-rabbit secondary antibody (Deko, Denmark) (diluted 1:4000 in 5% BSA in TBS-T) for an hour under agitation and then washed three times with TBS-T for 5 minutes each under agitation. SuperSignal® West Femto Maximum Sensitivity Substrate (ThermoFisher, USA), chemiluminescent substrate was used against the HRP enzyme conjugated secondary antibodies. After incubation of the membrane with the Supersignal substrate for 3 minutes, images were developed and analyzed using Odyssey® FC Dual–Mode Western Imaging (Li-COR Biosciences, UK) system at the chemiluminescence channel.

The constitutively expressed loading control β-actin was used to confirm that protein loading was the same across the gel and also to normalize the levels of protein detected. Here, initial washing of the membrane 3 times, 5 minutes each in TBS-T was done under agitation and incubated with mouse anti-β-actin antibody (sigma Aldrich, USA) (diluted with TBS-T at 1:5000) for 1 hour under agitation. The membrane was washed three times with TBS-T and incubated with goat anti-mouse HRP conjugated secondary antibody (Dako, Denmark) diluted in TBS-T at a ratio of 1: 5,000. Image was then developed after incubation with Supersignal substrate for 3 minutes using the chemiluminescent channel.

3.5 RNA isolation and real-time-RT-PCR

Real-time reverse transcriptase Polymerase Chain Reaction (qPCR) was performed with the aim of investigating the expression of mRNA for TLR 7, 8, and 9 as well as cytokines such as IL-2, IL-8, TNF-α, IL6, IFN-α in Jurkat E6.1 T cells and primary CD4+ T cells. This process starts with lysing of the cells and extracting purified RNA followed by synthesizing cDNA and performing polymerase chain reaction (PCR) to quantify the expression level of a gene of interest.

Cells (1×10^6), unstimulated or stimulated by different stimulants, were harvested from 24 well plate and centrifuged at 1,500 rpm for 6 minutes. Cell pellets were lysed with 350µl of RLT lysis buffer (Qiagen, Germany) in the presence of 1% beta-mercaptoethanol and stored at -80°C until RNA extraction. Total cellular RNA was isolated by using Rneasy mini kit (Qiagen, Germany) using QIAcube (Qiagen) automated robotic system system. Total RNA concentration and purity was determined using NanoDrop® ND-1000 spectrophotometer (Saveen Werner, Sweden).

High Capacity RNA-to-cDNA kit from Applied Biosystems was used to synthesize cDNA. Amount of less than or equal to 2 µg of the total RNA from each sample was subjected to

reverse transcription using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's protocol (Appendix: 1.1) in MicroAmp® Fast 8-tube strip 0.1ml vials with cap (Applied Biosystems). The C1000™ Thermal Cycler (Bio Rad) set up at 37°C for 1hr, 95°C for 5 minutes and finally 4°C indefinitely was used to synthesize cDNA. Final concentration of the synthesized cDNA was adjusted to 1ng/ul using sterile ion filtered water assuming that the total cDNA concentration equals total RNA concentration determined earlier.

3.5.1 TaqMan® assay

The real-time reverse transcriptase-PCR (qPCR) assay was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems) to detect GAPDH, TLR7, TLR8, TLR9, IL2, IL8, TNF-α, and IFN-α by TaqMan gene expression assays. Each PCR reaction occurred in 20µl containing 10 ul of PerfeCTa qPCR fast mix, UNG, Low ROX, (Quanta BioSciences), 1µl of TaqMan® primers/probes custom designed to amplify mRNAs for TLR7,8,9, IL-8, TNF or IFN-α (Applied Biosystems) and 9ul of 1ng/µl of cDNA. Quantification of mRNA for GAPDH (primer/probe from Applied Biosystems) was performed as a housekeeping gene and used to correct for variations in cDNA content among samples. Reaction cycle set up used was 45°C for 2 minutes, 95°C for 30seconds of holding stage, and 95°C for 1 sec and 60°C for 20 seconds for 40 cycles.

Data from real-time TR-PCR experiments were analyzed using relative quantification (Rq)-delta delta Ct method. Here, initially the ΔCt (delta cycle threshold) values of each target transcripts were determined by subtracting the Ct value of housekeeping gene from the target gene. Then the difference of ΔCt of a target transcript in treatment group to that a ΔCt of another untreated control sample (*i.e.* ΔΔCt) was used to determine the relative quantity of the target transcript using the $2^{-\Delta\Delta Ct}$ as described elsewhere (Livak & Schmittgen, 2001).

3.5.2 SYBRgreen assay

Real-time RT-PCR using SYBRgreen assay system was also performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems) for assessment of, TBP and TLR8 expression in Transduced/transfected cells. The Taqman probe is customized to target a region of TLR8 gene which is not included in the plasmid constract we used for Transduction/transfection experiment. The forward and reverse primers used for SYBRgreen qPCR amplification are 5'-GTTGGAACTACACGGAAACC-3' & 5'-GGACTGGCACAAATGACATC-3' respectively. These primer sequences were designed from NCBI reference sequence, NM-138636 and were

a kind gift from Lobke Gierman (postdoc, CEMIR). The reaction was set up at 95°C for 5 min holding stage, then 40 cycles at 95° for 5 seconds, 61°C for 10 seconds and 72°C for 8 seconds. Delta Ct values were calculated from the house keeping gene TBP which was run in the same assay.

3.6 Cytokine analysis using ELISA

IL-6, TNF-α and IL-8 secretion to culture medium were measured using paired antibodies Enzyme linked immunosorbent assays (ELISA) (Sandwich ELISA technique (Fig. 3.2)) from DuoSet® sandwich ELISA Development System (R&D Systems, Inc.) according to the manufacturer's instruction.

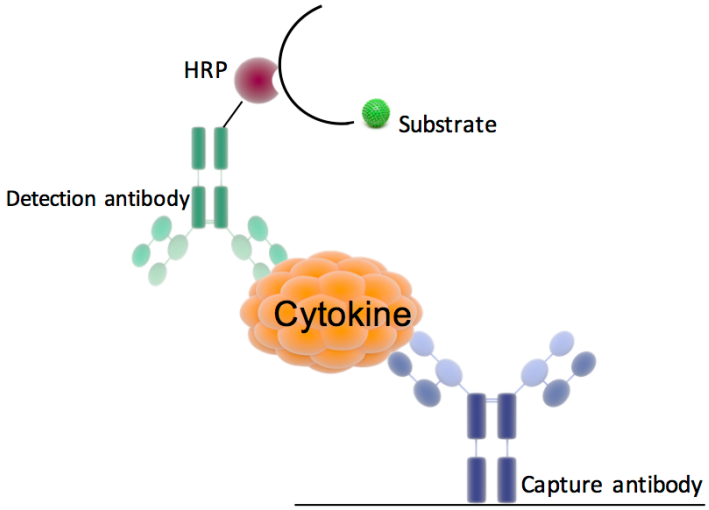


Figure 3.2: Schematic overview of Sandwich ELISA.
The cytokine of interest is immobilized by use of a capture antibody. The detection of the cytokine is then made possible by use of a biotinyilated detection antibody which then binds to HRP enzyme conjugated to streptavidin. Substrate oxidization is then detected by absorbance change using spectrophotometer. Figure created by author.

The human IL-6 and IL-8 kit (DuoSet® R&D Systems, Cat #: DY206 & DY208 respectively) were used. In general, the half volume 96 well ELISA microplates (Coster® Assay plate, 96 well half area) were coated with 50 µl of appropriate concentration of Capture Antibody for the cytokine of interest overnight at room temperature (RT). The plate was washed with 0.05% Tween in PBS three times using ELISA plate washer. Unspecific binding was then blocked using 150 µl of 1% BSA in PBS in each well and incubated for a minimum of 1 hour at RT. Plate was washed 3 times as described above and 50 µl of standards (7 point standard curve using 2-fold serial dilution in 1% BSA/PBS with starting highest standard concentration of

600ρg/ml for IL-6 and 2000ρg/ml for IL-8 as described on product manual), 1:2 diluted samples (in 1% BSA/PBS) and 1% BSA in PBS as blank were added to appropriate wells then incubated for 2 hours at RT. Then after washing three times, 50 μl of appropriate concentration of secondary (Detection antibody) was added to each well and incubated for 2 hours at RT. Three times washing was then repeated and 50μl of working dilution of Streptavidin-HRP was added to each well and incubated at RT in dark. After washing 3 times, 50μl of Tetramethylbenzidine (TMB) substrate solution was added to each well and incubated for 20 minutes at RT in dark. TMB is the substrate for streptavidin attached HRP (horseradish peroxidase). In the presence of the peroxidase activity TMB develops a blue reaction product. Since it is sandwich endpoint assay the reaction is stopped by addition of 25μl acid solution (1M H_2SO_4) forming a yellow reaction product. The plate was gently tapped to ensure thorough mixing. Optical Density (OD) of each well was measured using a microplate reader (iMark™ Microplate Reader, Bio Rad) set at wavelength of 450ηm. Correction for optical imperfections was made by taking another absorbance at 595ηm and subtracting it for the 450ηm measure.

3.6.1 Multiplex ELISA

Cytokine profile of Jurkat cells were determined without or after stimulation of the cells with the endosomal TLR ligands (CL264 (5ug/ml), CL75 (5ug/ml), R848 (5ug/ml), pU/pLA (5ug/ml), CpG (10uM)) or anti-CD3 with anti-CD28 antibody (1ug/ml) for 24 hours. Supernatant was collected and cytokine concentration determined using a 34plex human cytokine and chemokine multiplex ELISA kit (ProcartaPlex® Multiplex Immunoassay Panel, Affymetrix eBioscience, Cat. # EPX340-12167-901). Data was acquired by use of Luminex® xMAP® technology in Bio-Plex 200 system (Biorad) and rusults analyzed with the software in the system. The assay is basically similar to sandwich ELISA technique but uses a specialized dual laser flow cytometer like instrument detection system. Initially, different luminex beads each with its own spectral signature are created and attached to capture antibodies for a specific protein (analyte) from a sample liquid such as cell culture supernatant. Then different biotinylated detection antibodies and streptavidin-conjugated phycoerythrin substrate is added to the bead-immuno complex before analysis. The individual unique beads act like the specific wells in a traditional ELISA and allow the simultaneous assessment of a range of proteins at the same time. In this project 34plex kit used targets 34 different cytokines simultaneously. The list of cytokines targeted by the kit is presented in appendix 6.

3.7 Transfection and transduction of TLR8 gene into Jurkat cells

Transfection is process of introducing foreign nucleic acid into cells with the aim of generating phenotypic changes that are of interest for further investigation. A number of chemical and physical techniques have been developed to deliver genetic material into eukaryotic cells. Commonly used methods include, but not limited to, calcium phosphate precipitation, liposome-mediated transfer (lipofection), electroporation or viral methods (Hamm et al, 2002; Maurisse et al, 2010). The subsequent expression, that is transcription of the introduced gene with further production of protein is facilitated by use of expression vectors that carry our nucleic acid of interest to be delivered into cells. These vectors can be divided into two categories: plasmid vectors and viral vectors. When viral vectors are used the process is called transduction (Colosimo et al, 2000). In this project the Nucleofection ® (electrophoration) (Amaxa, Gaithersburg, MD) system using plasmid expression vector and the lentiviral system using viral vector have been used to transfect or transduce respectively, TLR 8 gene into Jurkat E6.1 T cells.

The plasmid expression vector used here was pLenti CMV PURO Dest w118-1 (Addgene). The plasmid contains ampicillin resistant loci for bacterial selection and also puromycin resistant loci for eukaryotic cell selection. TLR8 open reading frame cloned into the pLentiCMV PURO Dest w118-1expression vector was obtained from former master's student at CEMIR, I.C. Schrøder (2015) stored as glycerol stock in *E. coli* DH5-α at -80°C. Initially the bacteria containing the plasmid (both the empty back bone and TLR8 recombined) were cultured in LB-medium containing 100 μg/ml ampicillin, to which the plasmid containing bacteria is resistant, overnight at 37° C under agitation in round bottomed Falcon™ tube. On the next day, plasmid was purified from both sets of the bacteria culture (the empty back bone plasmid and TLR8 recombined plasmid) using the alternative PureYield plasmid miniprep protocol from Promega® (Appendix: 1.2). Then the concentration of purified plasmid was quantified using NanoDrop ND-1000 (Saveen Werner) and was ready for use in both Nucleofection® and Lentiviral transduction experiment.

However, before commencing with the Nucleofection® and the Lentiviral transduction experiments, puromycin cell killing curve for Jurkat E6.1 cell, and cell proliferation assay at different concentration of puromycin was done as described below.

3.7.1 Puromycin titer for selection of TLR8 transfected/transduced Cells

The pLenti CMV PURO Dest w118-1 expression plasmid used for transfection and transduction contains gene expressing puromycin resistance for eukaryote cells. Hence, puromycin resistance was utilized to select for cells that have successfully integrated TLR8 gene. Here, the minimum concentration of puromycin that is required to kill the un-transfected Jurkat E6.1 cells was determined by performing kill curve experiment. First 5 x 10^5 cells were plated into 24 well plate. The next day, the culture medium was replaced by medium containing a range of puromycin concentration; 0 μg/ml, 0.1 μg/ml, 0.25μg/ml, 0.5μg/ml, 1ug/ml, 2μg/ml. Cell count and viability was checked using Countess™ Automated Cell Counter (Invitrogen) by Tryphan blue dye exclusion assay after 72 hours of incubation at 37°C, 5% CO_2 incubator; and media was also changed every 3 days. The minimum concentration of puromycin that killed the cells were selected for use with the selection of TLR 8 transfected/transduced cells.

3.7.2 Cell Proliferation assay to identify optimal puromycin titer

Puromycin surviving and proliferating cells were also assessed by using CellTiter 96® AQueos One Solution Cell Proliferation Assay (Promega, USA) colorimetric method. Briefly, 5 x 10^4 cells were seeded in 100 μl media in flat bottomed 96 well plate in the presence of increasing concentration of puromycin (0 μg/ml, 0.1 μg/ml, 0.25 μg/ml, 0.5 μg/ml, 1μg/ml, 2 μg/ml) over night. Then 20 μl of Cell CellTiter 96® AQueos One Solution Reagent was pipetted into the wells and incubated at 37° C, 5% CO_2 atmosphere. Five absorbance reads were made every hour at 490 nm using a 96-well plate reader (iMark™ Microplate Reader, Bio Rad).

Here the principle is that Cell CellTiter 96® AQueos One Solution Reagent contains tetrazolium compound (MTS) and an electron coupling reagent (Phenazine ethosulfate; PES) which is bioreduced by actively proliferating cells into a colored formazan product that is measured at an absorbance of 490nm and is directly proportional to the number of living cells in culture.

3.7.3 Nucleofection transfection method to transfect Jurkat cell with TLR8 gene

Nucleofection® is an electroporation-based technique equipped with company custom made unique electrical parameters and buffer solution for specific cell type. The technique is able to deliver plasmid DNA or other gene of interest directly into target cell nucleolus. Hence, transfection of cells using this method is not cell division dependent (Aluigi et al, 2006). The Nucleofactor™ technology is reported to have high transfection efficiency both on primary and transformed cell line (Maurisse et al, 2010; T O'Neil et al, 2015).

Here, the Amaxa® Nucleofactor® Kit for Jurkat E6.1 cells (Catalogue # VPA-1003) was used following the manufacturers manual. Jurkat, 1 x 10^6 cells, were transfected with 2ug of pLenti CMV PURO Dest w118-1 expression plasmid recombined with or without TLR8 using the Nucleofactor® solution for Jurkat E6.1 and the program X-005 on the Nucleofactor® II Device. At the same time, Jurkat cells were transfected with pmaxGFP® Vector as a positive control. Each of the nucleofected cells were then transferred to a 12-well plate in total volume of 1.5ml media and incubated for 24 hours at 37° C at humidified 5% CO_2 atmosphere.

The pmaxGFP transfected cells were analyzed 24 hours post Nucleofection by flow cytometry. Untransfected and pmaxGFP transfected (500 μl from each) were sampled to determine transfection efficiency and viability. 1ul of Propidium Iodide (PI) (1:10 diluted in PBS) was added to both the tube just before the run. Viable and intact cells will not internalize PI and hence will not emit light at the PE channel (excitation maximum: 535nm, emission maximum 617nm). At the same time, efficiently transfected cells with pmaxGFP are expected to have green fluorescence at FITC channel compared to un-transfected cell.

3.7.4 Lentiviral system to transduce Jurkat cells with TLR8 gene

Retroviruses are very important tools for the transduction of a target cell, resulting in the integration of the foreign gene into target cell's genome. This process requires cell division for the integration of viral DNA into target cell. Subsets of retroviral vectors called lentiviral vectors (including HIV-1) have been very instrumental since they do not depend on cell division for completion of their replicative cycle and hence can transduce target cells regardless of cell cycle stage, (Naldini et al, 1996; Reiser et al, 1996).

Safety of handling lentivirus has been guaranteed by use of third-generation vector system. This is achieved by disintegration of HIV-1 genetic material into three different plasmid packages each with no virulence potential (Shearer & Saunders, 2015). Decreasing the probability of creating replication-competent lentivirus in target cell is achieved by deletion of segment from 3' long terminal repeat (3'-LTR) region of the transfer plasmid vector that contains sequences encoding for enhancer and promoter functions. This deletion does not affect generation of viral genome in the producer cell line, but results in "self-inactivation" of the lentivirus after transduction of the target cell (Yu et al, 1986). Therefore, once integrated into the transduced target cell, the lentiviral genome is no longer capable of producing packageable viral genome, hence no new replication-competent virus can be produced from transduced target cells (Yu et al, 1986).

In this project, the packaging system is split into three plasmid-DNA constructs (3rd generation lentiviral system); the packaging plasmid pMDLg/pRRE (Addgene®, #12251) contain Gag and Pol genes that codes for structural proteins and viral integration enzymes such as reverse transcriptase and integrase respectively; while the other packaging plasmid pRSV-REV (Addgene® #12253) codes for REV which is involved in nuclear export of provirus. The third is an envelop plasmid pMD2.G (ADDgene® #12259) and codes for the pseudotyped G glycoprotein from vesicular stomatitis virus (VSV-G) protein coat. VSV-G envelope is not HIV derived and is known to have a vast repertoire of cells that it can bind compared to HIV-envelop, and also known to enhance transfection (Okimoto et al, 2001). The transduction of target cells (Jurkat E6.1 T cells) was made with the three mentioned plasmids together with TLR8 recombined expression plasmid vector, pLenti CMV PURO Dest w118-1 (Addgene® #17452).

In short, 25 µl of OneShot *E. coli* DH5α competent cells were transformed by the three lentiviral packaging plasmids described above following a protocol described elsewhere (Untergasser, 2008). Then, 100 µl of transformed competent cells were plated on LB plate overnight at 37°C incubator. Three colonies, representing 3 biological repeats were selected the next day and each inoculated into 5ml LB-media and incubated at 37° under agitation overnight, then PureYield plasmid miniprep alternative protocol (Appendix 1.2) was used to purify the plasmid of interest and plasmid concentration determined using NanoDrop ND-1000. In parallel, TLR8 expression vector plasmid, which was already stored as glycerol stock in *E. coli* DH5α, competent was thawed, inoculated into 5 ml LB-media and incubated at 37° under agitation overnight and purified using PureYield plasmid miniprep alternative protocol.

Transfection of the packaging cells (HEK293T Cells) was carried out by GeneJuice® transfection reagent (Novagen) according to the manufacturer's instruction (Protocol Appendix-1.3). In short, 1 x 10^6 HEK293T cells per well in 6 well plate were cultured in 2 ml media 24 hours before transfection; to ensure exponential growth and 80% confluence. For each well, 100 µl of serum and antibiotic free DMEM media was mixed with 3µl GeneJuice transfection reagent (Novagen) and incubated for 5 minutes at room temperature. Cocktail of plasmid made to a final concentration of 1ug total DNA (Plasmid proportions of 4:2:1:1; pLenti CMV puro Dest w118-1 with/without TLR8, pMDLg/pRRE, pRSV-REV and pMD2.G respectively) was added to the GeneJuice reagent/serum-free medium mixture and mixed by gentle pipetting and incubated for 15 minutes at room temperature. The 24hr media was taken out from the 6 well plates and replaced by serum and antibiotic free DMEM media (1ml to each well) and transfected with the cocktail preparation. Four hours post transfection, the wells were

supplemented with 20% FCS and 2% Penicillin-streptomycin containing DMEM media (1 ml to each well). After 48 hours, the supernatant was harvested for viral particles, stored at -4°C and was replaced by 2ml fresh media (DMEM with 10% FCS and 1% Penicillin-Streptomycin), then 24 hours later the supernatant was again harvested and pooled together with the 48-hour time point harvest and the cell pellet discarded. The lentiviral stock was concentrated using the Lenti-X™ Concentrator (Clontech) following product protocol (Appendix:1.4).

