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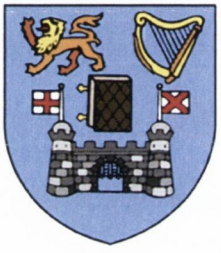
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Protein Kinase Cη: Regulation of expression and functional role in human T lymphocytes

By

Denise Triglia van Nierop



A dissertation submitted in partial fulfilment for the degree of

Doctor in Philosophy

Under the supervision of Aideen Long, Ph.D. at

Trinity College Dublin

2014

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Denise G. Triglia van Nierop

II Dedication

This work is dedicated to the anonymous donors of the primary cells. I hope they know that, as blood donors, they not only save lives, they also contribute a great deal to science.

III Acknowledgements

When I arrived in Ireland six years ago, I could not imagine what the future had in store for me. As it comes to the end of this Ph.D, I could not be more grateful for the outcome of my Irish life. I met incredible people during this journey who I will always be indebted to.

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Now I can finally switch Christmas FM on...

IV Summary

Protein kinase Cη (PKCη) is a member of the novel PKC subfamily of serine/threonine kinases that has been shown to play critical roles in cell differentiation, proliferation, secretion and apoptosis in several cell types. This isoform is primarily expressed in epithelial tissues but it has also been shown to be expressed at high levels in T cells. T cells are the centre of cell-mediated immunity, responsible for recognising, with high specificity through their T cell receptor (TCR), antigenic peptides presented by specialised cells and responding by orchestrating an effective immune response. Stimulation of the TCR and co-receptors triggers a complex signalling cascade that results in T cell activation, cytokine production, proliferation and differentiation into effector cells. Understanding the role of PKCη in the biology of T cells is in its early stages and has focussed on the PKCη knockout mouse model. The role of PKCη in human T cells has not been addressed. For this reason, the overall aim of this study was to investigate the regulation of expression and function of PKCη in human T cells using two human T cell lines (HuT78 and Jurkat) and primary lymphocytes. It was shown that levels of PKCη vary among the T cell types analysed with HuT78 cells expressing very low basal levels of PKCη compared to Jurkat T cells and primary $CD4^+$ T cells. Expression of PKCη was specifically up-regulated in the T cell lines following T cell activation while it was decreased at the protein level in primary $CD4^+$ T cells in response to the same stimulus. Moreover, activation-induced proteolysis of full-length PKCη in $CD4^+$ T cells corresponded to the accumulation of a lower molecular weight fragment of approximately 35 kDa which was blocked following treatment with the pan-caspase inhibitor Z-VAD-FMK, suggesting that PKCη is a substrate for caspase(s) in this context. Amino acid starvation also resulted in down-regulation of PKCη protein levels in both T cell lines and in primary lymphocytes indicating a possible role for PKCη in the regulation of T cell survival in response to stress conditions. The PI3K pathway was shown to be involved in regulation of PKCη expression following cell activation in the T cell lines but not in primary cells. On the other hand, basal and activation-induced expression of PKCη is regulated by PKC activity in all T cell types studied. The function of PKCη in the context of cell activation was also investigated in this study. PKCη activity was shown to be

required for IL-2 production following activation of the T cell lines but not for primary lymphocytes. A functional role for PKCη was analysed in more detail in the $CD4^+$ T cell subset of primary lymphocytes. Silencing of PKCη expression with siRNA in $CD4^+$ T cell had no effect on cell cycle progression following cell activation with anti-CD3/CD28 antibodies when analysed at 48 hours. However, cell proliferation in response to anti-CD3/CD28 stimulation was significantly impaired following silencing of PKCη expression. These observations suggest an essential PKCη-dependent event happening after 48 hours of cell activation in $CD4^+$ T cells, which corresponded with the timing of accumulation of the 35 kDa PKCη fragment. The results obtained in this study demonstrate that the PKCη isoform is an important player in the biology of human T cells. Distinct mechanisms in relation to PKCη expression and regulation of expression between the immortalised T cell lines and primary T cells were identified, and may provide new insights into the mechanistic control of cell survival in these cells. Also, the requirement of PKCη for proliferation of $CD4^+$ T cells in response to cell activation but not for regulation of entry into cell cycle, at a time corresponding to the appearance of a caspase-dependent lower molecular weight PKCη fragment may suggest a new mechanism for PKCη function in T cell activation.

V Abbreviations

Abbreviation	Name
4-IPBA	4-Iodophenylboronic Acid
Akt (PKB)	Protein Kinase B
AnV	Annexin V
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
Caspase	Cysteinyl Aspartate-Specific Proteases
CD	Cluster of Differentiation
cDNA	Complementary DNA
DAG	Diacylglycerol
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
eIF2α	Eukariotic initiation factor α
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FCS	Foetal calf serum
GFP	Green Fluorescent Protein
GSK3	Glycogen synthase kinase 3
h	Hour
HBSS	Hanks Buffered Salt Solution
HRP	Horseradish peroxidase
IFN-γ	Interferon- γ
IL	Interleukin
iNKT cells	Invariant Natural Killer T cell
IS	Immunological Synapse
ITAM	Immunoreceptor yyrosine-based activation motifs

kDa	Kilo Dalton
LAT	Linker for activation of T cells
MAPK	Mitogen Activated Protein Kinase
MFI	Mean Fluorescence Intensity
mRNA	Messenger Ribonucleic acid
MTOC	Microtubule Organising Centre
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PAGE	Polyacrylamide Gel Electrophoresis
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate buffered saline
PBTL	Peripheral Blood T Lymphocytes
PFA	Paraformaldehyde
PHA	Phytohaemagglutin
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PKC	Protein Kinase C·
PLCγ1	Phospholipase C γ1
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethanesulfonylfluoride
PTEN	Phosphatase and tensin homologue
PVDF	Polyvinylidene Fluoride
qRT-PCR	Real-time polymerase chain reaction
RNAi	RNA interference
SDS	Sodium Dodecyl Sulphate
S.E.M	Standard error of the mean
siRNA	Small interfering RNA
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
TBS	Tris Buffered Saline
TCR	T cell receptor
TNF- α	Tumor necrosis factor α
ZAP-70	ζ-chain associated protein kinase of 70 kDa

VI Keywords

PKCη

T lymphocytes

T cell activation

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1 Chapter 1 – General Introduction

1.1 General features of the immune response

The principal functions of the immune system are the recognition with subsequent elimination of foreign antigens, formation of immunologic memory, and development of tolerance to self-antigens. In vertebrates, the immune system is generally classified into two subsystems: the innate and adaptive immune systems. The innate immune system is the front line of defence and the main mechanism for immediate responses to infection and cellular injury (Janeway and Medzhitov 2002; Iwasaki and Medzhitov 2010). This system uses germline-encoded receptors expressed by various cells, such as dendritic cells, to recognise conserved molecular patterns shared by many infectious agents (Takeda, Kaisho et al. 2003; Kawai and Akira 2010). The adaptive immune system relies on the recognition of specific antigens by highly specific T cell receptors (TCR) and B cell receptors (BCR) generated somatically through site-specific DNA recombination (Vivier and Malissen 2005). Adaptive immune responses are carried out by lymphocytes in two stages. First, the antigen is presented to and recognised by the specific TCR or BCR leading to cell priming, activation and differentiation, which usually occurs within specialised lymphoid tissues. Second, the effector response takes place, either by the cell-mediated immunity with activated T cells leaving the lymphoid tissue and migrating to the site of inflammation or by the humoral immunity, with the release of antibodies from effector B cells (plasma cells) (Parkin and Cohen 2001; Chaplin 2010). In addition, once the foreign antigen has been cleared and the immune response resolved, a small number of these activated T and B cells become long-lived memory cells and protect the organism against recurring invasions (Sprent and Surh 2001; Good-Jacobson and Shlomchik 2010).

T cells and the event of T cell activation are the focus of this study and will be discussed in further detail.

1.2 T lymphocytes

T lymphocytes or T cells are the centre of the cell-mediated immune response. They are capable of recognising, with high specificity, an antigenic peptide bound to a major histocompatibility complex (MHC) molecule presented on the surface of an antigen-presenting cell (APC) and respond to this by mounting and effective immune response (Huang, Meyer et al. 2012). When T cells encounter their cognate antigen they become activated and undergo differentiation into distinct effector subtypes that play a major role in mediating immune response through the secretion of specific cytokines (Zhu, Yamane et al. 2010; Luckheeram, Zhou et al. 2012).

T cells are characterised by the expression of a surface T cell receptor (TCR) compose of variable α:β heterodimers, linked by a disulfide bond. A smaller population expresses an alternative form of the TCR composed of γ:δ chains (Girardi 2006). The TCR forms a complex with CD3 molecules, which are invariant accessory chains that serve to transduce signals when the TCR binds to antigen-MHC complexes (Salmond, Filby et al. 2009). These accessory chains make up the CD3 complex (Figure 1-1.A), consisting of non-polymorphic transmembrane CD3 proteins presented in dimers which can be CD3εδ, CD3εγ or CD3ζζ (Wucherpfennig, Gagnon et al. 2010).

Each TCR chain (α,β) is composed of a variable domain, which binds the antigenic peptide and a constant domain, followed by a transmembrane segment and a short cytoplasmic tail. The variable domain is generated from rearranging variable (V), diversity (D) and joining (J) gene segments (Gascoigne, Zal et al. 2001; Rudolph, Stanfield et al. 2006). The assembly of these gene elements is initiated by the lymphoid-specific recombinase-activating gene (RAG) 1 and RAG2 proteins, which cleave the DNA near the V, D and J segments and are spliced together using ligases (Gellert 2002). This DNA rearrangement gives rise to a large number of possible combinations and accounts for the high diversity of the receptor repertoire.

1.2.1 T cell development in the thymus

T cells are originally formed in the bone marrow and exit to the thymus to finish their development and become thymocytes (Germain 2002; Dzhagalov and Phee 2012). The thymic microenvironment with specialised stroma cells provides unique signals that are required for proper thymocyte differentiation (Takahama 2006). One of the first steps of the maturation process is the expression of the β and α chains of the TCR, followed by expression of the cell-surface co-receptors CD4 and CD8. The thymocytes are initially $CD4^-$ and $CD8^-$ (double negative) to then become $CD4^+$ and $CD8^+$ (double positive) and finally maturing into single-positive (SP) $CD4^+$ or $CD8^+$ T cells (Germain 2002; Zuniga-Pflucker 2004). It is not completely clear the mechanism by which the CD4/CD8 expression is chosen but the transcription factor ThPOK is described as a central regulator of CD4 commitment while the Runx factor Run1 and Run3 control CD8 differentiation (Ellmeier, Haust et al. 2013). The presence of these co-receptors on the cell surface are necessary to stabilize the T cell receptor (TCR)-antigen engagement and also to contribute in signal transmission when the receptor is activated (Yin, Wang et al. 2012)

Once the TCR is formed and delivered to the cell surface, T cells undergo two selective processes which will ensure the functionality of the recently created TCR. The first process is known as positive selection and test whether the TCR has sufficient affinity for self-MHC molecules presented by nearby cells but incapable of triggering a full activation response. If the lymphocyte fails this positive selection, it undergoes apoptosis and is cleared by thymic cortical macrophages. If not, the T cells are then tested for reactivity for an extensive array of tissue-specific proteins that are expressed by a population of thymic medullary epithelial cells. T cells that recognise the self-peptides expressed by these epithelial cells undergo negative selection and are eliminated by apoptosis (Janeway 2001; Chaplin 2010; Morris and Allen 2012).

Mature T cells that have survived both positive and negative selections then enter the bloodstream and re-circulate through the body and lymphoid organs until they encounter their cognate antigen. These cells are known as naive T cells. Two-photon imaging-based studies have shown that naive T cells are in constant motion, scanning the lymph nodes in search of an antigen with velocities achieving peaks of more than 25 micrometres per minute (Miller, Wei et al. 2002).

1.2.2 T cell subsets

The T lymphocyte population is divided in two major subpopulations according to the expression of the co-receptors CD4 or CD8.

$CD8^+$ T cells develop into effector cytotoxic T lymphocytes (CTLs) following cell activation and are fundamental to promote clearance of intracellular pathogens including viruses and bacteria. These cells are specialised in secretion of cytolytic granules containing perforin and granzymes, selectively destroying infected or transformed cells (Williams and Bevan 2007). In addition, activation of $CD8^+$ T cells can be also mediated by IL-12 or from signals supplied by TLR ligands and type I interferons (Jenkins and Griffiths 2010).

In turn, $CD4^+$ T cells play a central role in the function of the immune system: they help B cells make antibodies, enhance and maintain responses of $CD8^+$ T cells, orchestrate immune responses against various pathogens, and regulate/suppress immune responses both to control autoimmunity and to adjust the magnitude and persistence of responses (reviewed by Zu, Yamane et al.2010). They are also known as T helper (Th) cells and are further subdivided functionally by the pattern of cytokines they produce and function. Th1 cells are involved in cell-mediated immunity. They produce interferon-γ (IFN-γ) as their signature cytokine, which activates macrophages to kill intracellular pathogens and also induces cytotoxic natural killer cells (Del Prete 1992). An important marker for Th1 cells is the transcription factor T-bet, which is induced by signal transducer and transcription factor 1 (STAT1), and regulates transcription of the IFN-γ gene (Robinson and O'Garra 2002). The second classic group of Th cells are the Th2 cells. They are involved in humoral immunity, supporting B cell activation and maturation (Del Prete 1992). These cells produce interleukin (IL)-4, IL-5, IL-9, IL-13 and express the transcription factor GATA3. A Th2 response is usually formed to support humoral and allergic responses and is critical for the control of certain parasitic infections (Murphy and Reiner 2002). The third major effector population of $CD4^+$ T cells are the Th17 cells. They are characterised by the production of IL-17 as a signature cytokine but also produce IL-22 (Aggarwal, Ghilardi et al. 2003; Harrington, Hatton et al. 2005). IL-17 has a critical role in regulating chemokine expression and tissue inflammation and Th17 cells are therefore very important in inflammatory responses (Park, Li et al. 2005). The fourth

group of Th cells are the regulatory T cells (Tregs). Tregs have an important role in down modulating the immune response by secreting the immune suppressive cytokines IL-10 and TGF-β. There are two groups of Tregs. The first group develops its regulatory function in the thymus and is known as natural Tregs cells (nTreg). These cells are characterized by surface expression of CD4 and CD25 (IL-2 receptor) antigens and by nuclear expression of the forkhead box protein 3 (Foxp3) transcription factor that is essential for their regulatory function (Kriegel, Li et al. 2006). The second group, called induced Tregs (iTreg) can arise from peripheral naive $CD4^+$ T cells and in the presence of TGF-β, which is a potent inducer of Foxp3 expression (Chen, Lin et al. 2011). More recently, a fifth group of Th cells was added to the list. These are the T follicular helper cells (Tfh cells), described to reside within the germinal centres and be specialised in providing cognate help to B cells, by inducing antibody production and immunoglobulin class switch (Tsai and Yu 2013). In addition, new T cell subsets have been identified as potentially distinct lineage of $CD4^+$ T cells, such as Th9 cells and the regulatory type 1 cells (Tr1) but more research need to be conducted to get more insights on these cells (Gregori, Tomasoni et al. 2010; Staudt, Bothur et al. 2010).

1.2.3 Antigen recognition by T cells

The T cell receptor recognizes its cognate peptide displayed on the surface of an antigen presenting cells (APCs) in the context of a Major Histocompatibility Complex (MHC) molecule (Huang, Meyer et al. 2012) (Figure 1-1.B). APCs are specialized cells which engulf and process the antigen, and present them in the form of short peptides bound to MHC class I or class II molecules. This process is different for $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells only recognise antigens presented with MHC class II molecules and $CD8^+$ T cells with MHC class I (reviewed by Wang, Meijers et al. 2001; Li, Teng et al. 2013). Since $CD4^+$ and $CD8^+$ T cells have different functions, the MHC molecule that is used to present the antigen will determine the type of effector response generated. Antigenic peptides that are bound to MHC class I molecules are derived from protein synthesised within the cell bearing the class I molecule, described as endogenous peptides, and activate $CD8^+$ cytotoxic T cells. Endogenous peptides are usually derived from cells infected with a virus or other intracellular pathogen, or from abnormal cells carrying tumour antigens. On the other hand, $CD4^+$ T cells are activated by peptides bound to

MHC class II molecules, which leads to production of cytokines which in turn activate a wide range of cells around them. As only a small number of specialised APCs express MHC class II molecules, the antigen peptide loaded on these molecules molecules originates by the exogenous pathway that starts with endocytosis or phagocytosis of extracellular proteins. The exogenous antigens include antigenic proteins of extracellular pathogens, such as most bacteria, parasites and virus particles (Janeway 2001; Chaplin 2010).

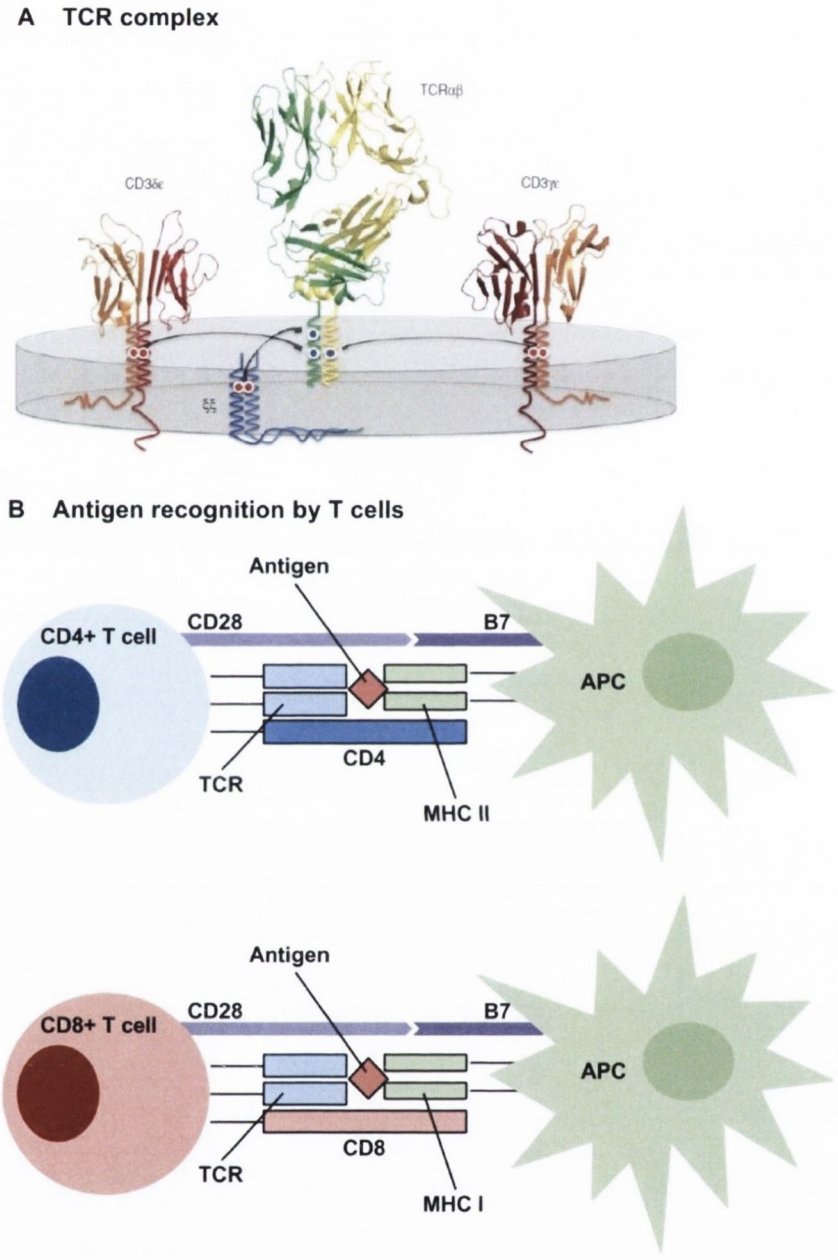


Figure 1-1 The T cell receptor (TCR) complex and antigen presenting cell (APC) interactions with T cells. A) Structure of the TCR complex. The TCR-CD3 complex is composed of two αβ chains and three dimmers CD3δε, CD3γε and CD3ζζ, which associate through intramembrane contacts to form the complex. *Adapted from Wucherpfenning et al. (2009).* B) APC present processed antigen peptides bound to MHC molecules on their surface and present these to T cells through a ligand-receptor complex involving the T cell receptor (TCR). Co-stimulation through CD28/B7 is required for optimal T cell activation.

1.2.4 TCR signalling

Ligation of the TCR with a cognate peptide displayed on the surface of an APC triggers a complex signalling cascade resulting in cell activation, proliferation and differentiation into effector cells (Figure 1-2). The molecules and pathways involved in this process have been extensively reviewed (Burbach, Medeiros et al. 2007; Smith-Garvin, Koretzky et al. 2009; Brownlie and Zamoyska 2013).

When the TCR-pMHC complex is formed, the local concentration of the SRC-family tyrosine kinases LCK and FYN increases and promotes phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the cytoplasmic tails of the CD3 subunits associated with the TCR (Salmond, Filby et al. 2009). It has been shown that LCK is constitutively associated with the cytoplasmic domains of the co-receptors CD4 and CD8 and is recruited to the site of interaction when the co-receptor binds to the MHC molecule to stabilise the TCR-pMHC complex and sustain the engagement (Veillette, Bookman et al. 1988). Phosphorylation of the ITAMs leads to recruitment and activation of the protein tyrosine kinase zeta-associated protein of 70 kDa (ZAP-70). ZAP-70 undergo conformational changes which promotes its kinase activity and, in association with activated LCK and FYN, phosphorylates multiple downstream substrates, including the adapter protein linker for activation of T cell (LAT) (Deindl, Kadlecek et al. 2007). Activation of LAT results in recruitment of several effector molecules to form a multiprotein complex known as the LAT signalosome which includes SRC homology 2 (SH2) domain-containing leukocyte phophoprotein of 76 kDa (SLP-76) and phospholipase C γ1 (PLCγ1) (Shim, Jung et al. 2011). The LAT signalosome organizes effector molecules in the right spatiotemporal manner to allow the activation of several signalling pathways (Balagopalan, Coussens et al. 2010).

Once bound to this signalling complex, PLCγ1 is activated by ITK and hydrolyzes the membrane lipid phosphatidylinositol-4,5-biphosphate (PIP_2) producing the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the activity of Ca^{2+}channels on the endoplasmic reticulum (ER) membrane leading to an influx of Ca^{2+} to the cytoplasm. Ca^{2+} ions are important second messengers in the cells and mediate the activation of Ca^{2+} and calmodulin-dependent transcription factors

including the nuclear factor of activated T cells (NFAT)(Smith-Garvin, Koretzky et al. 2009). Translocation to the nucleus of NFAT results in the expression of genes important for T cell activation including IL-2 (Macian 2005). The production of DAG following TCR ligation activates two main pathways involving Ras and members of the Protein Kinase C (PKC) family. Ras is a guanine nucleotide-binding protein required for activation of the mitogen-associated protein kinase (MAPK) signalling cascade which in turn results in phosphorylation of MAPK's extracellular signal-regulated kinase 1 (Erk1) and Erk2. Erk kinase activity causes the activation of the transcription factor Elk1, which contributes to the activation of the activator protein-1 (AP-1) transcription factor (Brownlie and Zamoyska 2013). Morover, DAG is responsible for recruitment and activation of members of the PKC family, in particular PKCθ which is critical for the regulation of the signalling pathway leading to IL-2 production through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) , Ca^{2+}/NFAT pathways and AP-1 leading to IL-2 production (Monks, Kupfer et al. 1997; Hayashi and Altman 2007; Vallabhapurapu and Karin 2009).

In addition to the TCR ligation, co-stimulatory signals are required to trigger an effective response in T cells. Several molecules can provide co-stimulation for T cells, including CD2, CD5, OX40, inducible costimulator (ICOS), and the integrin leukocyte function-associated antigen-1 (LFA-1) (Burbach, Medeiros et al. 2007; Smith-Garvin, Koretzky et al. 2009). However, the main co-stimulatory signal is delivered by the CD28 co-receptor and results in increased activation of the signalling pathways involved in T cell activation (reviewed by Riha and Rudd 2010).

1.2.5 CD28 co-stimulation

The engagement of the TCR itself is not sufficient to fully activate a naive T cell. Additional signals originated by the same APC are necessary to sustain the TCR engagement and overcome signalling thresholds required for an effective T cell response (Riha and Rudd 2010). The absence of a co-stimulatory signal results in a state of unresponsiveness to further antigen stimulation known as T cell anergy (Jenkins, Chen et al. 1990).

The main co-stimulatory molecule in T cells is an integral membrane protein homodimer named CD28. This co-receptor is expressed on the surface of both naive and activated T cells and binds to B7.1 (CD80) or B7.2 (CD86), molecules highly expressed by APCs (Sharpe and Freeman 2002; Sharpe 2009). Ligation of the co-receptor CD28 increases the sensitivity of the T cell to antigen receptor engagement and greatly augments the level of expression of genes targeted by TCR activation including cytokine production and in particular IL-2 (Rudd, Taylor et al. 2009). It also contributes to up-regulation of energy metabolism, cell cycle progression, protection from or enhancement of cell death and regulates cell proliferation (Acuto and Michel 2003; Riha and Rudd 2010). CD28-deficient mice showed reduced IL-2 production and cell proliferation in response to antigens (Shahinian, Pfeffer et al. 1993). Signalling downstream of CD28 mediated stabilization of IL-2 mRNA and other transcripts that are up-regulated upon TCR stimulation (Raghavan, Ogilvie et al. 2002). This increase in transcriptional response involves mainly genes targeted by the NFAT and NF-κB transcription factors (Diehn, Alizadeh et al. 2002; Tuosto 2011). Moreover, CD28-B7 ligation induces the expression of the pro-survival protein B-cell lymphoma extra-large (Bcl-$_X$L) protecting the cells from death signals (Watts 2010).

The main signalling cascade downstream of CD28 involves the activation of the phosphoinositide 3-kinase (PI3K) pathway (Riha and Rudd 2010). Ligation of CD28 by B7 molecules results in tyrosine phosphorylation at the YMNM proline-rich motif in its cytoplasmic tail. The phosphorylated YMNM motif subsequently interacts with the p85 subunit of PI3K via its SH2 domain (Boomer and Green 2010). PI3K induces the production of phosphatidylinositol (3,4)-biphosphate (PIP_2) and phosphatidylinositol (3,4,5)-triphosphate (PIP3)(Okkenhaug and Vanhaesebroeck 2003). These molecules recruit proteins containing pleckstrin homology (PH) domains such as phosphoinositide-dependent kinase 1 (PDK-1) and Akt (also known as protein kinase B). Activated Akt phosphorylates a number of substrates, including forkhead family transcription factors, glycogen synthase kinase-3 (GSK-3), caspase-9, and mammalian target of rapamycin (mTOR)(Rudd, Taylor et al. 2009).

Activation of Akt downstream of the CD28 co-receptor mediates cell proliferation and cell cycle progression by down-regulating the cyclin-dependent kinase inhibitor $p27^{kip1}$ (Appleman, van Puijenbroek et al. 2002) and increases cell metabolism by inducing the

levels of glucose uptake in the cell (Frauwirth, Riley et al. 2002). Conversely, inhibition of Akt phosphorylation can drive Foxp3 expression and therefore differentiation of the activated T cell into a Treg cell (Sauer, Bruno et al. 2008).