Transduction of Jurkat E6.1T cells by the concentrated lentiviral stock was carried out in the presence of polybrene. Polybrene is a cationic polymer known to increase retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes (Davis et al, 2002).

For the transduction, 5 x 10^5 Jurkat cells were centrifuged at 1500 rpm for 5 minutes in 6 x 15 ml conical tubes. The pellets were then resuspended in 200ul of viral concentrate plus 300µl of RPMI + 2mM L-glutamine and 1% HEPES (without serum nor antibiotic). Polybrene at concentration of 6ug/ml was included in the media. Here, the cell pellets in the 6 tubes were resuspended according to the 6 different conditions:

1. Cells only: resuspended using 500ul serum free media without any viral construct
2. Plasmid only: resuspended using 200ul of viral concentrate containing all the packaging plasmids plus 300ul of media
3. GFP-tagged: resuspended using 200ul of viral concentrate containing GFP-tagged GAPDH plasmid construct plus 300ul of media
4. TLR8A (Biological repeat 1): resuspended using 200ul of viral concentrate containing TLR8 recombined plasmid construct plus 300ul of media
5. TLR8B (Biological repeat 2): resuspended using 200ul of viral concentrate containing TLR8 recombined plasmid construct plus 300ul of media
6. TLR8C (Biological repeat 3): resuspended using 200ul of viral concentrate containing TLR8 recombined plasmid construct plus 300ul of media

The fluorescently tagged positive control, GFP-GAPDH gene containing viral concentrate, was kindly provided by Zekarias Ginbot, NTNU. The resuspended cells were then seeded to 12 well plate and incubated at 37°C in 5% CO_2 atmosphere. Four hours later, the cells were supplemented with 500ul of RPMI + 2mM L-glutamine, 1% HEPES plus 20% FCS and 2% Penicillin/streptomycin. After 48 hours, the media was changed by centrifuging the content of the wells at 1500rpm for 5 minutes and re-suspending the cells with Jurkat complete media (RPMI +2mM L-glutamine, 1% HEPES plus 10% FCS and 1% pen/strep) with 0.5ug/ml

puromycin to select for transduced cells. Three days later (72hr), the media was again changed with puromycin concentration of 0.5ug/ml. The GFP-GAPDH visual positive control was examined and pictures taken after 7 of transduction using EVOS® FL Auto Imaging System (Life Technologies).

The overall overview of the lentiviral transduction process is depicted in fig. 3.3.

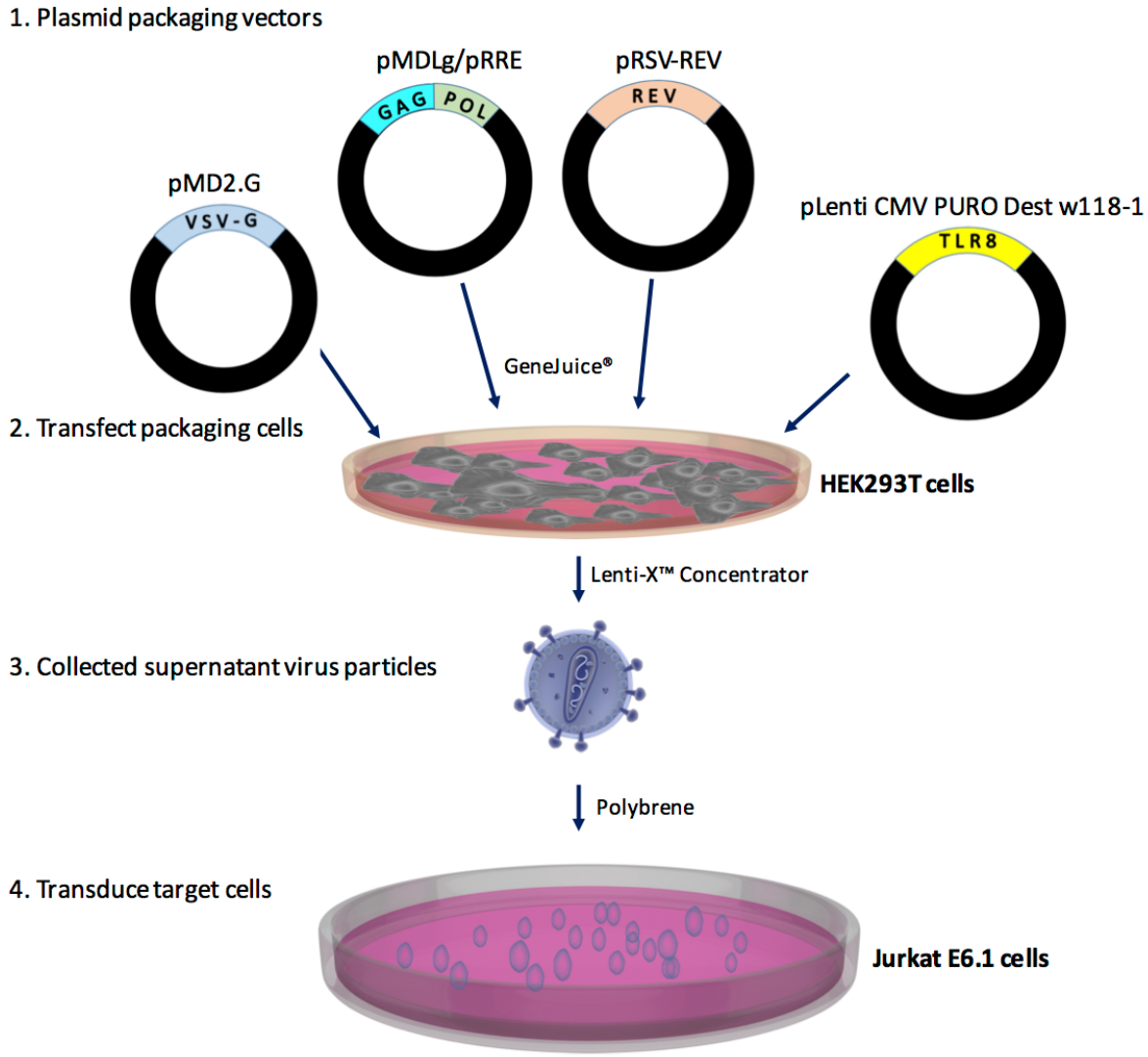


Figure 3.3:Third-generation Lentiviral system for transduction of Jurkat cells overview.
Essential viral genes were split into three packaging vectors; pMD2.G encodes pseudotyped VSV-G envelope protein, pMDLg/pRRE encodes Gag and Pol polyproteins, pRSV-REV encodes REV. The expression vector pLenti CMV PURO Dest w118-1 is cloned with TLR8 gene. The four plasmids were transfected into using HEK293T cells using GeneJuice®; at 48hr and 72hr viral particles were harvested from the supernatant, and stocked using Lenti-X™ concentrator and are used to transduce the Jurkat E6.1 target cells in the presence of Polybrene to enhance viral delivery into cell. Figure created by author.

3.8 Flowsight® Imaging Flow cytometry to asses nuclear translocation of NF-κB

Nuclear translocation of transcription factors is difficult to analyze by flow cytometry since this does not give information regarding cytosolic or nuclear localization of a protein. Accurate and easier investigation of nuclear translocation can be done with microscopy techniques. However, microscopy is a low-throughput method and quantification of results is quite difficult. Microscopic analysis is also more challenging for suspension cells such as Jurkat cells. Imaging flow cytometry combines flow cytometry and microscopy by taking a microscopy image of each cell that is analyzed with flow cytometric method. Thus imaging flow cytometery allows analysis of parameters that cannot be analyzed by normal flow-cytometer such as nuclear translocation, endocytosis, co-localization, and cell shape.

Nuclear translocation of NF-κB was analyzed using Amnis® FlowSight®; it is an imaging flow cytometer that combines a method of tracking moving cells with high resolution multispectral imaging system to acquire multiple images of each cell by use of Charge-Coupled Device (CCD) camera (Basiji et al, 2007). In this project, $5x10^5$ Jurkat cells unstimulated, stimulated with TLR8 ligand CL75 (5ug/ml) for 4 and 24 hours, or stimulated with anti-CD3 and anti-CD28 (1ug/ml) were washed by flow wash buffer, fixed and permeabilized following a method developed in our lab. Cells were then stained with anti-human NF-κB-FITC (ACS10000 kit, EMD Milipore). Prior to acquisition of the cells, Hoechst® (diluted 1:5,000) was added to stain the nuclear DNA. During analysis, 20,000 events were acquired with Flowsigh® (Amnis, EMD Millipore) instrument using Channel 2 to acquire FITC labelled anti human NF-κB and channel 7 to acquire Hoechst nuclear staining. Compensation for the samples and data analysis was done using IDEAS® software. NF-κB translocation to the nucleus was analayzed using the nuclear translocation application wizard for Jurkat cell treated with CL75 or anti CD3/antiCD28.

4 RESULTS

The Jurkat T cell leukemia line has been an important model cell line in the study of TCR signaling and in HIV infection studies (Cervantes-Acosta et al, 2001; Weiss et al, 1984). In this project, we aim to address if the Jurkat E6.1 T cell line is a suitable model to study the role of endosomal TLRs in sensing viral derived nucleic acids and synthetic ligands in CD4+ T cells. However, information regarding the general features of Jurkat cells, such as surface marker expression, activation markers and cytokine profile, is not complete. There is also limited information regarding expression pattern of TLRs in Jurkat cells.

Therefore, this projected started with determination for the expression level of endosomal TLR 7, 8 and 9 in both primary CD4+ T cell and Jurkat cell line using qPCR and western blotting methods. This was followed by a flow cytometric characterization of cell surface marker expression on Jurkat cells in comparison to primary CD4+ T cells. Of special interests were the analysis for the expression of the T cell activation markers CD25, CD40L and CD69 upon stimulation with T cell activators and TLR ligands.

4.1 Expression of endosomal TLR 7, 8 and 9 in Jurkat cells and primary CD4+ T cells

TLR7 and TLR9 gene expression in primary CD4+ T cells and Jurkat cell have been reported (Caron et al, 2005). However, studies about the expression of TLR8 in primary CD4+ T cells is contradicting. Here we investigated for the expression of these endosomal TLRs in primary CD4+ T cells and Jurkat cells. Determination of the expression level for TLR7, 8 and 9 genes was done on both primary CD4+ T cells and Jurkat cells using qPCR. Furthermore, western blotting was used to determine the protein expression of TLR8 in primary CD4+ T cells and Jurkat cells.

Using western blot analysis to determine TLR8 protein expression, we found that both the full length (140kDa) and the cleaved (80kDa) TLR8 were evident on primary CD4+ T cells. (Fig 4.1a). These CD4+ T cells were obtained by a negative ("untouched") isolation procedure using magnetic beads and were greater than 96% pure (Appendix 3). Of note here is that, our cell preparation was free from CD14+ monocytes (less than 0.6%), which are known to express abundant TLR8. Purity of isolated CD4+ T cells increases to more than 99% when the positive isolation method was utilized. Western blot analysis of TLR8 was then made on both positively and negatively isolated CD4+ T cells to check if method of preparation of CD4+ T cells

influence on TLR8 expression level. The protein for TLR8 was detected both on the positively and negatively isolated CD4+ T cells thus confirming that the TLR8 expression observed on the negatively isolated CD4+ T cells is not a result of contamination (Fig 4.1e).

The protein level expression of TLR8 by CD4+ T cells was also confirmed at mRNA level using PCR. Furthermore, mRNA for TLR7 and 9 were also detected on the primary CD4+ T cells. Generally, the expression pattern of TLR7, 8 and 9 was not or minimally influenced by stimulation of primary CD4+ T cells by plate bound anti-CD3 and soluble anti-CD28 in the presence or absence of TLR7, 8 and 9 agonists (Fig 4.1c).

On the other hand, we found that Jurkat cell lack TLR8 protein expression when examined using western blotting (Fig 4.1b). Consistent result was obtained at gene expression level, where mRNA for TLR8 was completely absent or undetermined using qPCR. On the other hand, expression for mRNA of TLR7 and 9 were detected on Jurkat cells. Furthermore, expression of TLR8 mRNA is not induced after stimulation of the cells by PHA for 24 hours. Treatment of the cells with TLR7, 8 and 9 agonists also did not induce TLR8 nor alter the expression pattern of TLR 7 and 9 (Fig 4.1d). Therefore, we concluded that the Jurkat cell clone we have is a natural knockout for TLR8 as the evidences are confirmed at both protein level and mRNA level.

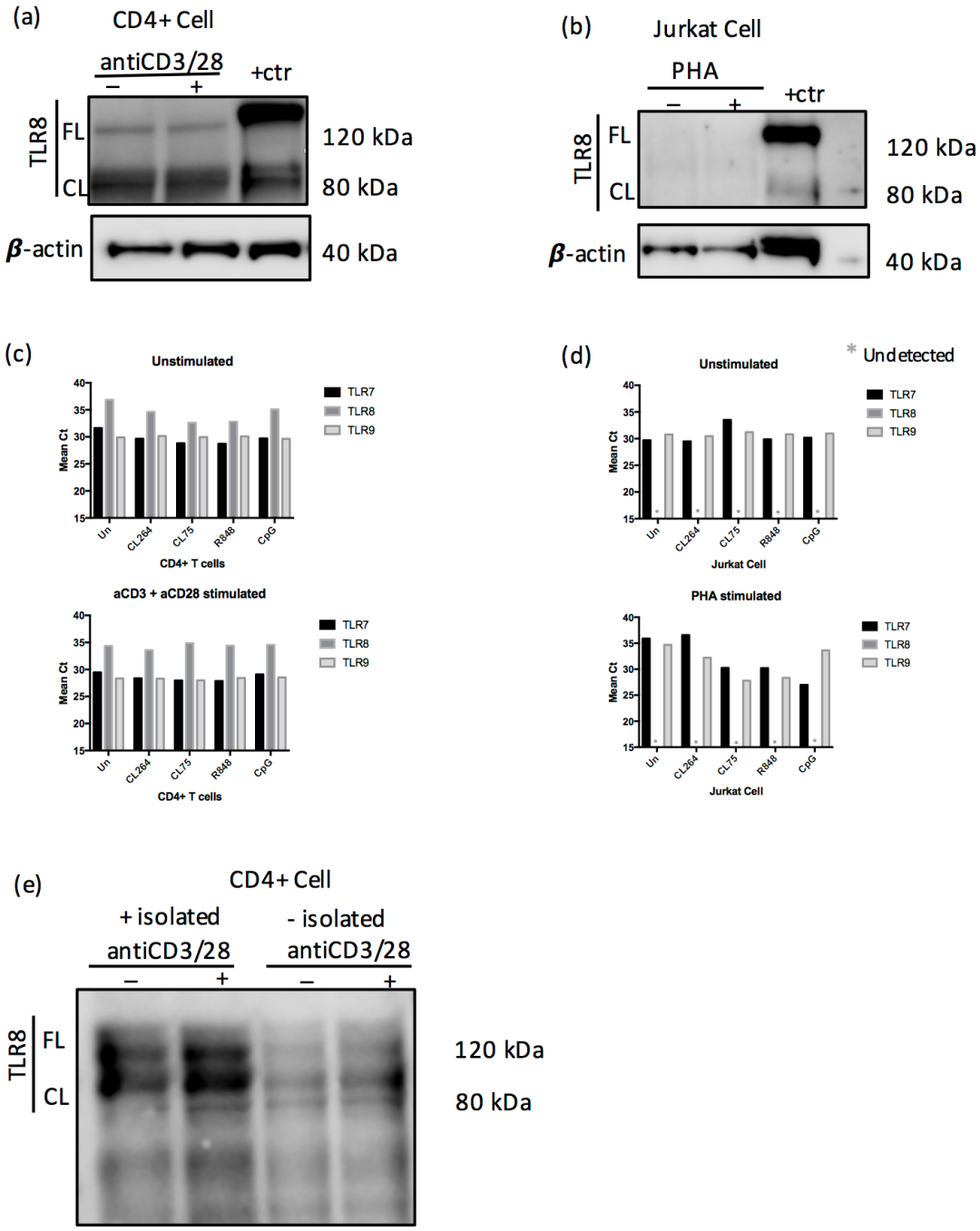


Figure 4.1 TLR7, 8 and 9 are expressed on primary CD4 T cells while Jurkat cell express TLR7 and 9 but not TLR8.
(a) Negatively isolated primary CD4+ T cells unstimulated or stimulated with plate bound anti-CD3 and soluble anti-CD28 for 24 hours were lysed and analyzed by western blot with TLR8 specific antibodies. (b) Jurkat cells unstimulated or stimulated with PHA (1ug/ml) for 24 hours were lysed and analyzed by western blot with TLR8 specific antibodies (Protein lysate from TLR8 transduced U373 cells were included as positive controls for the western blot run and β-actin housekeeping gene was used as loading control). (c) primary CD4+ T cells treated with CL264 (5ug/ml), CL75 (5ug/ml), R848 (5uM), and CpG(5ug/ml) in the presence or absence 1ug plate bound anti-CD3 with anti-CD28 analyzed for mRNA expression of TLR7, 8 and 9.using qPCR (d) Jurkat cells treated with CL264 (5ug/ml), CL75 (5ug/ml), R848 (5ug/ml), and CpG(10 uM) in the presence or absence 1ug PHA analyzed for mRNA expression of TLR 7,8 and 9 using qPCR (one representative experiment of two independent experiments shown, "" represent undetectable qPCR value), (e) Positive and negative isolated primary CD4+ T cells unstimulated or stimulated with plate bound anti-CD3 and soluble anti-CD28 for 24 hours were lysed and analyzed by western blot with TLR8 specific antibodies.*

4.2 Characterization of general T cell surface markers in Jurkat cell

We found that Jurkat cell line is TLR8 deficient but have TLR7 and TLR9. In addition, Jurkat cells have been reported to express functional TCR signaling feature together with the invariant signaling protein CD3 (Verweij et al, 1991). Furthermore, expression of both CD4 and the chemokine receptor CXCR4 on Jurkat cells have been attributed for Jurkat cell's candidacy as a suitable host for HIV infection (Cervantes-Acosta et al, 2001). In order to use them as a model we wanted to phenotype the level of TCR co-receptors CD3 and CD4 and the HIV co-receptors CXCR4 and CCR5 expression on the cell surface of Jurkat cells. We also investigated if expressions levels of TCR co-receptors are regulated by factors such as age of culture and cell activators. This was followed by investigation of changes in activation markers such as CD69, CD25 and CD40L upon activation of Jurkat cells in comparison with primary CD4+ T cells. Here, it was also assessed if treatment of the cells with endosomal TLR ligands would influence phenotypic changes in activation markers.

4.2.1 Level of CD4 and CD3 cell surface marker expression in Jurkat cells

In order to assess the expression levels of the T cell co-receptors CD3 and CD4 during the course of cell culture and in response to stimuli, freshly split Jurkat cells were cultured for a 5-day period with or without stimuli and phenotyped by flow-cytometry at different time points. Expression of CD3 on unstimulated Jurkat cells showed only little changes during the 5 day culture period with close to or more than 70% of Jurkat cells expressing CD3 at all time points (69%-80.1%, Fig 4.2a). In contrast, CD4 expression in unstimulated Jurkat cells was found relatively low (≈23.5%) on freshly split Jurkat cells (Fig. 4.2b). CD4 expression increased as the culture aged reaching highest level (≈58%) at 72-hour after splitting (Fig 4.2b). CD4 expression also seem to be modulated by activation of the cells with T cell stimuli, since stimulation of the cells with PHA decreased the expression of CD4 to a level of only 10% after 48-hours of stimulation (Fig 4.2d). However, expression of CD3 remained fairly stable also after treatment with PHA for 48 hours as more that 50% of Jurkat cells maintained their CD3 positive marker (Fig 4.2c)

Next we analyzed Jurkat cells for their expression levels of the chemokine receptors CXCR4 and CCR5, which are important for HIV entry. Flow cytometry method was used to assess for CXCR4 and CCR5 expression on Jurkat cells and was compared to their expression level on primary CD4+ T cells. The result showed that both primary CD4+ T cells as well as Jurkat cells express majorly the CXCR4 chemokine receptor (93.7% for primary CD4+ T cells and 98.7 %

for Jurkat cells), while the remaining 1% in Jurkat and 5.97% in primary CD4+ T cells express CCR5 (Fig 4.2e).