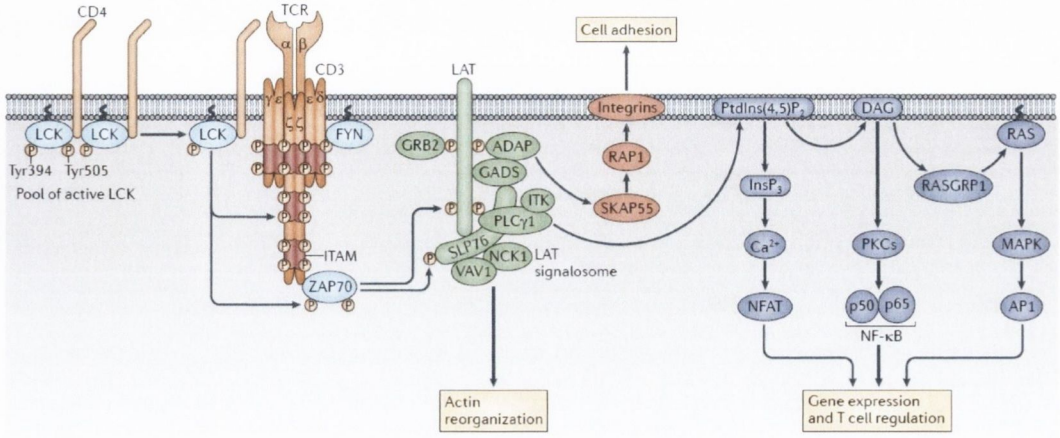


Figure 1-2 TCR signalling. Following TCR and co-receptor binding to peptide-MHC complexes presented on the surface of an APC, the tyrosine kinase Lck present on the cytoplasmic tail of the CD4/CD8 co-receptor phosphorylates the ITAM motifs of the CD3 complex. Phophorylation of ITAMs results in recruitment and activation of ZAP-70 which in turn recruits and phosphorylates the adaptor molecules LAT and SLP-76. These proteins contain several docking sites and form a backbone for the recruitment of other effector molecules such as Gads, Vav-1 and PLCγ1. PLCγ1 in turn hydrolyses PIP_2 into IP_3 inducing the production of the second messengers Ca2+ and DAG. Recruitment of these two molecules will lead to translocation of the transcription factors NFAT, AP-1 and NF-κB and subsequent expression of several genes necessary for T cell differentiation and proliferation. *Adapted from Browlie and Zamoyska, 2013.*

1.2.6 The immunological synapse

The immediate effect of TCR recognition of an antigen peptide presented by an APC is the formation of the immunological synapse (IS) at the point of contact between the T cell and the APC. The formation of the IS induces the reorganization of cell surface receptors and recruitment of cytoplasmic signalling effectors to the site of the contact. The IS is divided in two major regions according to the spatial distribution of the proteins recruited: the central supramolecular activation cluster (c-SMAC) and the peripheral cluster (p-SMAC). The TCR complex and costimulatory molecules such as CD28 are situated in the c-SMAC while molecules related to cytoskeletal rearrangements, such as lymphocyte function-associated antigen 1 (LFA-1) and talin are situated in the p-SMAC (Huppa and Davis 2003). Although the IS was initially proposed to be a plataform for T cell-APC signalling (Monks, Freiberg et al. 1998; Grakoui, Bromley et al. 1999), subsequent studies demonstrated that TCR activation precedes and is required for IS formation (Lee, Dinner et al. 2003). It is believed now that the IS does not function to trigger TCR signalling events but rather to modulate downstream T cell effector responses (Dustin, Chakraborty et al. 2010). The formation of the IS also results in reorientation of the microtubule organizing centre (MTOC) and the Golgi apparatus towards the T cell/APC contact site (Dustin 2003). MTOC translocation has been shown to be required to modulated the IS formation and control sustained T cell signalling and enable the directional secretion of cytokines, cytolic factors and other soluble molecules towards the antigen-presenting cell (Martin-Cofreces, Robles-Valero et al. 2008; Chemin, Bohineust et al. 2012; Huse, Le Floc'h et al. 2013).

1.3 Outcomes of the T cell activation

Following the ligation of the T cell receptor with its cognate antigen presented on the surface of an APCs in the context of a MHC molecule, the T cells become activated and undergo a process of clonal expansion and differentiation into effector T cells, followed by migration to the site of inflammation, where they can exert their effector function (Yuseff, Lankar et al. 2009). Hence, T cell activation is critical process for an effective immune response and requires several changes in the T cell biology, including enhanced cell metabolism, recruitment of transcription factors followed by expression of inflammatory cytokines, and cytoskeletal rearrangement (Fan, Nishanian et al. 1998; Chang, Curtis et al. 2013; Kumari, Curado et al. 2013).

T cell activation and proliferation can also be achieved *in vitro* by, among others, treatment of cells with phytohemagglutinin (PHA) (Ceuppens, Baroja et al. 1988) or with phorbol myristate acetate (PMA) and ionomycin (Io) . PMA activates protein kinase C directly while Io increases the levels of intracellular calcium, important second messengers in the signalling cascade (Blumberg 1988). Moreover, TCR and CD28 engagement can be mimicked by the use of specific antibodies against the CD3 complex and the CD28 molecule.

The activation of naive T cells can be divided in three distinct stages. The induction phase corresponds to the first 8 hours after the cells enter the lymph node. During this period, the T cells experience several short interactions with APCs which results in up-regulation of activation markers and gradual reduction of mobility until they come to a halt and are stimulated by a particular APC. The following 12 hours are marked by a strong attachment between T cell and APC and secretion of cytokines. Phase three or effector phase starts on the following day and corresponds to further cell differentiation and proliferation which culminates in destruction of targeted cells (Mempel, Henrickson et al. 2004).

The different processes that result from T cell activation are going to be discussed separately as follows:

1.3.1 Cell cycle progression and T cell proliferation

Several reviews have been written about the regulation of the cell cycle and a brief description will be given here (Cobrinik 2005; Malumbres and Barbacid 2005; Satyanarayana and Kaldis 2009). The cell cycle has been classically divided into four phases: Gap 1 (or G1 in which the cells prepare for DNA synthesis), S phase (in which the DNA is synthesised), Gap 2 (or G2 in which the cells prepare for division) and mitosis (or M phase, in which sister chromatids are separated and the cells divide).

Progression through cell cycle is regulated by a family of cyclin-dependent kinases (Cdks) that require binding of a regulatory subunit for their catalytic activity (Malumbres and Barbacid 2005). The activating partners of Cdks are molecules whose expression is strictly controlled and limited to a particular cell cycle phase known, as cyclins. There are four major classes of cyclins, each one binding to a specific Cdk and controlling a particular phase of the cell cycle. Entry of quiescent cells into the cell cycle and progression through early G1 is regulated by D-type cyclins (cyclins D1, D2 and D3), which binds to Cdk4 and Cdk6 (Musgrove, Caldon et al. 2011). Transit through late G1 and progression through S phase is regulated by cyclin E bound to Cdk2 (Hwang and Clurman 2005). The S phase transit and early G2 are regulated by cyclin A, which binds to Cdk1 or Cdk2. Finally, the cyclin B/Cdk1 complex regulates progression into M phase (Malumbres and Barbacid 2005). Conversely to the interaction with cyclins which promotes kinase activity, the Cdks are negatively regulated by two families of inhibitors named the INK4 family and the Cip and Kip family, the latter including $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. INK4 members bind and inhibit Cdk4 and Cdk6 proteins on their own, while Cip and Kip proteins inhibit activity of Cdk/cyclin complexes including Cdk2/cyclin E, Cdk2/cyclin A, Cdk1/cyclin A and Cdk1/cyclin B (Satyanarayana and Kaldis 2009).

In the absence of a stimulus, naive T cells are in a resting, quiescent state, and the cell cycle regulatory proteins are absent or expressed at very low levels in these cells. T cell activation rapidly induces the expression of cyclins D and E and their associated Cdks, allowing the cells to leave the G0 phase and entry the cell cycle (Appleman, Berezovskaya et al. 2000). Cyclins D2 and D3 are considered major players in T cells

and along with Cdk4 and Cdk6 are up-regulated as early as 12h upon CD3/CD28 stimulation at both mRNA and protein levels in $CD4^+$ T cells (White, Shore et al. 2006). At the same time, induction of cyclin E, cyclin A and Cdk2 is detected only after 24h of CD3/CD28 stimulation (Lupino, Buccinna et al. 2010). Expression of cyclin D3, cyclin E and Cdk2 in $CD4^+$ T cells is suggested to be controlled by I-κB kinase in response to CD28 costimulation in a mechanism independent of IL-2 (Lupino, Buccinna et al. 2010).

CD28 co-stimulation plays a critical role in cell cycle regulation. Stimulation of CD28 results in activation of the PI3K pathway, leading to phosphorylation of the 70kDa S6 kinase, which in turn activates the transcription factor E2F and consequent transcription of genes required for S phase entry, such as cyclin E (Brennan, Babbage et al. 1999). Furthermore, CD28 co-stimulation down-regulates the cyclin kinase inhibitor $p27^{Kip}$, allowing activation of the cyclin-dependent kinases Cdk4 and Cdk6 and therefore promoting G1 to S phase progression (Appleman, van Puijenbroek et al. 2002)

Interleukin-2 (IL-2) is considered a key mediator of T cell proliferation. The widely accepted model involves production of IL-2 and upregulation of the IL-2 receptor (IL-2R) in response to TCR engagement and CD28 costimulation and consequential interaction of IL-2 and IL-2R then driving extensive clonal expansion (Malek 2008). However, additional studies have shown that T cell proliferation can also occur in an IL-2-independent manner depending on the duration of the TCR/CD28 engagement. While short (20-24h) ligation of TCR results in T cell proliferation in response to IL-2, a prolonged engagement (72-96h) bypasses the need for IL-2 secretion and sustains IL-2 independent T cell proliferation (Colombetti, Basso et al. 2006).

1.3.2 Cytokine production by activated T cells

Cytokines are important mediators in the regulation of the immune response. They are differentially expressed depending on the type of T cell and mediate diverse immune functions including activation, proliferation, differentiation and survival of immune cells. The resting, naive T cells release very low levels of cytokines but gene expression is induced within 30 minutes of cell activation. Most cytokine mRNAs (i.e. IL-2, TNF-α, GM-CSF, IL-5) peak at 4-8h upon cell stimulation and then gradually decline, an

exception to this is IFN-γ, whose levels can be sustained for up to 96h (Abdalla, Kiaii et al. 2003).

Cytokines produced by T cells can be broadly divided in two groups: the pro-inflammatory cytokines, which interfere positively in the establishment of the immune response, such as IL-2, IL-1, IL-6, IFN-γ, TNF-α and the immune-suppressive cytokines, which control proliferation and cell differentiation, such as transforming growth factor-β (TGF- β) and IL-10.

The production of cytokines is a process tightly regulated at transcriptional, post-transcriptional and secretory levels so the cytokine release can be terminated as soon as the interaction with the target cell ends. Most cytokine genes are expressed transiently and at low levels in naive T cells and the production of these molecules is highly augmented upon T cell activation (Abdalla, Kiaii et al. 2003). The mRNAs encoding for cytokines are usually unstable and exhibit rapid degradation, which provides the cell with a general mechanism for rapidly turning gene expression off and thus down-regulating the immune response (Khabar 2007; Anderson 2008). Part of the instability of the cytokine mRNAs is due to the presence of AU-rich elements (ARE) present in the 3'-untranslated regions (3'-UTR) of the transcripts (Barreau, Paillard et al. 2005; Espel 2005). AREs are the best characterized example of a *cis*-agent that controls mRNA degradation by targeting transcripts for rapid degradation. For this reason, the transcripts need to undergo differential regulation to sustain their stability. In T cells, co-stimulatory molecules such as CD28 are important in the stabilization of the cytokine transcripts (Diehn, Alizadeh et al. 2002). Ligation of CD28 in addition to TCR engagement leads to stabilization of IL-2, IFN-γ, TNF-α transcripts as well as many other activation markers (Raghavan, Ogilvie et al. 2002; Sanchez-Lockhart, Marin et al. 2004). Moreover, AREs recruit several different ARE-binding proteins (ARE-BPs) that can promote mRNA decay or mediate mRNA stabilization. NF90, for example, is an ARE-BP that translocates from the nucleus into the cytoplasm upon T cell activation mediating stabilization of IL-2 mRNA (Pei, Zhu et al. 2008). In the same context, activation of the p38 mitogen-activated protein kinase (MAPK) pathway is described to be the main pathway involved in relief of ARE-dependent destabilization and translational silence. Many cytokines use p38 MAPK signalling to trigger mRNA stabilization (Khabar 2007).

Regarding the control of cytokine production at the level of secretion, two distinct pathways have been described: one that targets cytokines to the immunological synapse (IS) and another that releases them in a multidirectional way. In activated $CD4^+$ T cells, a group of cytokines including IL-2, IFN-γ and IL-10 is secreted towards the synapse area while TNF, IL-4 and other chemokines are originally synthesized in the Golgi apparatus situated in the IS region, but they then move away and scatter throughout the cell (Huse, Lillemeier et al. 2006). It has been suggested that cytoskeleton remodelling at the IS plays a role in the process of cytokine secretion. Silencing of Cdc42, a well-known regulator of cytoskeleton rearrangement, inhibits the secretion of several cytokines including IFN- γ in primary T cells (Chemin, Bohineust et al. 2012).

1.3.2.1 The Endoplasmic reticulum (ER) stress response

It has been described that T cells use components of the endoplasmic reticulum (ER) stress response in their differentiation into cytokine-secreting effector cells in response to antigen stimulation. Increased cellular production of proteins requires an increase in the protein folding capacity of the cells to prevent an accumulation of unfolded proteins. To neutralize this stress effect and return to cell homeostasis, a mechanism known as ER stress response is initiated. This mechanism induces expression of ER chaperones and activates specific stress kinases, which results in phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) and consequent attenuation of general protein translation (Ellgaard and Helenius 2003). For example, priming of $CD4^+$ T cells with peptide-MHC molecule resulted in increased phosphorylation of eIF2α, increased expression of stress response genes and accumulation of cytoplasmic granules associated with RNA-binding proteins, all features of the ER stress response (Scheu, Stetson et al. 2006). In addition, activation of $CD8^+$ T cells showed both *in vivo* and *in vitro* increased expression of the ER chaperones GRP94, GRP78, ERp72 and PD1 (Pino, O'Sullivan-Murphy et al. 2008).

1.3.3 Differentiation into effector T cells

Both $CD4^+$ and $CD8^+$ T cells differentiate into functionally distinct subsets after exposure to antigen. This is best described as the transition of naive T cells to effector populations. $CD8^+$ T cells differentiate into cytotoxic T cells that can kill antigen-bearing target cells using granzyme and perforin, and can rapidly produce anti-viral cytokines, such as IFN-γ, TNF-α (Williams and Bevan 2007; Parish and Kaech 2009). In turn, $CD4^+$ T cells can differentiate into several types of effector helper cells (see section 1.2.2; Figure 1.3) that regulate the immune response by production of specific cytokines.

Early after the TCR/CD28 stimulation, $CD4^+$ T cells begin to produce IL-2. As they respond to the activation signal, they progress toward polar extremes of differentiation designated Th cells depending on the cytokines present at the site of activation (Sallusto and Lanzavecchia 2009; Luckheeram, Zhou et al. 2012). IFN-γ and IL-12 produced by APCs and natural killer cells are critical cytokines initiating the signalling cascade to develop Th1 cells (Trinchieri, Pflanz et al. 2003; Iwasaki and Medzhitov 2010). The master transcription factor to coordinate Th1 differentiation is the T-box transcription factor (T-bet). T-bet is responsible for enhancing the production of IFN-γ while suppressing genes that regulate the development of opposing cell lineages such as Th2 and Th17 (Lugo-Villarino, Maldonado-Lopez et al. 2003; Lazarevic, Chen et al. 2011). IL-2 and IL-4 are critical for Th2 differentiation and the major transcription factor involved in this process is the IL-4-induced STAT6 (Kaplan, Schindler et al. 1996; Zhu, Guo et al. 2001). STAT6 up-regulates the expression of the master regulator GATA-3 (GATA-binding protein), which enhances Th2 cytokine production (IL-4, IL-9, IL-13, IL-10) and inhibits Th1 differentiation by interacting with T-bet (Zhu, Yamane et al. 2006). *In vivo*, GATA-3 is essential for Th2 responses, as differentiation of naive cells was diverted towards the Th1 lineage in GATA-3-deficient mouse (Zhu, Min et al. 2004). Differentiation into Th17 cells depends on the presence of IL-6, IL-21, IL-23 and TGF-β, which activates STAT3 and consequently the master regulator of this lineage the retinoic acid receptor-related orphan receptor gamma-T (RORγT) (Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006; Manel, Unutmaz et al. 2008). TGF-β is a critical signalling cytokine for Th17 differentiation but also plays a significant role in the

development of iTregs (Bettelli, Carrier et al. 2006). TGF-β at low concentrations, and in the presence of IL-6 induces Th17 differentiation, production of IL-21 and up-regulates the expression of the IL-23 receptor. However, TGF-β alone, at high concentration can divert lineage differentiation towards Treg development through the induction of Foxp3 (Zhou, Lopes et al. 2008). The forkhead transcription factor Foxp3 is the major transcription factor in Treg differentiation. Natural Treg cells (nTreg) are released from the thymus as a distinct lineage with Foxp3 already expressed, but this transcription factor is induced in $CD4^{+}CD25^{+}$ T cells in the presence of TGF-β and IL-2 leading to iTreg differentiation (Chen, Jin et al. 2003). IL-6 and IL-21 are the main cytokines that have been involved in the differentiation of Tfh cells (Tsai and Yu 2013). The transcription factor Bcl6 has been shown to be selectively expressed in Tfh cells and a key player in their differentiation. Bcl6 is activated downstream of IL-6 and IL-21 signalling and its overexpression induced Tfh differentiation while inhibiting opposing cell lineages (Nurieva, Chung et al. 2009).

The process of differentiation was believed to move in one direction from the naive cell towards the terminally differentiated effector cell. However, there is extensive evidence that Th1, Th2 and Th17 effector cells can return to a quiescent state with capacity for self-renewal and survival in the absence of an antigen peptide-MHC molecule signalling. For example, deleting *GATA-3* from Th2 cells allows the production of IFN-γ (Zhu, Min et al. 2004) and reduction of Foxp3 expression in Tregs enables them to gain a Th2 phenotype (Williams and Rudensky 2007). Moreover, Th17 in the presence of IL-12 switched to Th1 phenotype, and interaction with IL-4 led to the differentiation into Th2 cells (Bending, De la Pena et al. 2009; Lee, Turner et al. 2009).

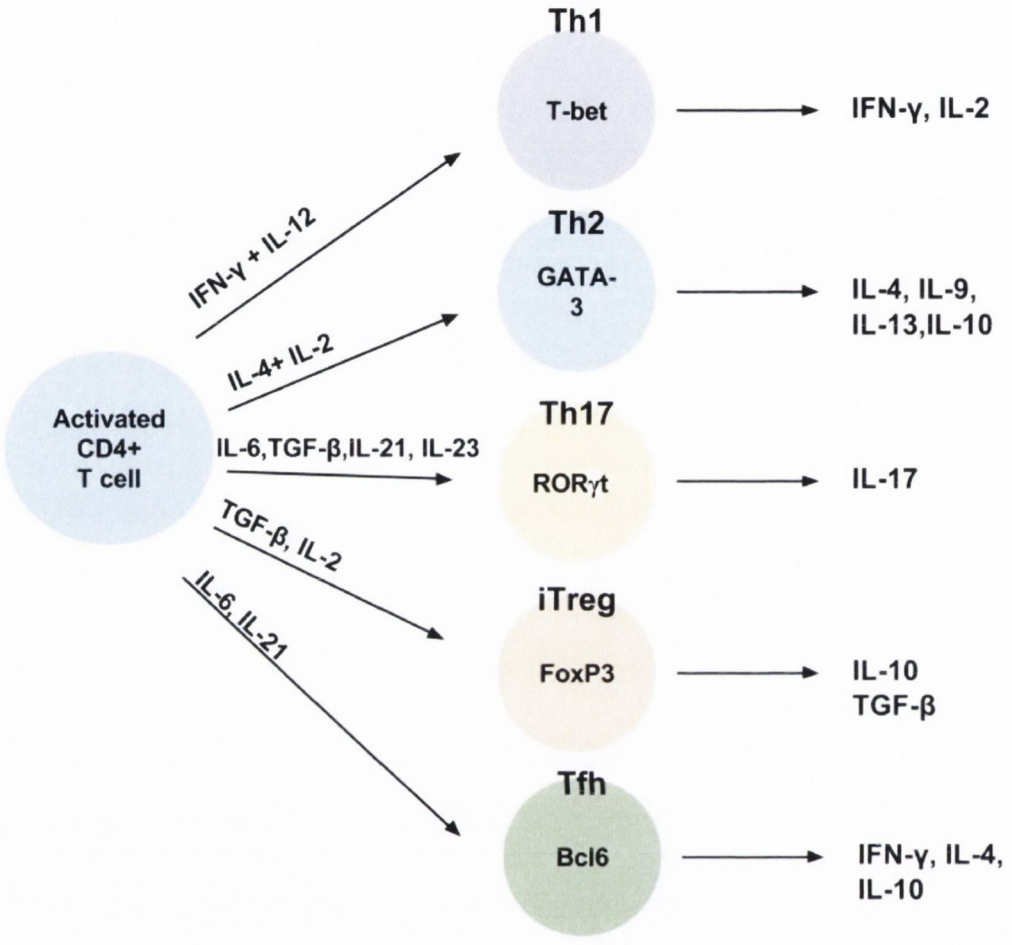


Figure 1-3 $CD4^+$ T cell differentiation. Cytokines play critical roles in differentiation and effector functions of Th1, Th2, Th17, iTreg and Tfh cells. Upon TCR activation triggered by antigen presenting cells, the activated $CD4^+$ T cell differentiates into distinct Th lineages in the presence of a particular cytokine milieu.

1.4 The Protein Kinase C family

Protein Kinase C (PKC) comprises a multigene family of related serine/threonine kinases that have been shown to be an essential component of the cell signalling machinery, playing a key role in several processes, including cell growth, differentiation and apoptosis (review by(Steinberg 2008; Newton 2010).

All PKC members share certain basic structural features. These kinases contain a highly conserved carboxyl-terminal kinase domain linked by a flexible hinge segment to an amino-terminal region containing regulatory activity. The regulatory domains have two key functions: targeting the enzymes to the appropriate cellular location by the C1 and C2 domains and controlling their kinase activity by pseudosubstrate (PS) sequence binding to the substrate cavity (Gould and Newton 2008; Newton 2010). The C1 domain is also binding site for tumor-promoting phorbol esters such as 12-myristate 13-acetate (PMA).

There are ten PKC isoforms divided into three subgroups according to differences in the structure of the amino-terminal domain which determines the cofactor regulation for each group: the conventional PKC (cPKCs), the novel PKC (nPKCs) and the atypical PKC (aPKCs) (Figure 1-4). The structural features and mechanism of activation of these isoforms have been reviewed in several reports (Newton 2003; Steinberg 2008; Newton 2010). The conventional PKC comprise four isozymes: PKCα, the alternatively spliced PKCβI and PKCβII, and PKCγ. cPKCs contain tandem C1 domains (C1A and C1B) that bind the lipid second messenger diacylglycerol (DAG) and a C2 domain that binds anionic phospholipids in a Ca^{2+}-dependent manner. The novel PKC (nPKCs), including PKCε, PKCθ, PKCδ and PKCη, also have two C1 domains that bind DAG but a C2 domain that lacks the Ca^{2+} coordinating residues. For this reason this group of isozymes is not sensitive to Ca^{2+} and regulated only by DAG. This variation in the C2 domain structure is the major difference from the conventional PKCs (Newton 2003). Novel PKCs have been shown to have a higher affinity for binding DAG compared to cPKCs due to a mutation on a single conserved residue (Tyr in cPKCs versus Trp in nPKCs) at position 22 in the C1b domain (Dries, Gallegos et al. 2007). This fact provides a molecular explanation as to why DAG alone is sufficient to activate nPKCs but not

cPKCs The atypical PKC (aPKCs) group includes the isoforms -ζ and –ι (known as λ in the mouse). This group lack C2 domains and contain an atypical C1 domain that binds neither DAG nor phorbol esters. aPKCs are thus not regulated by DAG or Ca^{2+} and rather protein-protein interactions control the function of these isoforms. This group also contains a distinct structural domain at the N-terminus, Phox/Bem 1 (PB1), a well conserved protein interaction module (Hirano, Yoshinaga et al. 2004).

For PKCs to be able to phosphorylate their substrate, they need first to be primed by transphosphorylation or autophosphorylation on serine/threonine residues, events that are required for maturation and stabilization of the enzyme (Newton 2003; Freeley, Kelleher et al. 2011). There are three main phosphorylation sites on the C-terminus of PKCs: the activation loop A (A-loop), the turn motif (TM) and the hydrophobic motif (HM) (Keranen, Dutil et al. 1995). Phosphorylation of the A-loop is required to generate a catalytically competent enzyme and contributes to stabilisation of the active conformation of these kinases (Xu, Chaudhary et al. 2004). Phosphoinositide-dependent kinase-1 (PDK-1) is described as the master kinase that phosphorylates the A-loop of many protein kinases (Balendran, Hare et al. 2000). The turn motif (TM) is highly conserved among all PKCs. The TM is located on the C4 kinase domain on the so-called C-terminal tail of the enzymes. Phosphorylation of the TM enables interaction between the motif and residues in the enzyme to help stabilise the enzyme (Messerschmidt, Macieira et al. 2005; Grodsky, Li et al. 2006). The hydrophobic motif (HM) is a conserved motif in cPKCs and nPKCs but is absent in aPKCs. Atypical PKCs contain an aspartic or glutamic acid mimicking a constitutively phosphorylated state (Messerschmidt, Macieira et al. 2005). Phosphorylation of the HM is associated with kinase core stabilisation rather than PKC function (Edwards and Newton 1997). Moreover, the sequencing surrounding the HM serves as a docking site for the A-loop kinase PDK-1 (McManus, Collins et al. 2004).

PKCs are activated by phosphorylation and regulated according to their subcellular localisation and specific cofactor binding. The isozymes are localized mainly in the cytosol and maintained in an inactive conformation by a pseudosubstrate (PS) binding site, which blocks the substrate-binding pocket in the kinase domain (Dutil and Newton 2000). Production of the lipid second messengers DAG and Ca^{2+} by hydrolysis of PIP_2, recruits the classical and novel isoforms to the plasma membrane through their C1 domain (Newton 2010). Interaction with the membrane is considered the primary event

for PKC activation and results in release of the PS from the substrate cavity, allowing substrate binding and phosphorylation (Dutil and Newton 2000). Atypical PKCs are insensitive to DAG and Ca^{2+} and are suggested to be activated by other lipids such as phosphatidylinositols, phosphatic acid, arachdonic acid and ceramide (Diaz-Meco and Moscat 2012). PKCs have also been shown to be regulated by adaptor proteins such as receptors for activated C kinases (RACKs) and A-kinase anchoring proteins (AKAPs) (Ron and Mochly-Rosen 1995; Klauck, Faux et al. 1996; Steinberg 2008).

Termination of PKC signalling is achieved by a process known as down-regulation. Conventional and novel PKC can be down-regulated simply by removal of second messengers and translocation away from the cellular membrane. In addition, prolonged activation, as occurs upon treatment with phorbol esters, results in rapid proteolytic degradation of several PKC isoforms (Parker, Bosca et al. 1995). Down-regulation of PKC can also occur by dephosphorylation. When in inactive conformation, the isozymes are relatively resistant to dephosphorylation, but their sensitivity to dephosphorylation increases when in the active, membrane-bound conformation (Dutil, Keranen et al. 1994). The activity of phosphatases has been shown to be involved in the process of dephosphorylation, and PKC-specific candidates includes the heterotrimeric type 2A phosphatase (PP2A) (Hansra, Bornancin et al. 1996) and the PH domain leucine-rich repeat protein phosphatase (PHLPP), which has been recently described to dephosphorylate the hydrophobic motif of cPKC and nPKC (Gao, Brognard et al. 2008).

Protein kinase C members have also been shown to be degraded via the ubiquitin-proteasome pathway. This mechanism involves labelling proteins with an ubiquitin tag which targets the protein to the proteasome, a multi-enzymatic complex, where they are proteolysed and degraded. The ubiquitin-proteasome system has been shown to be involved in degradation of the isoforms PKCα and PKCε in PMA-activated human fibroblasts (Lee, Smith et al. 1997) and PKCβII, mediated by the binding of a novel RING domain E3 ligase named RINCK (RING protein that interacts with C kinase) (Chen, Gould et al. 2007).

Additionally, proteasome-independent pathways have been involved in downregulation of PKCs. Signalling of PKCα can be terminated by endocytic trafficking and lysosome-mediated degradation of the fully phosphorylated protein (Lum, Pundt et al. 2013).

Moreover, the hinge domain of PKCδ, PKCθ, PKCε and PKCζ has been shown to be substrates for caspases in response to a range of apoptotic stimuli (Datta, Kojima et al. 1997; Mizuno, Noda et al. 1997; Anantharam, Kitazawa et al. 2002). The cleavage of the hinge domain by caspases results in the release of a catalytic domain fragments that are generally considered to be constitutively active in the absence of a lipid second messenger (Steinberg 2008). It has been shown that the catalytic fragment of PKCδ generated following cleavage by caspase-3 has a special role in induction of apoptosis (Yoshida 2007). In contrast, the catalytic domain fragment of PKCζ has been shown to be catalytically inactive (Smith and Smith 2002).

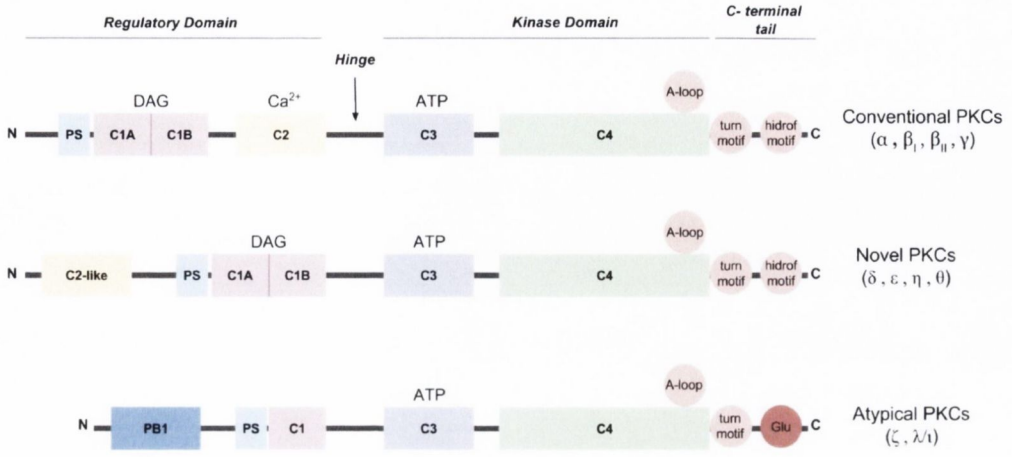


Figure 1-4 Structure of the PKC subfamilies. PKC members are classified into conventional PKC (cPKCs; PKCα, PKCβI, PKCβII and PKCγ), novel PKC (nPKCs; PKCε, PKCδ, PKCθ, PKCη) and atypical PKC (aPKCs; PKCζ, PKCλ/ι). cPKCs require Ca^{2+} and diacylglycerol (DAG) for activation, nPKCs are Ca2+ independent and aPKCs require neither Ca2+ or DAG for activation.

1.5 Role of the PKC family in T cell biology

Activation of T cells leads to a process involving differentiation of the naive T cell to an effector cell that actively secretes immune regulatory cytokines or cytotoxic granules (Yuseff, Lankar et al. 2009). The signalling cascade downstream the TCR receptor results in activation of PLCγ1 and subsequently cleavage of PIP_2, leading to the production of the second messenger diacylglycerol (DAG) and Ca^{2+} influx (see section 1.2.4). As mentioned before, these are key molecules in the recruitment and activation of conventional and novel PKCs. In addition, CD28 co-stimulation results in activation of the PI3K pathway, which causes phosphorylation of PDK-1 and Akt (Sharpe 2009; Riha and Rudd 2010). PDK-1 is a well established protein kinase upstream of PKCs (Balendran, Hare et al. 2000). As a result, several processes in T cell activation are mediated by PKCs, including cell activation, differentiation, adhesion and motility and cell survival (Baier 2003). It is also known that TCR signalling can be mimicked by treatment of T cells with phorbol ester (i.e. PMA) and Ca^{2+} ionophore (i.e. Ionomycin) which are direct activators of conventional and novel PKCs.

T cells express nine out of the ten isoforms described including PKCα, βI and βI, δ, ε, θ, η, ζ and λ/ι, which makes it difficult to determine the specific cellular functions of these individual isoforms in the context of T cell signalling (reviewed by Pfeifhofer-Obermair, Thuille et al. 2012). Some of the major findings on the contribution of individual PKC isoforms in T cell biology are outlined below:

1.5.1 PKCα

PKCα is a conventional PKC that plays an important role in induction of the immune response. It has been reported that PKCα activates the NF-κB signalling cascade (Trushin, Pennington et al. 2003) and, to a lesser extent, induces activity of AP-1 and NFAT in lymphoma cells lines (Genot, Parker et al. 1995). Interestingly, peripheral T cells from PKCα-knockout mice showed normal activation-induced IL-2 secretion, surface expression of CD25, CD44 and CD69, as well as activation of NFAT, NFκB and AP-1

but IFN-γ production was severely impaired in these cells (Pfeifhofer, Gruber et al. 2006). Transgenic thymocytes overexpressing PKCα showed a costimulation-independent increase in proliferation and IL-2 production (Iwamoto, Hagiwara et al. 1992). In addition, PKCα is associated with differentiation and expansion of immature thymocytes (Michie, Soh et al. 2001).

1.5.2 PKCβ

PKCβ is another member of the conventional PKCs and consists of two alternatively spliced forms PKCβI and PKCβII. PKCβI is suggested to play a major role in regulation of the TCR/CD28 signal transduction leading to IL-2 synthesis and production. (Dreikhausen, Gorf et al. 2003). Downregulation of PKCβ synthesis in Jurkat T cells showed suppression NFAT and NFκB activation and inhibition of IL-2 transcription and secretion. This isoform has also been shown to be necessary for stabilization of IL-2 transcripts through phosphorylation of the nuclear factor NF90 which increases its capability to stabilize the IL-2 mRNA (Zhu, Jiang et al. 2010). Studies with the PKCβ-deficient clone of HuT78, K-4, a T cell lymphoma cell line have shown that PKCβ is also critical to the export of IL-2 molecules from the T cells (Long, Kelleher et al. 2001). Furthermore, PKCβI has a important role in cytoskeleton rearrangement in LFA-1 triggered T cell migration (Volkov, Long et al. 2001).

1.5.3 PKCθ

PKCθ is a member of the novel PKCs and by far the most studied isoform in T cells. Since the finding that PKCθ was selectively recruited to the immunological synapse shortly after TCR/CD28 ligation (Monks, Kupfer et al. 1997), this isoform has been the focus of many studies regarding T cell activation and signalling networks (Isakov and Altman 2012). Studies with PKCθ deficient mice showed defects in TCR-induced proliferation, cytokine production and differentiation (Hayashi and Altman 2007). Once at the immunological synapse, PKCθ is activated by phosphorylation at different sites (Freeley, Volkov et al. 2005; Wang, Chuang et al. 2012). PKCθ plays an important role in the activation of transcription factors NF-κB, AP-1 and NFAT which are critical for T

cell activation and differentiation (Lin, O'Mahony et al. 2000; Manicassamy, Sadim et al. 2006; Pfeifhofer-Obermair, Thuille et al. 2012). Moreover, PKCθ has been shown to mediate the activation-adhesive capacity of T cells mediated by LFA-1 (Letschka, Kollmann et al. 2008). In the same context of T cell adhesion, PKCθ has also been shown to regulate L-selectin signal transduction through the TCR complex in association with the PKCα isoform (Kilian, Dernedde et al. 2004). Furthermore, PKCθ is essential for harmful inflammatory responses mediated by Th2 (allergies) and Th17 (autoimmunity) cells as well as for graft-versus-host disease and allograft rejection (Isakov and Altman 2012). On the other hand, PKCθ has also been shown to have a role in regulatory T (Treg) cells that suppress activity of effector T cells. Activation of Tregs resulted in sequestration of PKCθ away from the immunological synapse, and inhibition of PKCθ activity increased the suppressive activity of Tregs (Zanin-Zhorov, Ding et al. 2010).

1.5.4 PKCε

PKCε is classified as a novel PKC isoform, highly expressed in neuronal, hormonal and immune cells. In T cells, it has been described that this isoform has a positive role in regulation of the transcription factors NFAT-1 and AP-1 leading to IL-2 production (Genot, Parker et al. 1995). Additional studies showed that stimulation of primary T cells with anti-TCR antibody results in translocation of PKCε to the plasma membrane (Keenan, Volkov et al. 1997) and that inhibition of PKCε decreased CD3-mediated IL-2 production (Szamel, Appel et al. 1998). Altogether these findings suggest a critical function of PKCε in TCR/CD28 activated T cells. Interestingly, Gruber et al. (2005) reported no alteration in function and maturation of PKCε-deficient T cells. Proliferative response, IL-2 production, as well as transactivation of NF-kB and induction of CD25, CD44 and CD59 in activated T cells from PKCε null mice were comparable to control T cells, suggesting that PKCε loss of function is compensated by other isoforms of the PKC family. However, a specific role for PKCε in $CD4^+$ T cell proliferation and response to TGF-1β has been suggested by Mirandola et al. (2011). Silencing of PKCε by siRNA decreased IL-2 receptor chain expression and cell proliferation and reduced the expression levels of the surface marker CD25 and NF-kB1 and NF-kB2 gene expression upon TCR/CD28 stimulation. It was also reported that the inhibitory effect of TGF-1β was augmented by PKCε gene knockdown.

1.5.5 PKCδ

PKCδ was the first member of the novel PKCs to be identified. It has been described to a have a negative role in cellular functions by inhibiting proliferation and promoting apoptosis (Yoshida 2007). In T cells, PKCδ is a negative regulator of TCR signalling. T cells from PKCδ-deficient mice showed a significant increase in IL-2 secretion upon CD3 stimulation and enhanced proliferative response to allogenic MHC. On the other hand, levels of secreted IL-4, IFN-γ, TNF-α and IL-6 were unaffected in response to CD3 stimulation. Thymocyte numbers and development were also normal in PKCδ-deficient mice (Gruber, Barsig et al. 2005). Additional studies with Jurkat T cells showed that knockdown of PKCδ potentiated TCR-triggered NF-κB activation and IL-2 secretion while overexpression of PKCδ inhibited NF-κB activation through inhibition of caspase recruitment domain-containing membrane–associated guanylate kinase 1 (CARMA-1) (Liu, Song et al. 2012). CARMA-1 is an important scaffold protein in the signalling cascade downstream of TCR, responsible for the recruitment of several molecules that results in the congregation of a specific TCR-induced signalosome that triggers NF-κB activation (Rawlings, Sommer et al. 2006).

PKCδ has also been described to have a pro-apoptotic role in T cells, as mediator of Fas-induced apoptosis in Jurkat T cells (Sen, Sashwati et al. 1999). Moreover, studies with PMA-stimulated $CD4^+$ T cells from patients with systematic lupus erythematosus (SLE) suggested that impared PKCδ phosphorylation may contribute to the development of this condition (Gorelik, Fang et al. 2007).

1.5.6 PKCζ

PKCζ is a member of the atypical PKC family, which are insensitive to activation by DAG or Ca^{2+}. Naive T cells from PKCζ knockout mice showed no major alterations in proliferation upon cell activation with TCR/CD28 antibodies (Martin, Duran et al. 2002). However, further studies on the differentiation of T cell subsets described an important role for PKCζ in IL-4 signalling and Th2 differentiation (Diaz-Meco and Moscat 2012).

In addition to the fact that PKCζ levels were increased during Th2 differentiation, lack of PKCζ impaired the secretion of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 and the expression of GATA3. Moreover, PKCζ-deficient T cells showed reduced nuclear translocation and phosphorylation of STAT6 and JAK1 activation, well characterised downstream targets of IL-4 signalling (Martin, Villares et al. 2005). PKCζ was also found to be phosphorylated and to interact directly with PKCθ in the pathway leading to NF-κB activation in TCR/CD28 stimulated Jurkat T cells (Gruber, Fresser et al. 2008). In addition, T cells expressing a PKCζ mutant failed to polarize and showed affected scanning behaviour in response to activation with the chemokine CCL19, suggesting that PKCζ plays also a role in T cell polarization (Real, Faure et al. 2007). PKCζ has been also shown to be involved in T cell polarization and migration in response to chemokine stimulation through phosphorylation of L-plastin, a leukocyte-specific F-actin bundling protein (Freeley, O'Dowd et al. 2012).

1.5.7 PKCλ/ι

PKCι (known as PKCλ in mice) is the second member of the atypical PKC family. Due to its high similarity to the other atypical isoform, PKCζ, it has been difficult to determine specific roles for the individual isozymes. Therefore, not much is known about the function of PKCι in T cells. PKCλ/ι has been shown to activate NF-κB in Jurkat cells in response to stimulation with the proinflammatory cytokines TNF-α and IL-1β (Bonizzi, Piette et al. 1999). A role for PKCλ/ι in T cell adhesion has been suggested after this isoform was found to be associated with the cytoplasmic domain of L-selectin in Jurkat T cells treated with PMA (Kilian, Dernedde et al. 2004). Soloff et al. (2004) described a critical role for PKCλ/ι in embryogenesis but found no abnormalities in T cell development and function. However, it was later described that in addition to PKCζ, PKCλ/ι is also phosphorylated by PKCθ in the signalling pathway leading to NF-κB activation in TCR/CD28 stimulated T cells, suggesting that PKCι might play a compensatory role in this case (Gruber, Fresser et al. 2008).

Subsequently, studies with PKCλ/ι-deficient $CD4^+$ T cells from mutant mice suggested an important role for PKCλ/ι in the Th2 response. Although PKCλ/ι was not necessary for Th2 differentiation in vitro, the T cells lacking this isoform showed reduced Th2-cytokine

secretion (IL-4 and IL-13) and proliferation upon stimulation with CD3/CD28 antibodies. Nuclear levels of the transcription factor GATA-3 (marker for Th2 cells), NFAT and STAT6 were also reduced in these cells. In addition, PKCλ/ι mutant mice did not mount a Th2 response in ovalbumin-induced allergic airway inflammation. Furthermore, the study has also found that PKCλ/ι deficiency in activated T cells leads to impaired polarity during late T cell activation (Yang, Leitges et al. 2009).

1.6 Protein kinase Cη

PKCη was first described by Osada et al. (1990) and classified as a member of the novel PKC family that showed the highest sequence similarity to the PKCε isoform. PKCη was shown to be highly expressed in epithelial tissues including lung, skin, oesophagus, stomach, and at low levels in the brain, suggesting an important role for this PKC isoform in epithelial differentiation (Osada, Hashimoto et al. 1993) . Similarly to the other members of the novel PKC family, PKCη has a C1 domain that binds diacylglycerol (DAG) and phorbol esters and a C2 regulatory domain lacking the binding site for Ca^{2+} (Hashimoto, Osada et al. 1992). It has been shown that the C1b domain and the pseudosubstrate region are required for localisation of PKCη to membranous systems (Maissel, Marom et al. 2006). The structure of the PKCη C2 domain has been solved. It was shown to contain two potential autophosphorylation sites, suggesting that autophosphorylation at these residues may act as means of self-regulation in PKCη (Littler, Walker et al. 2006).

Regulation of PKCη expression appears to be unique among the PKC isoforms. Prolonged treatment with phorbol esters results in down-regulation of many PKC isoforms, primarily as a result of proteolysis of activated PKC isoforms rather than diminished synthesis (Parker, Bosca et al. 1995). However, PKCη is resistant to this mechanism, and its levels remain constant after prolonged treatment with phorbol esters in macrophages, astrocytes and human keratinocytes (Murakami, Chida et al. 1996; Chen, Wang et al. 1997). In addition, prolonged treatment with phorbol esters results in up-regulation of PKCη in EL4 mouse thymoma cells and MCF-7 breast cancer cells among other cell types (Resnick, Luo et al. 1997; Pal, Outram et al. 2012). Studies in fibroblasts proposed that the failure to PKCη to down-regulate upon treatment with phorbol esters

was due to limited activation of this isoform and showed that constitutively activated PKCη can undergo rapid degradation to generate several lower molecular weight fragments. This fact indicates the requirement for kinase activity and an active conformation for down-regulation of PKCη (Kang, French et al. 2000). In agreement with this idea, it was shown that over-expression of a constitutively active PKCη form resulted in a much faster degradation of the protein when compared with that of normal PKCη (Resnick, Kang et al. 1998).

Expression of PKCη has been shown to be controlled at the translational level both under normal growth and stress conditions (Raveh-Amit, Maissel et al. 2009). The 5' untranslated region (5' UTR) of the PKCη gene was described to be unusually long and GC rich, characteristics of many oncogenes and growth regulatory genes. In addition, this region contained two upstream open reading frames (uORFs), which maintained low basal levels of PKCη in growing cells while up-regulating its expression under stress conditions imposed by amino acid starvation. PKCη was also shown to be rapidly phosphorylated and activated by type I interferons (IFN-α and IFN-β) upon stimulation of their related receptor in normal and leukemic cell lines (Redig, Sassano et al. 2009).

More recently, Pal et al. (2012) have shown that the novel PKC isoform PKCε was involved in post-transcriptional regulation of PKCη expression in breast cancer cells. Additional studies by the same group demonstrated that the protein levels of PKCη were also regulated by the PI3K pathway in addition to PKC members. However, the down-regulation observed in PKCη used two distinct pathways: while the PKCη down-regulation caused by the PI3K inhibitor LY294002, through the inhibition of PDK-1 was via a ubiquitin proteasome-mediated pathway, the down-regulation caused by inhibition of PKC activity was proteasome-independent. (Pal, Outram et al. 2013).

Soon after PKCη was shown to be highly expressed in epithelial tissues, an important role for PKCη in regulation of keratinocyte differentiation was described (Ohba, Ishino et al. 1998; Kashiwagi, Ohba et al. 2002). Moreover, expression of PKCη in NIH-3T3 fibroblasts primed these cells to undergo differentiation into adipocytes (Livneh, Shimon et al. 1996).

Studies have suggested that PKCη also plays an important role in secretory processes (Gundersen, Kohan et al. 2002; Diaz Anel and Malhotra 2005; Byrne, Foran et al. 2010). PKCη has been shown to co-localise with the Golgi apparatus and endoplasmic reticulum (ER) in breast cancer cells (Maissel, Marom et al. 2006). Moreover, it has been shown that because of its localisation in the Golgi apparatus, activation of PKCη is necessary for trafficking of vesicles between the *trans*-Golgi network (TGN) and the cell surface in HeLa cells (Diaz Anel and Malhotra 2005).

PKCη has been also described to be a major regulator of cell cycle and cell proliferation in several cell types. Induced expression of PKCη in MCF-7 breast cancer cells resulted in increased expression of the G1 phase cyclins D and E and the cyclin-dependent kinase inhibitor p21, enhancing cell growth (Fima, Shtutman et al. 2001). PKCη also increases cell proliferation in glioblastoma cells, through activation of Akt and the mammalian target of rapamycin (mTOR) as well as the ERK pathway (Aeder, Martin et al. 2004; Uht, Amos et al. 2007). On the other hand, overexpression of PKCη results in cell cycle arrest at the G1/S transition in pro-B-cells (Morrow, Muljo et al. 1999) and induces growth arrest via up-regulation of $p27^{Kip1}$ and switch to differentiation in keratinocytes (Hara, Miyazaki et al. 2011). PKCη has been also shown to physically associate with cell cycle regulators. In keratinocytes, PKCη was associated with the cyclin E/Cdk2/p21 complex and was responsible for phosphorylation of p21 in this complex. This resulted in the inhibition of Cdk2 kinase activity, leading to G1 arrest (Kashiwagi, Ohba et al. 2000). In addition, the complex PKCη/cyclin E/Cdk2 was also found in the perinuclear area of other cell types including NIH-3T3 fibroblast cells, the breast cancer cell line MCF-7 and the mammary non-transformed HC11 cells (Shtutman, Hershko et al. 2003).

A role for PKCη in resistance to apoptosis in different cell lines has also been described. In cells derived from Hodgkin's Lymphoma and MCF-7 breast cancer cells, PKCη functions as an anti-apoptotic agent and might serve as a target to reduce resistance to anti-cancer treatment (Abu-Ghanem, Oberkovitz et al. 2007; Rotem-Dai, Oberkovitz et al. 2009). It has been suggested that PKCη confers protection against apoptosis by activation of the NF-κB pathway, which has been shown to be used by tumour cells to acquire resistance to anticancer drugs and radiation (Raveh-Amit, Hai et al. 2011). In addition, it has been shown that PKCη translocates to the nuclear envelope in response to the DNA-damaging agent, etoposide and that the C1b domain of PKCη is responsible for it

(Tamarkin, Zurgil et al. 2011). On the other hand, studies with pro-B-cells have shown that PKCη is cleaved in response to various pro-apoptotic stimuli and that the fragments generated were kinase-active and presented pro-apoptotic function (Morrow, Muljo et al. 1999).

Among other functions for this isoform, a role for PKCη was suggested in lipid metabolism in a hepatocarcinoma cell line, as PKCη was shown to constitutively accumulate around lipid droplets in these cells (Suzuki, Iio et al. 2013). Moreover, PKCη can be used as a prognostic marker for non-small cell lung cancer, in which overexpression of PKCη was associated with advanced stages of the disease (Krasnitsky, Baumfeld et al. 2012) and for hepatocellular carcinoma, in which downregulation of this isoform was associated with poorer long term survival (Lu, Chou et al. 2009). Furthermore, PKCη has been described to regulate occludin phosphorylation and epithelial tight junction integrity, revealing an important role for PKCη in maintenance of epithelial integrity (Suzuki, Elias et al. 2009).

1.6.1 PKCη function in T cells

Although PKCη is known to be primary expressed in epithelial tissues (Osada, Hashimoto et al. 1993), this isoform has been shown to be also highly expressed in T cells (Baier 2003). Also, PKCη gene expression is induced in thymocytes during positive selection (Mick, Starr et al. 2004), suggesting that PKCη might be an important player in the biology of T cells.

In resting T cells, PKCη presents a diffuse cytoplasmic distribution (Keenan, Long et al. 1997). Expression of PKCη was shown to be up-regulated at both protein and mRNA levels following stimulation with phorbol esters in EL4 mouse thymoma cells (Resnick, Luo et al. 1997). Further studies in the same cells suggested that while PKCθ contributed to cell cycle progression and IL-2 production, PKCη was involved in actin cytoskeletal reorganization and morphological changes (Resnick, Kang et al. 1998). More recently, it has been shown that levels of PKCη were significantly down-regulated in the lipid raft compartment in Jurkat T cells upon apoptosis induction (Solstad, Bjorgo et al. 2010)

Until recently, the role of PKCη in T cell biology at a mechanistic level had not been investigated. Studies by Quann et al. (2011) using murine T cells showed for the first time that TCR ligation induced accumulation of PKCη and PKCε at the immunological synapse within seconds after cell stimulation and that this event was required for recruitment of PKCθ to this area and consequent IL-2 production. This fact was confirmed by combined knockdown of PKCη and PKCε in these cells, which decreased the production of IL-2 upon stimulation.

Fu et al. (2011) found an important role for PKCη in murine T cell activation and homeostatic proliferation. Comparing T cells from wild-type, $PKC\eta^{-/-}$,$PKC\theta^{-/-}$ and double knockout mice they were able to show distinct and redundant functions of these two isoforms. Although both PKCθ and PKCη were recruited to the immunological synapse upon TCR stimulation, their distinctive localisation pattern suggested different functions of these two isoforms in signal transduction. $PKC\eta^{-/-}$ T cells showed impaired cell proliferation, calcium influx, NF-κB translocation, and cytokine production (IL-2 and IFN-γ) in response to cell activation; all characteristics shared to some extent with $PKC\theta^{-/-}$ T cells. However, PKCη rather than PKCθ had an important role for T cell homeostatic proliferation, which requires recognition of self-antigen.

1.7 Aims and objectives of this thesis

Recent studies indicate that PKCη plays a potentially important role in T cell biology. It is fact that T cells express high levels of PKCη and that PKCη expression is induced during the process of positive selection in thymocytes (Baier 2003; Mick, Starr et al. 2004). In other cell types, PKCη has been described to play a major role in several cellular mechanisms that are also initiated in T cells upon TCR stimulation, including regulation of secretory processes, cell cycle progression, cell proliferation and differentiation, and apoptosis. While the more recent studies were carried out in mouse models, information about the role of PKCη in human T cells is still scarce.

For this reason, the overall aim of this study was to investigate the role of PKCη in human T cells. Therefore, the objectives were to:

- compare the expression of PKCη in the resting state and regulation of PKCη expression in response to T cell activation in two T cell lines (HuT78 and Jurkat) and in primary human lymphocytes;
- investigate cell signalling pathways that regulate PKCη expression at the basal level and following cell activation in the T cell lines and in primary lymphocytes;
- investigate a functional role for PKCη in the context of T cell activation in relation to cytokine production, cell cycle progression, cell proliferation and cell differentiation into effector T cell subsets.

2 Chapter 2 - Materials and Methods

2.1 Reagents and antibodies

For a complete list of all reagents, inhibitors and antibodies used in this study, please refer to Appendix.

2.2 Plasmids and siRNA

Control pcDNA and pcDNA-PKCηFLAG constructs were kindly donated by Prof Vivek Malhotra (Centre de Regulació Genômica, Barcelona, Spain).

The small interfering RNAs (siRNAs) used in the PKCη gene silencing experiments were purchased from Dharmacon (Lafayette, CO, USA). Both Control and PKCη siRNAs were produced according to the ON-TARGETplus SMARTpool technology which targets four mRNA regions at once, reducing off-target effects and enhancing the potency of the assay. The ON-TARGETplus Control – Non-targeting pool was used as negative control. The ON-TARGETplus SMARTpool Human PRKCH was used to target the PKCη gene and is comprised of a mixture of the following four siRNAs: #1 GCACCUGUGUCGUCCAUAA; #2 CCACGAGGGUCACUGUAAA; #3 UAACCAGGAUGAGUUUAGA; #4 CAUAUUCGAUGUCAAGCGA.

2.3 Cell culture

2.3.1 Human HuT78 lymphoblast T cell line

The human HuT78 cell line (American Type Culture Collection (ATCC), Manassas, VA, USA) was established from peripheral blood of a 53-year old white male with Sezary syndrome and has a mature helper T cell phenotype. HuT78 T cells were cultured in RPMI-1640 with 10% v/v Foetal Calf Serum (FCS) and 50 U/ml Penicillin/Streptomycin (PenStrep) supplemented with 1.5 mg/ml Sodium Bicarbonate and 1 mM Sodium Pyruvate in a humidified atmosphere at 37°C and 5% CO_2. The cells were kept in T-25 or

T-75 cm^2 flasks and cell density was monitored with a haemocytometer and maintained between $2x10^5$ to $1x10^6$ cells/ml. Fresh medium was added to the cells every 2-3 days.

2.3.2 Human Jurkat leukemic T cell line

The Jurkat cell line (ATCC, Manassas, VA, USA), subclone E6.1 was derived from a 14-year-old boy with T cell acute lymphoblastic leukaemia and has characteristics of immature thymocytes. The Jurkat cells were maintained in RPMI-1640 medium with 10% v/v FCS and 50 U/ml PenStrep in T-25 or T-75 cm^2 flasks. Cells were kept in T-25 or T-75 cm^2 flasks and cell density was monitored with a haemocytometer and maintained between $2x10^5$ to $1x10^6$ cells/ml. Fresh medium was added to the cells every 2-3 days.

2.3.3 Jurkat T cell PTEN Tet-On clones

The Jurkat T cell PTEN Tet-On clones were obtained from Dr Ronald Wange (described in (Seminario, Precht et al. 2003) and were maintained in RPMI-1640 containing 10% v/v FCS and 50 U/ml PenStrep in a humidified atmosphere at 37°C and 5% CO_2. The antibiotics G418 (100 µg/ml) and hygromycin (100 µg/ml) were added fresh to the medium of these T cell clones every 2 days while in culture. These antibiotics are necessary to select the cells expressing the stable Tet-On plasmids. Cell density was maintained between $2x10^5$ to $1x10^6$ cells/ml. The expression of PTEN was induced in these cells by the addition of 1 µg/ml doxycycline (Dox) for 48 hours after which time the cells were ready for experimentation.

2.3.4 $CD28^+$ HuT78 and wild type HuT78 clones

The HuT78 T cell clone stably transfected with a plasmid encoding for the human CD28 co-receptor ($CD28^+$ HuT78) and wild type HuT78 (CD28 negative) were kindly donated by Dr Frédérique Michel (Institut Pasteur, Paris, France). These cells were maintained in RPMI-1640 with 10% v/v FCS and 50 U/ml PenStrep supplemented with 1.5 mg/ml Sodium Bicarbonate and 1 mM Sodium Pyruvate in addition to 400 ug/ml of G418 in a

humidified atmosphere at 37°C and 5% CO_2. Addition of the antibiotic G418 to the cell culture medium was necessary to select the cells expressing the plasmid. Cell density was monitored with a haemocytometer and never allowed to exceed $1x10^6$ cells/ml. Fresh medium was added every 2-3 days.

2.4 Primary Human Lymphocytes

A diagram of the isolation process of all populations of primary human lymphocytes used in this study is shown in Figure 2-1.