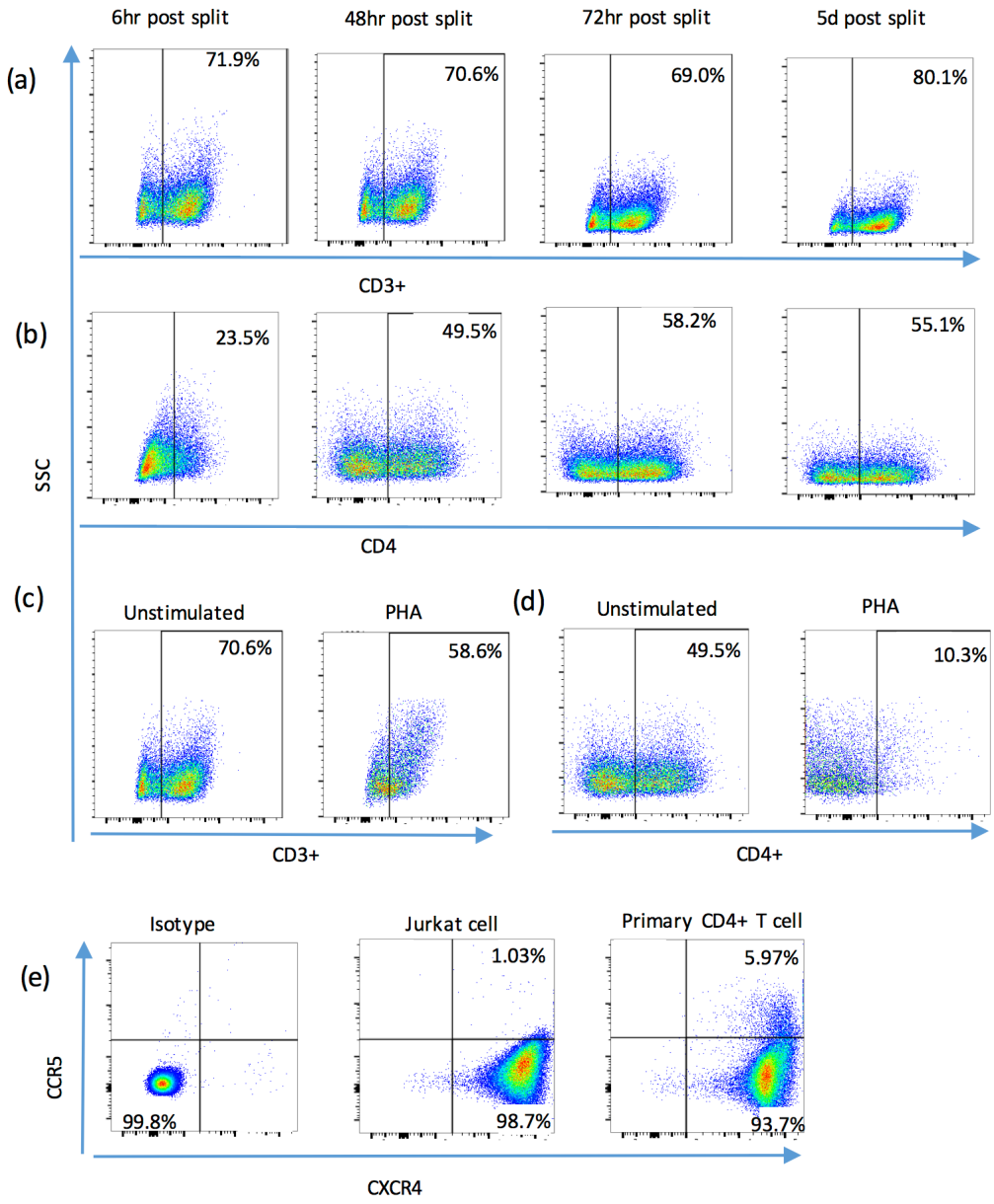


Figure 4.2: Flow cytometric analysis of CD3, CD4, CCR5 & CXCR4 expression on Jurkat cells.
Jurkat cells were stained for CD3, CD4, CXCR4 and CCR5 detection using the corresponding florescent tagged monoclonal antibodies as described in section 3.3 (a) Staining example for the level of CD3 expression on Jurkat cells after 6hr, 48hr, 72hr and 5days after splitting. (b) staining example for the level of CD4 expression on Jurkat cells after 6hr, 48hr, 72hr and 5days after splitting. (c &d) Analysis of CD3 and CD4 expression on Jurkat cells plated in the presence or absence of 1ug/ml of the T cell mitogen PHA for 48hrs.(e) CXCR4 and CCR5 expression analysis on the cell surface of Jurkat cells (middle) and primary CD4+ T cells (right). Cells were stained with fluorescent tagged monoclonal antibodies specific for CXCR4 and CCR5 receptors or with matching fluorescence labelled isotype antibodies (left), one representative figure showed for repeated experiment with similar finding.

4.2.2 Activation marker expression in Jurkat cells and primary CD4+ T cells in response to T cell activation and TLR stimulation

Activation markers are upregulated on the surface of T cells after TCR stimulation. We wanted to test if the activation marker expression in stimulated (activated) Jurkat cells parallels expression of these markers in stimulated primary CD4+ T cells. For this purpose, we chose to analyze the expression of the T cell activation markers CD25, CD40L CD69 and MHC class II on Jurkat as well as primary CD4+ T cells using flow cytometry. Jurkat cells and primary T cells were stimulated with plate bound anti-CD3 plus anti-CD28, PHA or PMA/Ionomycine, which all are known to induce antigen-independent T cell signaling and activation, but through different mechanisms (Section 3.2). Since prolonged stimulation of the cells with PMA/Ionomycin was found to be toxic to the cells, it was only used for 24 hours of stimulation.

Furthermore, we also wanted to asses if the endosomal TLR ligands CL264 (TLR7 ligand), CL75 (TLR8 ligand), R848 (TLR7/8 ligand) and CpG (TLR9 ligand) have any effect on the expression pattern of the cell surface activation markers.

Increased expression of CD25, CD40L and CD69 was observed when Jurkat cells were stimulated with all the T cell activators; anti-CD3/CD28, PHA and PMA/ionomycin (Fig. 4.3b, c and d). The highest increase in cell surface activation marker expression was exhibited by CD69 (≈60-98% at 24 hour and later time points) followed by CD40L (≈15-35% at 24 hour and later time points). Upregulation of CD69 in response to PHA was also found to be fastest with more than 60% of Jurkat cells expressing CD69 after only 6 hours of PHA stimulation; wile with PMA/Ionomycin and anti-CD3/anti-CD28 lesser response was observed at the 6-hour point (< 10%) followed by steep increase after 24 hours of stimulation (93% after stimulation with PMA/Ionomycine and 71% after stimulation with anti-CD3/anti-CD28) (Fig 4.3c and d). Here, only a marginal increase in the expression of MHC class II on Jurkat cells was observed when stimulated with PHA; with less than 5% MHC II expression in PHA-activated Jurkat cells at all time-points compared to around around 2% expression in untreated cells (Fig 4.3b).

Treatment of Jurkat cells with PHA upregulated expression of CD25 to ≈22 % at 24 hours from ≈3% at 6-hour point. However, upregulation of CD25 was reduced when Jurkat cells were treated with PHA for more than 24 hours (9.5% after 48 hours and 5.81% after 72 hours of stimulation with PHA) (Fig 4.3b).

Investigation of the expression pattern for the above cell activation markers on primary CD4+ T cells demonstrated an increase in the expression of CD25, CD40L, CD69 and MHC II when cells were treated with anti-CD3/anti-CD28.

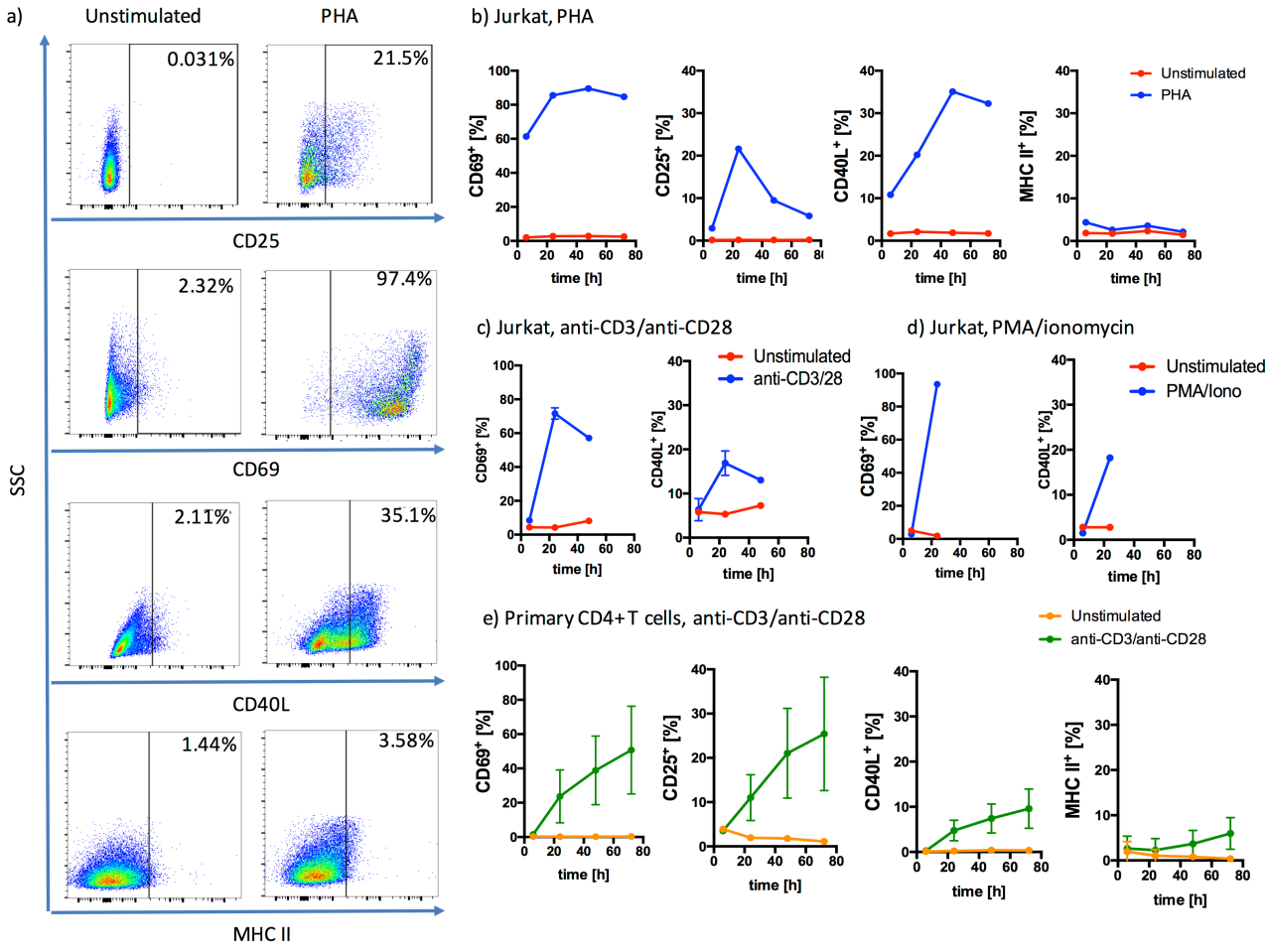


Figure 4.3: Flow cytometric analysis of activation markers on Jurkat cells and primary CD4+ T cells in response to T cell activators.
Flow cytometric analysis of activation markers on Jurkat cells Jurkat cells were stimulated with anti-CD3/anti-CD28, PHA or PMA/Ionomycin. Unstimulated cells were used as controls. At different time points post stimulation, cells were harvested and stained for activation marker expression with CD25-BV510, CD69-PE, CD40L-BV605, MHC II-BV785 fluorescent antibodies (a) Representative staining examples of flow cytometric analysis of activation marker expression on the surface of Jurkat cells 48h post stimulation. Expression examples of CD25 (upper), CD69 (medium), CD40L(medium) and MHC II (lower) on Jurkat cells unstimulated or stimulated with PHA (1ug/ml) are shown. Cells were gated on their FSC/SSC properties, activation marker gates were chosen according to staining with a matched isotype antibody for each channel (b) Quantification of activation marker for expression of MHC II, CD40L, CD25 and CD69 after Jurkat cells were unstimulated or stimulated with PHA (1ug/ml), for 6, 24, 48 and 72 hours. (c) Flow cytometer analysis for expression of CD69 and CD40L on Jurkat cells untreated or stimulated with anti-CD3/anti-CD28 (1ug/ml)) for 6, 24 and 48 hors (data representative of 3 independent experiments, n=3, m±SEM) (d) Flow cytometer analysis for expression of CD69 (left)and CD40L (right) on Jurkat cells untreated or stimulated with PMA (100ng/ml) and Ionomycin (1ug/ml) for 6 and 24 hours (one representative experiment of two independent experiments shown) (e) Quantification of activation marker for expression of MHC II, CD40L, CD25 and CD69 after primary CD4+ T cells were unstimulated or stimulated with anti-CD3/anti-CD28 (1ug/ml), for 6, 24, 48 and 72 hours. (Experiment on primary CD4+ T cells done by M. Haug/H. Ibrahim; data representative of 3 independet experiments from 3 different donors, n=3, m±SEM)

To investigate if treatment of Jurkat cells with endosomal TLR agonists influences activation marker responses, Jurkat cells were stimulated with TLR7 (CL264, 5ug/ml), TLR8 (CL75 5ug/ml), TLR7/8 (R848 5ug/ml) and TLR9 (CpG 10uM) agonists. The result shows that stimulation of the cells with any of the endosomal TLR agonists used was not found to affect cell surface expression of any of the studied activation markers (Fig 4.4). Also combining endosomal TLR stimulation (TLR8 ligand CL75) with T cell activation (anti-CD3/anti-CD28 or PMA/Ionomycin), as expected since Jurkat cells do not express TLR8, did not result in enhanced expression of the activation markers CD69 or CD40L (data not shown). Here, similar to Jurkat cells, treatment of primary CD4+ T cells with endosomal TLR ligands did not result in upregulation of CD25, CD40L, CD69 or MHC II.

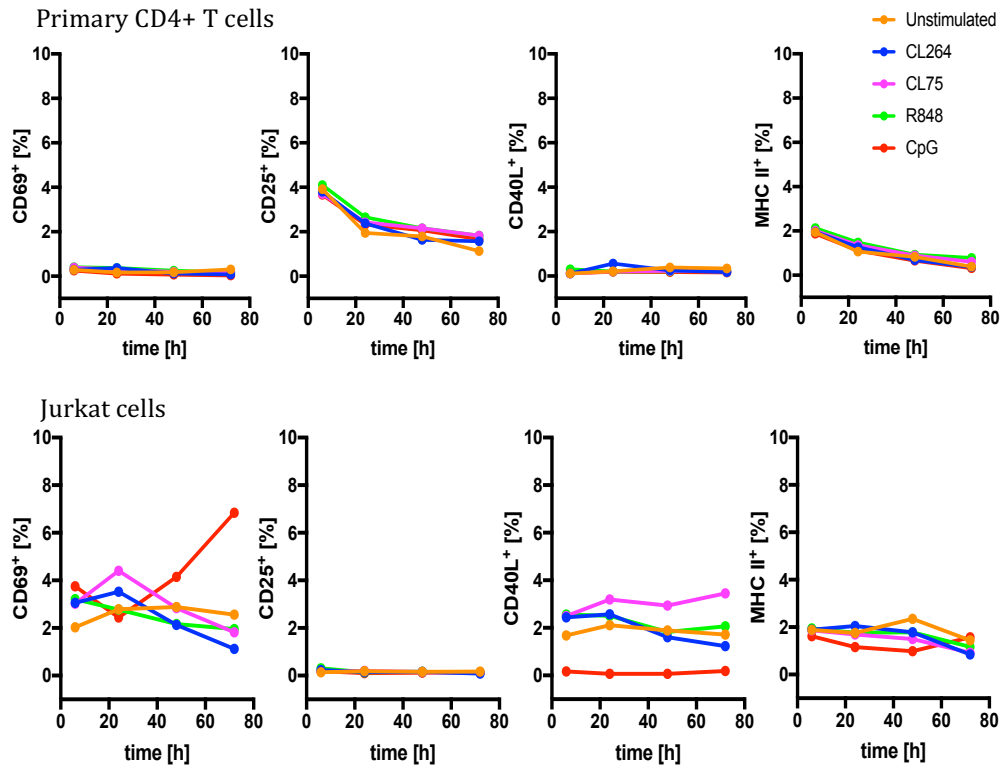


Figure 4.4:Flow cytometric analysis of activation markers on Jurkat cells and primary CD4+ T cells in response to TLR stimulation.
Flow cytometric analysis of activation markers on primary CD4 + T cells and Jurkat cells. Both primary CD4+ T cells and Jurkat cells were stimulated with TLR7(CL264, 5ug/ml), TLR8 (CL75, 5ug/ml) TLR7/8 (R848, 5ug/ml) and TLR9 (CpG, 10uM) ligands. Unstimulated cells were used as controls. At different time points post stimulation, cells were harvested and stained for activation marker expression with CD25-BV510, CD69-PE, CD40L-BV605, MHC II-BV785 fluorescent antibodies

4.3 Cytokine production profile of Jurkat cells in response to stimulation with TLR ligands and T cell activators

In the experiments above we did see upregulation of activation markers in Jurkat cells upon stimulation with T cell activators but not in response to endosomal TLR-ligands. Another hallmark of T cell activation is the production and secretion of effector cytokines. Typical CD4+ T cell cytokines include IFN-γ, TNF, IL-2, IL-4 and IL-17 amongst others. Jurkat cells have been reported to produce high amount of IL-2 and IL-8 upon TCR activation (Bartelt et al, 2009). We wanted, therefore, to test next which cytokines Jurkat cells produce in response to TCR activators as well as endosomal TLR ligands. The cytokine profile of Jurkat cells was assessed by qPCR and multiplex ELISA.

In experiments with purified primary CD4+ T cells we found that primary CD4+ T cells upregulate IL-6 production when stimulated with TLR8 (CL75) and TLR7/8 (R848) ligands in the absence of TCR stimulation (Fig. 4.5a, left). We chose, therefore, to assess IL-6 mRNA levels also in Jurkat cells in response to endosomal TLR stimulation with or without additional T cell activation using PHA (Fig 4.5 a middle and right). Jurkat cells did not upregulate IL-6 mRNA in response to stimulation with TLR7 (CL264), TLR8 (CL75), TLR7/8 (R848) or TLR9 (CpG) ligands. Additional stimulation of Jurkat cells with the T cell activator PHA also could not induce IL-6 mRNA production in Jurkat cells (Fig 4.5a middle and right). The absence of IL-6 production from Jurkat cells in response to endosomal TLR or combined TLR and T cell activator stimulation was also confirmed at protein level: Supernatants from Jurkat cell cultures were analyzed several times in IL-6 ELISA and multiplex experiment and revealed in all experiments that IL-6 concentrations was below the detection limit (data not shown). In contrast to Jurkat cells the primary CD4+ T cells show more than 500 times more induction of IL-6 when stimulated with TLR8 ligand (CL75) compared to unstimulated ones. This induction was doubled when CD4+ T cells were treated with the TLR7/8 ligand R848. No increased induction of IL-6 was not obtained when CD4+ T cells were stimulated with TLR7 (CL264) or TLR9 (CpG) ligands (Fig 4.5a, left). These findings were confirmed by ELISA on the protein level (experiments performed by Markus Haug, not shown)

Since IL-6 was not produced by Jurkat cells neither in response to TLR ligands nor to T cell activators, we assessed for the production of other cytokines that might possibly be produced by Jurkat cells upon TLR or TCR stimulation. First we addressed IFN-α, IFN-β, TNF-α, IL-8 and IL-2 production by qPCR quantification of mRNA. IL-2 and IL-8 were chosen to be

analyzed since they have been described to be produced by stimulated Jurkat cells (Bartelt et al, 2009), the other cytokines were chosen based on our results from stimulation of primary CD4+ T cells (PhD project Hany Ibrahim). Basal levels of IFN-α mRNA were detected in primary CD4+ T cells but seemed not to be regulated by treatment with endosomal TLR ligands CL264 (TLR7), CL75 (TLR8), R848 (TLR7/8) or CpG (TLR9) (Fig. 4.5b left). However, IFN-α mRNA could neither be detected in unstimulated Jurkat cells nor in Jurkat cells that were stimulated with endosomal TLR ligands with the presence or absence of T cell activator PHA (Fig 4.5b middle and right).

In contrast to IL-6 and IFN-α, we were able to detect IL-8, IL-2, TNF-α and IFN-β mRNA in Jurkat cells (Fig. 4.5c). Upon TCR-stimulation (anti-CD3/anti-CD28), the highest mRNA upregulation was found for IL-8 (≈32 fold) followed by IL-2 and TNF-α (≈8 fold). IFN-β levels seemed not to be affected by TCR stimulation (Fig 4.5c). Here, treatment of the cells with endosomal TLR7, 8 and 9 ligands (CL264, CL75, R848 and CpG) was not found to affect mRNA expression of all the cytokines tested: IL-8, IL-2, TNF-α and IFN-β.