2.4.1 Isolation of Peripheral Blood Lymphocytes (PBL)

PBL were isolated from buffy coat blood packs that were generously provided by the Irish Blood Transfusion Service at St. James's Hospital Dublin. PBL were isolated using the Lymphoprep™ density gradient centrifugation technique. The content of the buffy coat packs was initially diluted in an equal volume of Phosphate Buffered Saline (PBS). The mixture was then carefully layered onto an equal volume of Lymphoprep™ solution and the phases were not allowed to mix. The samples were centrifuged for 20 minutes at 1200xg at room temperature with the brake of the centrifuge set to 0. After centrifugation, the white layer containing the white blood cells was collected and placed into a new tube and washed three times with an excess of fresh medium. The cell pellet was re-suspended in fresh medium and added to a T175 cm^2 flask and incubated at 37°C for one hour in order to deplete the number of monocytes and macrophages in the cell population. These cells adhere to the bottom of the flask and can be removed in this way while the lymphocytic population remain in suspension. A second round of cell adhesion was performed by transferring the suspension cells to a new T175 cm^2 flask and incubating the cells at 37°C for another hour. At the end of the second round, the non-adherent PBL were collected and counted. The cells were maintained in a density of $1\text{-}2x10^6$ cells/ml in RPMI-1640 with 10% v/v FCS and 50U/ml PenStrep at 37°C and 5% CO_2 until they were used in experimental procedures.

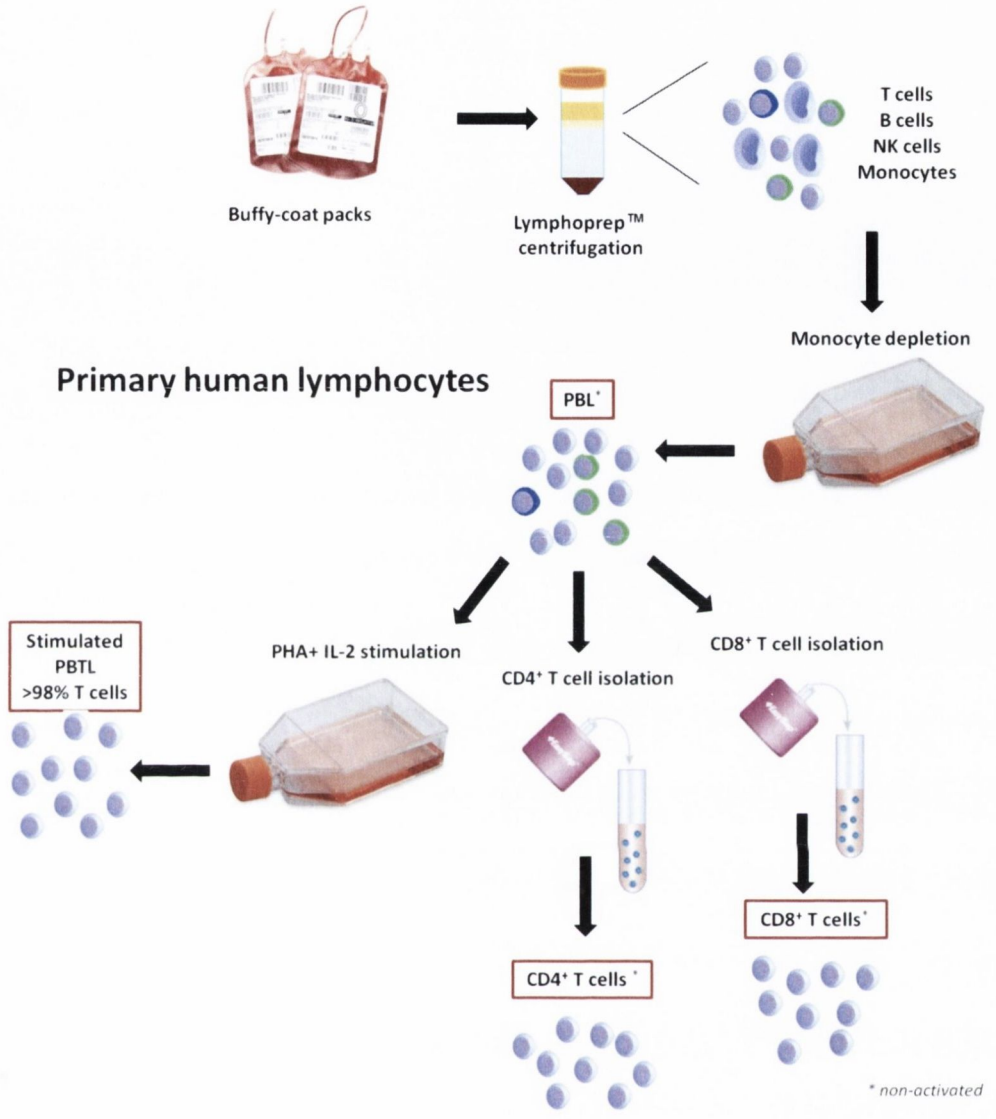


Figure 2-1 Isolation of primary human lymphocytes. Primary human lymphocytes were isolated from buffy coat packs derived from blood donations of healthy donors. Initially, mononuclear white blood cells were isolated by density gradient centrifugation over Lymphoprep™. The cells then went through a round of monocyte depletion by adherence to plastic and the resultant cell population was called peripheral blood lymphocytes (PBL). These PBL were then used for experimentation as a model of resting primary cells or were further processed to isolate non-activated $CD4^+$ or $CD8^+$ T cell subsets by negative selection using magnetic beads (StemCell Technology). In addition, PBL were stimulated with phytohaemagglutinin (PHA) and exogenous IL-2, which generated a cell population comprised mainly of activated T cells named stimulated peripheral blood T lymphocytes (PBTL).

2.4.2 Expansion of stimulated Peripheral Blood T lymphocytes (PBTL)

This technique was used to obtain a population of stimulated T cells comprised by >98% $CD3^+$ T cells, as detected by flow cytometry (Lefort and Kim 2010).

After the isolation of PBL described in section 2.4.1, the cells were seeded in complete medium (RPMI-1640 with 10% v/v FCS and 50 U/ml PenStrep) at a density of $2x10^6$ cells/ml and expanded in the presence of 2 µg/ml phytohaemagglutin (PHA) for 72 hours at 37°C. Cells were recovered and washed 3 times with complete medium and seeded into new flasks at a density of $1\text{-}2x10^6$ cells/ml in complete medium supplemented with 20 ng/ml recombinant human IL-2. Cells were cultured for a further 5 days and fresh medium with IL-2 was added every 2-3 days. After the 5-day period of IL-2 stimulation, the PBTL were ready to be used in experimentation for another 7 days.

2.4.3 Isolation of $CD4^+$ T cells

$CD4^+$ T cells were isolated using the EasySep Negative Selection Human $CD4^+$ T cell Enrichment Kit (Cat# 19052, StemCell, Grenoble, France) according to the manufacturer's instructions. This kit is designed to isolate $CD4^+$ T cells from fresh or frozen peripheral mononuclear cells by negative selection. Tetrameric antibody complexes and dextran-coated magnetic particles are used to label unwanted cells which are attracted by a magnetic field; allowing only the $CD4^+$ population to be poured off into a new tube.

Freshly isolated PBL were re-suspended at a concentration of $5x10^7$ cells/ml in RoboSep buffer (StemCell, 0.2g/L Potassium Chloride, 0.2g/L Potassium Phosphate Monobasic, 8.0g/L Sodium Chloride, 1.15g/L Sodium Phosphate Dibasic, 2% v/v FCS and 1 mM EDTA in PBS) and placed in a sterile 5 ml polystyrene tube. 50 µl/ml of the EasySep Human $CD4^+$ T cell Enrichment Cocktail (containing antibodies recognizing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ and glycophorin A) was added to the cell suspension; mixed and incubated at room temperature for 10 minutes.

The EasySep D Magnetic Particles were then added at a concentration of 100 µl/ml to the cells and incubated at room temperature for 5 minutes. After the incubation, RoboSep buffer was added to the cells to a final volume of 2.5 ml. The cell suspension was mixed gently and the tube was placed into the Purple EasySep magnet for 5 minutes. The EasySep magnet creates a high-gradient field which processes samples without the use of columns. After this period, the $CD4^+$ T cells were poured into a new tube by inverting the magnet with the cell suspension in one continuous motion. The magnetically-labelled $CD4^-$ T cells/non- T cells remained in the original tube (held by the magnetic field), while the $CD4^+$ T cells were transferred to the new tube and were ready to be used in the study.

During experimentation with $CD4^+$ T cells, the cells were maintained in a density of 1-$2x10^6$ cells/ml in RPMI-1640 with 10% v/v FCS and 50U/ml PenStrep at 37°C and 5% CO_2.

2.4.4 Isolation of $CD8^+$ T cells

$CD8^+$ T cells were isolated using the EasySep Human $CD8^+$ T cell Enrichment Kit (Cat# 19053, StemCell, Grenoble, France StemCell). This technique follows the same approach described in section 2.4.3 for isolation of $CD4^+$ T cells. However, the EasySep Human $CD8^+$ T cell Enrichment Cocktail contains tetrameric antibody complexes recognizing CD4, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ and glycophorin A.

During experimentation using $CD8^+$ T cells, the cells were maintained in a density of 1-$2x10^6$ cells/ml in RPMI-1640 with 10% v/v FCS and 50U/ml PenStrep at 37°C and 5% CO_2.

2.5 Isolation and expansion of invariant natural killer T(iNKT) cells

iNKT cells are cytotoxic T cells which express an invariant T cell receptor as well as NK cell markers. Experiments with iNKT cells were done in collaboration with Dr. Derek Doherty (Trinity College Dublin). The iNKT cells were prepared by Ms Yasmeen

Ghnewa as outlined in O'Reilly et al. (2011) . Briefly, iNKT cells were enriched from peripheral blood mononuclear cells (PBMC) obtained from buffy coat packs by magnetic bead separation using 6B11 coated magnetic beads (Miltenyi Biotec, Germany). iNKT cells were then purified by cell sorting of $CD3^+V\alpha24^+V\beta11^+$ cells using a cell sorter. Sorted iNKT cells were then expanded for a minimum of three weeks with 1μg/ml of PHA and 250 U/ml IL-2 in the presence of an excess of allogeneic PBMC prepared from two different donors. During this period, iNKT cells were maintained in RPMI-1640 containing 0.05 mM L-glutamine, 10% v/v FCS, 1% penicillin-streptomycin, 1% fungizone, 25mM HEPES, 50 μM 2-mercaptoethanol, 1mM sodium pyruvate, 1% non-essential amino acid mixture and 1% essential amino acid mixture. Every 2 days, fresh medium containing 250 U/ml IL-2 was added to the cell culture.

2.6 T cell Activation

2.6.1 Antibody-mediated cell activation

For T cell activation with antibodies, the cells were stimulated with antibody against the T cell receptor-associated CD3 complex (anti-CD3) alone or with a combination of anti-CD3 and anti-CD28 antibodies. For this stimulation, wells in a 24, 48 or 96-well plate were first coated with 0.5μg/ml of goat anti-mouse IgG antibody in sterile PBS and incubated overnight at 4°C. Unbound solution was removed and the wells were washed once with sterile PBS. The second coating was performed with 10 μg/ml of anti-CD3 ± 0.1 μg/ml of anti-CD28 for 1-2 hours at 37°C. The unbound solution was carefully removed and the wells were washed one more time with sterile PBS. T cells that were previously re-suspended at a concentration of $1x10^6$ cells/ml in complete medium were added to the wells and incubated at 37°C with 5% CO_2 for the indicated time points. In experiments using stimulated PBTL, these cells were starved of serum for 2 hours before activation and starved of IL-2 for the duration of the experiment. Cells were then harvested and centrifuged for 4 minutes at 2000*x*g. The cell supernatant was stored at -20°C and analysed for secreted IL-2 by Enzyme-linked immunosorbant assay (ELISA) (see section 2.13) and the cell pellet was processed to produce a cell lysate (see section 2.8) or mRNA isolation (see section 2.12).

2.6.2 Cell activation with Phorbol 12-myristate 13-acetate (PMA)

PMA is a phorbol ester commonly used in cancer research as a tumour promoter. It is a well known activator of the Protein Kinase C (PKC) family of serine/threonine kinases due to its similarity to diacylglycerol (DAG), one of the natural activators of PKC.

For activation of T cells with PMA, the cells were re-suspended at a concentration of $1x10^6$ cell/ml in complete medium and 50 ng/ml of PMA was added to the cell suspension and gently mixed. In experiments using stimulated PBTL, the cells were serum starved for 2 hours before activation with PMA. The cells were then incubated at 37°C for the indicated time points and harvested by centrifugation for 4 minutes at 2000*x*g. The cell supernatant was stored at -20°C and analysed for secreted IL-2 by ELISA (see section 2.13) and the cell pellet was processed for production of a cell lysate (see section 2.8) or mRNA isolation (see section 2.12).

2.6.3 Stimulation of iNKT cells with α-Galactosylceramide (α-GalCer).

iNKT cells recognise antigens presented on CD1d molecules on the surface of antigen presenting cells (APC). α-Galactosylceramide is a glycolipid antigen identified to specifically activate the invariant T cell receptor exclusively expressed on NKT cells (Kawano, Cui et al. 1997). Therefore, iNKT cells can be stimulated *in vitro* with α-GalCer-pulsed APC.

In this study, CD1d-transfected HeLa (HeLa-CD1d) cells were pulsed with α-GalCer and used as the antigen/CD1d complex necessary to activate the iNKT. The procedure was done in collaboration with Dr. Derek Doherty (Trinity College Dublin) and the cells and materials used were kindly donated by him. First, HeLa-CD1d were seeded at a concentration of 100,000 cells per well in a 96-well plate. α-GalCer stock (at 1 mg/ml kept at -70°C) was then prepared to be used in the HeLa-CD1d cells by thawing followed by heating to 80°C for 2 minutes, sonication for 10 minutes and vortexing for 1 minute. Dilution to a concentration of 0.2 μg/ml was then made in iNKT medium pre-warmed at

37°C followed by further vortexing for 1 minute, heating at 80°C for 2 minutes and sonicating for 5 minutes. The α-GalCer dilution was added to the HeLa-CD1d cells to a final concentration of 100 ng/ml and the cells were left to recover for 18 hours at 37°C to allow for antigen processing and presentation. After this period, half volume of the medium in the well was removed carefully without disturbing the HeLa-CD1d cells and the iNKT cells (100,000 cells) were then added to the well. HeLa-CD1d cells without pulsing with α-GalCer were used as negative control. iNKT cells were stimulated for 24 hours at 37°C. After that, iNKT were processed for intracellular staining of the cytokines IL-2, IL-4, IFN-γ and TNF-α (section 2.15.2) or the cell supernatant was harvested and analysed for secreted IFN-γ and IL-10 by ELISA (section 2.13).

2.7 Treatment with inhibitors

When indicated, T cells were pre-incubated with pharmacological inhibitors prior to activation with anti-CD3/CD28 antibodies or PMA. Concentrations used and time of pre-incubation for each inhibitor are listed in Table 2-1. Dimethyl Sulphoxide (DMSO) was used as vehicle control in these experiments with its final concentration never allowed to exceed 0.1% v/v.

Table 2-1 List of inhibitors used in the study.

Inhibitor	Function	Concentration	Pre-incubation time
LY294002	PI3K inhibitor	10 μM	1 hour
Wortmannin	PI3K inhibitor	100 nM	1 hour
Rapamycin	PI3K inhibitor	100 nM	2 hours
Bisindolylmaleimide I	PKC inhibitor	10 μM	30 minutes
Gö6976	PKC inhibitor	5 μM	30 minutes
LY333531	PKCβ inhibitor	5 μM	30 minutes
PD98059	MAPK inhibitor	25 μM	30 minutes
Lithium Chloride	GS3K inhibitor	20 mM	30 minutes
Bio X	GS3K inhibitor	5 μM	30 minutes
Z-VAD(OMe)-FMK	Caspase inhibitor	50 μM	3 hours
MG-132	Proteasome inhibitor	10 μM	1 hour
Staurosporine	Protein kinase inhibitor	1 μM	30 minutes
Salubrinal	eIF2α inhibitor	50 μM	2 hours
Cyclosporin A	Calcineurin inhibitor	1 μM	30 minutes
PKCη pseudosubstrate inhibitor	PKCη inhibitor	10 μM	30 minutes

2.8 Preparation of cell lysate

2.8.1 Cell lysate with NP-40 lysis buffer

T cell lines and primary T cells were harvested at the end of the experiments by centrifugation. Cell supernatant was discarded or kept at -20°C when necessary for analysis of secreted IL-2 by ELISA (see section 2.13). The cell pellet was lysed by addition of 50µl per 10^6 cells of ice-cold NP-40 cell lysis buffer freshly supplemented with protease inhibitors (1 mM PMSF, 10 µg/ml leupeptin and 1 mM Na_3VO_4). Components of the NP-40 cells lysis buffer are described in Appendix. After addition of lysis buffer to the cell pellet, the samples were incubated on ice for 30 minutes with vortexing of the sample every 5 minutes to promote efficient cell lysis. Unlysed cells, nuclei, and detergent-insoluble material were removed by centrifugation at 14,000x*g* for 5 minutes at 4°C. The resulting supernatant was stored on ice and the protein content was quantified using the Pierce BCA Protein Assay kit (see section 2.9). The samples were then mixed with an equal volume of 2X SDS sample buffer (see Appendix) and incubated for 5 minutes at 100°C. The samples were then cooled and used immediately for Western blotting (see section 2.10) or were stored at -20°C.

2.8.2 Total cell lysate with Radioimmunoprecipitation assay (RIPA) buffer

$CD4^+$ T cells were harvested at the end of the experiments by centrifugation. Cell supernatant was discarded or kept at -20°C when necessary for analysis of secreted IL-2 by ELISA (section 2.13). The cell pellet was lysed by addition of 50µl per 10^6 cells of ice-cold RIPA cell lysis buffer freshly supplemented with protease inhibitors (1 mM PMSF, 10 µg/ml leupeptin and 1 mM Na_3VO_4). Components of the RIPA cells lysis buffer are described in Appendix. After addition of the lysis buffer, samples were incubated on ice for 30 minutes with vortexing of the samples every 5 minutes to promote efficient cell lysis. Detergent-insoluble material was removed by centrifugation at 14,000x*g* for 15 minutes at 4°C. The resulting supernatant was stored on ice and the protein content was quantified using the Pierce BCA Protein Assay kit (see section 2.9). The samples were then mixed with an equal volume of 2X SDS sample buffer (see

Appendix) and incubated for 5 minutes at 100°C. The samples were then cooled and used immediately for Western blotting (see section 2.10) or were stored at -20°C.

2.9 Calculation of Protein Concentration of Cell Lysates

The protein concentration of the cell lysates was estimated using the Pierce BCA Protein Assay Kit (Thermo Scientifc, USA) following the manufacturer's instructions for microplate procedure. This procedure utilises 10µl of each preparation of unknown protein concentration. Firstly, 1:3 dilutions of the samples were prepared in distilled water to a final volume of 20 µl. The content of one Albumin Standard (BSA) ampoule with known concentration of 2000 µg/ml provided in the kit was used to prepare the standard curve for this assay ranging from 0 to 2000µg/ml of BSA (see table 2-2 below). The BCA working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B. The BSA standards and the samples were loaded in duplicate in a 96-well plate and 200 µl of the BCA working reagent was added to each well. The plate was then incubated at 37°C for 30 minutes. The colour change correlated with the protein concentration with dark purple reflecting the highest quantity and light green the lowest. The absorbance of the standards and samples was read at 570 nm using a Microplate Reader and a standard curve of BSA was determined by plotting the optical density (OD) at 570 nm versus protein concentration of BSA. The equation of the trend line produced by the standard curve was calculated using Microsoft Excel software. The average of each duplicate was calculated and corrected for the blank absorbance. The corrected absorbance values of the samples were entered into the equation to calculate the protein concentration. The value obtained was then adjusted by the dilution factor of the sample.

Table 2-2 Preparation of BSA Standard Curve for determination of protein concentration.

Final BSA Concentration (μg/ml)	Source of BSA (μl)	Volume of distilled water (μl)
2000	300 of stock	0
1500	375 of stock	125
1000	325 of stock	325
750	175 of 1500 μg/ml dilution	175
500	325 of 1000 μg/ml dilution	325
250	325 of 500 μg/ml dilution	325
125	325 of 250 μg/ml dilution	325
25	100 of 125 μg/ml dilution	400
0	0	400

2.10 Western blot analysis

2.10.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were separated by 10% SDS-PAGE gels according to their size and molecular weight using the Atto gel electrophoresis system (Atto, Japan). The components of the resolving and stacking gels as well as buffers used during electrophoresis are provided Appendix. Firstly, the resolving gel was prepared and poured between two glass plates, overlaid with isobutanol and allowed to set for approximately 40 minutes. The isobutanol was removed and the gel was washed with distilled water. The stacking gel was made and poured on top of the resolving gel. A plastic comb was inserted into the stacking gel to make loading wells and the gel was allowed to set for approximately 40 minutes at room temperature. The gels were placed in the gel electrophoresis box and the box was filled with 400 ml of 1X SDS-PAGE Running Buffer (see Appendix). Equivalent amounts of protein for each cell type (5 μg for primary T cells, 10 μg for Jurkat cells and 20 μg for HuT78 T cells unless otherwise stated) were loaded into the wells and the gels were run at 100 V/ 25 mAmp per gel until the samples

reached the bottom of the gel (approximately 1.5h). The separated proteins were then ready to be transferred to a PVDF membrane. The PageRuler™ Unstained Broad Range Protein Ladder (Thermo Scientific, USA) containing identifiable bands ranging in size from 5 kDa to 250 kDa was used as molecular weight marker to guide the correct identification of the proteins studied in this project.

2.10.2 Western Blotting

After the completion of the gel electrophoresis, the protein samples in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer technique according to the manufacturer's instructions. (Atto, Japan). Previous to the procedure, the PVDF membrane and filter papers were cut to the same size as the resolving gel. The PVDF membrane was activated by soaking it in methanol for 30 seconds and then immersing in transfer buffer (see section 2.1 of Appendix). A gel sandwich was made by soaking a set of five sheets of filter paper in transfer buffer and placing on the anode of the transfer apparatus. The PVDF membrane was placed on top of the filter papers. The resolved SDS-PAGE gel was removed from the glass plates and the stacking gel was removed with a scalpel. The resolving gel was immersed briefly in transfer buffer and placed on top of the PVDF membrane. The sandwich was finished by placing another set of pre-soaked filter papers on top of the resolving gel. Any air bubbles were removed and the sandwich was kept moist by addition of more transfer buffer but avoiding an excess of the same to prevent the system from short-circuiting. The transfer of the protein samples to the PVDF membrane was carried out for 1 hour at 100 V/100 mAmp per sandwich at room temperature.

Once the transfer was finished, the lane containing the molecular weight marker was removed from the membrane and stained with Comassie stain solution (Appendix) for 10 minutes followed by incubation with Comassie destain solution (Appendix) until the bands could be clearly seen on the membrane. The non-protein bound sites of the PVDF membrane containing the protein samples were blocked by soaking the membrane in 5% w/v milk powder in TBS-T (1X TBS with 0.1% v/v Tween-20) for 1 hour at room temperature with constant gentle agitation. The membrane was then washed 3 times for 5

minutes with TBS-T and was ready to be incubated with an antibody recognising the protein of interest.

2.10.3 Detection of proteins of interest by Enhanced Chemiluminescence (ECL)

For detection of a protein of interest, the membrane was incubated with a specific antibody diluted according to the manufacturer's suggestion in 5% w/v BSA in TBS-T containing 0.05% Sodium Azide overnight at 4°C with gentle agitation. Unbound antibody was then removed by washing the membrane 3 times for 5 minutes with TBS-T. Following this, the membrane was incubated with the appropriate species-specific Horseradish Peroxidase (HRP)-linked antibody diluted in 5% w/v milk powder in TBS-T for 1-2 hours at room temperature with gentle agitation. Unbound secondary antibody was then removed by washing the membrane 3 times for 5 minutes with TBS-T. Detection of antigens was carried out with a HRP detection solution as described by Haan and Behrmann (Haan and Behrmann 2007) (the components of this solution are also described in Appendix).

The membrane was incubated in this solution for 60 seconds, placed between two acetate sheets and taped into a cassette. The membrane was exposed to light-sensitive film for various times, ranging from 1 second to 5 minutes. The film was developed using an automated film developer (Curix 60, AGFA, Agfa-Gevaert, Munich, Germany).

2.10.4 Stripping of western blot membranes

In some experiments where indicated, the PVDF membrane was stripped of its primary and secondary HRP-linked antibodies and reprobed with antibodies to different proteins of interest. This procedure was carried out by incubating the membrane with pre-warmed Restore™ Plus Western Blot Stripping Buffer (Cat# 46428, Thermo Scientific, USA) at 37°C under gentle agitation for 30 minutes. The membrane was then washed 3 times for 5 minutes with TBS-T for removal of the stripping solution and incubated in 5% w/v milk

powder in TBS-T for 1 hour. Incubation with primary and HRP-labelled secondary antibodies and detection of proteins of interest was carried out as described in section 2.10.3.

2.10.5 Densitometric Analysis

Densitometric analysis of the Western blots was performed using the gel analysis option of ImageJ software (version 1.44, National Institute of Health, Bethesda, USA). Briefly, each band on the blot can be visualised by the software as a peak, which is proportional to the intensity of the band. The area under the peak is then selected and measured and the value is normalised to the corresponding value measured for a loading control, such as β-actin, α-Tubulin or Talin.

2.11 Transfection of T lymphocytes

The process of introducing any negatively charged molecule (e.g., DNA and RNA) into cells is defined as transfection. This method is extensively used to study gene function and protein expression in the context of a cell. In this study, electroporation or nucleofection was used to transfect T cell lines and primary T cells, respectively, with small interfering RNA (siRNA) or DNA vectors. Transfection of T cells with siRNA was carried out in order to transiently suppress gene expression in T cells, whereas transfection with a DNA vector was used to overexpress a protein of interest in T cells.

2.11.1 Delivery of siRNA to T cell lines by electroporation.

Both HuT78 and Jurkat cell lines were transfected with siRNA using a BTX 830 squarewave electroporator (BTX Molecular Delivery Systems, Holliston, MA, USA). Cells were re-suspended at a concentration of $2.5x10^6$ cells/ml in cold RPMI-1640 with 20% FCS. Control siRNA or PKCη siRNA (Dharmacon, Lafayette, CO, USA) was added to the cells to a final concentration of 500nM. The cell suspension was then transferred to a 2 mm gap BTX cuvette and electroporated with one pulse of 12 milliseconds duration at

130V. The cells were transferred to a well in a 12-well plate containing 2 ml of pre-warmed RPMI-1640 with 10% v/v FCS with no antibiotics and incubated for 24 hours at 37°C. The cells were then harvested and re-electroporated under the same conditions and incubated at 37°C for 48h. The efficiency of gene knockdown was determined by Western blot 72 hours after the first electroporation (see section 2.10).

2.11.2 Delivery of siRNA to freshly-isolated primary lymphocytes by nucleofection.

A nucleofection procedure was used to transfect freshly isolated PBL as well as $CD4^+$ T cells and $CD8^+$ T cells. Cells were re-suspended at a concentration of $5x10^6$ cells in 100 µl Ingenio Electroporation Solution (Mirus Bio, Madison, WA, USA) and mixed with Control siRNA or PKCη siRNA (Dharmacon, Lafayette, CO, USA) to a final concentration of 1000nM. This cell suspension was then transferred to Amaxa electroporation cuvettes. Nucleofection was carried out with the Amaxa™ Nucleofector device (Lonza, Cologne, Germany) using the program U-14 for non-activated T cells. The cells were transferred to a well in a 12-well plate with 2 ml of pre-warmed RPMI-1640 with 10% v/v FCS with no antibiotics and incubated for 72 hours at 37°C. Efficiency of gene knockdown was assessed by Western blot 72hs after electroporation (see section 2.10).

2.11.3 Delivery of siRNA to stimulated peripheral blood T lymphocytes (PBTL) and iNKT cells by nucleofection.

In order to prepare stimulated PBTL to be used in the electroporation process, these cells were harvested on day 5 of IL-2 stimulation (see section 2.4.2) and viable cells were isolated by density gradient centrifugation using Lymphoprep™ solution by centrifugation at 1200 xg for 5 minutes with the brake of the centrifuge set to 4. The viable stimulated PBTL were then counted using a haemocytometer and $5x10^6$ cells were separated to be used per transfection. Regarding the use of iNKT cells in the technique, the cells were processed as described in section 2.5. iNKT cells were then harvested by

centrifugation,counted as described above and $5x10^6$ cells were separated to be used per transfection.

Stimulated PBTL or iNKT cells ($5x10^6$ cells per transfection) were washed in sterile PBS and re-suspended in 100µl of Ingenio Electroporation solution (Cat# 50112, Mirus Bio, Madison, WI, USA). Control siRNA or PKCη siRNA (Dharmacon, Lafayette, CO, USA) was added to the cell suspension to a final concentration of 1000 nM. The cells were then transferred to Amaxa electroporation cuvettes and electroporated in the Amaxa™ Nucleofector device using program T-07. Cells were removed from the cuvette and transferred to wells in a 12-well plate containing 2 ml of pre-warmed RPMI-1640 with 10% v/v FCS without antibiotics. IL-2 (20 ng/ml) was added 2-4 hours after transfection and the cells were incubated at 37°C for 72 hours. After 72 hours, the cells were harvested, washed in sterile PBS and re-suspended in 100 µl of Ingenio Electroporation solution. Control siRNA or PKCη siRNA was one more time added to the cell suspension at a final concentration of 1000 nM and a second electroporation was performed under the same conditions as described above. Cells were transferred to new wells in a 12-well plate containing 2 ml of pre-warmed RMPI without antibiotics and 20 ng/ml of IL-2 was added to the cells 2-4 h after transfection. The cells were then incubated for a further 48 hours at 37°C. After this period, the cells were harvested for experimentation and the efficiency of the knockdown was assessed by Western blotting (section 2.10).

2.11.4 Transfection of $CD4^+$ T cells with vector DNA

Transfection of $CD4^+$ T cells with FLAG-PKCη vector was carried out using the Amaxa™ Human T Cell Nucleofector Kit (Lonza, Cologne, Germany) in order to overexpress the PKCη gene in these cells.

Freshly isolated $CD4^+$ T cells were harvested, washed in sterile PBS and seeded at a concentration of $5x10^6$ cells per transfection. The cell pellet was re-suspended in 100 µl of Nucleofector solution at room temperature and 5 µg of pcDNA Control or FLAG-PKCη was added to the cells. The cell suspension was transferred to Amaxa cuvettes and electroporated in the Amaxa™ Nucleofector device using program U-14. The cells were

removed from the cuvette and transferred to a well in a 12-well plate containing 2 ml of pre-warmed RPMI-1640 with 10% v/v FCS without antibiotics. The transfection efficiency was assessed 24 hours later by Western blot (see section 2.10).

2.12 Relative gene expression analysis

The expression of PKCη and IL-2 at the mRNA level was analysed by real-time RT-PCR (qRT-PCR). RNA was isolated from cells using NucleoSpin RNA II kit (Cat# 740955.50, Macherey-Nagel, Germany) following the manufacturer's instructions. Briefly, $1x10^6$ cells were lysed in 350 µl Buffer RA1 provided with the kit supplemented with 3.5 µl β-mercaptoethanol. The viscosity of the lysate was reduced by filtration through a NucleoSpin Filter provided in the kit. The RNA was precipitated from the cell lysate with the addition of 350µl of 70% ethanol. This solution was then loaded into a NucleoSpin Column, also provided with the kit, which has a silica membrane that binds RNA. The silica membrane in which the RNA is bound was then treated with 350µl of the Membrane Desalting Buffer provided in order to remove any salt from the membrane and make the following DNA digest step more effective. Subsequently, an on-column DNA digestion was performed by addition of the rDNase (recombinant DNAse) solution provided with the kit to remove contaminating DNA. After two wash steps with buffers RA2 and RA3 provided, the RNA was eluted from the membrane with 40µl RNase-free water and quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Total complementary DNA (cDNA) was synthesized from 500 ng of RNA using the Tetro cDNA Systhesis Kit (Cat# BIO-65043, Bioline Reagents, London, UK) following the manufacturer's instructions. Per reaction, enough RNase-free water was added to 500 ng of RNA to a final volume of 12 µl. To this solution, the following reagents provided with the kit were added, comprising a total volume of 20µl: 1 µl of random hexamers, 1 µl of 10mM dNTP mix, 4 µl of 5X RT Buffer, 1 µl of Ribosafe RNase inhibitor and 1 µl of Tetro Reverse Transcriptase. The samples were then placed in the thermocycler TC-3000 (Techne, Burlington, NJ, USA) and cDNA synthesis was performed incubating samples for 10 minutes at 25°C followed by 1 hour incubation at 45°C. The reaction was

terminated by incubating the samples for 5 minutes at 85°C followed by chilling the samples on ice. The samples were stored at -20°C until used for the qRT-PCR reaction.

The qRT-PCR reaction was carried out in 384-well clear reaction plates using TaqMan® Gene Expression Assay for PKCη, IL-2 and Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) provided by Applied Biosystems (Carlsbad, CA, USA). Details of the primers are provided in Table 2-3.

Table 2-3 TaqMan® Gene Expression Assays used for relative gene expression analysis.

Gene name/Symbol	Assay number
PKCη/ PRKCH	Hs00178933_m1
IL-2	Hs00174114_m1
GAPDH	Hs99999905_m1

The TaqMan® Gene Expression Assays contain an unlabelled PCR primer pair and a dye labelled probe containing a nonfluorescent quencher. The labelled probe binds to the target DNA between the two unlabelled PCR primers. During PCR the Taq polymerase, directed by the template strand, extends the unlabelled primers. The labelled probe is cleaved by the polymerase, which releases the quencher allowing the dye to fluoresce. The level of fluorescence detected reflects the expression level of the target DNA.

For the qRT-PCR reaction, cDNA samples were diluted 1:10 in RNase-free water and 4.5 µl of this dilution was loaded in triplicate in a 384-well plate. 5.5 µl of a mix containing 5µl of Taqman Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA, USA) and 0.5 µl of primers/probe was added to each well containing cDNA. A GAPDH control was also run in triplicate for each cDNA sample. qRT-PCR was performed with the ABI Prism 7900T real time thermocycler (Applied Biosystems, Carlsbad, CA, USA). Cycling conditions for the reactions were maintained as suggested by the manufacturer, with an initial 10 minutes at 95°C step to activate the polymerase followed by 40 cycles of 15s at 95°C and 60s at 60°C. Ct values were determined by automated threshold analysis.

Fold change in gene expression was calculated using the ΔΔCt method where GAPDH and 18S rRNA were used as the endogenous controls for the T cell lines and primary cells respectively. Untreated resting cells were used as the calibrator for fold change. The

relative quantification analysis technique was used in these experiments to determine expression levels of the genes of interest. This method involved a comparison of the cycle threshold (Ct) value of the gene of interest with the Ct value of the endogenous control to obtain a delta Ct value (ΔCt). The fold change of the gene of interest was calculated with the formula $2^{(-\Delta\Delta Ct)}$ which was derived from the delta-delta Ct value (ΔΔCt). The ΔΔCt value represents the change in ΔCt of the gene of interest between a sample that has been exposed to a treatment/activation and a resting untreated sample which is used as calibrator (Livak and Schmittgen 2001).

2.13 Detection of secreted IL-2

The levels of secreted IL-2 were measured in the supernatants of T cells after 24 hours of cell stimulation with either anti-CD3/CD28 antibodies or PMA by ELISA using the Human IL-2 DuoSet® kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

ELISA is a widely used plate-based colorimetric assay designed to detect and quantify the presence of a particular protein in a liquid sample, such as cell culture supernatants. The Human IL-2 DuoSet kit contains the basic components required for a sandwich ELISA (Figure 2-2). In this type of assay, the protein of interest is immobilised between a capture antibody, which had been previously adsorbed to the plate and a detection antibody, which has a biotin tag, both antibodies recognize different epitopes in the IL-2 molecule. A HRP enzyme coupled to streptavidin is then added to the samples. Streptavidin has high affinity for biotin and therefore binds to the capture antibody-IL-2-detection antibody complex. In the following step, the enzymatic substrate tetramethylbenzidine (TMB) is added and oxidized by the HRP into a blue colour proportional to the quantity of the target protein present in the sample. The reaction is terminated by addition of a stop solution, usually an acid, which will turn the colour to yellow. The absorbance of the colour is measured in a plate reader at 450 nm and the amount of IL-2 in the sample is calculated from this value using a standard curve.

To determine the levels of IL-2 secreted in the cell supernatant by activated T lymphocytes, wells in a flat-bottomed 96-well plate were firstly coated with 100µl of

capture antibody diluted to a working concentration of 4 μg/ml in PBS. The plate was then sealed and incubated at room temperature overnight. The following day, unbound capture antibody was removed and the wells were washed 3 times with 250 μl wash buffer (0.05% Tween-20 in PBS, pH7.2-7.4). After the last wash, any remaining buffer was removed by inverting the plate and blotting it against clean paper towel. The plate was then blocked with 200 μl blocking buffer (1% w/v BSA in PBS) and incubated for 1 hour at room temperature. The blocking buffer was aspirated and the plate was washed for a total of three times with wash buffer. Recombinant IL-2 standards of known concentration and cell culture supernatants of unknown IL-2 concentration were prepared and 100 μl of each were loaded in duplicate onto the plate. A seven point standard curve of recombinant human IL-2 standard was prepared by making a 2-fold serial dilution in reagent diluent (0.1% w/v BSA, 0.05% Tween-20 in 1X TBS pH 7.2-7.4, 0.2 μm filtered) with a the top standard concentration of 1000 pg/ml. Samples of unknown IL-2 concentration in the T cell supernatants were left either undiluted or diluted 1:5 with reagent diluent. The plate was incubated for 2 hours at room temperature. Unbound standards or samples were aspirated from the plate and the plate was washed three times with wash buffer. The detection antibody was diluted to a working concentration of 200 ng/ml with reagent diluent and each well was coated with 100 μl of this solution and the plate was incubated for 2 hours at room temperature. Unbound detection antibody was aspirated and the plate was washed 3 times with wash buffer. A working stock of Streptavidin-HRP was prepared (1/200 dilution in reagent diluent) and 100 μl of this solution was added to each well. The plate was incubated in the dark for 20 minutes at room temperature. After aspiration of unbound Streptavidin-HRP and three washes of the plate, 100 μl of Substrate Solution (TMB soluble, Cat#613544, Calbiochem, Darmstadt, Germany) was added to each well and incubated in the dark for 20 minutes. 50 μl of stop solution (0.5 M H_2SO_4 in distilled water) was added to each well of the plate and the absorbance values of each well were immediately read on a microplate reader at 450 nm wavelength. A standard curve of recombinant IL-2 was constructed by plotting the absorbance (OD) at 450 nm versus concentration of IL-2 (pg/ml). The equation of the line of the standard curve was obtained using Microsoft Excel software. The concentration of IL-2 in the unknown samples was determined by using the equation of the line from the standard curve. Samples which had been diluted to use in the assay were adjusted and had their concentration values multiplied by the dilution factor used.

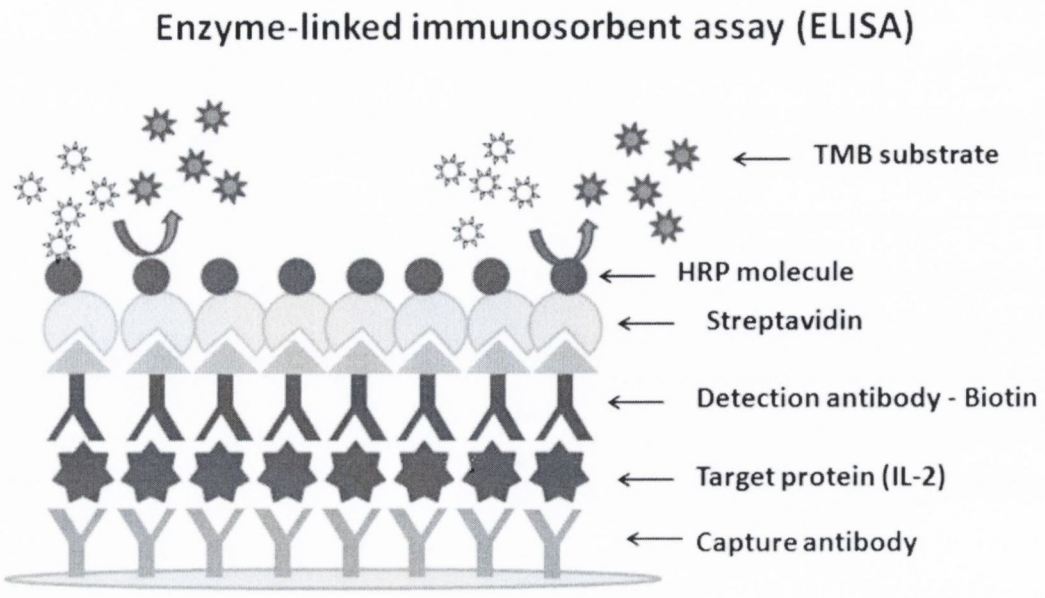


Figure 2-2 Schematic representation of the sandwich ELISA assay for detection of secreted IL-2. Briefly, the cell supernatant is added into a well in a culture plate pre-coated with a capture antibody which recognizes the target protein to be measured in the supernatant (in this case IL-2). After several wash steps, a biotinylated detection antibody is added to the well and recognizes the target protein bound to the capture antibody, forming a sandwich. In the sequence, a conjugate of streptavidin with the enzyme horseradish peroxidase (HRP) is added to form a complex with the antibody sandwich. This is mediated by binding of the streptavidin with the biotin molecule that is attached to the detection antibody. The substrate TMB is then added to the well and converted by the enzyme HRP into a colour signal which is proportional to the quantity of the target protein present in the sample.

2.14 Differentiation of human T cells in vitro

The different subsets of T helper cells, including Th1, Th2, Th17 and iTreg can be differentiated *in vitro* by activation of naive $CD4^+$ T cells with anti-CD3/anti-CD28 in the presence of a particular cytokine milieu. The distinct subsets can be then defined by the type of cytokine produced and detection of specific transcription factors expressed by each subtype. It is not yet clear of how the individual T cells can adopt a specific phenotype during the skewing conditions, with evidence suggesting that the cytokines added to the medium can act both ways by selecting a pre-differentiated population or inducing the transcription factors that determine differentiation (Zhu, Yamane et al. 2010).

2.14.1 Differentiation of Th1 cells

Differentiation into Th1 cells was performed by stimulation of freshly isolated PBL with anti-CD3/CD28 antibodies for 4 days in the presence of 50 ng/ml IL-12. Control cells (Th0) were also stimulated with anti-CD3/CD28 but cultured without the Th1 polarization cytokine IL-12. After this period the cells were harvested and analysed by flow cytometry to detect the surface co-receptor CD4 (as described in section 2.15.1) and production of intracellular IFN-γ (as described in section 2.15.2). Cells that were positive for both CD4 and IFN-γ in the flow cytometric analysis were considered Th1 cells.

2.14.2 Differentiation of Th17 cells

Differentiation into Th17 cells was performed by stimulation of freshly isolated PBL for 4 days with anti-CD3/CD28 antibodies in the presence of 50 ng/ml IL-23, 30 ng/ml IL-6 and 50 ng/ml IL-1β. Control cells (Th0) were also stimulated with anti-CD3/CD28 but cultured without the Th17 polarization cytokines described above. After this period the cells were harvested and analysed by flow cytometry to detect the surface co-receptor CD4 (as described in section 2.15.1) and production of intracellular IL-17 (as described in

section 2.15.2). Cells that were positive for both CD4 and IL-17 in the flow cytometric analysis were considered Th17 cells.

2.14.3 Differentiation of FoxP3$^+$ Regulatory T cells (Treg)

Differentiation into Treg was performed with stimulation of freshly isolated PBL with anti-CD3/CD28 antibodies for 4 days in the presence of 20 ng/ml IL-2 and 5 ng/ml TGF-β. Control cells (Th0) were also stimulated with anti-CD3/CD28 but cultured without TGF-β and IL-2. The cells were then harvested and analysed by flow cytometry to detect the CD4 co-receptor on the cell surface (as described in section 2.15.1) and the transcription factor FoxP3 (as described in section 2.15.3). Cells that were positive for both CD4 and FoxP3 in the flow cytometric analysis were considered iTreg cells.

2.15 Flow cytometric analysis

Flow cytometry is a laser-based technology that measures, and analyses simultaneously, multiple characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, granularity and relative fluorescence intensity. The size of the particle is measured by the Forward Scatter detector (FSC) while the granularity/complexity of the particle is measured in the Side Scatter detector (SSC). In addition, one or more fluorescence detectors can detect fluorescent chemicals in the particle or attached to it. With this, flow cytometry can be used for cell counting, measurement of cell viability, immunophenotyping, analysis of total DNA content, among others.

Flow cytometric analysis was carried out within a minimum of 10,000 cells per sample in the bench top DAKO CyAn ADP Analyser (Beckman Coulter Diagnostics Limited, Lismeehan, Ireland). This flow cytometer is equipped with three excitation lasers (488 nm, 405 nm and 642 nm) and 11 parameters (FSC, SSC, FL1-FL9) which allow detection of a broad range of fluorochromes. The data were analysed by DAKO Summit Software (version 4.3) or FlowJo software (version 7.6.5).

When more than one fluorochrome was used in the assay, the use of compatible dyes was taken into account so two dyes with the same colours were not used in the same tube. Negative controls were used for all fluorochromes in the experiments and consisted of unstained cells. In cases where cells were stained with two fluorochromes, single stained controls were also set-up in parallel in order to perform the compensation of the fluorochrome signal for the experiment.

During the analysis, FSC and SSC parameters were adjusted so the lymphocyte population could be clearly identified. A gate was drawn around these cells and all the following analyses were focused in this population.

2.15.1 Detection of cell surface markers

For the detection of markers present on the cell surface, at least 150,000 T cells were harvest at the end of the experiment by centrifugation and washed with 1 ml of ice cold PBS. The supernatant was discarded and the cell pellet re-suspended in 100 µl of PBS Staining of cell surface molecules was performed by addition of 2 µl of the specific fluorescently-labelled antibodies against these molecules (e.g. CD3, CD4, CD8, CD69, CD25, CD71) to the cells. The samples were then vortexed and incubated on ice for 10 minutes in the dark. After this period, the cells were washed one more time with 1 ml PBS, re-suspended in 400 µl and immediately analysed with a flow cytometer or proceeded for fixation and intracellular cytokine staining (section 2.15.2).

2.15.2 Detection of Cytokine Production by Intracellular Staining

Production of cytokines by activated T cells was assessed by intracellular staining and flow cytometric analysis. To do so, the cells were stimulated (or re-stimulated depending on the experiment) with plate-bound anti-CD3/CD28 antibodies or 50 ng/ml PMA and 1 µg /ml Ionomycin (Io) for 4 hours at 37°C in the presence of 10 µg/ml Brefeldin A. Brefeldin A is known to inhibit transport of proteins from the ER to the Golgi apparatus resulting in accumulation of the cytokines produced inside the cell. After this period, the cells were transferred to 1.5 ml tubes and stained for cell surface markers as described in

section 2.15.1. After staining for the surface markers, the cells were washed once with 1.5 ml PBA buffer (PBS containing 1% BSA and 0.02% sodium azide) and fixed with 500 µl of 4% paraformaldehyde (PFA) in the dark for 10 minutes at room temperature. The cells were washed again with PBA and permeabilised with 1 ml of permeabilisation buffer (0.2% w/v saponin in PBA) in the dark for 10 minutes at room temperature. The cells were then stained for intracellular cytokines by addition of 10 µl of fluorescently labelled anti-cytokine antibodies (e.g., IL-2, IFN-γ, IL-10, IL-4 or IL-17) diluted in 100 µl permeabilisation buffer for 20 minutes in the dark at room temperature. After this period, the cells were washed one more time with 1.5 ml PBA, re-suspend in 500 µl PBA buffer and transferred to flow cytometry tubes. The samples were kept in the dark at 4°C until analysis by flow cytometer which was carried out immediately or the next day.

2.15.3 Detection of FoxP3 transcription factor by flow cytometry

To detect the presence of the FoxP3 transcription factor in the Treg cell population, the cells were first harvested and stained for the CD4 co-receptor as described in section 2.15.1. The cells were then fixed and permeabilised using the FoxP3 Fix/Perm Buffer Set (BioLegend, US) according to the manufacturer's instruction. Firstly, the FoxP3 Fix/Perm buffer 4X and the 10X FoxP3 Perm buffer, both provided in the kit, were diluted to 1X working concentration with PBS. After staining the cells for surface markers, as described in section 2.15.1, 1 ml of 1X FoxP3 Fix/Perm buffer was added to the tubes with the cells. The tubes were vortexed and the cells were incubated at room temperature in the dark for 20 minutes. Thereafter, the cells were washed once with 1 ml of the 1X FoxP3 Perm Buffer and re-suspended in 1 ml of 1X FoxP3 Perm Buffer and then incubated for 15 minutes in the dark at room temperature. The cells were centrifuged, the supernatant was discarded and the cell pellet re-suspended in 100 µl of 1X FoxP3 Perm Buffer. Fluorescently-labelled FoxP3 antibody (1 µl) was then added to the cells, mixed and incubated for 30 minutes in the dark at room temperature. After this period, cells were washed two times with 1 ml of PBA buffer (PBS containing 1% BSA and 0.02% sodium azide) and re-suspended in 400 µl of PBA buffer and transferred to flow cytometry tubes. The samples were kept in the dark at 4°C until analysis in the flow cytometer which was carried out immediately or the next day.

2.15.4 Cell Proliferation Assay with Carboxyfluorescein Diacetate, Succinimidyl Ester (CFSE) Staining

CFSE is a dye that passively diffuses into the cells and interacts covalently with intracellular molecules. It is used to track cell proliferation due to its progressive halving in concentration within daughter cells following each cell division. Analysis of cell proliferation in T cells upon anti-CD3/anti-CD28 activation was carried out using CFSE staining. In the experiments involving stimulated PBTL, the cells were incubated in the absence of serum and IL-2 for 4 hours before staining with CFSE.

To perform the analysis, primary T cells were re-suspended at a concentration of $1x10^6$ cells/ml in pre-warmed 0.1% BSA in PBS and incubated with 10 μM of CFSE for 10 minutes at 37°C. The staining was quenched by addition of 5 volumes of ice-cold complete medium to the cells and incubation on ice for 5 minutes. The cells were pelleted by centrifugation and washed 3 times with an excess of fresh medium. After the washing steps the cells were re-suspended in complete medium at a concentration of $1x10^6$ cells/ml and activated with plate-bound anti-CD3/CD28 antibodies. Cells were allowed to proliferate for 5 days while unstimulated cells were used as undivided control. Upon 5 days of stimulation, the cells were harvested, pelleted by centrifugation and re-suspended in 400 μl of PBS. The flow cytometric analysis was carried out immediately after harvest of the cells using the 488 nm excitation laser and FL1 channel for detection of the CFSE dye. Histograms were created using the Summit software (version 4.3). A gate was drawn around the live lymphocytic population using the FCS vs SSC histogram and cell division was visualised by plotting the log intensity of the CFSE against event counts. The number of peaks found in the analysis corresponded to the number of divisions that the cells underwent throughout the 5-day period.

2.15.5 Cell Cycle Analysis

Staining with Propidium iodide (PI) was used to evaluate the DNA content and analyse cell cycle progression in primary T cells following activation with anti-CD3/anti-CD28 antibodies. PI is a dye which binds to DNA and the intensity of the dye is proportional to the amount of DNA present in the cells. In this way, cells in the S phase will have more

DNA than the cells in G1 and less than the cells in G2 phase, which in turn will be approximately twice as bright as cells in G1 because they have doubled their DNA content.

$CD4^+$ T cells and PBL were stimulated for 48 or 72 hours respectively with plate-bound anti-CD3/anti-CD28 antibodies. After this period, these cells were harvested, washed one time with PBS and re-suspended in 500 µl of PBS. The cells were then fixed by careful addition of the cell suspension to 4.5 ml of pre-chilled 70% ethanol followed by incubation at 4°C for a minimum of 2 hours. The cell suspension was centrifuged for 5 minutes at 200*x*g and the ethanol was thoroughly decanted. The cells were washed with 5 ml PBS and then re-suspended in 500 µl of PI/RNase staining buffer (BD Pharmingen, San Jose, CA, USA) for 1 hour at room temperature. After incubation, the cells were transferred to flow cytometry tubes and analysed immediately.

The DNA content of stained cells was accessed with the DAKO CyAn ADP (Beckman Coulter Diagnostics Limited, Lismeehan, Ireland) benchtop flow cytometer using the 488 nm excitation laser and FL3 channel for detection of the PI. Analysis was carried out using Dako Summit (version 4.3) software. Firstly, a gate was drawn in the lymphocyte population followed by gating on single cells using the FSLin *versus* FSArea plot. A histogram was then created for these cells with the PI *versus* Event counts parameters to analyse the cell cycle profile of the samples. The resulting histograms of cells which progressed through cell cycle consisted of two connected peaks (as shown in Figure 2-3). The peak on the left of the graph showed the DNA content of a cell in the G0/G1 phase of the cell cycle and the peak on the right hand side had double the fluorescence value of peak one and corresponded to dividing cells or those in G2/M phase. Cells with PI intensity between those two values are considered to be in the S phase. Apoptotic cells were detected in the subG1 phase due to degradation of their DNA content. The percentage of cells in each of the cell cycle phases was calculated by the Dako Summit (version 4.3) software.

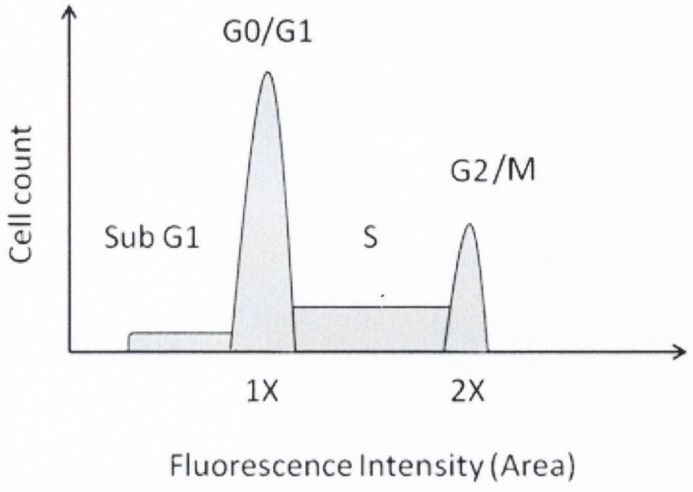


Figure 2-3 Representation of DNA histogram acquired during flow cytometric analysis of cell cycle. Cells that are in the G2/Mitosis (G2/M) phase of cell cycle present double the amount of DNA/PI intensity of the cells in G0/G1 phase. Cells in S phase have intermediate values while apoptotic cells have a high level of DNA degradation and therefore are in the Sub G1 phase.

2.15.6 Flow cytometric analysis of cell apoptosis.

Flow cytometry was also used to investigate the presence of cell death and apoptotic cells using Annexin V and Propidium Iodide (PI) staining. Phosphatidylserine is a phospholipid component located on the inner side of the cell membrane in healthy cells. In early phases of apoptosis, the cell membrane loses its polarity and the phosphatidylserine molecule is exposed on the external side of the membrane. This event is therefore considered an early marker of cell death by apoptosis. Annexin V, an endogenous protein, has high affinity in binding to phosphatidylserine and has been extensively used to detect this protein on the cell surface (van Engeland, Nieland et al. 1998). In turn, PI is excluded from cells that have an intact and functional membrane. In dead cells or cells in late apoptosis the membrane becomes unable to exclude this dye from the cell and it binds to DNA. Combining staining for phosphatidylserine with Annexin V and PI allows detection of early apoptosis, late apoptosis and cells death.

For this assay, $CD4^+$ T cells were left resting or activated with anti-CD3/CD28 as described in section 2.6.1 and then harvested at 24, 48 and 72 hours upon cell activation. The cells were then washed one time with Annexin V binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH7.4) and re-suspended in 100 µl of the same buffer. Fluorescently labelled Annexin V (2 µl) and a final concentration of 1 µg/ml of PI were then added to the cells. The samples were then mixed and incubated at room temperature in the dark for 15 minutes. After incubation, 300 µl of Annexin V binding buffer was added to the cells and samples were transferred to flow cytometry tubes to be analysed. $CD4^+$ T cells that had been previously fixed with 4% paraformaldehyde (PFA) were used as a control for PI positive cells. For Annexin V positive control, $CD4^+$ T cells were treated for 4 hours with 1 µM of staurosporine, a known apoptosis-inducing agent (Belmokhtar, Hillion et al. 2001), prior to analysis.

2.16 Statistical Analysis

Quantitative data are presented as mean ± SEM of number (N) of experiments. The statistical significance of differences between experimental groups was assessed with Student's t-test. A $p<0.05$ was considered to be statistically significant. Data was first analysed in Microsoft Excel and then transferred to Graph Pad Prism (version 4) for statistical analysis and production of graphs.