In order to detect Jurkat-produced cytokines on the protein level, we performed a multiplex analysis from supernatants of stimulated Jurkat cells. A range of cytokine production can be analyzed simultaneously by the use of a Multiplex ELISA. The multiplex kit used in this study allows parallel analysis of 34 different immune-cell related cytokines from a single supernatant sample (Appendix: 6). This would allow us to eventually find additional cytokines that might be secreted by Jurkat cells during TLR or TCR stimulation. Therefore, supernatants from Jurkat cells untreated or treated for 24-hour with endosomal TLR ligands (CL264 (TLR7), CL74 (TLR8), R848 (TLR7/8), CpG (TLR9) and polu-U complexed with poly-L-arginine (pU/pLA) (TLR8)) or TCR activator (plate bound anti-CD3 with soluble anti-CD28) were subjected for multiplex ELISA to identify further potential functional response cytokine markers. Out of the 34 cytokines analyzed by the Multiplex assay (appendix 6) only IL-2, IL-8, TNF-α, IL-10, TNF-β and MIP1-α were found to be secreted by Jurkat cells in response to TCR stimulation with anti-CD3 and anti-CD28 (Fig.4.5d). However, none of these cytokines were found to be secreted by Jurkat cells at detectable levels in response to stimulation with any of endosomal TLR ligands (CL264, CL75, R848, CpG and pU/pLA) for 24h (Fig.4.5d).

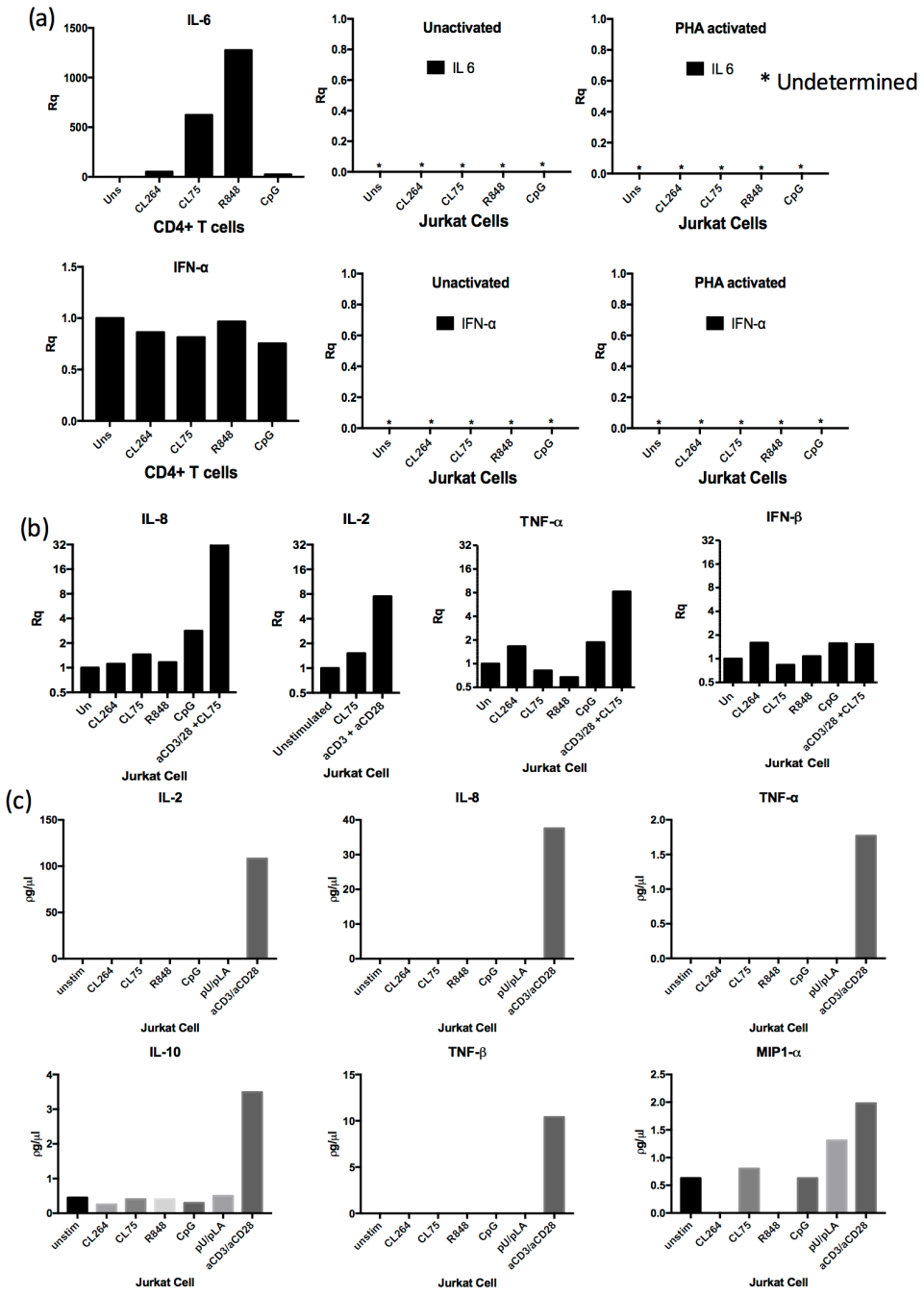


Figure 4.5:Cytokine production profile of Jurkat cells stimulated with TLR ligands and T cell activators. (a)qPCR analysis of IL-6 and IFN-α production on primary CD4 cells (left)as well as un-activated (middle) and PHA-activated (right) Jurkat cells. Cells were untreated or treated for 24hr with CL264 (5ug/ml), CL75 (5ug/ml) R848 (5ug/ml) and CpG (10 uM) before mRNA isolation and qPCR analysis. The experiment was performed in the presence or absence of 1ug/ml PHA for Jurkat cells. (b) qPCR analysis of IL-8, TNF-α and IFN-β production in Jurkat cells untreated or treated with CL264 (5ug/ml) CL75 (5ug/ml) R848 (5ug/ml) CpG (10 uMl) and anti-CD3/anti-CD28 (1ug/ml) for 24hrs. IL-2 determination was done only after stimulation with CL75 (5ug/ml) and anti-CD3/antiCD28 for 24 hours (c) Multiplex ELISA analysis of cell culture supernatants from Jurkat cells. Jurkat cells were unstimulated or stimulated with CL264 (5ug/ml) CL75 (5ug/ml) R848 (5ug/ml), CpG (10 uM) and anti-CD3/anti CD28 (1ug/ml) for 24hrs before supernatant harvesting. Out of 34 different cytokines tested by the Multiplex assay (appendix: 6) only the displayed cytokines were secreted at detectable levels IL-6 ELISA was performed for several experiments, the majority of qPCR experiments were repeated at least twice with similar outcome (qPCR results were normalized to the housekeeping gene GAPDH and Relative quantification (Rq) values calculated by the delta-delta Ct method taking the unstimulated cells as baseline) the Multiplex analysis was performed once."" represent undetectable values on qPCR*

4.4 Transfection and lentiviral transduction to achieve functional TLR 8 protein expression in Jurkat cells

From qPCR and Western Blot results we concluded that Jurkat cells lack TLR8 (section 4.1). Therefore, our next plan was to introduce the TLR8 gene in order to generate a TLR8 expressing Jurkat cell line. We tried to achieve this aim using two different approaches; by the use of a lentiviral system for transduction and the Nucleofection® method of transfection.

4.4.1 Determination of the optimal concentration of puromycin in the selection medium

The TLR8 gene we used for both transduction and transfection is packaged in the expression plasmid pLenti CMV PURO Dest w118-1. This plasmid construct has an antibiotic resistance gene for puromycin. Therefore, the minimum concentration of puromycin to prevent growth of Jurkat cells had to be determined before proceeding with transfection and transduction.

First 6 x 10^5 Jurkat cells were plated in 1 ml of 24 well plate in the presence of increasing concentration of puromycin from 0 μg/ml to 2 μg/ml. Counting of viable cells was performed every 72 hours by a trypan blue exclusion method using Countess™ Automated Cell Counter. On the 6^{th} day Jurkat cells treated with puromycin in concentrations of 0.5 μg/ml and higher did not increase in cell number (Fig 4.5a). Since 0.5 μg/ml of puromycin inhibited growth of un-transfected/ un-transduced Jurkat cells, it indicated the optimal concentration to select for Jurkat cells that are efficiently transfected/transduced with the plasmid construct containing TLR8 gene.

Growth inhibition effect of 0.5μg/ml puromycin on Jurkat cell was also confirmed by cell proliferation assay method using CellTiter 96® AQueos One Solution cell proliferation assay. The principle of the assay rests on the fact that metabolically active cells can bioreduce the tetrazolium compound MTS found in CellTiter 96® AQueos One Solution into a colored formazan product. 5 x 10^4.cells were seeded in 100 μl medium per well in a 96 well plate in the presence of gradient increase in concentration of puromycin concentration from 0 μg/ml to 2μg/ml and incubated for 6 days before the MTS tetrazolim compound was added. The cells were then incubated for additional 5hrs where by the accumulation of the formazan product formation by actively proliferating cells was monitored after each hour during the 5-hours period. The results indicated that cells treated with 0.5 μg/ml puromycin or higher concentration over the 6-day period completely inhibited Jurkat cell proliferation and thus confirmed our findings from the viable cell counting (Fig 4.5b).

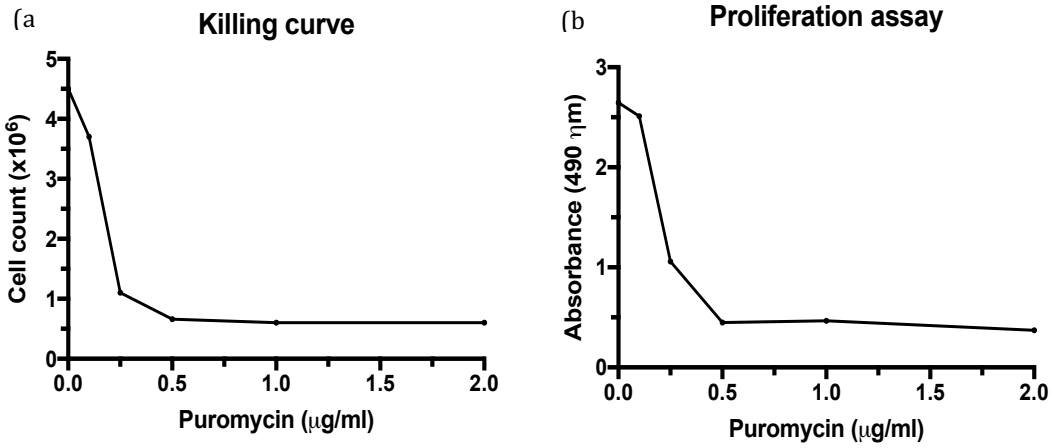


Figure 4.6: Determination of the minimum puromycin concertation that kills Jurkat cells
(a) Puromycin killing curve for Jurkat cells. 6 x 10^5 Jurkat cells were plated in 1 ml of media in 24 well plate and cultured for 6-days in the presence of 0, 0.1ug/ml, 0.25ug/ml, 0.5ug/ml, 1ug/ml and 2ug/ml of puromycin. On day 6 viable cells were counted by trypan blue exclusion method using the Countess™ Automated cell counter. (b) Jurkat cell proliferation assay to assess puromycin growth inhibition of Jurkat cells 5 x 10^4 Jurkat cells were seeded in 100 μl media per flat bottomed 96 well plate in the presence of 0, 0.1ug/ml, 0.25ug/ml, 0.5ug/ml, 1ug/ml and 2ug/ml of puromycin and incubated for 6days at 37°C, 5% CO_2. Then 20 μl of Cell CellTiter 96® AQueos One Solution Reagent was pipetted into the wells and incubated for 5 more hours at 37° C,. Five Absorbance reads were made every hour at 490 nm using a 96-well plate reader (section 3.6). The 3h-point read presented here, with consistent result for all time point reads. A puromycin concentration of 0.5 ug/ml and above stopped proliferation of Jurkat cells in both assays.

4.4.2 Transfection of Jurkat cells with TLR 8 gene by Nucleofection method

Once the puromycin killing curve for Jurkat cell was determined, our next plan was to transfect the cells using nucleofection transfection method. This is an electroporation based method with abilities to transfer genetic materials direct into cell nucleus thereby eliminating the need for cell division dependent genetic material into transfected cells.

During the experiment 1 x 10^6 Jurkat cells were transfected with 2 μg of pLenti CMV PURO Dest w118-1-TLR8 expression plasmid as well as pmaxGFP® vector control as described in section 3.7.3. The pmacGFP® control plasmid construct contains gene for green fluorescent protein (GFP) expression, thus cells efficiently transfected with pmacGFP® can be measured by flow cytometry as percentage of cells expressing GFP. Furthermore, by use of propidium iodide (PI) that stains the nucleus of non-viable cells, viability of the cells can be checked.

After 24 hours of incubating nucleofected and non-nucleofected Jurkat cells, the cells were stained with PI and assessed using flow cytometry. Jurkat cells were gated on the bases of their FSC/SSC properties (Fig. 4.7a and b, left). This gated population was further analyzed for PI

negative (*i.e.* viable) and GFP positive (*i.e.* transfected) cells (Fig. 4.7a and b, right). 50% of the FSC/SSC gated pmacGFP® vector nucleofected cells express GFP (*i.e.* efficiency of 50%), almost all of these cells were viable (PI-negative): While 11.4% of FSC/SSC-gated nucleofected cells were non-viable (PI-positive) (Fig 4.7b, right). As expected none of the FSC/SSC-gated non-nucleofected Jurkat cells were GFP positive and only 4.63 % of them were non-viable (PI-positive (Fig4.7a, right).

In parallel, the pLenti CMV PURO Dest w118-1-TLR8 expression plasmid nucleofected cells were put under 0.5µg/ml puromycin containing media for selection 24-hours post nucleofection. During the beginning of puromycin selection, most cells did not survive the antibiotic but a few surviving cells started growing slowly. After maintenance of Jurkat cells under selection condition for about 5 more weeks, enough puromycin resistant cells were expanded for further experiments and to cryopreserve aliquots.

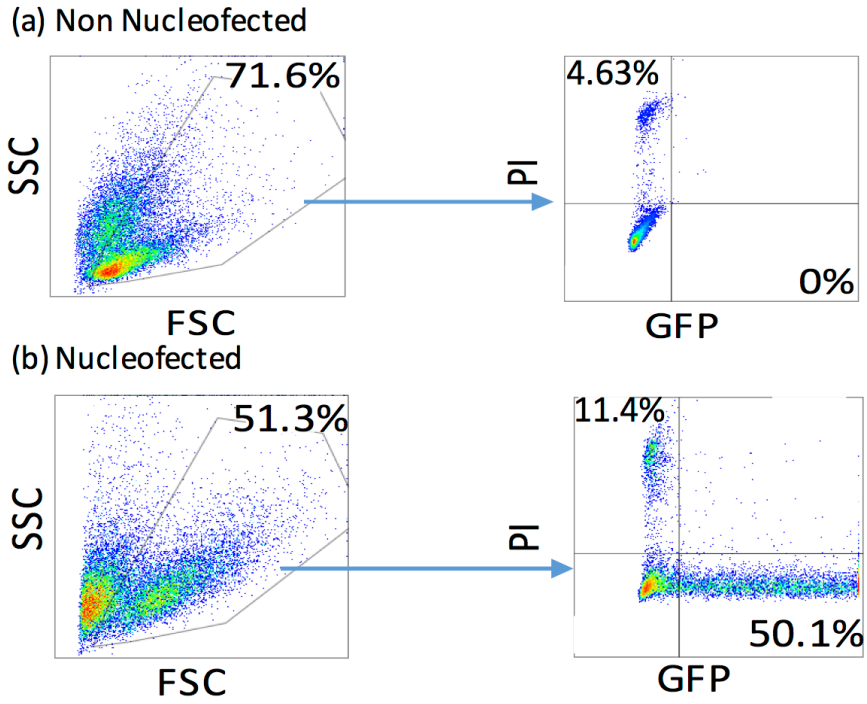


Figure 4.7: Efficiency and viability of nucleofection transfection in Jurkat cells. Jurkat cells non-nucleofected and nucleofected were analyzed 24 hours post Nucleofection by flow cytometry after staining with the nuclear stain propidium iodide (PI). Jurkat cells were gated on FSC/SSC properties(left) and further analyzed for PI negative and GFP positive cells (right) (a) Dot plot representation of flow cytometric analysis of non-nucleofected Jurkat cells. Stained with PI (b) Dot plot representation of flow cytometric analysis of Jurkat cells nucleofected with 2ug of pmaxGFP® vector control

4.4.3 Lentiviral transduction of Jurkat cells with TLR8 gene

Alongside the nucleofection experiment, Jurkat cells were transduced with a lentiviral vector system. The aim was to stably transduce Jurkat cells with TLR8 containing pLenti CMV PURO Dest w118-1-TLR8 expression plasmid (the same expression plasmid used for nucleofection experiment). Since the TLR8 expression plasmid does not have a fluorescence marker, we used a GFP-tagged expression plasmid as a control for transduction efficiency. The GFP-tagged expression plasmid was also constructed from the same expression vector (pLenti CMV PURO Dest w118-1) that was used to package TLR8 gene.

Three biological replicates of viral concentrates were used to transduce Jurkat cells with TLR8. These viral concentrates represent three different clones of packaging plasmid. The detailed process of developing these clones is presented in section 3.7.4. The viral concentrates for the three TLR8-expression clones (referred to as clone A, B and C) as well as the GFP-tagged construct were added to 5 x 10^5 Jurkat cells. 24 hours post transduction with virus concentrates, the cells were placed under 0.5 μg/ml puromycin selection. After 7 days of selection, the transduction efficiency was analyzed from the cells that were transduced with the GFP-tagged construct by fluorescence microscopy. The fluorescence microscopy image indicates that the lentiviral transduction has worked since we could detect GFP positive cells. although the number of fluorescent cells was low (Fig 4.8).

Similar to the Nucleofected cells (section 4.4.2), lentiviral transduced Jurkat cells were cultured under puromycin selection for over 1 month in order to expand enough puromycin resistant Jurkat cells for further experiments. Two rounds of lentiviral Jurkat cell transduction were performed. In the first transduction experiment Jurkat cells transduced with clone B (slow) and C (fast) but not A were found to grow under puromycin selection. In the second experiment Jurkat cells from all three clones A, B and C were growing, indicating possible expression of TLR8 in this cells. When enough cells were grown from a clone, aliquots were cryopreserved as well as analyzed for TLR8 expression and function in further experiments.

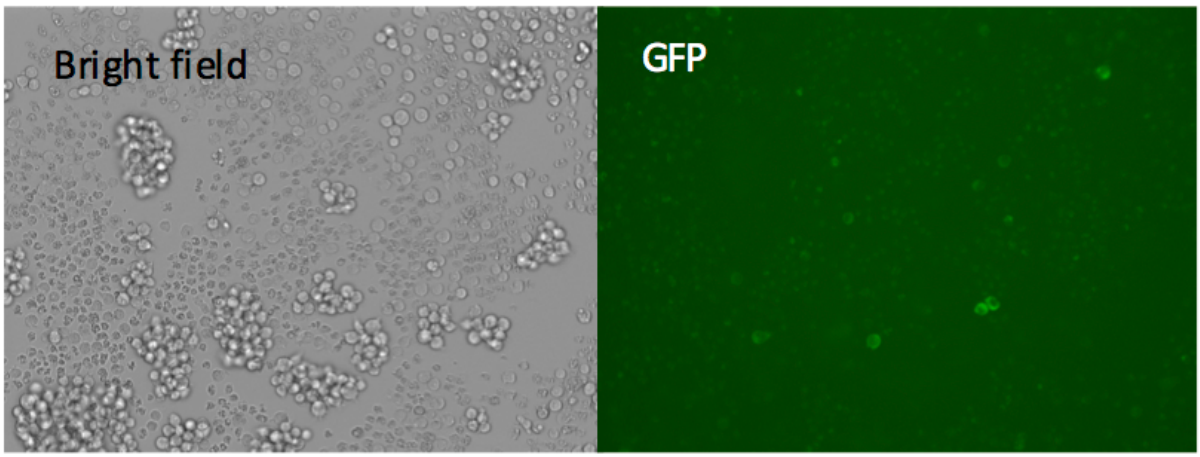


Figure 4.8:Microscopic analysis of Jurkat cells transduced with s pLenti-CMV-PURO-Dest-w118-1-GFP. Jurkat cells transduced with GFP containing plasmid construct were analyzed 7 days post Transduction using bright field and fluorescence microscopy by use of EVOS® FL Imaging Systems.

4.5 TLR 8 mRNA and protein expression analysis on transduced and nucleofected Jurkat cells

Once enough TLR 8 gene transfected/transduced Jurkat cells were expanded, expression of TLR8 in Jurkat cells was assessed. qPCR and western blot analyses were done on the TLR8 transduced (clone A, B, and C) and the nucleofected (Nu) Jurkat cells. Since we found that the Taqman probe/primer we have does not target the TLR8 gene segment used in our expression plasmid construct, SYBRgreen qPCR method was applied using primers designed at CEMIR. Primer pairs and protocols for the SYBRgreen qPCR method were obtained from Lobke Gierman (postdoc, CEMIR).