3 Chapter 3 – Regulation of PKCη expression and function in T cell activation

A comparison between T cell lines and primary lymphocytes

3.1 Introduction

T cells control the focus and intensity of the adaptive immune response. These cells become activated by the ligation of their T cell receptor (TCR) with an antigen bound to a major histocompatibility (MHC) complex presented on the surface of antigen presenting cells (APC) (Sundberg, Deng et al. 2007; Brownlie and Zamoyska 2013). To become fully activated and capable of producing a response to foreign pathogens while at the same time tolerating self, T cells require an extra activation signal provided by co-stimulatory molecules such as the co-receptors CD28 or CTLA-4 (Rudd, Taylor et al. 2009). Stimulation of the TCR and co-receptors initiates a complex signalling cascade which will result in T cell proliferation and differentiation (see Figure 1-1 for details). In addition to activation of tyrosine kinases, three main pathways are activated during T cell activation, the phosphoinositide 3-kinase (PI3K) pathway is principally activated downstream of CD28 and involved in cytokine production, cell proliferation and survival (Kane and Weiss 2003; Rudd, Taylor et al. 2009; Riha and Rudd 2010); the Mitogen-activated protein kinase (MAPK) pathway, which includes the ERK pathway is reported to play an important role in regulation of T cell activation and differentiation (Smith-Garvin, Koretzky et al. 2009) and the Protein kinase C (PKC) pathway, described to be crucial for signal transduction and activation of transcription factors (Wang, Chuang et al. 2012).

The PKC family of kinases is constituted of ten isoforms divided in three groups based on the second messengers required for activation. The conventional PKCs (cPKCs), PKCα, PKCβI, PKCβII and PKCγ, require both diacylglycerol (DAG) and calcium (Ca^{2+}) for activation. The novel PKCs (nPKCs), isoforms δ, η, θ and ε require DAG but are Ca^{2+} - independent and lastly the atypical PKCs (aPKCs), PKCζ and PKCλ/ι which are both DAG and Ca^{2+} independents (Pfeifhofer-Obermair, Thuille et al. 2012).

The novel PKCθ is the most studied isoform in the context of T cell activation. This isoform has been shown to be activated downstream of the ligated TCR and recruited to the immunological synapse, the site of contact between the T cell and APC. In addition, PKCθ has been characterised as a key enzyme in regulating the activation of the

transcription factors NF-AT, AP-1 and NF-kB (Isakov and Altman 2012; Wang, Chuang et al. 2012).

Recently, PKCη, another member of the novel PKC isoforms, has been described to have a potentially important role in T cell biology. Using murine $CD4^+$ T cells, Quann et al. (2011) were the first to show that PKCη together with PKCε also accumulate in the immunological synapse and these two isoforms act redundantly in recruiting PKCθ to the site. Moreover, they have demonstrated that simultaneous knockdown of the PKCη and PKCε genes decreases the production of IL-2 by activated T cells. Another study using PKCη knockout mice has described some redundant but also specific roles for PKCθ and PKCη in T cells. $PKC\eta^{-/-}$ T cells showed an impaired proliferative response following stimulation with anti-CD3 antibody as well as a decrease in up-regulation of the activation markers CD25 and CD69, both characteristics shared with $PKC\theta^{-/-}$ T cells. However, only $PKC\eta^{-/-}$ T cells showed defective homeostatic proliferation, which requires self-antigen recognition (Fu, Hu et al. 2011).

Human T cells can be studied in vitro using a range of available T cell lines and primary T cells isolated from blood. T cell activation can be mimicked by stimulation of the TCR and co-receptors with antibodies that specifically bind to these molecules or treatment of the cells with phorbol-esters, such as phorbol 12-myristate 13-acetate (PMA) which directly activates members of the PKC family. For a long time T cell lines have been extensively used in research as model systems for studying the activation and function of human T cells, since they offer an unlimited source of experimental material which can be maintained at low cost and stored for long periods. Among the wide variety of T cell lines available, the two most used T cell lines include the Jurkat T cell and HuT78 T cell. The Jurkat T cell clone was isolated in the early 1980s from a 14-year-old boy with T cell acute lymphoblastic leukemia and present characteristics of immature thymocytes. Jurkat T cells have contributed greatly to the understanding of characteristics and function of many TCR signalling molecules (Abraham and Weiss 2004). The HuT78 T cells were isolated in the late 1970's from a patient with Sézary syndrome, a type of cutaneous lymphoma, and have a mature, $CD4^+$ T-helper phenotype. The subclone H9 of the HuT78 T cell line has historical importance, being the cell used in the identification and characterisation of the HIV retrovirus (Bunn and Foss 1996).

Despite being useful for several reasons, it is known that these T cell lines present several abnormalities. It has been shown that Jurkat T cells are defective in the expression of phosphatase and tensin homologue (PTEN) and SH2-domain-containing inositol polyphosphate 5' phosphatase (SHIP), two lipid phosphatases important in the regulation of PI3K function (Astoul, Edmunds et al. 2001). The loss of PTEN and SHIP results in high basal levels of $PI(3,4,5)P_3$ and constitutive phosphorylation of PKB/Akt (Freeburn, Wright et al. 2002). In addition, the lack of PTEN causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation (Shan, Czar et al. 2000). HuT78 T cells also present some defects, including lack of CD28 expression, abnormal function of the proto-oncongene *MYC*, over expression of the Bcl-xL protein and constitutively active NF-κB (Finger, Huebner et al. 1988; O'Connell, Cleere et al. 1995; Vallejo, Nestel et al. 1998; Uddin, Hussain et al. 2004).

The use of primary lymphocytes isolated from peripheral blood provides a more real model to study T cell biology, but there are several limitations regarding ethical considerations of experimentation and the genetic variability from donor to donor can lead to inconsistent results. A study comparing TCR-induced proximal signalling in immortalized and primary T cells showed that both Jurkat and HuT78 T cell lines have similarities and differences compared to activated peripheral blood T cells and highlighted the importance of keeping these differences in mind when choosing a model system for studying the biology of T cells (Bartelt, Cruz-Orcutt et al. 2009).

As discussed above, recent findings describe PKCη as a potentially important player in T cell activation. However, very little is known about regulation and function of this PKC isoform in T cell biology, particularly in human T cells. Keeping in mind the differences and similarities between the model systems used to study T cell activation *in vitro* with human T cells, we decided to further investigate a role for PKCη in the context of T cell activation while at the same time comparing the response to activation and regulation of PKCη in these models.

3.2 Aims and objectives

The overall objective of this chapter was investigate and compare the regulation of expression of PKCη following T cell activation in the T cell lines HuT78 and Jurkat and in peripheral blood lymphocytes (PBL). Also, I was interested in investigating if PKCη plays a role in IL-2 secretion in response to activation in these cells. Thus, the aims of this study were to:

- Determine the expression of PKCη at both protein and mRNA levels in HuT78, Jurkat and PBL in resting cells and in response to cell activation;
- Investigate possible regulators of PKCη expression within main signalling pathways involved in T cell activation using pharmacological inhibitors against key effector kinases in these pathways.
- Use a siRNA approach to silence PKCη expression, in addition to a PKCη-specific pseudosubstrate kinase inhibitor to investigate if this isoform has a role in IL-2 secretion following cell activation.

3.3 Regulation of PKCη expression upon T cell activation in T cell lines and peripheral blood lymphocytes (PBL).

3.3.1 Expression of PKCη is distinctly regulated among T cell models in the context of T cell activation.

The expression of PKCη following T cell activation was investigated in the T cell lines HuT78 and Jurkat and in freshly isolated PBL. Both cell lines mentioned are known to be T cells while PBL are a mixed cell population consisting mainly of lymphocytes (T cells, B cells and NK cells). Please refer to section 2.4 for more information on the technique used to isolate PBL in this study.

All three cell types studied expressed PKCη at the protein level, including when in the resting state. HuT78 T cells showed very low basal PKCη protein levels, which increased after 24 hours of cell activation with a mix of anti-CD3/anti-CD28 antibodies or PMA (Figure 3-1.A). The basal level of PKCη expression was higher in the Jurkat T cell line than in HuT78 (Figure 3-1.B) and consequently the induction of PKCη expression following activation was not as evident as in HuT78. Densitometric analysis of the immunoblots for Jurkat cell lysates (Figure 3-1.B, right panel) showed a slight increase in PKCη protein levels at 24 hours in cells activated with anti-CD3/CD28 or PMA, although this was inconsistent.

PKCη protein levels were also increased in PBL at 24 hours upon cell activation with anti-CD3/CD28 antibodies or PMA (Figure 3-1.C). Interestingly in this case, an induction of PKCη protein levels was also observed in cells left resting for 24 hours, although this induction was to lesser extent when compared to activated cells.

Relative gene expression for PKCη was also measured upon cell activation of the three cell types HuT78, Jurkat and PBL (Figure 3-2). PKCη messenger RNA (mRNA) levels were analysed in resting HuT78 T cells and those activated with anti-CD3/CD28 antibodies for 24 hours. PKCη mRNA levels were increased 2-fold in activated HuT78 in

comparison with resting cells (Figure 3-2.A). In Jurkat T cells, PKCη mRNA levels were measured at 0, 6 and 24 hours of cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA. In accordance with the other T cell line HuT78, PKCη mRNA levels in Jurkat T cells were significantly increased (2-3 fold) at 24 hours in cells activated with anti-CD3/CD28 antibodies as well as with anti-CD3 antibody alone in comparison with the 0 hour time point (Figure 3-2.B). Although not statistically significant, stimulation with anti-CD3 alone showed a more potent induction of PKCη gene expression than the combination of anti-CD3/anti-CD28. Activation of PBL with anti-CD3/CD28 antibodies, but not with anti-CD3 antibody alone, also resulted in an increase (although not statistically significant) in PKCη mRNA levels. (Figure 3-2.C). These findings correspond with the increase in PKCη protein levels observed for this cell population (Figure 3-1.C).

Taken together, these results demonstrate that PKCη is up-regulated at the protein level in response to cell activation in the T cells models studied. Although the increase in PKCη expression was clear in HuT78 at protein and mRNA levels, up-regulation of PKCη was confirmed in Jurkat T cells at the mRNA levels. In PBL, both protein and mRNA levels of PKCη were up-regulated in response to cell activation. Moreover, PKCη were also slightly up-regulated in these cells when they were kept in culture with no other type of stimulation.

Because T cells express nine out of the ten PKC isoforms (Pfeifhofer-Obermair, Thuille et al. 2012) it was important to determine if PKCη was being specifically induced upon cell activation or if expression of other isoforms was also modulated. The expression levels of the PKC isoforms were analysed by western blotting in total protein lysates obtained from resting and activated cells (anti-CD3/CD28 or PMA). It is known that T cells do not express PKCγ and unfortunately antibodies against the isoforms PKCε and PKCλ were not available at the time of the experiment. Therefore, it was investigated if the expression levels of the conventional PKCα and PKCβ (a pan-PKCβ antibody which recognizes both βI and βI isoforms was used), the novel PKCδ and PKCθ and the atypical isoform PKCζ were changed following T cell activation. The expression of these PKC isoforms was first determined in the T cell lines HuT78 and Jurkat (Figure 3-3 A and B respectively). The results showed that the protein levels of any of the PKC isoforms tested were significantly altered when HuT78 and Jurkat T cells were stimulated with

anti-CD3/anti-CD28 for a period of 24 hours. However, activation of both T cell lines with PMA for 24 hours caused down-regulation of the isoforms PKCα, PKCβ and PKCθ. Proteolysis of certain PKC isoforms in response to prolonged exposure to phorbol esters has been described before and was therefore expected (Liu and Heckman 1998). Interestingly, PKCθ protein levels were decreased in Jurkat cells, but not in HuT78 cells, at 24 hours upon cell activation with anti-CD3/anti-CD28 antibodies (Figure 3-3.B). Following on from this, the expression levels for the mentioned PKC isoforms was investigated in PBL (Figure 3-4). Using the same methodology, PBL that were resting or were activated with anti-CD3/CD28 antibodies or PMA for 24 hours were lysed and the protein extracts were analysed by western blotting for expression of the given PKC isoforms. Interestingly, PKCη was not the only isoform to be up-regulated in activated PBL. Activation of PBL with anti-CD3/CD28 increased levels of most of the isoforms analysed, with the exception of PKCβ. Treatment with PMA also caused up-regulation of PKCζ, PKCδ and PKCθ and down-regulation of PKCα, PKCβ (Figure 3-4).

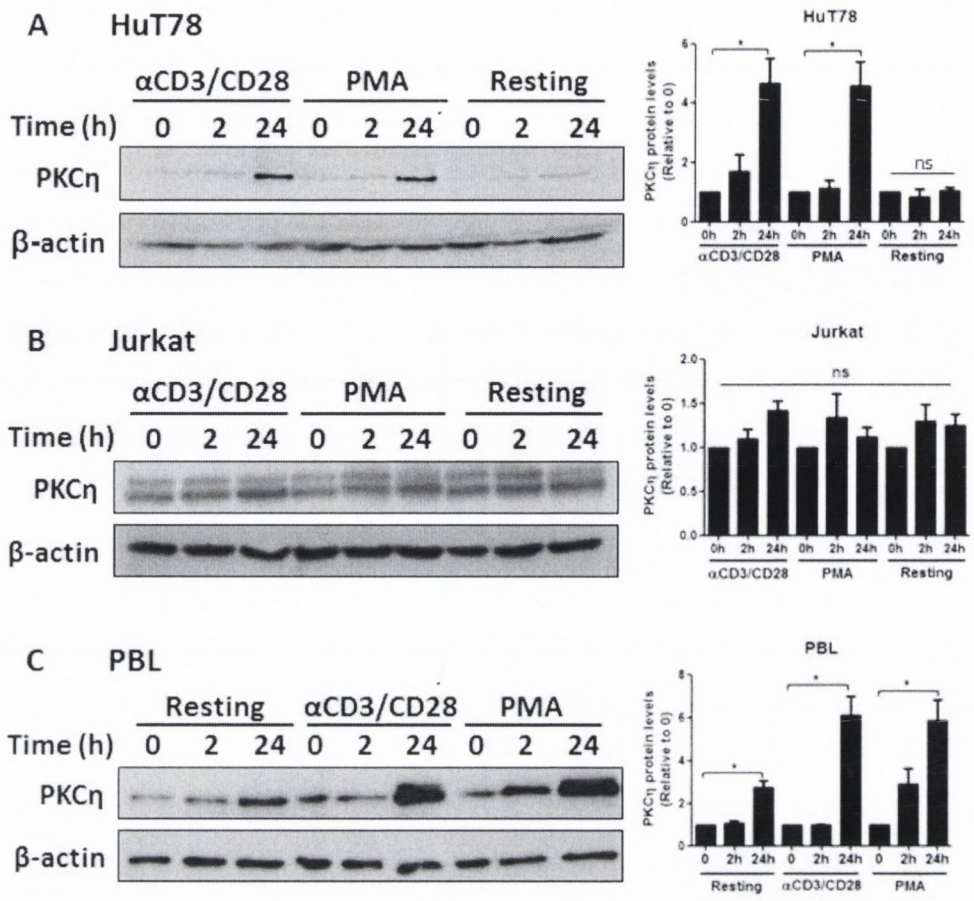


Figure 3-1 Regulation of PKCη protein expression upon T cell activation in different T cell types. Western blot analysis (left panel) and densitometric analysis (right panel) of PKCη protein levels at 0, 2 and 24 hours in resting and anti-CD3/CD28 or PMA activated T cells. Equal amounts of protein (considering 20 μg for HuT78, 10 μg for Jurkat and 5 μg for PBL) were loaded per lane and protein levels of β-actin were used as loading control. Western blots are one representative and graphs show mean ± S.E.M. of at least three individual experiments. *$p<0.05$ using Student's t test as samples significantly differ from 0h time point. A) HuT78 T cells; B) Jurkat T cells; C) PBL.

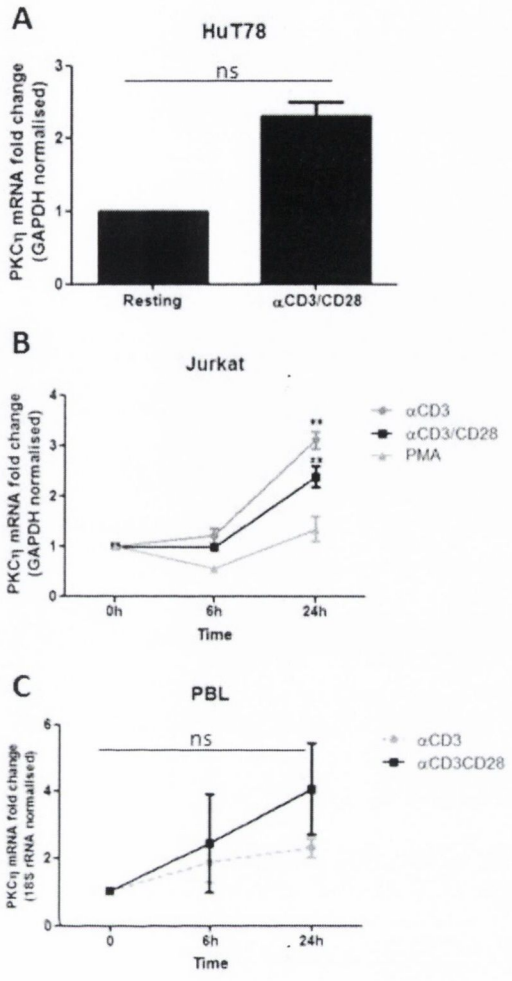


Figure 3-2 Regulation of PKCη expression at the mRNA level in T cells upon cell activation. A) Analysis of PKCη mRNA levels in the HuT78 T cell line at 24 hours in resting and activated cells with anti-CD3/CD28 antibodies. Graph shows mean ± S.E.M. of two independent experiments. B) Analysis of PKCη mRNA levels in the Jurkat T cell line at 0h (before activation) and at 6 and 24 hours following cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA. Graph shows mean ± S.E.M. of three independent experiments. C) Analysis of PKCη mRNA levels in PBLs at 0h (before activation) and at 6 and 24 hours upon cell activation with anti-CD3 antibody alone or a mix of anti-CD3/CD28 antibodies. Graph shows mean ± S.E.M. of three independent experiments. $^{**}p<0.01$ using Student's t test as samples significantly differ from 0h time point.

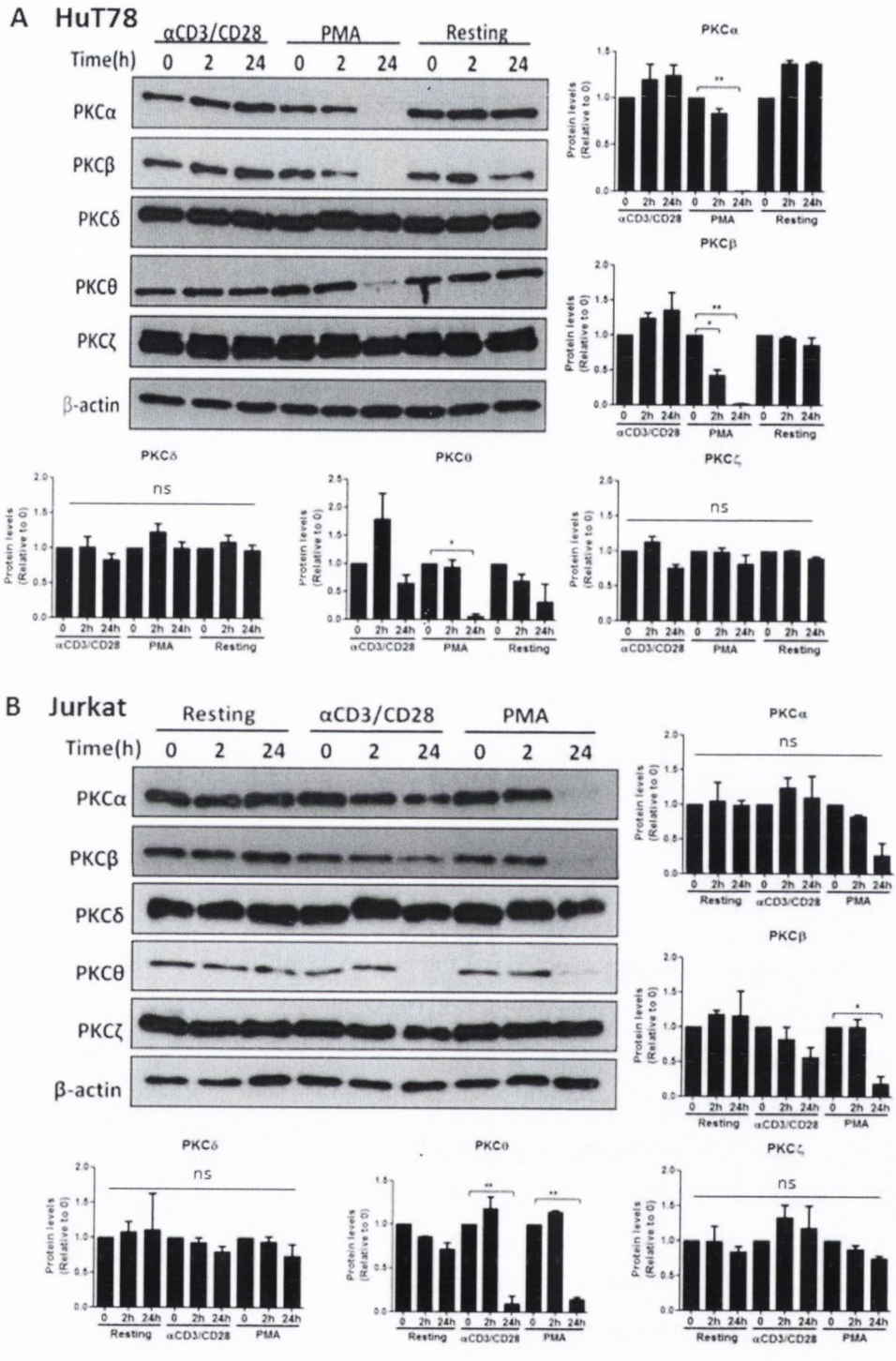


Figure 3-3 Expression of PKC isoforms in HuT78 and Jurkat T cells upon activation. Western blot analysis of protein levels of different PKC isoforms and densitometric analysis (bar charts) in activated and resting T cells. Cells were left unstimulated (resting) or activated with anti-CD3/CD28 or PMA and protein levels of conventional (PKCα, β), novel (PKCθ, δ) and atypical (PKCζ) PKC isoforms were analysed at 0, 2 and 24 hours. Equal amounts of cell lysates were loaded per lane (20 μg for HuT78 and 10 μg for Jurkat) and β-actin protein levels were used as loading control. A) Analysis as described for HuT78 T cells. PKCη protein levels of the same

samples can be found in Figure 3-1.A. B) Analysis as described for Jurkat T cells. PKCη protein levels of the same samples can be found in Figure 3-1.B. Western blots are one representative and graphs show mean ± S.E.M. of at least two independent experiments. *$p<0.05$ and **$p<0.01$ using Student's t test as samples significantly differ from 0h time point.

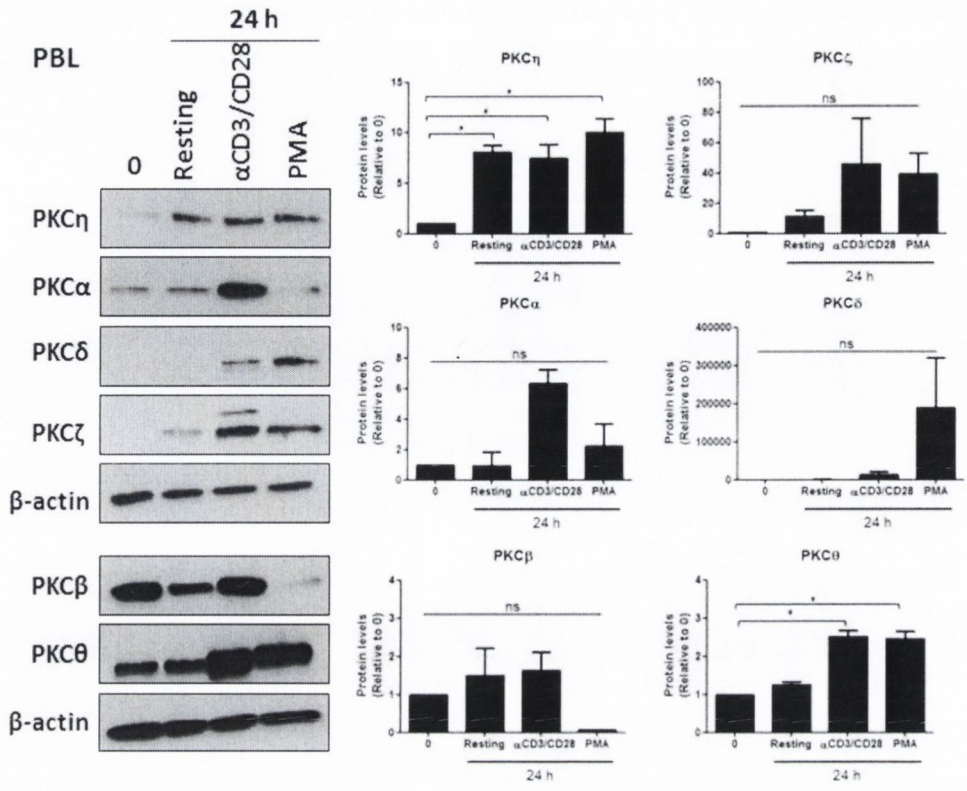


Figure 3-4 Expression of PKC isoforms in PBL upon activation. Western blot analysis of protein levels of different PKC isoforms and their correspondent densitometric analysis (bar charts) in activated and resting PBL. Cells were left unstimulated (resting) or activated with anti-CD3/CD28 or PMA for 24 hours and protein levels of conventional (PKCα, β), novel (PKCη,θ, δ) and atypical (PKCζ) PKC isoforms were analysed. Equal amounts of cell lysates were loaded per lane (5 μg for PKCα, η, δ and ζ and 15 μg for PKCβ and PKCθ) and β-actin protein levels were used as loading control. Western blots are one representative and graphs show mean ± S.E.M. of three experiments with different donors. *p<0.05 using Student's t test as samples significantly differ from 0h time point.

3.3.2 Signalling pathways involved in regulation of PKCη expression following T cell activation.

Interaction of the TCR with an antigen-MHC on the surface of an antigen presenting cell initiates a complex signalling cascade that rapidly results in activation of several pathways including the Phosphoinositide-3 kinase (PI3K), the Protein kinase C (PKC) and the Mitogen-activated protein kinase (MAPK)/ERK pathways.

Although distinct responses were observed across the different T cell lines and primary cells studied, expression of PKCη at both protein and mRNA levels changed following T cell activation and therefore it is important to investigate which pathways are involved in regulation of PKCη expression in both resting and activated cells.

3.3.2.1 The PI3K pathway is involved in regulation of PKCη protein expression in activated T cells

As mentioned above, the Jurkat T cell line showed a distinct pattern with regard to PKCη expression in comparison with HuT78 and PBL. While Jurkat T cells presented high basal levels of PKCη which did not change significantly in response to cell activation, HuT78 and PBL showed low basal levels of PKCη that increased significantly following cell activation (Figure 3-1). An important characteristic of the Jurkat T cell line is that it exhibits a constitutively phosphorylated Protein Kinase B (PKB), also known as Akt, which is activated downstream of the PI3K pathway. As a consequence, Jurkat T cells have an abnormal PI3K pathway response. This is due to a mutation in the gene encoding for the phosphatase and tensin homolog (PTEN), which results in lack of expression of PTEN by Jurkat T cells (Astoul, Edmunds et al. 2001). PTEN antagonises the activity of PI3K by dephosphorylating phosphoinositide substrates and therefore inhibits downstream signalling through PKB/Akt. For this reason the role of the PI3K pathway in the regulation of PKCη expression following T cell activation was investigated.

Also included in this investigation was another important kinase related to PI3K, the mammalian target of rapamycin (mTOR) which is a target of Akt and has been involved in regulating T cell activation, differentiation and function (Gamper and Powell 2012).