Analysis of qPCR denoted that TLR8 expression was induced in all the transduced clones as well as nucleofected Jurkat cells. This was demonstrated by a reduction in the mean CT value of the modified Jurkat cells in comparison with the wild type Jurkat cells, which had an undetermined or very high Ct value (Ct value of >35) (Fig 4.9 a & b).

Absolute Ct values for TLR8 gene (Fig 4.9a) as well as delta Ct values, obtained by subtracting the Ct values for the house keeping gene TBP from Ct values for TLR8 gene (Fig 4.9b), showed that there was a 5 times or more reduction in the qPCR cycle number needed to detect TLR 8 mRNA expression in the transduced (clone A, B, C) and nucleofected cells compared to the wild type Jurkat cells. The TLR8 mRNA expression in the transduced and transfected cells was found to increase when the cells were treated with TCR stimulation (anti-CD3 plus anti-CD28, Fig 4.8a and b, gray stripe bars)

Since we observed TLR8 mRNA expression in transduced and nucleofected Jurkat cells, we wanted to test next if these cells also express TLR8 protein. This was done by western blot analysis for TLR8. However, contrary to expectations, western blot analysis of transduced/transfected Jurkat cells was found to be negative for TLR8 protein. Moreover, treatment of the cells with the TLR7 ligand (R837) and the TLR8 ligand (CL75) was not found to induce expression of TLR8 protein in the Jurkat cells transduced with clone A, B or C. Activation of transduced (clone A, B and C) Jurkat cells with the diacylglycerol analog PMA together with the ionophore Ionomycin did also not induce TLR8 protein expression (Fig. 4.9c). Similar results were obtained when transduced and transfected Jurkat cells were stimulated with a T cell activator anti-CD3 plus anti-CD28 (Fig 4.9d).

These results indicate that both the transfection and transduction methods were able to induce TLR8 mRNA expression in Jurkat cells. However, protein level expression of TLR8 could not be detected in any of the modified Jurkat cells and we were not able to induce detectable levels of TLR8 protein by TLR or TCR stimulation of the modified cells.

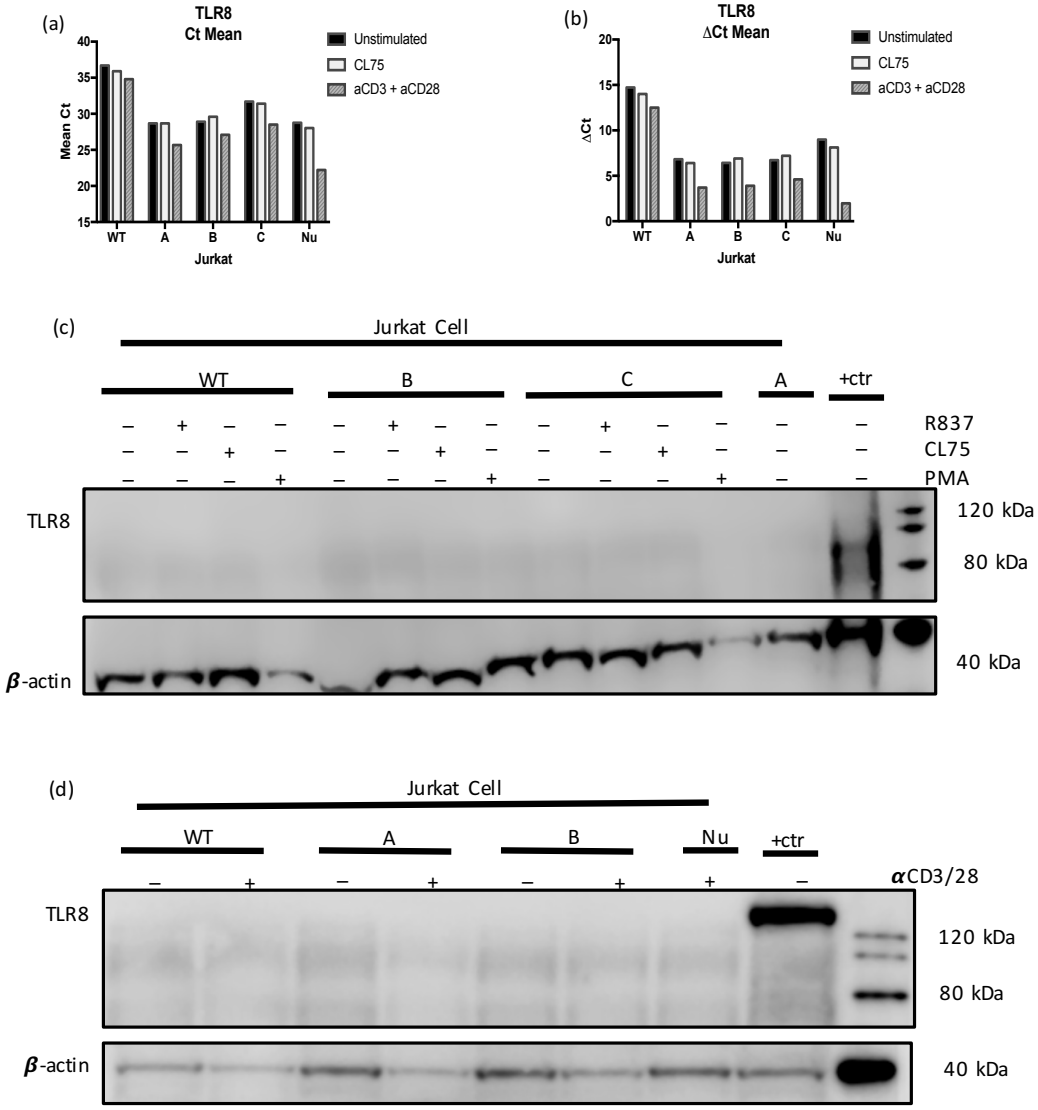


Figure 4.9: TLR8 mRNA and protein expression analysis in lentiviral transduced and nucleofected Jurkat cells. (a, b) TLR8 mRNA expression analysis in Jurkat cells by qPCR. Unmodified Jurkat cells (WT), Transduced (clone A, B and C) and Nucleofected (Nu) Jurkat cells were stimulated with TLR7 ligand (R837, 5ug/ml), TLR8 ligand (CL75, 5ug/ml) or plate coated anti-CD3 with soluble anti CD28 (1ug/ml) for 24 hours. RNA was then extracted, reverse transcribed and SYBRgreen qPCR was performed using specific primers for TLR8. Data presented as (a) actual mean CT value of TLR8 expression direct without housekeeping gene subtraction or (b) delta CT value by subtracting the CT value of the house keeping gene TBP from TLR8 expression (one representative experiment of two independent experiments shown for WT, A and B clones). (c) TLR8 protein expression analysis in Jurkat cells by Western Blot. WT, and the modified Jurkat cell clone A, B and C were stimulated by TLR7 agonist R837 (5ug/ml), TLR8 agonist CL75 (5ug/ml) and PMA 100 ng/ul with Ionomycin (1ug/ml) for 24 hours. TLR8 expression was analyzed by western blot. Protein lysate for U373 cell line transduced with TLR8 gene was run as a positive control, and β-actin housekeeping gene was used as loading control (d)TLR8 protein expression analysis in Jurkat cells by Western Blot as in (c). Nucleofected Jurkat cells were analyzed in addition to WT and transduced (clone A and B) Jurkat cells. TLR8 protein was analyzed in untreated cells as well as cells stimulated with plate coated anti-CD3 with soluble anti-CD28 (1ug/ml) for 24 hours. Protein lysate for U373 cell line transduced with TLR8 gene was run as a positive control andβ-actin housekeeping gene was used as loading control

4.6 Functional analysis of TLR8 responses in TLR8 transduced and nucleofected Jurkat cells

Protein detection of TLRs using western blotting may have some challenges, for example in terms of sensitivity and specificity of available antibodies. Since we could not detect protein level expression of TLR8 but found TLR8 mRNA induction (section 4.5), it might be possible that the engineered Jurkat cells possess functional protein that could not be detected during our western blot analysis. Therefore, we wanted to test if we could detect any functional TLR8 responses in the modified Jurkat cells lines. For this purpose we tested cytokine responses as well as TLR8 induced NF-κB signaling responses in the TLR8 transduced and nucleofected Jurkat cells.

4.6.1 Effector cytokine responses of TLR 8 nucleofected and transduced Jurkat cells in response to TLR8 stimulation.

To investigate for a potential functional success of our TLR8 gene transfection and transduction, we analyzed the cytokine production of the transformed cells in response to TLR8 ligand stimulation. To this end, we focused on the limited cytokines that were shown to be secreted by wild type Jurkat cells upon stimulation with T cell activators (Fig 4.4), *i.e.* IL-2, TNF-α and IL-8.

All the transduced (clone A, B and C), the nucleofected (Nu) as well as wild type (WT) Jurkat cells were stimulated with the TLR8 ligand CL75. For comparison, in addition to CL75 (TLR8), unstimulated and TCR stimulated (anti-CD3 plus anti-CD28) were included as controls. We found that 24 hour treatment with the TLR8 ligand CL75 did not induce a considerable increase in IL-2, IL-8 or TNF-α production (Fig 4.10). Cytokine production for IL-2, IL-8 and TNF-α was investigated using qPCR on the mRNA level (Fig. 4.10a,b and c), IL-8 secretion was in addition analyzed on the protein level for clone B and C using ELISA (Fig. 4.10d).

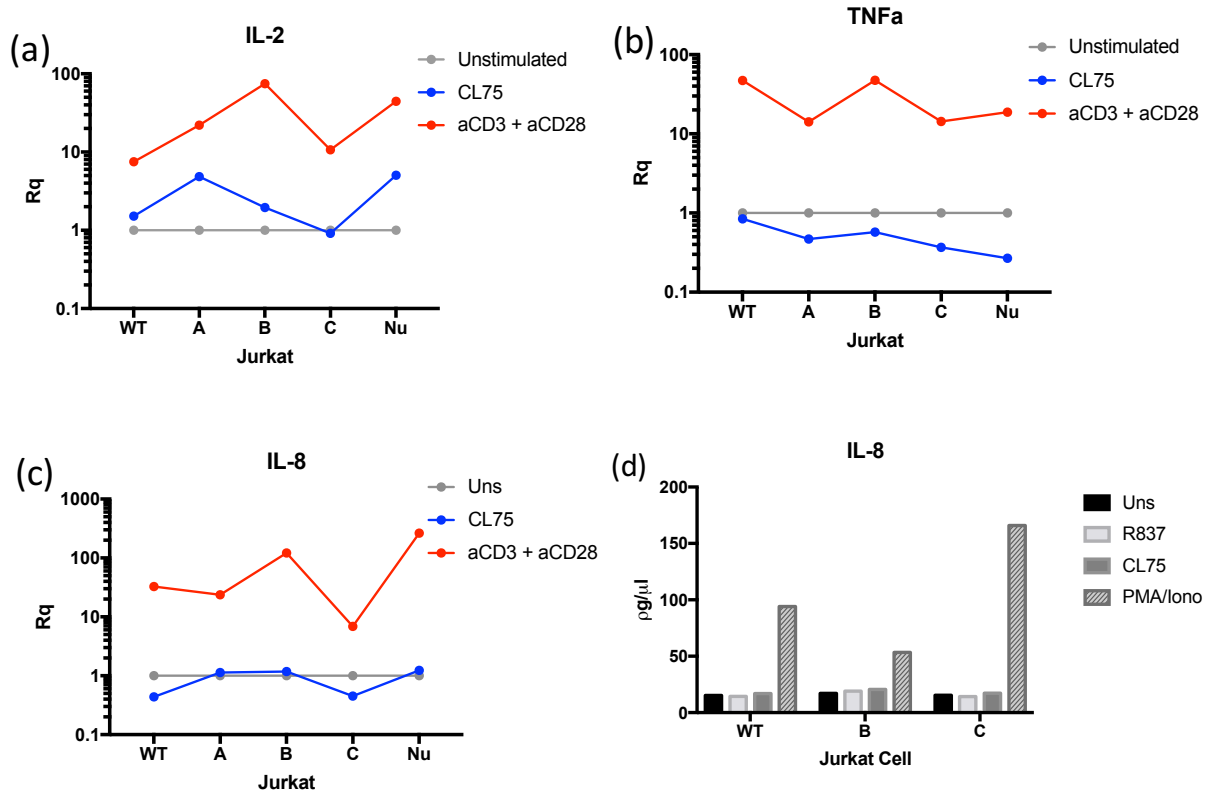


Figure 4.10:TLR8 stimulation does not upregulate cytokine production in TLR8 gene transfected and transduced Jurkat cells
(a, b, c) qPCR analysis of cytokine mRNA in Jurkat cells (WT), transduced (clone A, B and C) and nucleofected (Nu). Jurkat cells were stimulated with the TLR8 ligand CL75 (5ug/ml) and plated coated-anti-CD3 with soluble anti-CD28 (1ug/ml) for 24 hours. RNA was extracted, reverse transcribed and qPCR run with Taqman primer/probe for IL-2, TNF-α and IL-8. Results were normalized to the housekeeping gene GAPDH and Relative quantification (Rq) values calculated by the delta-delta Ct method taking the unstimulated cells as baseline (d) IL-8 protein analysis by ELISA in supernatants from Jurkat cells. (WT) and transduced (clone B and C) Jurkat cells were stimulated with TLR7 ligand R837 (5ug/ml), TLR8 ligand CL75 (5ug/ml) and with PMA (100ng.ml) plus Ionomycin (1ug/ml) for 24 hours. Supernatants were collected after 24 hours and ELISA performed to analyze IL-8 secretion.

4.6.2 Intracellular signaling analysis of TLR8 nucleofected and transduced Jurkat cells in response to TLR8 stimulation.

Since we could not detect functional cytokine responses to TLR8 ligand stimulation of the TLR8 transduced/transfected Jurkat cells, we finally planned to investigate if TLR8 mediated signaling is actually initiated in the modified cells. The TLR8 signaling pathway is known to induce nuclear translocation of the transcription factor NF-κB (Bergstrom et al, 2015). To test a possible NF-κB nuclear translocation upon TLR8 stimulation, two of the transduced clones, A and B together with the wild type Jurkat cells were stimulated with TLR8 ligand CL75 (5μg/ml) for 4 and 24 hours. The cells were also stimulated with plate coated anti-CD28 with soluble anti-CD28 for 4 hours as positive control. Early time point (4 hour) for NF-κB nuclear translocation assay was selected since it has been described that nuclear translocation of NF-κB starts as early as 1hour and reaches maximal level at 5 hour post stimulation in T cells (Ouellet et al, 2003). Nuclear translocation of NF-κB was then analyzed by use of imaging flow cytometry following a method established in our group (section 3.8).

The four hours treatment with anti-CD3 plus anti-CD28 resulted in increased percentages of Jurkat cells with nuclear translocated NF-κB across all the transduced and un-transduced cells (close to 13% compared to ≈5% basal level). However, stimulation of the cells with the TLR8 ligand CL75 was not found to upregulate the nuclear translocation of NF-κB even when the cells were treated for a prolonged period of time (24hr) (Fig. 4.11c). This indicates that none of the TLR8 transduced Jurkat cells induced functional signaling upon TLR8 stimulation.

In conclusion it seems that with TLR8 transduction/nucleofection we were able to induce TLR8 mRNA expression in Jurkat cells. However, none of the employed methods resulted detectable levels of TLR8 protein expression or functional TLR8 responses in Jurkat cells as measured by TLR8 specific signaling induction or cytokine production.

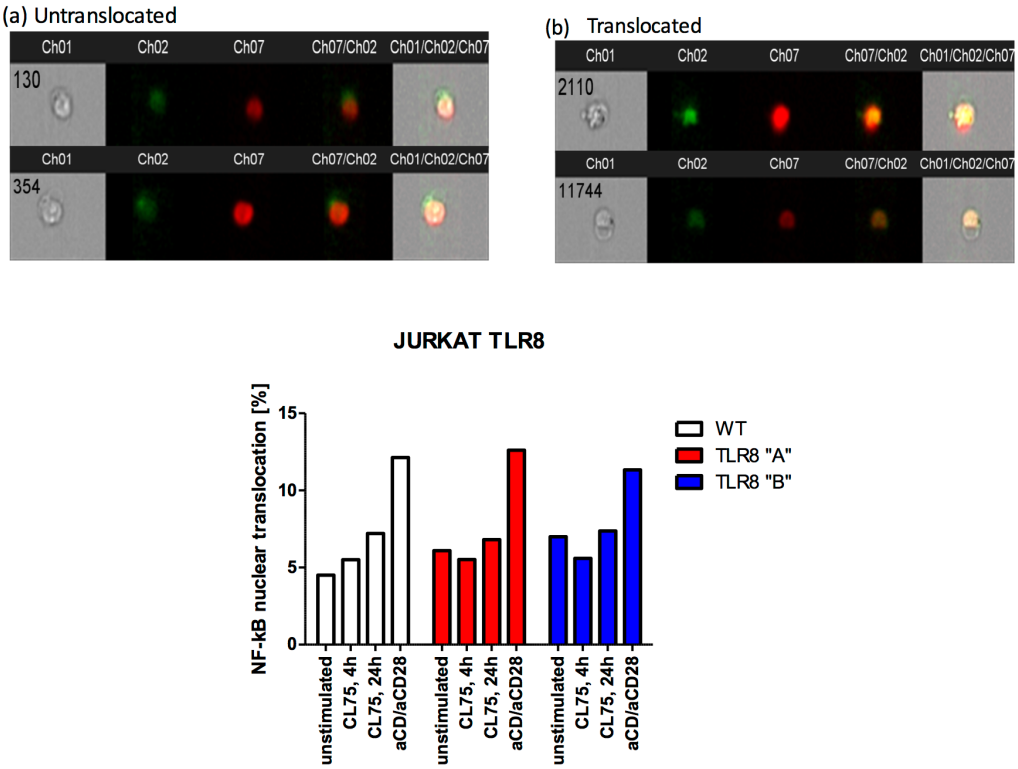


Figure 4.11: Stimulation of TLR8 transduced Jurkat cells with CL75 does not induce NF-κB nuclear translocation. NF-κB nuclear translocation analysis by imaging flow-cytometry WT and transduced (clone A and B) were stimulated with CL75 (5ug/ml) for 4 and 24hr as well as with plate coated anti-CD3 with soluble anti-CD28 for four hours. The cells were then stained with FITC fluorescent tagged anti-human NF-κB antibody and Hoechst nuclear stain and acquired and gated using Flowsight® analyser and data analyzed using IDEAS® software (see 3.8). NF-κB was stained in green (FITC, ch02) and nucleus was stained in red (Hoechst, ch07), when these two colors overlap they give rise to yellow fluorescence at the ch07/ch02 channel, the software calculates the similarity between the nuclear and the NF κB stain (similarity Ch2/Ch7) which can be quantified from all cells. Typical example image of (a) un-translocated NF-κB and (b)nuclear translocated NF-κB cells. (c) Quantification of NF-κB translocation in un-transduced (WT) and transduced (clone A and B) Jurkat cells unstimulated or stimulated with CL75 (5ug/ml, 4h, 24h) as well as with plate coated anti-CD3/anti-CD2 (4h). Nuclear translocation of NF-κB was driven by treatment of the cells with anti-CD3/anti-CD28 but not by treatment with CL75 for 4hr and 24hr

5 DISCUSSION

TLRs are PRRs that are essential for early detection of microbes by the innate immune system. Expression pattern and signaling pathways of TLRs in general and endosomal TLRs in particular appears to be well elucidated in APCs, such as dendritic cells (Hemmi & Akira, 2005). Interestingly, expression for most of TLRs have also been described in adaptive immune cells such as T cells (Hornung et al, 2002). However, only a few published reports have indicated the existence for direct roles of the endosomal TLR7 and 8 on primary CD4+ T cells (Caron et al, 2005; Dominguez-Villar et al, 2015), and in depth studies about endosomal TLR mediated CD4+ effector functions in the context of HIV infection is still scarce.

Our group is investigating the molecular mechanisms and associated immune responses behind sensing of HIV derived nucleic acids by endosomal TLRs in human primary CD4+ T cells, with an emphasis on TLR8. In line with this, availability of a cell line model system would greatly assist in overcoming some of the challenges of working with primary cells, such as donor variability, demand for fresh blood donor for each experiment and difficulty of gene modification in primary cells. While it is clear that the human Jurkat CD4+ leukemia cell line shares basic TCR-signaling mechanisms with primary CD4+ T cells (Smeets et al, 2012), information regarding cell biomarkers, cytokine profile and TLR expression pattern in Jurkat cells is not complete. Therefore, in this project we assessed if the Jurkat E6.1 clone could be a suitable model system to investigate endosomal TLR signaling and exhibit similar properties as primary human CD4+ T cells.

Expression of TLR7, 8 and 9 in primary CD4+ T cells is still under scrutiny and contradicting results have been published regarding the expression of TLR8 in CD4+T cells. In this project, qPCR assessment of primary CD4+ T cells showed positive gene expression for TLR7, 8 and 9. This finding agrees well with a study by Hornung et al (2002), that characterized TLR expression pattern of different subsets of PBMC including total T cells, as well as with another report (Zarember & Godowski, 2002) that described TLR expression profile of CD4+ T cells in comparison with CD14+ monocyte cells isolated from PBMC. However, contrary reports have also been published that indicated absence of TLR8 expression on CD4+ T cells isolated from PBMC and tonsil (Caron et al, 2005; Mansson et al, 2006). All the studies about TLR8 expression mentioned above are based on findings from PCR analysis of negatively isolated CD4+ T cells. To our knowledge there is no study so far that investigated protein level expression of TLR8 on CD4+ T cells. Accordingly, here we showed TLR8 protein expression

on western blot analysis from protein lysate of negatively isolated CD4+ T cells. However, as discussed earlier in section 1.4.3, there have been some speculations regarding purity of CD4+ T cell isolations and that TLR expression in CD4+ T cells is may be detected due to contamination with other cell types. We therefore investigated whether the purification method of CD+ T cells influences TLR8 expression results on western blot. We found that both, negatively isolated CD4+ T cells with >96% purity and positively isolated CD4+ T cells with >99% purity showed protein level expression of TLR8. This is to our knowledge the first report showing the presence of protein level expression of TLR8 in primary human CD4+ T cells from isolates demonstrated to be free from CD14+ monocyte contaminants using two methods. The presence of functional TLR8 in primary CD4+ T cells is further supported by evidence from our laboratory showing that primary CD4+ T cells provide distinct functional responses when exposed to TLR8 ligand. Other reports mentioning a functional role of TLR8 in primary CD4+ T cells have also been published (Caron et al, 2005; Dominguez-Villar et al, 2015).

However, the Jurkat model cell line we investigated was consistently negative for TLR8 expression when analyzed at the level of mRNA (using qPCR) and at the level of protein (using western blotting). Thus, Jurkat E6.1 clone can be considered a natural TLR8 knockout model. Other features of Jurkat cell we investigated was immunophenotyping for biomarkers. CD3 and CD4 surface antigens are identifiers for the CD4+ subpopulation of T cells. They are involved in TCR interaction with antigens presented via MHC class II and initiation of downstream signaling upon TCR stimulation. Furthermore, together with CXCR4 or CCR5 as co-receptors, HIV uses CD4 to gain entry into host T cells. Therefore, characterization of these antigen markers is important in studies that revolve around T cell signaling and HIV pathophysiology. We observed that close to 70 to 80% of Jurkat cell express CD3. A similar result was published in a study that demonstrated 73% of Jurkat cells expressed the CD3 T marker (Shatrova et al, 2015). However, CD4 expression seemed to be low (expressed in ≈20-25% of Jurkat cells) and varied greatly with the age of the culture and the activation status of Jurkat cells. Such s reduced expression of CD4 in the E6.1 clones of Jurkat cell was also reported by Cervantes-Acosta et al (2001), but in this study, Jurkat cells have been shown to host productively infective HIV despite the decreased level of CD4+ expression. Furthermore, Lee et al (1999) have also reported that the level of CD4 expression does not correlate with the relative capabilities of Jurkat cells as HIV host. In line with this, multiple studies have used the E6.1 clone Jurkat cell as HIV infection host to study processes such as inflammatory pathways triggered upon HIV binding to CD4/CXCR4, the role of CD4 down-modulation on HIV entry and mechanism of

HIV induced autophagy initiation in CD4+ T cells (Conti et al, 2000; Popik et al, 1998; Wang et al, 2012). We also have observed that Jurkat cell has a very strong expression of CXCR4 (≈99%). A study has demonstrated that the CXCR4 expressed in Jurkat cells is biologically active and therefore can bind and respond to its natural ligand-stroma-derived factor (SDF-1) (Hesselgesser et al, 1998).

Measurement of lymphocyte activation status is a useful tool to understand and asses the level of immune responses and immune reactivity during T cell studies. In this regard, T cell surface activation markers and cytokine responses are important tools to define T cell activation status. Stimulation of T cells *in vitro* does not rely on antigen specific interaction of the TCR with pMHC, but rather can be polyclonaly initiated by T cell activator reagents. The use of agonistic antibodies to the TC-complex and co-stimulatory molecules (anti-CD3 in combination with anti CD28) or generalized cross-linking and agglutinating of cell surface glycosylated proteins using lectins such as PHA, or by acting directly on intracellular molecules involved in T cell activation with the DAG analogue PMA, in combination with the calcium ionophore ionomycin have been used to achieve T cell activation (Chatila et al, 1989; Frantz et al, 1994; O'Flynn et al, 1986). However, each method differs on the exact mechanism of T cell activation. We assessed for the expression of activation markers on Jurkat cells using these different T cell activators. Our results demonstrated that Jurkat cells upregulate expression of CD25, CD69 and CD40L following stimulation with PHA. Similar pattern of increased expression of CD69 and CD40L were also achieved after stimulation of Jurkat cells with plate coated anti-CD3 with anti CD28 and also by PMA with ionomycin. Parallel experiments were also performed (in our lab by M. Haug/H. Ibrahim) in primary CD4+ T cells, where CD25, CD40L and CD69 are all upregulated when CD4+ T cells are stimulated by anti-CD3/anti-CD28 antibody stimulation. Regulation of CD25, CD69 and CD40L markers expression can, then, reliably be used to detect activation status of both Jurkat cells and primary CD4+ T cells.

However, the particular response and kinetics observed among the activation markers slightly differed between the different T cell activators as well as between Jurkat cells and primary CD4+ T cells. CD25 is the α-subunit of IL-2 receptor and its assembly with β and γ subunits increases affinity of T lymphocytes to IL-2, which is a key event in regulation of T cell activation and proliferation. Here, we showed that stimulation of Jurkat cell by PHA for 24 hours increased CD25 expression to ≈22% from 3% at 6-hour. A slightly higher result was reported by Shatrova et al (2015) (32.3±3.4 %). This increased CD25-expression might be

attributed to the increased concentration of PHA (5μg/ml) they used; compared to the 1μg/ml of PHA used in this study.

CD40 is constitutively expressed in APCs while its ligand CD40L is induced in T cells following stimulation. Lack of functional expression of CD40L has been attributed to defective antigen-specific T cell expansion and immune responses (Grewal et al, 1995). CD40L induces proliferation and isotype-switching in B lymphocytes which constitutively express CD40 (Ramesh et al, 1993). Here, we demonstrated that CD40L is modulated by stimulation of Jurkat cells with PHA, PMA/Ionomycin or anti-CD3/CD28. After 24 hours of stimulation, PHA appears to drive higher expression (25%) of CD40L than PMA/Ionomycin (18%) and anti-CD3/CD28 (17%).

CD69 is a T cell surface antigen which is known to be one of the earliest upregulated surface markers following T cell stimulation (Ziegler et al, 1994). Our lab and other study have indicated that expression of CD69 is increased when CD4+ T cells are stimulated with anti-CD3 with anti-CD28, PHA or PMA (Lindsey et al, 2007). Consistently, we also observed a strong expression of CD69 when Jurkat cells are stimulated with PHA, PMA or anti-CD3 with anti-CD28. Similar finding was reported by (Yiemwattana et al, 2012) in another stream of research, where Jurkat cells were indicated to respond to and increase CD69 expression when stimulated with PHA, PMA or anti-CD3/anti-CD28 antibodies.

It appears that CD25, CD40L and CD69 are not upregulated when Jurkat cells are stimulated with the endosomal TLR ligands; CL264 (TLR7), CL75 (TLR8), R848 (TLR7/8) and CpG (TLR9). Same finding was seen with the primary CD4+ T cells that also do not increase cell surface activation markers when stimulated by these endosomal TLR ligands in the absence of TCR activation (anti-CD3/anti-CD28). However, our lab has shown that TLR8 ligands in primary CD4+ T cells have a costimulatory role; since combination of TCR activation (anti-CD3/anti-CD28) in combination with TLR8-stimulation leads to an increase in upregulation of cell surface activation markers (experiments done in our lab by M. Haug/H. Ibrahim). However, in our experiments using Jurkat cells, we did not see increased activation marker expression when TCR activation was combined with TLR stimulation.

In primary CD4+ T cells, only the TLR8 ligand pU/pLA was found to upregulate expression of T cell activation markers in the absence of TCR stimulation. (experiments done in our lab by M. Haug/H. Ibrahim). This indicates that poly uridine (pU) oligonucleotide, the more natural ligand to TLR8, if complexed with the poly cationic polypeptide poly-L-arginine (pLA) for intracellular entrance, is a TLR8 ligand that is capable of inducing T cell activation marker

without TCR activation. However, investigations with pU/pLA were not performed with Jurkat cells, since they lack TLR8 expression and we did not succeed in introducing functional TLR8 protein expression in Jurkat cells in the course of this project.

Activation of CD4+ cells not only results in upregulation of cell surface activation markers but also secretion of effector cytokines. TLR signaling has been attributed for pro-inflammatory cytokine production mainly by APCs (Cros et al, 2010). Although reports of TLR-mediated cytokine responses in CD4+ T cells have not been studied in depth, a couple of studies published have reported increased production of IFN-γ when primary CD4 T cells are treated with TLR8 agonists (Caron et al, 2005; Dominguez-Villar et al, 2015). In this study, increase in pro-inflammatory innate cytokine IL-6 in response to TLR8 stimulation in primary CD4+ T cells was demonstrated by qPCR. Moreover, our laboratory has also shown a range of cytokines upregulated by exposure of primary CD4+ T cells to the TLR8 ligand agonists in the absence of TCR stimulation, this cytokines include; IL-6, IL-1β, IFN-α, IFN-β and IFN-γ (unpublished observations; H. Ibrahim and M. Haug). From these findings it seems that TLR8 stimulation in the absence of TCR activation induces production of pro-inflammatory cytokines in primary CD4+ T cells and suggests a potential involvement of TLR8 in sensing of HIV derived ssRNA in non-productively infected CD4+ T cells.

However, regarding cytokine responses upon activation in general and with TLR stimulation in particular, we found that Jurkat cells are not a good model system. Jurkat cells did not mirror the cytokine profile secreted by primary CD4+ T cells when activated by T cell activators and secreted a small range of cytokines, mainly IL-2, IL-8 and TNF. Although, IL-6 secretion by Jurkat cells have been reported Khalaf et al (2010), repeated experiments with different T cell activators did not induce IL-6 production neither on mRNA nor on protein level in our experiments. Furthermore, none of the cytokines were upregulated when Jurkat cells were stimulated with endosomal TLR ligands. Since Jurkat cells do not have TLR8, it is not expected to observe any cytokine responses from TLR8 ligand. However, we did not detect any cytokine response to the TLR7 and 9 ligands on Jurkat cells either. This might be a consequence of the limited cytokine profile we found in Jurkat cells compared to primary CD4+ T cells. For instance, none the cytokines upregulated during TLR8 stimulation of primary CD4+ T cells (IL-1β, IL-6, IFN-α, IFN-β IFN-γ) are secreted in detectable level in Jurkat cells even when they are stimulated with T cell activator. Therefore, the anticipated cytokines to be secreted in response to TLR7 and 9 ligand stimulation might virtually be ascent in Jurkat cells. Of note here is that, TLR7 stimulation in primary CD4+ T cells has been found to induces an anergic

phenotype and results in complete absence of immune responses (Dominguez-Villar et al, 2015). Another approach for the investigation of TLR mediated cytokine response in Jurkat cells might be to use the TLR ligands as co-stimulator together with anti-CD3, rather than expecting a direct role of TLR ligands as in the primary CD4+ T cells. The TLR5 ligand (flagelin), for instance, has been reported to act as a co-stimulant for Jurkat cells treated with plate bound anti-CD3 antibody and trigger IL-2 secretion (Ye & Gan, 2007). However, the TLR5 ligand should be alone if IL-8 production is needed from Jurkat cells, since combination of flagelin with anti-CD3/anti-CD28 has actually been reported to decrease Il-8 secretion (Akhade & Qadri, 2015). Therefore, it appears that optimization experiments should be done to find the effective way a specific TLR ligand triggers a specific cytokine response in Jurkat cells.

In any event, the exact functional response of TLR8 in Jurkat cells can only be investigated if a TLR8 expressing Jurkat cell line is generated. Both a physical method (nucleofection) and a biological method with lentivirus system have been utilized to integrate the TLR8 gene into Jurkat cells. We also investigated the optimal puromycin titer to select for Jurkat cells with successful integration of TLR8 expression plasmid with the resistance gene to puromycin. Parallel experiments of unmodified Jurkat cells maintained in 0.5 μg/ml of puromycin revealed a complete halt in their growth as confirmed by both cell counting and cell proliferation assays. The lentiviral transduction and the nucleofection methods seemed successful as cells generated with both methods survived puromycin selection. We also found mRNA level expression of TLR8 in the transduced/nucleofected cells. Furthermore, expression of TLR8 mRNA seemed to be increased when Jurkat cells are stimulated with anti-CD3/anti CD28 antibody. However, western blot analysis revealed no band for TLR8 protein in any of the nucleofected or TLR8 transduced Jurkat cells.

The fact that cells survived antibiotic selection and showed mRNA expression of TLR8 prompted us into thinking possibly the modified Jurkat cells expressed functional TLR8 protein that was not detectable with our western blot method. We therefore performed functional assays to test for TLR8 signaling induction and nuclear translocation of transcription factors in response to TLR8 stimulation. However, we found that production of IL-2, IL-8 and TNF cytokines did not differ between TLR8 transfected/transduced and the wild-type Jurkat cells. Furthermore, intracellular signaling, investigated by assessing nuclear translocation of NF-κB, was not induced in TLR8 modified Jurkat cells in response to stimulation with TLR8 agonist.

These findings with undetected protein as well as absence of cytokine production and transcription factor translocation in response to TLR8 stimulation imply that even if we

succeeded in integration of the TLR8 gene in Jurkat cells, functional TLR8 expression was not achieved. This calls for a suspicion about the intactness of the coding region for the gene of interest in our transduced/transfected Jurkat cells. However, sequencing of the first ≈1,000 nucleotides of the expression vector, using a primer targeting the promotor region for the recombined TLR8 gene, revealed a 99% sequence identity to the original TLR8 gene sequence used to construct the plasmid. Furthermore, the same expression plasmid was used to transduce the U373 cell lines in our lab (Schrøder, 2015) and protein lysates of this cell line showed a positive band on western blot and this cell line has been used as a positive control during western blot analysis in this study.

Success in TLR8 expression in Jurkat cells might be possible if a different expression vector would be constructed to rule out the possibility of impaired transcription or mRNA processing or the possibility that high protein turnover might have occurred. However, since we found that Jurkat cells are not an optimal model to study TLR signaling in CD4+ T cells especially in terms of cytokine responses, it might be better to change to a different CD4+ T cell line or to primary CD4+ T cells for further studies. Knock-down of proteins in primary T cells has been shown to be difficult, but can be achieved. Due to associated toxicity of transfection and transduction method and consequently a decreases in the number of viable cells for further downstream experiments has been a big challenge. This makes modifying genes in primary cells in general and in primary CD4+ T cells in particular more challenging compared to the robust growth ability of cancer cell lines such as Jurkat cells. However, optimized protocols are available regarding the development of microRNAs/shRNA and CRISPR-Cas9 system that are specifically customized for primary CD4+ T cells gene modifications (Bilal et al, 2015; Schumann et al, 2015). Moreover, shRNA knockdown of TLR7 using a lentiviral system in primary CD4+ T cells have been utilized to investigate the exact role of TLR7 in HIV infection (Dominguez-Villar et al, 2015). Direct knockdown of TLR8 expression in primary CD4+ T cells using one of these methods would allow investigate directly the role and molecular mechanisms of TLR8 signaling in CD4+ T cells. This would also open opportunities to distinctively study the implication behind sensing of HIV derived ssRNA after endocytoses by TLR8 in endosomes of CD4+ T cells and analysis of TLR8-mediated downstream signaling. Such studies could unravel new aspects in CD4+ T cell function that are mediated by receptors of the innate immune systems, especially in the context of HIV infection.

6 CONCLUSION AND FUTURE PERSPECTIVE

The novel direct role of endosomal TLRs in CD4+ T cells may unlock a new paradigm about pathophysiology of HIV infection. Here we demonstrated that primary CD4+ T cells express TLR8 and our group found that triggering of TLR8 in primary CD4+ T cells elicits pro-inflammatory responses. On the other hand, we also showed that the leukemic Jurkat T cell line lacks TLR8 expression. Availability of a TLR8 knock-out T cell line opens a promising prospect for a model system to study and understand the mechanism of TLR8 mediated immune responses in CD4+ T cells, if insertion of TLR8 gene is succeeded. Since Jurkat cells lack TLR8, we, hence, aimed at generating a TLR8 gene expressing Jurkat T cell line. Availability of TLR8-negative and TLR8-positive Jurkat cells could be valuable tools to analyze the function of TLR8 in CD4+ T cells. These model systems could also be used as HIV trans-infection host to investigate the molecular mechanisms of viral derived genetic material in eliciting innate immune responses in T cells.

In this study we characterized the responses of Jurkat cells to TLR7,8 and 9 ligands as well as T cell activators and compared responses to primary human CD4+ T cells. Increased expression of CD25, CD40L and CD69 was observed in Jurkat cells following stimulation with T cell activators. Similar pattern of CD25, CD40L and CD69 upregulation is also apparent in primary CD4+ T cells. However, along with primary CD4+ T cells, Jurkat cells did not upregulate expression of these markers when treated with endosomal TLR ligands. Furthermore, investigation of functional immune responses showed that Jurkat cells secreted a limited range of cytokines when stimulated. Therefore, we concluded that direct translation of the cytokine profile from primary CD4+ T cells to studies done on Jurkat cells is not possible.

Here, we successfully transfected and transduced Jurkat cells with TLR8 gene and showed that TLR8 mRNA expression is increased when the modified cells are activated by TCR engagement. However, protein level expression of TLR8 was not obtained from western blot analysis. Also we did not find differences in functional responses during analysis of cytokine production and NF-κB nuclear translocation between wild type and the TLR8 transduced/transfected cells. Together, these findings suggest that expression of functional TLR8 protein was not achieved in both the transfection and transduction methods.

Possibilities of faulty transcription or mRNA processing during our transfection or transduction of TLR8 could in future experiments be ruled out by trying a different expression vector and use of newly designed primers to amplify and clone the TLR8 gene.

Alternatively, knocking-down of TLR8 expression in primary CD4+ T cells would be more physiologically relevant considering the substantial differences observed between primary CD4+ T cells and Jurkat cells regarding cytokine responses upon stimulation. Since, findings in our laboratory show a direct role of TLR8 in modulating immune responses in primary CD4+ T cells, a more thorough approach would be to compare these findings directly against TLR8 knockdown primary CD4+ T cells. Transfection experiments might be more challenging in primary CD4+ T cells due to toxic effects of transfection or transduction techniques and as a consequence low yield of cells for further downstream experiments. However, with the advancement of techniques such as the CRISPR-Cas9 system and availability of microRNA/shRNA mediated knockdown protocols specifically designed for primary CD4+ T cells (Bilal et al, 2015; Schumann et al, 2015), it is becoming more attainable to modify gene expression in these cells and thus opening more opportunities to do more comparative studies directly on primary CD4+ T cells.