To investigate the involvement of the PI3K pathway in the regulation of PKCη expression, both T cell lines HuT78 and Jurkat as well as PBL were treated with PI3K inhibitors (Wortmannin and LY294002) or the mTor inhibitor rapamycin prior to stimulation with anti-CD3/CD28 or PMA and the protein levels of PKCη were analysed by western blotting in these cells (Figures 3-5 and 3-6). In addition, levels of phosphorylated Akt (pAkt) were verified in these samples in order to test the efficiency of the treatment with the PI3K inhibitors. Interestingly, pAkt was not detected in any of the time points tested in either HuT78 or PBL (data not shown). It has been described that resting primary T cells have very low or undetectable levels of pAkt, which rapidly increase and peak within 5 minutes of TCR stimulation (Lafont, Astoul et al. 2000; Astoul, Edmunds et al. 2001). Hence, the time points chosen (0, 2h and 24h) to investigate levels of PKCη in this experiment might not be optimal for detection of pAkt in HuT78 and PBL. Pre-treatment with LY294002 blocked the up-regulation of PKCη in HuT78 activated with anti-CD3/CD28 but not with PMA at 24 hours. In contrast, pre-treatment with wortmannin or rapamycin did not have any significant effect in PKCη regulation in Hut78 (Figure 3-5.A). In Jurkat T cells, the levels of PKCη were decreased at 24 hours following anti-CD3/CD28 stimulation in cells pre-treated with LY294002 or rapamycin (Figure 3-5.B). However, the lower levels of PKCη observed in these cases did not correlate with inhibition of Akt phosphorylation. At 24 hours following anti-CD3/CD28 the level of pAkt was comparable to that found in control cells. In PBL, none of the inhibitors used caused a significant effect on regulation of PKCη expression (Figure 3-6). The densitometric analysis of the western blots suggested a partial inhibition of PKCη up-regulation in response to anti-CD3/CD28 activation in PBL pre-treated with LY294002 or wortmannin while pre-treatment with rapamycin caused no effect whatsoever.

Taken together, the results using the LY294002 PI3K inhibitor suggest that the PI3K pathway is involved in regulation of expression of PKCη in the context of T cell activation in the T cell lines HuT78 and Jurkat. This relationship is not so clear for PBL,

suggesting that the PI3K pathway is only partially involved in regulation of PKCη expression following cell activation.

Since the effect of the PI3K inhibitor LY294002 was consistent in down-regulating PKCη protein levels when used prior to anti-CD3/CD28 stimulation of HuT78 and Jurkat T cells, the effect of this inhibitor on PKCη gene expression was also assessed in these T cell lines by real time PCR (Figure 3-7). Gene expression of PKCη was increased at 24 hours upon cell activation with anti-CD3/CD28 in both cell lines as described before. Interestingly, treatment with LY294002 suppressed the activation-mediated increase in PKCη mRNA in HuT78 T cells (Figure 3-7.A) but not in Jurkat T cells (Figure 3-7.B). These results suggest distinct mechanisms of regulation of PKCη expression in HuT78 and Jurkat T cells. Pre-treatment of HuT78 with LY294002 inhibits the activation-induced up-regulation of PKCη expression at mRNA levels, while the PKCη protein down-regulation observed in activated Jurkat T cells following treatment with this inhibitor is a result of post-translational mechanisms.

Given the hypothesis that in Jurkat T cells the high basal levels of PKCη could be associated with the known constitutive activity of PKB/Akt, it was decided to further investigate the involvement of the PI3K pathway in regulation of PKCη expression in this T cell line using Jurkat PTEN Tet-On clones (Figure 3-8). In the Tet-On technology, the expression of the gene of interest (in this case PTEN) can be induced in these cells by addition of Doxycycline (Dox) to the cell culture (Gossen and Bujard 1992). Induction of PTEN expression in Jurkat Tet-On inhibits the constitutive activation of PKB/Akt and therefore permits the investigation of this inhibition in the regulation of PKCη expression in these cells. In this study, three clones of the PTEN-inducible Tet-On Jurkat were used; clones number 12 and 17 were PTEN-inducible while clone number 18 was a non-PTEN-expressing control (Seminario, Precht et al. 2003).

Addition of Dox to the cell culture resulted of induction of PTEN in clones 12 and 17 but not in clone 18 (Figure 3-8.A). In the absence of Dox, all clones showed a lower constitutive expression of PKCη which was inducible by stimulation with anti-CD3/anti-CD28 or PMA for 24h. Following treatment with Dox and stimulation with anti-CD3/anti-CD28 or PMA for 24h, we observed the induced expression of PTEN in clones 12 and 17 as expected. In addition, the levels of PKCη detected in those cells were very

low or non-detectable. However, the control clone 18 also showed undetectable levels of PKCη even in the absence of PTEN, suggesting that Dox had an effect in the regulation of PKCη expression (Figure 3-8.B). For this reason, it was not possible to make a conclusion about the role of the PI3K pathway in TCR-induced PKCη expression using this model.

Taken together, these results suggest that the PI3K pathway is at least partially involved in the regulation of PKCη expression in the context of T cell activation. This fact was more evident in T cell lines; in which pre-treatment with LY294002 blocked the activation induced up-regulation of PKCη in HuT78 at both protein and mRNA levels. This same inhibitor did not affect PKCη mRNA levels in Jurkat T cells, but it caused considerable down-regulation of PKCη protein levels in activated cells. It is important to note that in the experiments with HuT78 and PBL, it was not possible to detect levels of phosphorylated Akt at the time points investigated. Also, the phosphorylation levels of Akt observed in Jurkat cells were comparable between control and LY294002 treated cells at the time point in which down-regulation of PKCη was observed. As a result, it was not possible to show a direct association between Akt activity and down-regulation of PKCη. However, as Akt is transiently activated, it may be regulating PKCη expression in a different timing. Also, the fact that treatment with rapamycin (which inhibits a target downstream of Akt) resulted in down-regulation of PKCη protein levels in Jurkat indicates a possible involvement of other elements of the PI3K pathway in the regulation of PKCη expression in activated T cells.

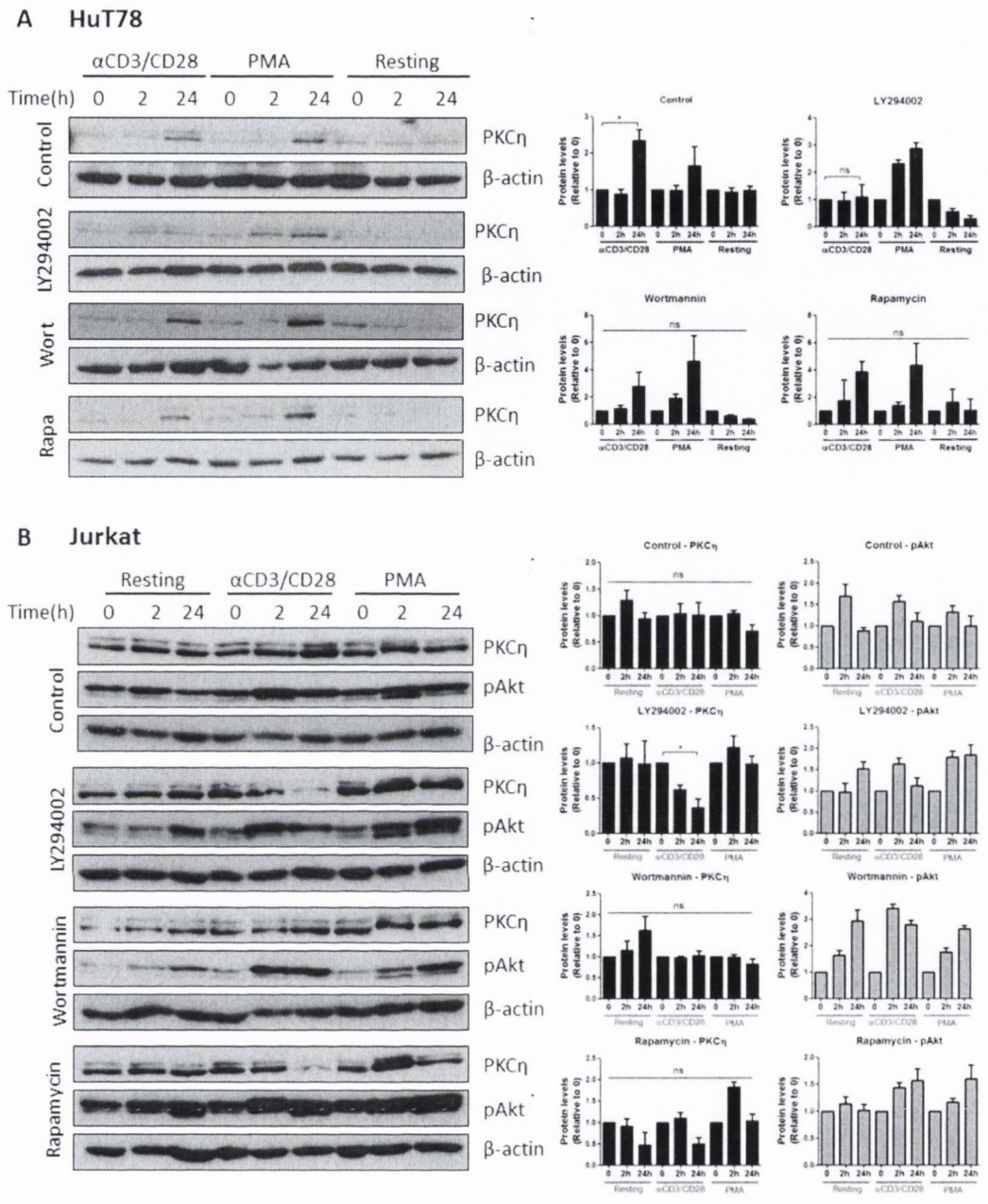


Figure 3-5 Role of the PI3K pathway in regulation of PKCη expression in HuT78 and Jurkat T cell lines. Western blot analysis and corresponding densitometric analysis of PKCη protein levels in A) HuT78 and B) Jurkat pre-treated with the PI3K inhibitors LY294002 (10 μM) and Wortmannin (100 nM) or the mTOR inhibitor Rapamycin (100nM). Control cells were pre-treated with DMSO. Cells were left unstimulated (resting) or activated with anti-CD3/CD28 antibodies or PMA. Protein extracts were analysed at 0, 2 and 24 hours. Equal amounts of cell lysate (20 μg for HuT78 and 10 μg for Jurkat cells) were loaded per lane and β-actin protein levels were used as loading control. Western blots are one representative and graphs show mean ± S.E.M. of at least three independent experiments. *p<0.05 using Student's t test as samples significantly differ from 0h time point.

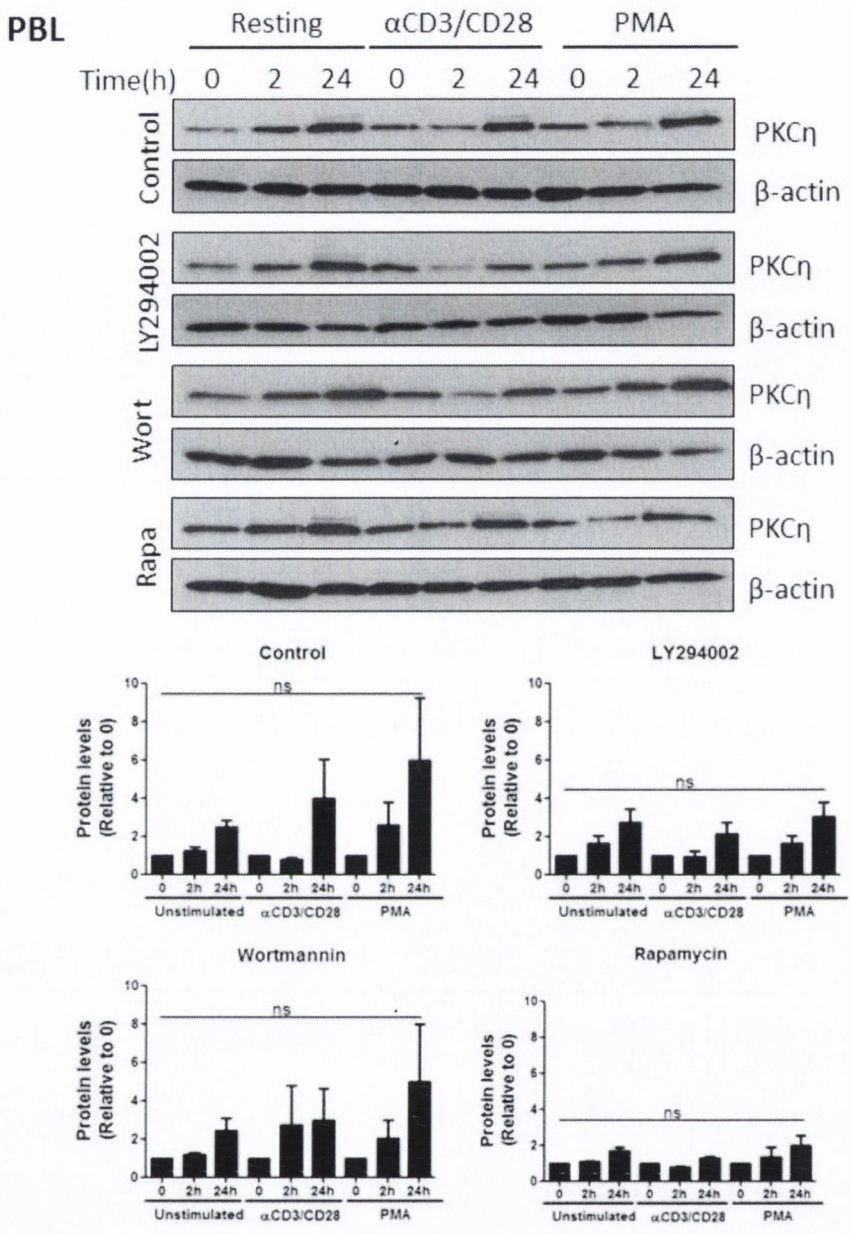


Figure 3-6 Role of the PI3K pathway in regulation of PKCη expression in PBL. Western blot analysis and corresponding densitometric analysis of PKCη protein levels in PBL pre-treated with the PI3K inhibitors LY294002 (10 μM) and Wortmannin (100 nM) or the mTOR inhibitor Rapamycin (100nM). Control cells were pre-treated with DMSO. Cells were left unstimulated (resting) or activated with anti-CD3/CD28 antibodies or PMA. Protein extracts were analysed at 0, 2 and 24 hours. Equal amounts of cell lysate (5 μg) were loaded per lane and β-actin protein levels were used as loading control. Western blots are one representative and graphs show mean ± S.E.M. of three experiments with different donors. Ns= not significant using Student's t test as samples do not differ significantly from 0h time point.

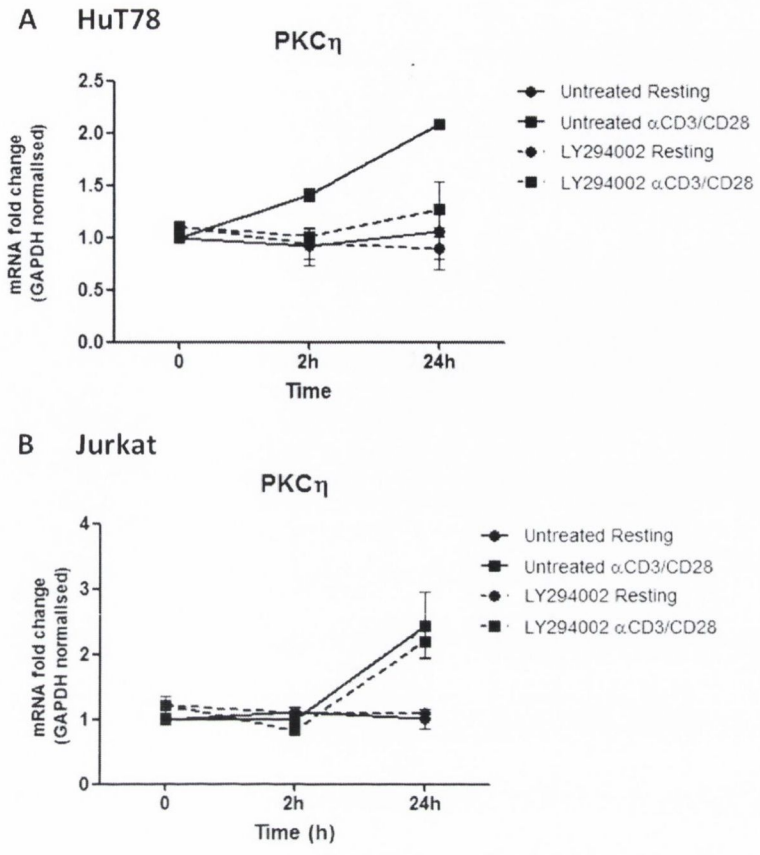


Figure 3-7 Role of the PI3K inhibitor LY294002 in regulation of PKCη mRNA expression in HuT78 and Jurkat T cells. Analysis of PKCη mRNA levels in A) HuT78 and B) Jurkat T cells treated with LY294002 prior to anti-CD3/CD28 activation and in resting cells. HuT78 and Jurkat T cells were pre-treated or not for 30 minutes with 10 μM of LY294002. Cells were then left unstimulated (resting) or activated with anti-CD3/CD28 antibodies and PKCη mRNA were analysed by real time PCR at 0, 2 and 24 hours. Graph shows mean ± S.E.M. of two independent experiments. Samples do not differ significantly from 0 time point using Student's t test.

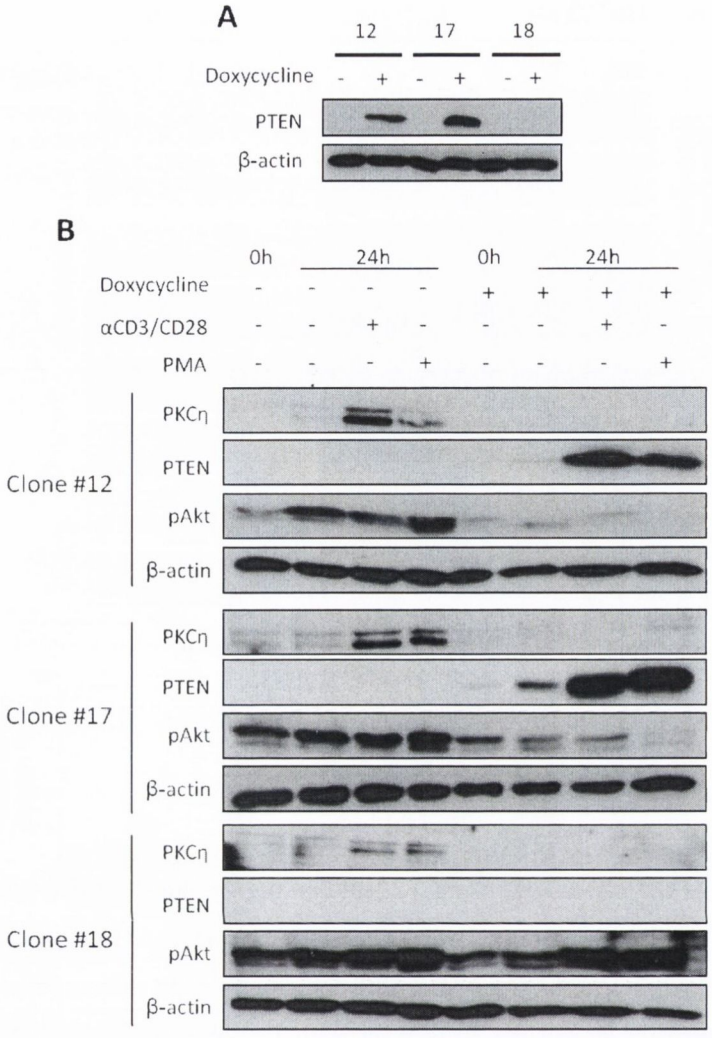


Figure 3-8 PTEN induction in Jurkat Tet-On clones and regulation of PKCη protein expression. A) Western blot analysis of PTEN expression following treatment of PTEN-inducible Tet-On Jurkat clones with 1μg/ml of Doxycycline for 48 hours. B) Western blot analysis for protein levels of PKCη, PTEN and phosphorylated Akt (pAkt) in cells pre-treated or not with Doxycycline followed by cell activation for 24 hours with anti-CD3/CD28 antibodies or PMA. β-actin protein levels were used as loading control. Data is one representative of four independent experiments

3.3.2.2 Investigation of the involvement of the Mitogen-activated protein kinase (MAPK) pathway in regulation of PKCη expression.

The MAPK family of kinases, including the extracellular signal-regulated kinase (ERK), is involved in the transduction of signals downstream T cell receptor (Smith-Garvin, Koretzky et al. 2009; Brownlie and Zamoyska 2013).

The PKCη isoform has been associated with the MAPK/ERK pathway in several reports. In glioblastoma cells, ERK is described to be a downstream target of PKCη, which induces cell proliferation through this pathway (Uht, Amos et al. 2007). Moreover, expression of PKCη in NIH-3T3 cells resulted in IL-6 production in a process mediated by the MAPK/ERK pathway (Fima, Shahaf et al. 1999). Also, ERK activity is described to be essential for IL-2 production in a late phase of T cell activation. It has been shown that inhibition of the ERK pathway 2-6 hours following TCR stimulation significantly reduced the production of IL-2 by activated cells, while the same treatment during the first 2 hours had no effect (Koike, Yamagishi et al. 2003). Based on these observations, it was decided to investigate the involvement of the MAPK/ERK pathway in regulation of PKCη expression in the context of T cell activation taking into consideration both early and late stages of cell activation.

In order to determine if the MAPK/ERK pathway is involved in the regulation of PKCη expression, the T cell lines HuT78 and Jurkat T cells as well as PBL were treated with the ERK inhibitor PD98059 for 30 minutes prior stimulation and also at 2 hours following stimulation with anti-CD3/anti-CD28. Expression of PKCη was then determined by western blotting in the T cell lines (Figure 3-9) and PBL (Figure 3-10).

Western blot analysis showed that in normal conditions (control cells), cell activation with anti-CD3/CD28 antibodies induced levels of phorphorylated ERK (pERK) although in a different pattern and extent across the all cell types analysed. An increase in ERK phosphorylation was observed in all the cell types in resting cells at 24 hours. This induction could be explained for the presence of growth factors in the culture medium, which have been also described to activate ERK (Rubinfeld and Seger 2005; Raman, Chen et al. 2007). Moreover, treatment with PD98059 efficiently blocked the

phosphorylation of ERK and consequently inhibited the activation of the signalling pathway in HuT78, Jurkat and PBL. However, no effect was observed in the regulation of PKCη protein expression in any of the cell types investigated. This was the case in either cells pre-treated or treated at 2 hours following anti-CD3/CD28 with PD98059. These observations therefore excluded the involvement of the MAPK/ERK pathway in the regulation of PKCη following T cell activation at both early and late stages.

As described above, the MAPK/ERK pathway is not involved in regulation of PKCη expression following T cell activation. However, the contrary relationship between these two factors has also been described, that is, in glioblastoma cells, ERK is a downstream target of PKCη (Uht, Amos et al. 2007). For this reason, it was decided to investigate if silencing of the PKCη gene by nucleofection with small interfering RNA (siRNA) would affect activation of the MAPK/ERK pathway following T cell activation. Hence, HuT78, Jurkat and PBL were transfected with a PKCη siRNA or a control siRNA prior to activation with anti-CD3/CD28 antibodies and the levels of PKCη and phosphorylated ERK were analysed by Western blotting (Figure 3-11). The analysis showed that knockdown of the PKCη gene did not affect the phosphorylation levels of ERK following activation in HuT78, Jurkat cells or PBL, indicating that activation of the MAPK/ERK pathway is not regulated by PKCη in this context.

Altogether, these findings suggest that PKCη is not related to MAPK/ERK signalling in response to T cell activation.

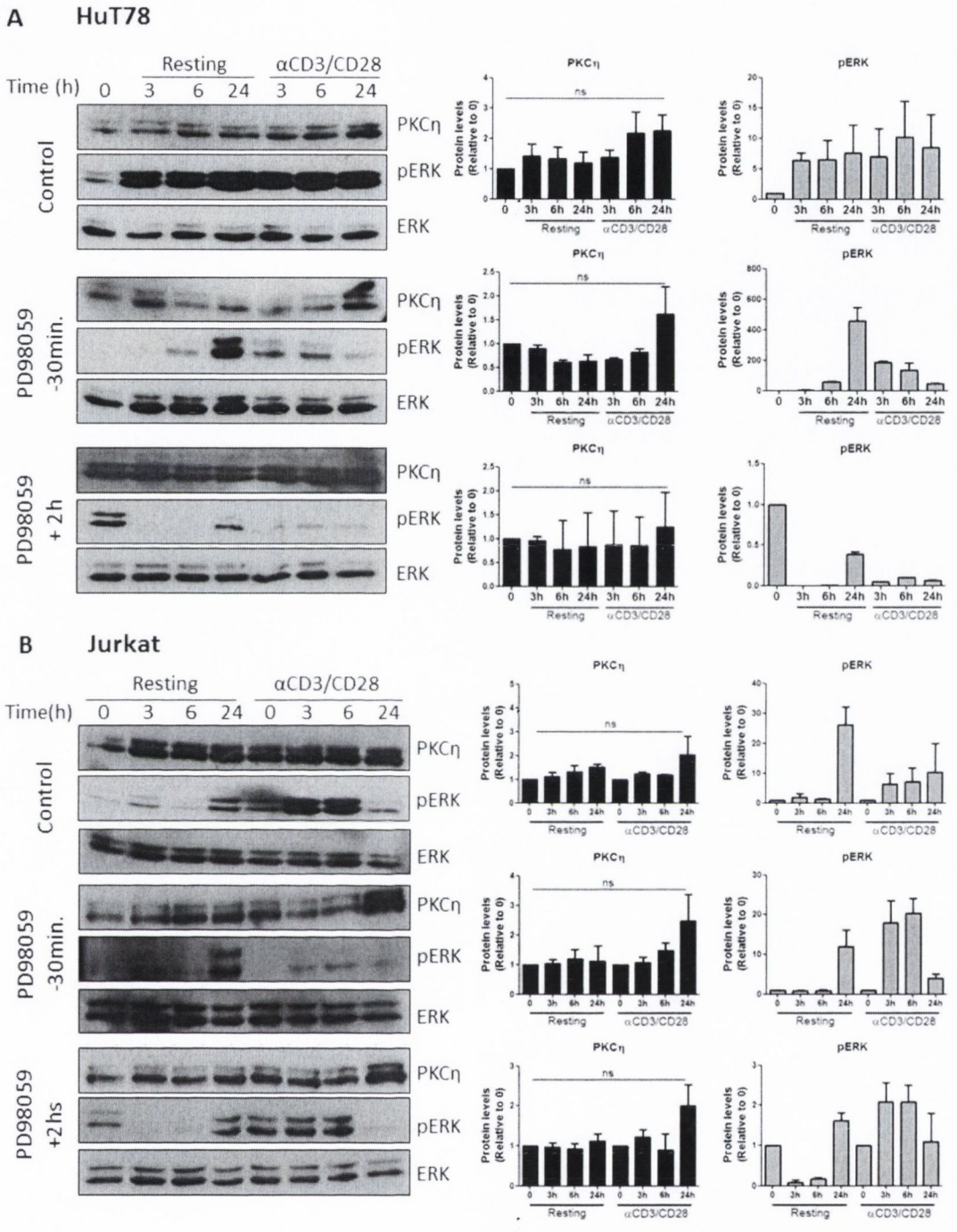


Figure 3-9 The MAPK pathway does not regulate PKCη protein expression in HuT78 or Jurkat T cells in response to cell activation. Western blot analysis and densitometric analysis of PKCη protein levels upon treatment of cells with 25 μM of the Erk inhibitor PD98059 at 30 minutes prior cell activation with anti-CD3/CD28 antibodies or at 2 hours upon cell activation. Protein extracts were prepared at 0, 3, 6 and 24 hours for each treatment method and for control cells treated with DMSO. Levels of phosphorylated Erk were also assessed to assure efficiency of the inhibitor and total protein levels of Erk were used as loading control. A) Immunoblots for HuT78 T cell samples. 20 μg of total protein lysates were loaded per lane. Data is one

representative of two independent experiments. B) Immunoblots for Jurkat T cell samples. 10 μg of total protein lysates were loaded per lane. Western blots are one representative and graphs show mean ± S.E.M. of three independent experiments. Ns= not significant using Student's t test as samples do not differ significantly from 0h time point.