REFERENCES

Abraham RT, Weiss A (2004) Jurkat T cells and development of the T-cell receptor signalling paradigm. *Nat Rev Immunol* **4:** 301-308

Akhade AS, Qadri A (2015) T-cell receptor activation of human CD4(+) T cells shifts the innate TLR response from CXCL8(hi) IFN-gamma(null) to CXCL8(lo) IFN-gamma(hi). *Eur J Immunol* **45:** 2628-2637

Akira S, Takeda K, Kaisho T (2001) Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* **2:** 675-680

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* **124:** 783-801

Aluigi M, Fogli M, Curti A, Isidori A, Gruppioni E, Chiodoni C, Colombo MP, Versura P, D'Errico‐Grigioni A, Ferri E (2006) Nucleofection is an efficient nonviral transfection technique for human bone marrow–derived mesenchymal stem cells. *Stem Cells* **24:** 454-461

Astoul E, Edmunds C, Cantrell DA, Ward SG (2001) PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol* **22:** 490-496

Bartelt RR, Cruz-Orcutt N, Collins M, Houtman JC (2009) Comparison of T cell receptor-induced proximal signaling and downstream functions in immortalized and primary T cells. *PLoS One* **4:** e5430

Basiji DA, Ortyn WE, Liang L, Venkatachalam V, Morrissey P (2007) Cellular image analysis and imaging by flow cytometry. *Clin Lab Med* **27:** 653-670, viii

Beignon A-S, McKenna K, Skoberne M, Manches O, DaSilva I, Kavanagh DG, Larsson M, Gorelick RJ, Lifson JD, Bhardwaj N (2005) Endocytosis of HIV-1 activates plasmacytoid dendritic cells via Toll-like receptor–viral RNA interactions. *J Clin Invest* **115:** 3265-3275

Bella J, Hindle K, McEwan P, Lovell S (2008) The leucine-rich repeat structure. *Cell Mol Life Sci* **65:** 2307-2333

Bergstrom B, Aune MH, Awuh JA, Kojen JF, Blix KJ, Ryan L, Flo TH, Mollnes TE, Espevik T, Stenvik J (2015) TLR8 Senses Staphylococcus aureus RNA in Human Primary Monocytes and Macrophages and Induces IFN-beta Production via a TAK1-IKKbeta-IRF5 Signaling Pathway. *J Immunol* **195:** 1100-1111

Bilal MY, Vacaflores A, Houtman JC (2015) Optimization of methods for the genetic modification of human T cells. *Immunol Cell Biol* **93:** 896-908

Blanco J, Bosch B, Fernández-Figueras MT, Barretina J, Clotet B, Esté JA (2004) High level of coreceptor-independent HIV transfer induced by contacts between primary CD4 T cells. *J Biol Chem* **279:** 51305-51314

Blasius AL, Beutler B (2010) Intracellular Toll-like Receptors. *Immunity* **32:** 305-315

Botos I, Segal DM, Davies DR (2011) The structural biology of Toll-like receptors. *Structure* **19:** 447-459

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55:** 611-622

Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J (2003) Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* **197:** 403-411

Caron G, Duluc D, Frémaux I, Jeannin P, David C, Gascan H, Delneste Y (2005) Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-γ production by memory CD4+ T cells. *J Immunol* **175:** 1551-1557

Cervantes-Acosta G, Cohen ÉA, Lemay G (2001) Human Jurkat lymphocytes clones differ in their capacity to support productive human immunodeficiency virus type 1 multiplication. *J Virol Methods* **92:** 207-213

Chatila T, Silverman L, Miller R, Geha R (1989) Mechanisms of T cell activation by the calcium ionophore ionomycin. *J Immunol* **143:** 1283-1289

Chattergoon MA, Latanich R, Quinn J, Winter ME, Buckheit RW, 3rd, Blankson JN, Pardoll D, Cox AL (2014) HIV and HCV activate the inflammasome in monocytes and macrophages via endosomal Toll-like receptors without induction of type 1 interferon. *PLoS Pathog* **10:** e1004082

Chopra RK, Nagel JE, Chrest FJ, Boto WM, Pyle RS, Dorsey B, McCoy M, Holbrook N, Adler WH (1987) Regulation of interleukin 2 and interleukin 2 receptor gene expression in human T cells: I. Effect of Ca2+-ionophore on phorbol myristate acetate co-stimulated cells. *Clin Exp Immunol* **69:** 433-440

Chuang TH, Ulevitch RJ (2000) Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw* **11:** 372-378

Colonna M (2007) TLR pathways and IFN-regulatory factors: to each its own. *Eur J Immunol* **37:** 306-309

Colosimo A, Goncz K, Holmes A, Kunzelmann K, Novelli G, Malone R, Bennett M, Gruenert D (2000) Transfer and expression of foreign genes in mammalian cells. *BioTechniques* **29:** 314-331

Conti L, Varano B, Gauzzi MC, Matarrese P, Federico M, Malorni W, Belardelli F, Gessani S (2000) Impairment of human immunodeficiency virus type 1 (HIV-1) entry into Jurkat T cells by constitutive expression of the HIV-1 vpr protein: role of CD4 down-modulation. *J Virol* **74:** 10207-10211

Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levings MK (2005) Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J Immunol* **175:** 8051-8059

Cros J, Cagnard N, Woollard K, Patey N, Zhang SY, Senechal B, Puel A, Biswas SK, Moshous D, Picard C, Jais JP, D'Cruz D, Casanova JL, Trouillet C, Geissmann F (2010) Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* **33:** 375-386

Davis HE, Morgan JR, Yarmush ML (2002) Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. *Biophys Chem* **97:** 159-172

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

Dolganiuc A, Garcia C, Kodys K, Szabo G (2006) Distinct Toll-like receptor expression in monocytes and T cells in chronic HCV infection. *World J Gastroenterol* **12:** 1198-1204

Dominguez-Villar M, Gautron A-S, de Marcken M, Keller MJ, Hafler DA (2015) TLR7 induces anergy in human CD4+ T cells. *Nat Immunol* **16:** 118-128

Ewald SE, Engel A, Lee J, Wang M, Bogyo M, Barton GM (2011) Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J Exp Med* **208:** 643-651

Frantz B, Nordby EC, Bren G, Steffan N, Paya CV, Kincaid RL, Tocci MJ, O'keefe S, O'neill E (1994) Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. *EMBO J* **13:** 861

Fredericksen BL, Wei BL, Yao J, Luo T, Garcia JV (2002) Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. *J Virol* **76:** 11440-11446

Fukata M, Breglio K, Chen A, Vamadevan AS, Goo T, Hsu D, Conduah D, Xu R, Abreu MT (2008) The myeloid differentiation factor 88 (MyD88) is required for CD4+ T cell effector function in a murine model of inflammatory bowel disease. *J Immunol* **180:** 1886-1894

Gelman AE, Zhang J, Choi Y, Turka LA (2004) Toll-like receptor ligands directly promote activated CD4+ T cell survival. *J Immunol* **172:** 6065-6073

Gorden KB, Gorski KS, Gibson SJ, Kedl RM, Kieper WC, Qiu X, Tomai MA, Alkan SS, Vasilakos JP (2005) Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol* **174:** 1259-1268

Grewal IS, Xu J, Flavell RA (1995) Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* **378:** 617-620

Gringhuis SI, van der Vlist M, van den Berg LM, den Dunnen J, Litjens M, Geijtenbeek TB (2010) HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. *Nat Immunol* **11:** 419-426

Guerrero S, Batisse J, Libre C, Bernacchi S, Marquet R, Paillart JC (2015) HIV-1 replication and the cellular eukaryotic translation apparatus. *Viruses* **7:** 199-218

Guo H, Gao J, Taxman DJ, Ting JP, Su L (2014) HIV-1 infection induces interleukin-1beta production via TLR8 protein-dependent and NLRP3 inflammasome mechanisms in human monocytes. *J Biol Chem* **289:** 21716-21726

Hamm A, Krott N, Breibach I, Blindt R, Bosserhoff AK (2002) Efficient transfection method for primary cells. *Tissue Eng* **8:** 235-245

Hashimoto C, Hudson KL, Anderson KV (1988) The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* **52:** 269-279

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S (2004) Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* **303:** 1526-1529

Hemmi H, Akira S (2005) TLR signalling and the function of dendritic cells. *Chem Immunol Allergy* **86:** 120-135

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. *Nat Immunol* **3:** 196-200

Hemont C, Neel A, Heslan M, Braudeau C, Josien R (2013) Human blood mDC subsets exhibit distinct TLR repertoire and responsiveness. *J Leukoc Biol* **93:** 599-609

Hesselgesser J, Liang M, Hoxie J, Greenberg M, Brass LF, Orsini MJ, Taub D, Horuk R (1998) Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. *J Immunol* **160:** 877-883

Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, Endres S, Hartmann G (2002) Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* **168:** 4531-4537

Ishii N, Funami K, Tatematsu M, Seya T, Matsumoto M (2014) Endosomal localization of TLR8 confers distinctive proteolytic processing on human myeloid cells. *J Immunol* **193:** 5118-5128

Itoh H, Tatematsu M, Watanabe A, Iwano K, Funami K, Seya T, Matsumoto M (2011) UNC93B1 physically associates with human TLR8 and regulates TLR8-mediated signaling. *PLoS One* **6:** e28500

Janeway Jr CA, Medzhitov R (2002) Innate immune recognition. *Annu Rev Immunol* **20:** 197-216

Jenkins MK, Khoruts A, Ingulli E, Mueller DL, McSorley SJ, Reinhardt RL, Itano A, Pape KA (2001) In vivo activation of antigen-specific CD4 T cells. *Annu Rev Immunol* **19:** 23-45

Jolly C, Kashefi K, Hollinshead M, Sattentau QJ (2004) HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *J Exp Med* **199:** 283-293

Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S (2002) Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat Immunol* **3:** 499-499

Kader M, Smith AP, Guiducci C, Wonderlich ER, Normolle D, Watkins SC, Barrat FJ, Barratt-Boyes SM (2013) Blocking TLR7- and TLR9-mediated IFN-alpha production by plasmacytoid dendritic cells does not diminish immune activation in early SIV infection. *PLoS Pathog* **9:** e1003530

Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* **3:** 984-993

Kawai T, Akira S (2006) TLR signaling. *Cell Death Differ* **13:** 816-825

Kawai T, Akira S (2007) TLR signaling. In *Seminars in immunology*, Vol. 19, pp 24-32.

Kawai T, Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* **21:** 317-337

Khalaf H, Jass J, Olsson P-E (2010) Differential cytokine regulation by NF-κB and AP-1 in Jurkat T-cells. *BMC Immunol* **11:** 26

Kim YM, Brinkmann MM, Paquet ME, Ploegh HL (2008) UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* **452:** 234-238

Klein L, Hinterberger M, Wirnsberger G, Kyewski B (2009) Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* **9:** 833-844

Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY (2004) TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci U S A* **101:** 3029-3034

Korneyeva M, Stalhandske P, Asjo B (1993) Jurkat-tat but not other tat-expressing cell lines support replication of slow/low type HIV. *J Acquir Immune Defic Syndr* **6:** 231-236

Krieg AM, Vollmer J (2007) Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. *Immunol Rev* **220:** 251-269

Kulkarni R, Behboudi S, Sharif S (2011) Insights into the role of Toll-like receptors in modulation of T cell responses. *Cell Tissue Res* **343:** 141-152

Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW (1999) Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proceedings of the National Academy of Sciences* **96:** 5215-5220

Lee BL, Barton GM (2014) Trafficking of endosomal Toll-like receptors. *Trends Cell Biol* **24:** 360-369

Lemaitre B, Nicolas E, Michaut L, Reichhart J-M, Hoffmann JA (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86:** 973-983

Lindsey WB, Lowdell MW, Marti GE, Abbasi F, Zenger V, King KM, Lamb LS, Jr. (2007) CD69 expression as an index of T-cell function: assay standardization, validation and use in monitoring immune recovery. *Cytotherapy* **9:** 123-132

Lingnau K, Egyed A, Schellack C, Mattner F, Buschle M, Schmidt W (2002) Poly-L-arginine synergizes with oligodeoxynucleotides containing CpG-motifs (CpG-ODN) for enhanced and prolonged immune responses and prevents the CpG-ODN-induced systemic release of pro-inflammatory cytokines. *Vaccine* **20:** 3498-3508

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25:** 402-408

Lu H, Dietsch GN, Matthews M-AH, Yang Y, Ghanekar S, Inokuma M, Suni M, Maino VC, Henderson KE, Howbert JJ (2012) VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res* **18:** 499-509

Luckheeram RV, Zhou R, Verma AD, Xia B (2012) CD4(+)T cells: differentiation and functions. *Clin Dev Immunol* **2012:** 925135

Maki W, Morales RE, Carroll VA, Telford WG, Knibbs RN, Stoolman LM, Hwang ST (2002) CCR6 colocalizes with CD18 and enhances adhesion to activated endothelial cells in CCR6-transduced Jurkat T cells. *J Immunol* **169:** 2346-2353

Mansson A, Adner M, Cardell LO (2006) Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. *Respir Res* **7:** 36

Maurisse R, Semir D, Emamekhoo H, Bedayat B, Abdolmohammadi A, Parsi H, Gruenert DC (2010) Comparative transfection of DNA into primary and transformed mammalian cells from different lineages. *BMC Biotechnol* **10:** 1

Melikyan GB (2014) HIV entry: a game of hide-and-fuse? *Curr Opin Virol* **4:** 1-7

Moresco EMY, LaVine D, Beutler B (2011) Toll-like receptors. *Curr Biol* **21:** R488-R493

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263-267

Nüsslein-Volhard C, Wieschaus E, Kluding H (1984) Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. *Wilhelm Roux Arch Entwickl Mech Org* **193:** 267-282

O'Flynn K, Russul-Saib M, Ando I, Wallace DL, Beverley PC, Boylston AW, Linch DC (1986) Different pathways of human T-cell activation revealed by PHA-P and PHA-M. *Immunology* **57:** 55-60

Ohto U, Tanji H, Shimizu T (2014) Structure and function of toll-like receptor 8. *Microbes and infection / Institut Pasteur* **16:** 273-282

Okimoto T, Friedmann T, Miyanohara A (2001) VSV-G envelope glycoprotein forms complexes with plasmid DNA and MLV retrovirus-like particles in cell-free conditions and enhances DNA transfection. *Mol Ther* **4:** 232-238

Ouellet M, Roy J, Barbeau B, Geleziunas R, Tremblay MJ (2003) NF-kappaB induction by bisperoxovanadium compounds requires CD45, p36(LAT), PKC, and IKK activity and exhibits kinetics of activation comparable to those of TCR/CD28 coengagement. *Biochemistry* **42:** 8260-8271

Pan X, Baldauf HM, Keppler OT, Fackler OT (2013) Restrictions to HIV-1 replication in resting CD4+ T lymphocytes. *Cell Res* **23:** 876-885

Pandey S, Kawai T, Akira S (2015) Microbial sensing by Toll-like receptors and intracellular nucleic acid sensors. *Cold Spring Harb Perspect Biol* **7:** a016246

Parson W, Kirchebner R, Muhlmann R, Renner K, Kofler A, Schmidt S, Kofler R (2005) Cancer cell line identification by short tandem repeat profiling: power and limitations. *FASEB J* **19:** 434-436

Peng G, Guo Z, Kiniwa Y, shin Voo K, Peng W, Fu T, Wang DY, Li Y, Wang HY, Wang R-F (2005) Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* **309:** 1380-1384

Piccinini A, Midwood K (2010) DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm* **2010**

Poltorak A, He X, Smirnova I, Liu M-Y, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282:** 2085-2088

Popik W, Hesselgesser JE, Pitha PM (1998) Binding of human immunodeficiency virus type 1 to CD4 and CXCR4 receptors differentially regulates expression of inflammatory genes and activates the MEK/ERK signaling pathway. *J Virol* **72:** 6406-6413

Rahman AH, Taylor DK, Turka LA (2009) The contribution of direct TLR signaling to T cell responses. *Immunol Res* **45:** 25-36

Ramesh N, Fuleihan R, Ramesh V, Lederman S, Yellin MJ, Sharma S, Chess L, Rosen FS, Geha RS (1993) Deletions in the ligand for CD40 in X-linked immunoglobulin deficiency with normal or elevated IgM (HIGMX-1). *Int Immunol* **5:** 769-773

Reiser J, Harmison G, Kluepfel-Stahl S, Brady RO, Karlsson S, Schubert M (1996) Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles. *Proc Natl Acad Sci U S A* **93:** 15266-15271

Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A (2005) The evolution of vertebrate Toll-like receptors. *Proc Natl Acad Sci U S A* **102:** 9577-9582

Schlaepfer E, Speck RF (2011) TLR8 activates HIV from latently infected cells of myeloid-monocytic origin directly via the MAPK pathway and from latently infected CD4+ T cells indirectly via TNF-alpha. *J Immunol* **186:** 4314-4324

Schneider U, Schwenk HU, Bornkamm G (1977) Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. *Int J Cancer* **19:** 621-626

Schrøder IC (2015) Estabilishing tools to study the involvement of Keap1 in inflammatory signaling upon infection with M. avium. MSc Thesis, Cancer Research and Molecular Medicine, CEMIR, NTNU

Schumann K, Lin S, Boyer E, Simeonov DR, Subramaniam M, Gate RE, Haliburton GE, Ye CJ, Bluestone JA, Doudna JA, Marson A (2015) Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc Natl Acad Sci U S A* **112:** 10437-10442

Shan X, Czar MJ, Bunnell SC, Liu P, Liu Y, Schwartzberg PL, Wange RL (2000) Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol Cell Biol* **20:** 6945-6957

Sharma N, Akhade AS, Qadri A (2013) Sphingosine-1-phosphate suppresses TLR-induced CXCL8 secretion from human T cells. *J Leukoc Biol* **93:** 521-528

Sharma N, Akhade AS, Qadri A (2016) Src kinases central to T-cell receptor signaling regulate TLR-activated innate immune responses from human T cells. *Innate Immun*

Shatrova A, Mityushova E, Aksenov N, Marakhova I (2015) CD25 expression on the surface of Jurkat cells. *Cell tissue biol* **9:** 364-370

Shearer RF, Saunders DN (2015) Experimental design for stable genetic manipulation in mammalian cell lines: lentivirus and alternatives. *Genes Cells* **20:** 1-10

Smeets RL, Fleuren WW, He X, Vink PM, Wijnands F, Gorecka M, Klop H, Bauerschmidt S, Garritsen A, Koenen HJ, Joosten I, Boots AM, Alkema W (2012) Molecular pathway profiling of T lymphocyte signal transduction pathways; Th1 and Th2 genomic fingerprints are defined by TCR and CD28-mediated signaling. *BMC Immunol* **13:** 12

Smith-Garvin JE, Koretzky GA, Jordan MS (2009) T cell activation. *Annu Rev Immunol* **27:** 591-619

Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF (2008) The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *Oncologist* **13:** 859-875

Sowinski S, Jolly C, Berninghausen O, Purbhoo MA, Chauveau A, Kohler K, Oddos S, Eissmann P, Brodsky FM, Hopkins C, Onfelt B, Sattentau Q, Davis DM (2008) Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat Cell Biol* **10:** 211-219

Stevenson M (2003) HIV-1 pathogenesis. *Nat Med* **9:** 853-860

T O'Neil R, Wei Q, Condie BG (2015) High efficiency transfection of thymic epithelial cell lines and primary thymic epithelial cells by Nucleofection. *bioRxiv***:** 030221

Takahama Y (2006) Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* **6:** 127-135

Takeda K, Akira S (2015) Toll-like receptors. *Curr Protoc Immunol* **109:** 14.12.11-14.12.10

Tanji H, Ohto U, Shibata T, Miyake K, Shimizu T (2013) Structural reorganization of the Toll-like receptor 8 dimer induced by agonistic ligands. *Science* **339:** 1426-1429

Tanji H, Ohto U, Shibata T, Taoka M, Yamauchi Y, Isobe T, Miyake K, Shimizu T (2015) Toll-like receptor 8 senses degradation products of single-stranded RNA. *Nat Struct Mol Biol* **22:** 109-115

Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA (2011) Pattern recognition receptors and the innate immune response to viral infection. *Viruses* **3:** 920-940

Tiefenthaler G, Hunig T (1989) The role of CD2/LFA-3 interaction in antigen- and mitogen-induced activation of human T cells. *Int Immunol* **1:** 169-175

Tomita T, Kanai T, Fujii T, Nemoto Y, Okamoto R, Tsuchiya K, Totsuka T, Sakamoto N, Akira S, Watanabe M (2008) MyD88-dependent pathway in T cells directly modulates the expansion of colitogenic CD4+ T cells in chronic colitis. *J Immunol* **180:** 5291-5299

Troutman TD, Bazan JF, Pasare C (2012) Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell cycle (Georgetown, Tex)* **11:** 3559-3567

Untergasser A (2008) Transformation of Chemical Competent Cells [Online].Untergasser's Lab. Available: http://www.untergasser.de/lab/protocols/competent_cells_chemical_trafo_v1_0.htm [Accessed on 11/02 2016].

Verweij C, Geerts M, Aarden LA (1991) Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-kB-like response element. *J Biol Chem* **266:** 14179-14182

Wang X, Gao Y, Tan J, Devadas K, Ragupathy V, Takeda K, Zhao J, Hewlett I (2012) HIV-1 and HIV-2 infections induce autophagy in Jurkat and CD4+ T cells. *Cell Signal* **24:** 1414-1419

Watters TM, Kenny EF, O'Neill LA (2007) Structure, function and regulation of the Toll/IL-1 receptor adaptor proteins. *Immunol Cell Biol* **85:** 411-419

Weiss A, Wiskocil RL, Stobo JD (1984) The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level. *J Immunol* **133:** 123-128

Wilen CB, Tilton JC, Doms RW (2012) Molecular mechanisms of HIV entry. *Adv Exp Med Biol* **726:** 223-242

Ye Z, Gan Y-H (2007) Flagellin contamination of recombinant heat shock protein 70 is responsible for its activity on T cells. *J Biol Chem* **282:** 4479-4484

Yeung ML, Houzet L, Yedavalli VS, Jeang K-T (2009) A genome-wide short hairpin RNA screening of jurkat T-cells for human proteins contributing to productive HIV-1 replication. *J Biol Chem* **284:** 19463-19473

Yiemwattana I, Ngoenkam J, Paensuwan P, Kriangkrai R, Chuenjitkuntaworn B, Pongcharoen S (2012) Essential role of the adaptor protein Nck1 in Jurkat T cell activation and function. *Clin Exp Immunol* **167:** 99-107

Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF, Gilboa E (1986) Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc Natl Acad Sci U S A* **83:** 3194-3198

Zarember KA, Godowski PJ (2002) Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* **168:** 554-561

Zhang W, Samelson LE (2000) The role of membrane-associated adaptors in T cell receptor signalling. *Semin Immunol* **12:** 35-41

Ziegler SF, Ramsdell F, Alderson MR (1994) The activation antigen CD69. *Stem Cells* **12:** 456-465

APPENDICES

Appendix 1: Protocols

Appendix 1.1: High-Capacity RNA-to-cDNA Protocol, Applied Biosystems

<table>
<tr><th>STEP</th><th colspan="2">ACTION</th></tr>
<tr><td>1</td><td>Input Amount of Total RNA</td><td>Use up to 2 µg of total RNA per 20 µL reaction.</td></tr>
<tr><td>2</td><td>Preparing the RT reaction</td><td>
Prepare the RT reaction mix using the kit components before preparing the reaction plate.
To prepare the RT reaction mix (per 20 µL reaction):
a. Allow the kit components to thaw on ice.
b. Referring to the following table, calculate the volume of components needed to prepare the required number of reactions.
<table>
<tr><th>Component</th><th colspan="2">Volume/Reaction (µL)</th></tr>
<tr><td></td><td>+RT</td><td>-RT</td></tr>
<tr><td>2× RT Buffer</td><td>10.0</td><td>10.0</td></tr>
<tr><td>20× RT Enzyme Mix</td><td>1.0</td><td>—</td></tr>
<tr><td>Nuclease-free H_2O</td><td>Q.S.† to 20 µL</td><td>Q.S. to 20 µL</td></tr>
<tr><td>Sample</td><td>up to 9 µL</td><td>up to 9 µL</td></tr>
<tr><td>Total per Reaction</td><td>20.0</td><td>20.0</td></tr>
</table>
† Quantity Sufficient

Note: Prepare the RT reaction on ice. Include additional reactions in the calculations to provide excess volume for the loss that occurs during reagent transfers.

WARNING CHEMICAL HAZARD. 2× RT Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate eyewear, clothing, and gloves.
</td></tr>
<tr><td>3</td><td>Preparing the cDNA Reverse Transcription Reactions</td><td>
To prepare the cDNA RT reactions:
a. Aliquot 20 µL of RT reaction mix into each well, or tube.
b. seal the plates or tubes.
c. Briefly centrifuge the plate or tubes to spin down the contents and to elimiate any air bubbles.

Place the plate or tubes on ice until you are ready to load the thermal cycler or Applied Biosystems Real-Time PCR system.
</td></tr>
</table>

<table>
<tr><th>STEP</th><th colspan="2">ACTION</th></tr>
<tr><td>4</td><td>Performing Reverse Transcription</td><td>To perform reverse transcription:
a. Using one of the required thermal cyclers listed in “Materials and Equipment” in High Capacity RNA-to-cDNA Kit Protocol (part number 4387951), program the thermal cycler conditions:
IMPORTANT! These conditions are optimized for use with the High Capacity RNA-to-cDNA Kit.
<table><tr><th></th><th>Step 1</th><th>Step 2</th><th>Step 3</th></tr><tr><td>Temperature (°C)</td><td>37</td><td>95</td><td>4</td></tr><tr><td>Time</td><td>60 min</td><td>5 min</td><td>∞</td></tr></table>b. Set the reaction volume to 20 µL.
c. Load the reactions into the thermal cycler or Applied Biosystems Real-Time PCR system.
d. Start the reverse transcription run.</td></tr>
<tr><td>5</td><td>Storing cDNA Reverse Transcription Reactions</td><td>You can store cDNA RT plates or tubes prepared using theHigh Capacity RNA-to-cDNA Kit for short-term or long-term storage.
<table><tr><th>Storage Duration</th><th>Storage Temperature (°C)</th></tr><tr><td>Short-term (up to 24 hours before use)</td><td>2 to 8</td></tr><tr><td>Long-term</td><td>–15 to –25</td></tr></table>If required, briefly centrifuge the archive plates or tubes before storing to spin down the contents and to eliminate any air bubbles.</td></tr>
</table>

Appendix 1.2: PureYield Miniprep Protocol, Promega:

PureYield™ Plasmid Miniprep System

INSTRUCTIONS FOR USE OF PRODUCTS A1220, A1221, A1222 AND A1223

Quick PROTOCOL

Solution Preparation

Before lysing cells and purifying DNA, prepare the Column Wash Solution by adding ethanol. Cap tightly after addition. See Technical Bulletin #TB374 for detailed instructions.

DNA Purification by Centrifugation

Prepare Lysate

1. Add 600µl of bacterial culture to a 1.5ml microcentrifuge tube.
   Note: For higher yields and purity use the alternative protocol below to harvest and process up to 3ml of bacterial culture.
2. Add 100µl of Cell Lysis Buffer (Blue), and mix by inverting the tube 6 times.
3. Add 350µl of cold (4–8°C) Neutralization Solution, and mix thoroughly by inverting.
4. Centrifuge at maximum speed in a microcentrifuge for 3 minutes.
5. Transfer the supernatant (~900µl) to a PureYield™ Minicolumn without disturbing the cell debris pellet.
6. Place the minicolumn into a Collection Tube, and centrifuge at maximum speed in a microcentrifuge for 15 seconds.
7. Discard the flowthrough, and place the minicolumn into the same Collection Tube.

Wash

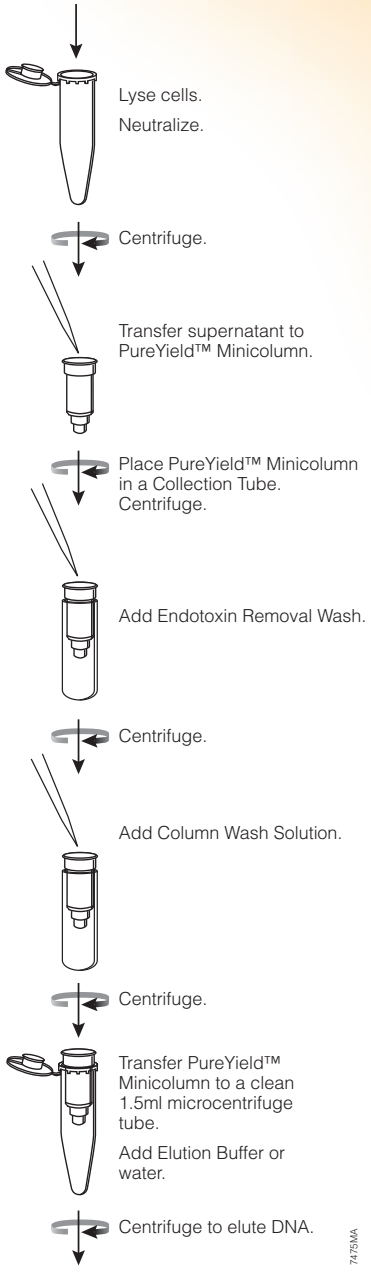
8. Add 200µl of Endotoxin Removal Wash (ERB) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 15 seconds.
9. Add 400µl of Column Wash Solution (CWC) to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 seconds.

Elute

10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 30µl of Elution Buffer or nuclease-free water directly to the minicolumn matrix. Let stand for 1 minute at room temperature.
11. Centrifuge for 15 seconds to elute the plasmid DNA. Cap the microcentrifuge tube, and store eluted plasmid DNA at –20°C.

Alternative Protocol for Larger Culture Volumes

1. Centrifuge 1.5ml of bacterial culture for 30 seconds at maximum speed in a microcentrifuge. Discard the supernatant.
2. Add an additional 1.5ml of bacterial culture to the same tube and repeat Step 1.
3. Add 600µl of TE buffer or water to the cell pellet, and resuspend completely.
4. Proceed to Step 2 of the standard protocol above.



Appendix 1.3: GeneJuice® transfection procedure

Transfection Procedure (35 mm plates)

The following procedures facilitate efficient introduction of DNA into adherent and suspended eukaryotic cells. These methods are suitable for a range of cell types, but may require optimization for individual cell lines, growth conditions, and other application-specific variables. Refer to *Optimization* on p 2. The DNA to be transfected should be at a concentration of 0.5–1 µg/µl. If the DNA concentration is lower, decrease the volume of serum-free medium in the transfection mix to compensate for the larger volume of DNA.

Transfection of adherent cells

1. The day before transfection, plate 1–3 × 10^5 cells in complete growth medium per 35-mm dish. Incubate at 37°C (5% CO_2) overnight. Cells should be 50–80% confluent before transfection.
2. For each 35-mm dish to be transfected, place 100 µl serum-free medium (for example, RPMI 1640 or Opti-MEM®) into a sterile tube. Add 3 µl GeneJuice® Transfection Reagent drop-wise directly to the serum-free medium. Mix thoroughly by vortexing. Volumes can be scaled up for transfection of multiple dishes with the same DNA.
3. Mix thoroughly by vortexing.

Note: *For most cell lines, the optimal ratio of GeneJuice reagent to DNA is 3 µl reagent to 1 µg DNA. However, the ratio can be varied from 2–6 µl per µg DNA during optimization.*

4. Incubate at room temperature for 5 min.
5. For each 35-mm dish to be transfected, add 1 µg DNA to GeneJuice reagent/serum-free medium mixture. Mix by gentle pipetting.
6. Incubate GeneJuice reagent/DNA mixture at room temperature for 5–15 min.
7. Add entire volume of GeneJuice reagent/DNA mixture drop-wise to cells in complete growth medium. Distribute drops over entire surface of dish. Gently rock dish to ensure even distribution. Do not swirl plate, as doing so will concentrate transfection mixture in center of plate.
   Optional: Remove transfection mixture after 2–8 h incubation and replace with complete growth medium.
8. Incubate cells for 24–72 h at 37°C (5% CO_2).
9. Harvest cells for analysis.

Note: *For stable cell line selection, subculture the cells (dilute at least 1:5) in complete growth medium plus selective agent. Continue incubation for 1–2 weeks, allowing for growth and selection of desired cells.*

Transfection of suspension cells

1. The day before transfection, dilute cells to a density of 0.5–2.5 × 10^5 cells per ml, so they will be in log phase growth the following day. Incubate cells at 37°C (5% CO_2) overnight.
2. Plate 3 ml cells at a density of 1–2 × 10^6 cells/ml in a 35-mm dish.
3. For each 35-mm plate to be transfected, place 100 µl serum-free medium (e.g., RPMI 1640 or Opti-MEM) into a sterile tube. Add 3 µl GeneJuice Transfection Reagent drop-wise directly to serum-free medium. Mix thoroughly by vortexing. Volumes can be scaled up for transfection of multiple dishes with the same DNA.
4. Incubate at room temperature for 5 min.
5. For each 35-mm dish to be transfected, add 1 µg DNA to GeneJuice reagent/serum-free medium mixture. Mix by gentle pipetting.
6. Incubate GeneJuice reagent/DNA mixture at room temperature for 5–15 min.
7. Add entire volume of GeneJuice reagent/DNA mixture drop-wise to cells in complete growth medium. Distribute drops over entire surface of the dish. Gently rock dish to ensure even distribution. Do not swirl plate, as doing so will concentrate transfection mixture and cells in center of plate.
8. Incubate cells for 24–72 h at 37°C (5% CO_2).
9. Harvest cells for analysis.

Appendix 1.4: Lenti-X™ concentrator protocol

Lenti-X™ Concentrator Protocol-at-a-Glance

(PT4421-2)

A. Summary

The Lenti-X Concentrator (Cat. Nos. 631231 & 631232) provides a fast and simple method for concentrating lentiviral stocks. Concentration is achieved by mixing a lentiviral supernatant with this concentration reagent, followed by a short incubation step and centrifugation in a standard centrifuge. The process is easily scaled up to accomodate larger supernatant volumes. No ultracentrifugation is required. The concentration procedure can be completed in as little as 1 hour, or for convenience, longer incubation times can be used. The Lenti-X Concentrator is designed for use with all lentiviral supernatants, including all of Clontech's Lenti-X vectors. Using this reagent increases vector titer (IFU/ml) by 1–2 logs in a short amount of time with minimal loss of material.

B. Protocol

Viral supernatant is collected from your virus-producing cell line and centrifuged to remove cells and debris. It is then mixed with the Lenti-X Concentrator and incubated for a short time at 4°C. The mixture is then centrifuged at low speed to obtain a high-titer virus-containing pellet which can then easily be resuspended and used for transduction of your intended target cells.

1. Harvest the lentivirus-containing supernatants. (Caution: supernatants contain live lentivirus.) Pool similar stocks, if desired. Centrifuge briefly (500 x g for 10 min) or filter through a 0.45 µm filter.

   NOTE: If filtering, use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters. Nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus.

2. Transfer clarified supernatant to a sterile container and combine 1 volume of Lenti-X Concentrator with 3 volumes of clarified supernatant. Mix by gentle inversion. Larger volumes may be accomodated through the use of larger (i.e., 250 ml or 500 ml) centrifuge tubes.

   NOTE: For easy calculation of the amount of Lenti-X Concentrator to use, simply measure the amount of viral supernatant to be concentrated, divide by 3 and add the resulting amount of Lenti-X Concentrator to your viral supernatant.

3. Incubate mixture at 4°C for 30 minutes to overnight.

   NOTE: We have tested incubation times as short as 15 minutes and up to 1 week at 4°C with minimal losses observed. Thorough cooling of the sample is essential, so larger volumes (>100 ml) may require longer incubation times.

4. Centrifuge sample at 1,500 x g for 45 minutes at 4°C. After centrifugation, an off-white pellet will be visible.

5. Carefully remove supernatant, taking care not to disturb the pellet. Residual supernatant can be removed with either a pipette tip or by brief centrifugation at 1,500 x g.

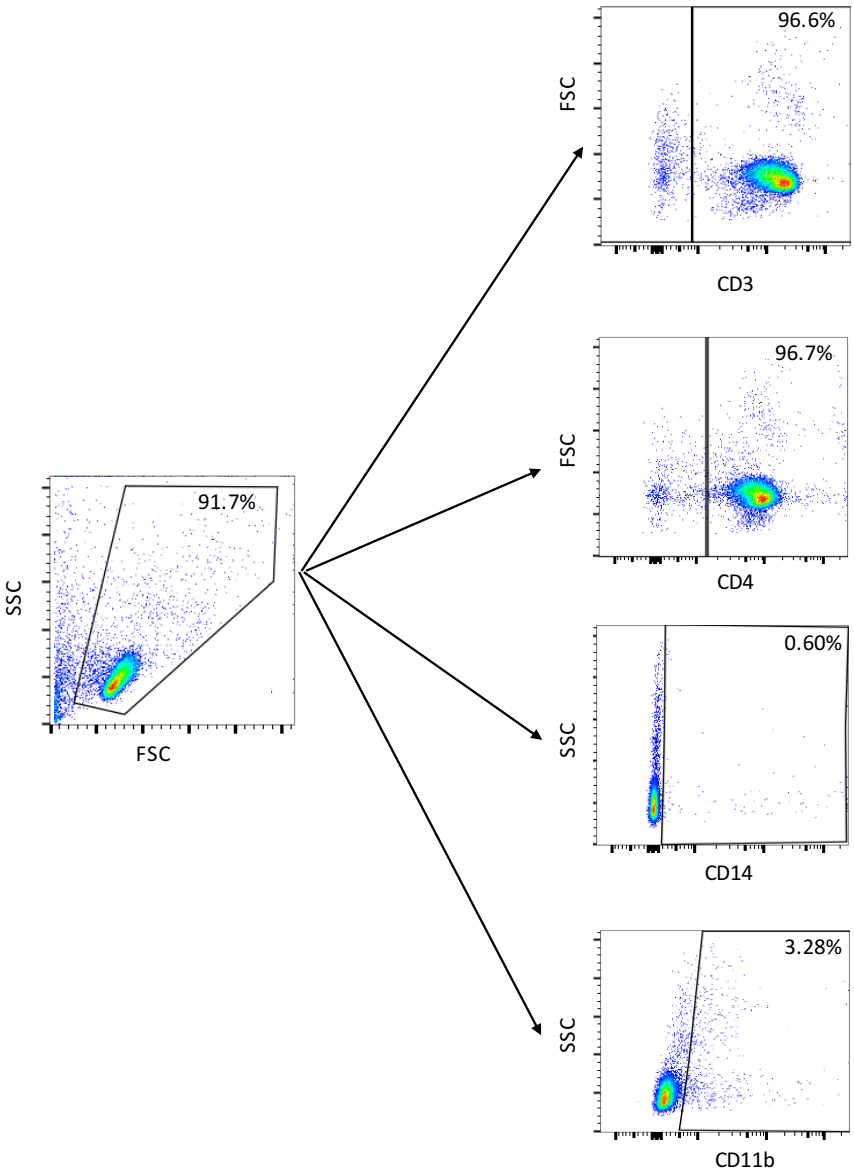
6. **Gently** resuspend the pellet in 1/10 to 1/100th of the original volume using complete DMEM, PBS, or TNE. The pellet can be somewhat sticky at first, but will go into suspension quickly.

7. Immediately titrate sample or store at –70°C in single-use aliquots.

Appendix 2: Antibodies for Flow cytomery

Marker - Color	Clone	Provider
CD4- Brilliant violet 711™	RPA-T4	BioLegend®
CD3-eFluor®	OKT3	eBioscience Inc, Sandiago, CA
CD40L-Brilliant violet 605™	23-31	BioLegend®
CD69-PE	FN50	BioLegend®
CD25-Brilliant violet 510™	M-A251	BioLegend®
CXCR4-PE/Cy5	12G5	BioLegend®
CCR5-PE/Cy7	J418F1	BioLegend®
HLADR-Brilliant violet 785™	L243	BioLegend®

Appendix 3: Purity of primary CD4+ T used for qPCR and Western Blotting



Flow cytometry purity layout for negatively isolated primary CD4+ T cells. Negatively isolated primary CD4+ T cells from PBMC were stained with anti-CD3, anti-CD4, anti-CD14 and anti-CD11b and analyzed using flow cytometry

Appendix 4: LB media and plate recipe

LB Broth (for 1 liter)

- NaCl 10g
- Bactotryptone 10g
- Yeast extract 5g
- Adjust pH to 7.0 with NaOH
- Add deionized water to final volume of 1 liter
- Autoclave

LB plate (for 1 liter)

- NaCl 10g
- Bactotryptone 10g
- Yeast extract 5g
- Bactoagar 20g
- Adjust pH to 7.0 with NaOH
- Add deionized water to final volume of 1 liter
- Autoclave

Appendix 5: Western Blot buffer and solution preparation

Buffer	Stock solution	Volume
Lysis buffer (2x) • *Keep in -20°C* • *Dilute 1:1 with fresh bezonase and proteinase inhibitor cocktail solution*		For 100ml:
	Glycerol 87%	23ml
	NaF 0.5M	20ml
	Tris/HCl (pH 8.0) 1M	10ml
	EDTA (pH 8.0) 0.2M	1ml
	EGTA 0.2M	1ml
	NaCl 5M	15.4ml
	Triton X-100 10%	20ml
	Na_3VO_4 0.2M	1ml
	Sodium Deoxychelat 10%	10ml
	MiliQ water	Up to 100ml
Benzpnase and proteinase inhibitor cocktail solution in 8M Urea • *Make before use*		For 5ml:
	Benzonase 0.25U/ml	1.3µl
	Protein inhibitor cocktail	1 tablet
	8M urea in MiliQ water	5ml
Tris buffer saline-with Tween (TBS-T) • *Stored at room temperature*		For 1000ml:
	Tris (pH 7.5) 1M	9.9ml
	Tween-20	1ml
	NaCl 5M	19.8ml
	Deionized water	Up to 100ml

Appendix 6: 34plex Multiplex ELISA: List of cytokines targeted

ProcartaPlex® Multiplex Immunoassay Panel: Product number: EPX340-12167-901

#	Target Name
1	Ecotaxin/CCL11
2	GM-CSF
3	GROα/CXCL1
4	IFN-α
5	IFN-γ
6	IL-1β
7	IL-1α
8	IL-1RA
9	IL-2
10	IL-4
11	IL-5
12	IL-6
13	IL-7
14	IL-8/CXCL8
15	IL-9
16	IL-10
17	IL-12p70
18	IL-13
19	IL-15
20	IL-17A
21	IL-18
22	IL-21
23	IL-22
24	IL-23
25	IL-27
26	IL-31
27	IP-10/CXCL10
28	MCP-1/CCL2
29	MIP-1α/CCL3
30	MIP-1β/CCL4
31	RANTES/CCL5
32	SDF1α/CXCL12
33	TNFα
34	TNFβ/LTA