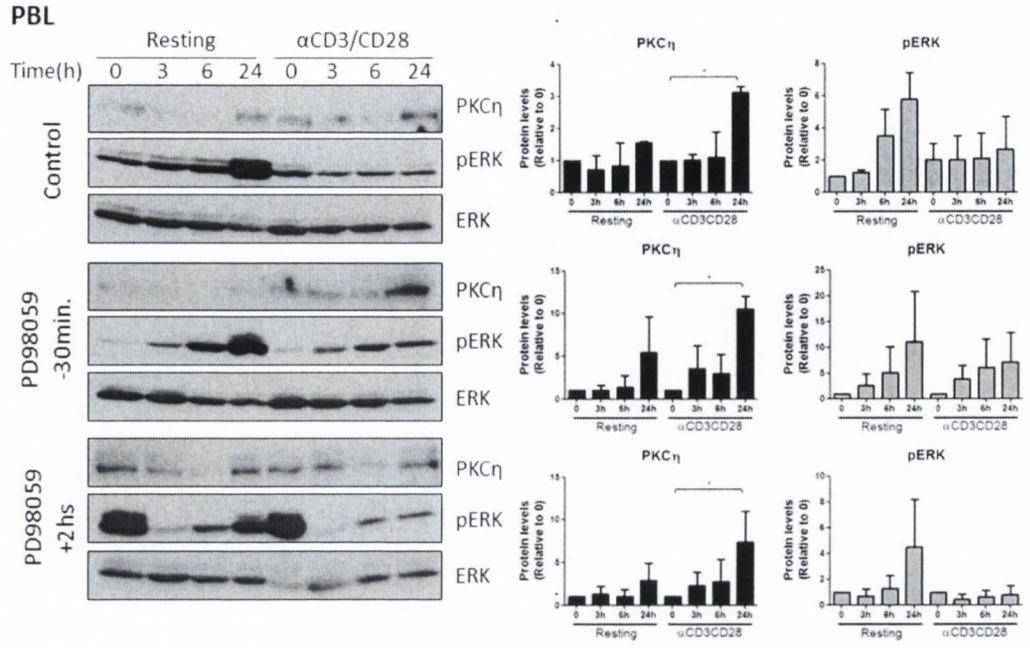


Figure 3-10 The MAPK pathway does not regulate PKCη protein expression in peripheral blood lymphocytes (PBL) in response to cell activation. Western blot analysis and densitometric analysis of PKCη protein levels upon treatment of PBL with 25 μM of the Erk inhibitor PD98059 at 30 minutes prior cell activation with anti-CD3/CD28 antibodies or at 2 hours upon cell activation. Protein extracts were prepared at 0, 3, 6 and 24 hours for each treatment method and for control cells treated with DMSO. Levels of phosphorylated Erk were also assessed to assure efficiency of the inhibitor and total protein levels of Erk were used as loading control. 5 μg of total protein lysates were loaded per lane. Western blots are one representative and graphs show mean ± S.E.M. of three experiments with different donors. $*p<0.05$ using Student's t test as sample significantly differ from 0h time point.

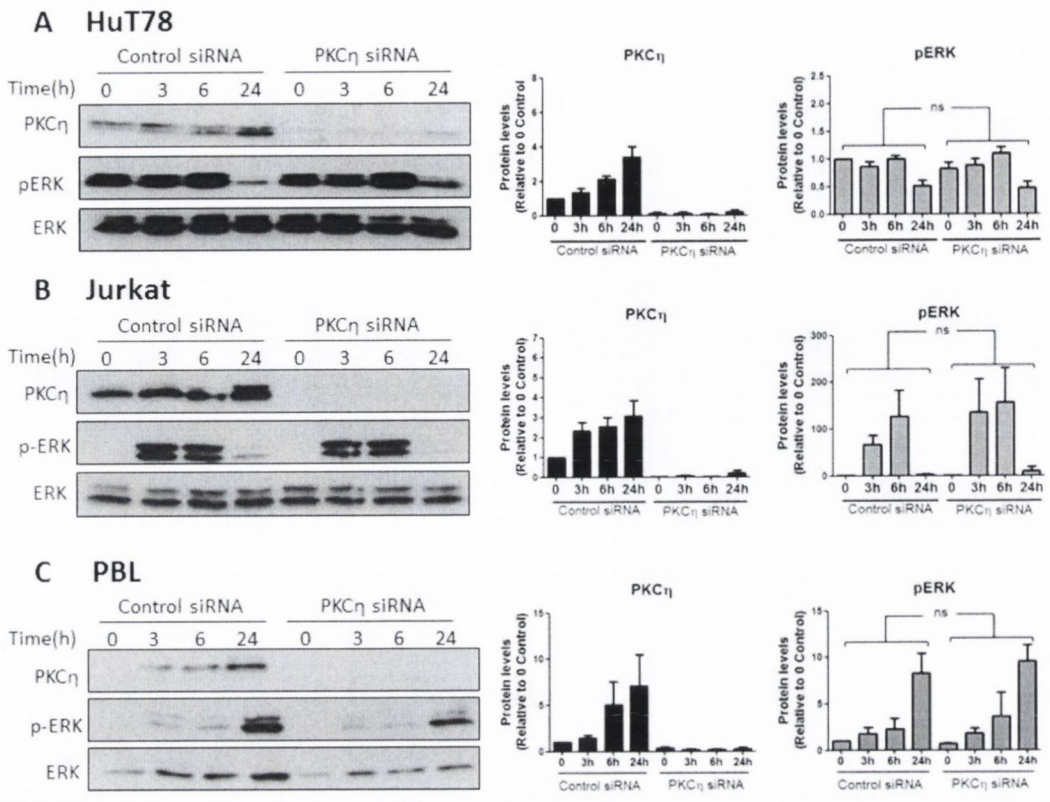


Figure 3-11 PKCη does not regulate the MAPK pathway in the context T cell activation. Western blot analysis of the levels of phosphorylated ERK (pERK) in response to T cell activation following silencing of the PKCη gene. A) HuT78, B) Jurkat T cell and C) PBL were transfected with either control or PKCη siRNA and then activated with anti-CD3/CD28 antibodies for the given time points. Equal numbers of cells (350,000) was lysed and protein extracts were resolved by SDS-PAGE. Levels of total ERK were used as loading control. Antibody dilutions used were PKCη (1:500), pERK and ERK (1:1000). Western blots are one representative and graphs show mean ± S.E.M. of three independent experiments. Ns= not significant using Student's t test as samples transfected with Control siRNA do not differ significantly from samples transfected with PKCη siRNA at the same time points.

3.3.2.3 Basal and activation-induced expression of PKCη requires PKC activity in T cells.

Among other very important intracellular events, stimulation of the T cell receptor results in influx of Ca^{2+} from outside the cell and production of diacylglycerol (DAG), two factors that are essential in activation of signalling pathways involving PKC members (Smith-Garvin, Koretzky et al. 2009). For this reason, together with the finding that expression of PKCη was increased in response to PMA stimulation, it was decided to investigate whether other PKC isoforms were involved in regulation of PKCη expression following activation of T cells. Hence, the T cell lines HuT78 and Jurkat and also PBL were treated with the PKC inhibitors Bisindolymaleimide (BIS) I, Gö6976 and LY333531 prior to cell activation with anti-CD3 antibody alone, anti-CD3/anti-CD28 or PMA for 24 hours and the levels of PKCη were analysed by Western blotting (Figure 3-12). BIS I is described to be a broad spectrum inhibitor of PKC isoforms while Gö6976 inhibits the Ca^{2+}-dependent isoforms (PKCα and PKCβ) and LY333531 has greater specificity for PKCβ.

Interestingly, pre-treatment with all PKC inhibitors had a significant effect in PKCη expression in all T cell types studied. In HuT78, PKCη protein levels were dramatically down-regulated at 24 hours following treatment with all the PKC inhibitors in resting and also following activation with anti-CD3 antibody alone, anti-CD3/CD28 or PMA (Figure 3-12.A). Treatment with Gö6976 was less effective in Jurkat T cells and protein levels of PKCη were only partially down-regulated at 24 hours in cells activated with anti-CD3/anti-CD28 or PMA when compared to samples treated with BIS I and LY333531 (Figure 3-12.B). In PBL, pre-treatment with BIS I and LY333531 also resulted in down-regulation of PKCη protein levels in both resting and activated cells. On the other hand, pre-treatment with Gö6976 did not show a significant effect on regulation of PKCη expression in these cells (Figure 3-12.C). To note, the down-regulation in the PKCη protein levels observed following treatment with the PKC inhibitors in the T cell lines that had been activated with PMA was less pronounced than the down-regulation of PKCη observed in HuT78 and Jurkat activated with anti-CD3/CD28 antibodies. However, this fact was not observed in PBL, in which pre-treatment with BIS I and

LY333531 inhibited the up-regulation of PKCη induced by both PMA and anti-CD3/CD28 antibodies.

These results suggested that basal and activation-induced expression of PKCη is dependent on PKC activity and maybe regulated *in-cis* or by other PKC isoforms in T cells.

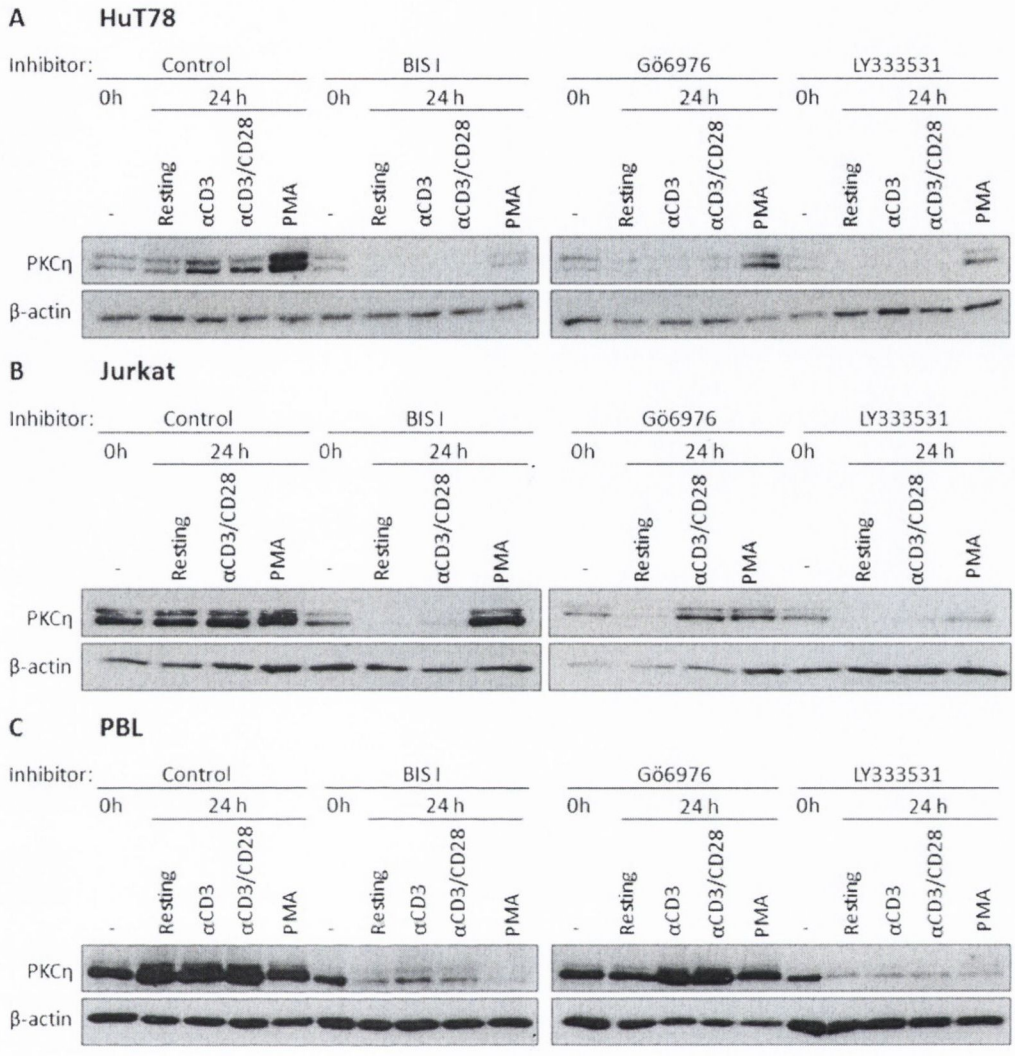


Figure 3-12 Basal expression of PKCη is dependent on PKC activity in T cells. Western blot analysis of PKCη protein levels in resting and activated A) HuT78, B) Jurkat and C) PBL pre-treated with PKC inhibitors. The cells were treated for 30 minutes with 10 μM Bisindolylmaleimide (Bis) I, 5 μM Gö6976 or 5 μM LY333531 prior to cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA. Control cells were pre-treated with DMSO. Protein extracts were prepared at the beginning of the experiment and at 24 hours upon cell activation. All western blots are one representative of three independent experiments.

3.3.2.4 PKCη and the Endoplasmatic reticulum (ER) stress response.

The ER stress response is a cellular mechanism initiated in response to an accumulation of unfolded proteins in the ER lumen in order to enhance its capacity of protein production. Two main events that characterize this process are the up-regulation of ER chaperone proteins such as Glucose-regulated protein-78 (GRP78) and GRP94, responsible for improving the protein folding capacity of the ER and the phosphorylation of eukaryotic translation initiation factor 2 α (eIF2α), which reduces protein translation (Chakrabarti, Chen et al. 2011). It has been described that T cells undergo an ER stress response upon TCR activation and that the PKC family is involved in this process (Pino, O'Sullivan-Murphy et al. 2008).

Studies using the MCF-7 human breast adenocarcinoma cell line showed that the expression of PKCη is regulated during normal cell growth and under cell stress. The gene for PKCη is suppressed under normal growth conditions but this suppression is relieved and PKCη is induced following amino acid starvation (Raveh-Amit, Maissel et al. 2009). It is possible, therefore, that the up-regulation of PKCη following T cell activation is associated with the TCR-induced ER stress response and consequently in regulation of protein production.

To address this hypothesis, it was first investigated if activation of T cells with anti-CD3/anti-CD28 or PMA also led to activation of the ER stress response in these cells by analysing the phosphorylation status of eIF2α and protein levels of GRP94 following cellular activation. Western blot analysis for HuT78 (Figure 3-13.A), Jurkat (Figure 3-13.B) and PBL (Figure 3-13.C) activated with anti-CD3/anti-CD28 for 24 hours did not show induction of phosphorylation of eIF2α in any of these cell types studied. Interestingly, the levels of phosphorylated eIF2α (p-eIF2α) observed in resting HuT78 and Jurkat T cells at 24 hours were slightly increased in comparison to the 0h time point while the opposite was observed in PBL. The densitometric analysis for the GRP94 protein levels suggested that they were slightly increased upon activation with anti-CD3/anti-CD28 in HuT78 and Jurkat T cells but not in PBL. These results imply that none of these cell types studied regulated TCR-induced protein production through

activation of the ER stress response and therefore the up-regulation of PKCη observed in these cells is not associated with this process.

Culture of cells in an amino acid free medium has been reported to induce cellular stress and consequent increase in phosphorylation of eIF2α due to nutrient deprivation (Holcik and Sonenberg 2005). Amino acid starved MCF-7 cells showed increased levels of PKCη in parallel to an increase in phosphorylation of eIF2α (Raveh-Amit, Maissel et al. 2009).

Expression of PKCη in amino acid starved cells was then analysed in HuT78, Jurkat and PBL. For this purpose, the cells were cultured in Hank's balanced salt solution (HBSS), which is nutrient and amino acid free and analysed at time points indicated (Figure 3-14). An increase in phosphorylation of eIF2α confirmed that cells were undergoing cellular stress. However, contradicting what was expected, levels of PKCη were decreased at 24 hours in all cell types. Moreover, Jurkat cells did not tolerate amino acid starvation and most of the cells were dead after 24 hour of culture in HBSS, fact that was observed under microscopic analysis (data not shown) and confirmed by the dramatic decrease in β-actin levels showed in the western blot analysis.

These observations also suggest that the up-regulation of PKCη at 24 hours following activation is not necessarily associated with an increase in ER stress markers. In addition, further investigation is necessary to elucidate the mechanisms in which absence of amino acids down-regulates the levels of PKCη in these T cells.

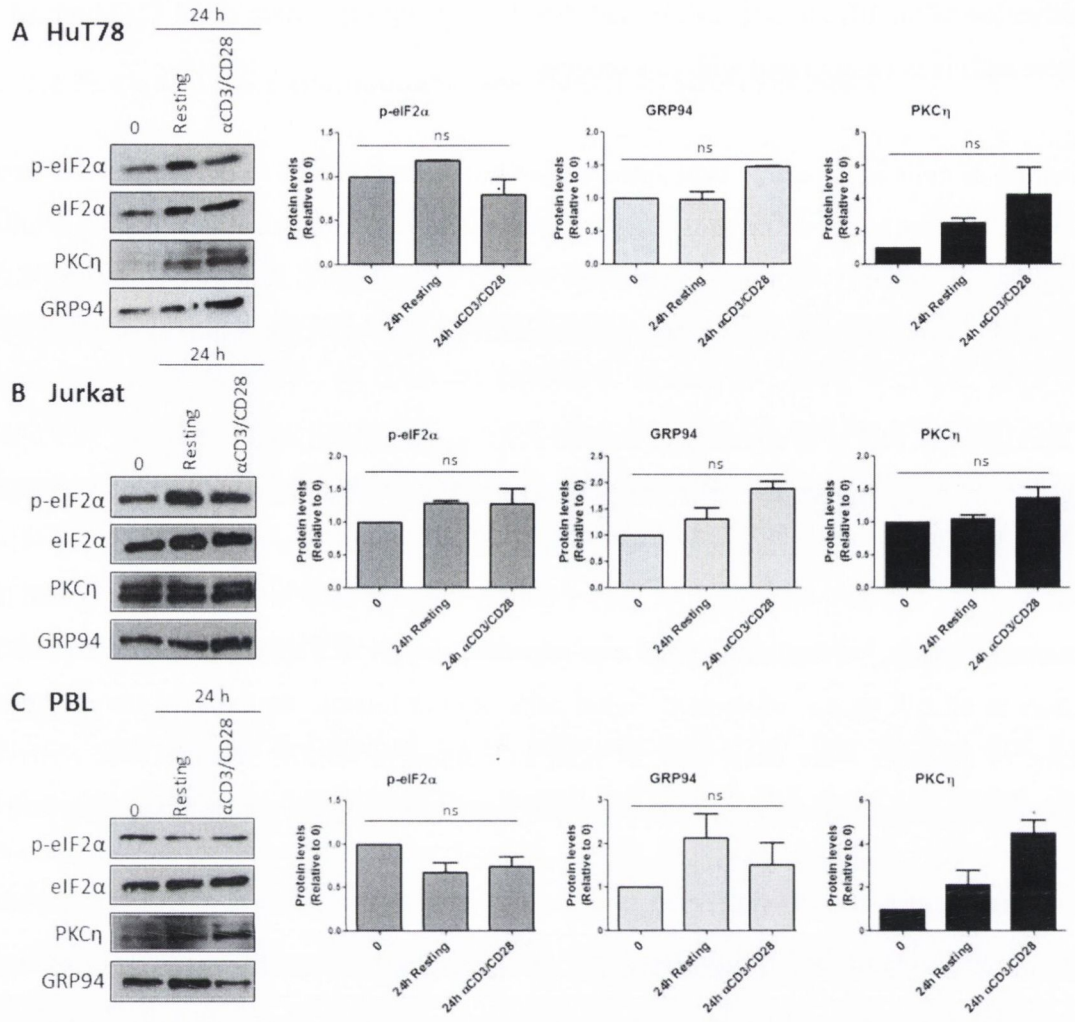


Figure 3-13 Relationship between ER stress response and PKCη expression. Western blot analysis and corresponding densitometric analysis for the ER stress markers phospho-eIF2α and GRP94 and protein levels of PKCη upon T cell activation. A) HuT78, B) Jurkat T cells and C) PBLs were left unstimulated (resting) or activated with anti-CD3/CD28 antibodies for 24 hours. Cell lysates were prepared and equal amounts of protein were loaded per lane (20 μg for HuT78, 10 μg for Jurkat T cells and 5 μg for PBLs). Total eIF2α protein levels were used as loading control. Western blots are one representative and graphs show mean ± S.E.M. of at least two independent experiments. *$p<0.05$ using Student's t test as sample significantly differ from 0h time point.

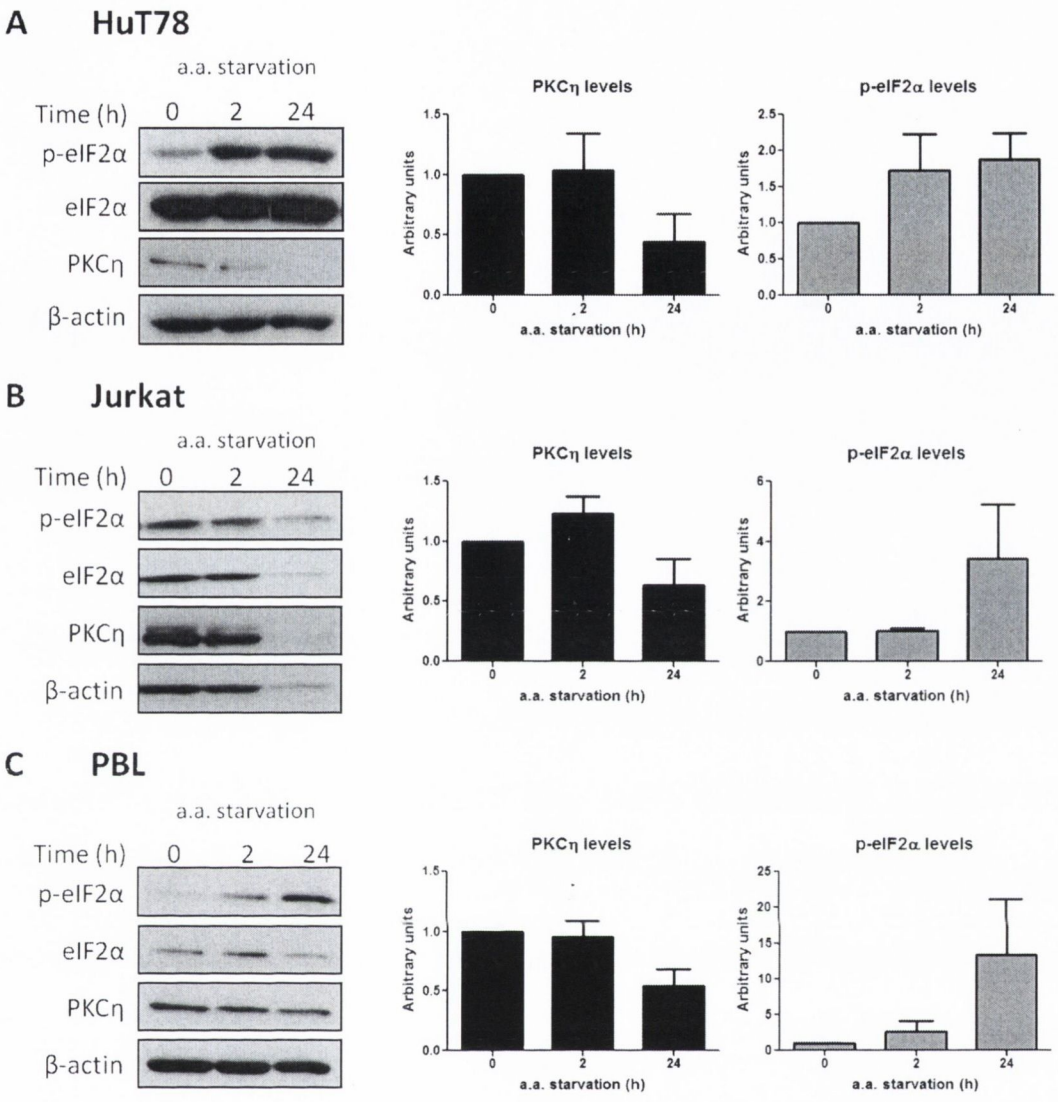


Figure 3-14 Amino acid starvation results in down-regulation of PKCη protein levels. Western blot analysis of protein levels of phosph-eIF2α and PKCη in A) HuT78, B) Jurkat and C) PBL upon amino acid starvation. The cells were amino acid starved by culture in Hank's Balanced Salt Solution (HBSS) for the indicated time points. Cells were then harvested and protein extracts were prepared. Equal amounts of cell lysates (20 μg for HuT78, 10 μg for Jurkat and 5 μg for PBL) were loaded per lane onto the gel and protein levels of PKCη and phospho-eIF2α were detected with specific antibodies. Levels of β-actin and eIF2α were used as loading control for PKCη and phospho- eIF2α respectively. Bar charts on the right show relative levels of the indicated proteins in relation to 0h time point. They represent mean values ± S.E.M (n=3) obtained in the densitometric analysis performed in the immunoblots obtained in the experiments for each cell type. Immunoblots are representative of three independent experiments.

3.4 Investigation of a role for PKCη in IL-2 production by activated T cells.

One of the key markers of T cell activation is the production of Interleukin-2 (IL-2) by the activated T cells. Production of IL-2 is essential for T cell growth, proliferation and differentiation (Malek 2008; Boyman and Sprent 2012). Also, IL-2 is the only cytokine to be produced in detectable quantities by all three HuT78, Jurkat T cells and PBL in response to cell activation (Bartelt, Cruz-Orcutt et al. 2009). Expression of IL-2 message RNA (mRNA) can be detected as early as 30 minutes following T cell activation, with maximum accumulation of IL-2 mRNA described to occur between 4-8 hours of activation (Abdalla, Kiaii et al. 2003). However, the peak for IL-2 secretion by T cells will only be achieved between 24-48 hours after the cells are activated (Sojka, Bruniquel et al. 2004). The results obtained with the investigation of PKCη expression in the context of T cell activation in HuT78, Jurkat T cells and PBL showed that PKCη levels were up-regulated at 24 hours following cell activation, which correlates with the peak of IL-2 secretion by activated T cells. Regardless of the fact that up-regulation of PKCη was not clear at the protein levels in activated Jurkat T cells, the real-time PCR analysis confirmed that PKCη mRNA levels were increased in this T cell line in response to cell activation. Multiple PKC isoforms have been shown to be involved in T cell activation leading to IL-2 production, in particular the novel PKCθ isoform (Szamel, Appel et al. 1998; Baier 2003). In addition, following activation of murine T cells, PKCη and PKCε have been shown to be necessary for the recruitment of PKCθ in a pathway that leads to IL-2 production (Quann, Liu et al. 2011). PKCη has already been involved in secretory processes in other cell types (Fima, Shahaf et al. 1999; Gundersen, Kohan et al. 2002). It has been described that PKCη co-localises with the Golgi apparatus and to regulate protein transport from the trans-golgi-network (TNG) to the cell surface (Diaz Anel and Malhotra 2005; Maissel, Marom et al. 2006). These facts combined suggest that PKCη could be involved in IL-2 production in human T cells, potentially at the level of mRNA regulation and/or protein secretion. The involvement of PKCη in regulation of IL-2 production in response to T cell activation was therefore investigated in the T cell lines HuT78 and Jurkat and in PBLs.

To determine the function of PKCη in IL-2 production following T cell activation, the expression of the PKCη gene was knocked down using an RNAi approach. In this technique, HuT78, Jurkat and PBL were transfected with a control siRNA or a siRNA targeting the PKCη gene prior to experimentation in order to silence the expression of the PKCη gene. Upon transfection of the cells with PKCη siRNA, the protein levels of PKCη were successfully down regulated on average by 75% in HuT78 and Jurkat and by 40% in PBL (Figure 3-15.A). Efficiency of PKCη gene knockdown was also measured at mRNA levels by real-time PCR, which showed that PKCη mRNA levels were decreased on average 75% in HuT78, 60% in Jurkat T cells and 50% in PBL after transfection with PKCη siRNA (Figure 3-15.B).

3.4.1 Effect of silencing of PKCη gene on IL-2 production by activated T cells was different for each cell type analysed.

It was first investigated if silencing of the PKCη gene would affect the ability of HuT78, Jurkat and PBL to produce IL-2 following T cell activation. Hence, these cells were transfected with either PKCη siRNA or control siRNA and the concentration of IL-2 secreted by the cells was measured by ELISA (see section 2.13) in the cell supernatant 24 hours after cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA (Figure 3-16).

The T cell lines tested (HuT78 and Jurkat) responded differently to activation in relation to IL-2 production. HuT78 cells produced comparable amounts of IL-2 when activated with anti-CD3 antibody alone or with a combination of anti-CD3/CD28. This is not surprising as HuT78 cells express negligible levels of CD28 (Bartelt, Cruz-Orcutt et al. 2009). On the other hand, Jurkat T cells produced very low or undetectable levels of IL-2 when stimulated with anti-CD3 antibody alone. In response to PMA, HuT78 was the only cell line to produce detectable levels of IL-2 (Figure 3-16 A and B). PBL showed similar responses to Jurkat cells and produced very low or undetectable levels of IL-2 when stimulated with anti-CD3 alone or PMA (Figure 3-16.C).

In HuT78 and PBL, knockdown of the PKCη gene produced an inconsistent result, in which levels of the IL-2 secreted by the PKCη knockdown cells oscillated from higher to lower levels when compared to control cells, or even resulted in no effect on the secretion

of IL-2 by the cells in which the PKCη gene had been silenced (Figures 3-16.A and C). In contrast, knockdown of the PKCη gene consistently resulted in a reduction in IL-2 production by activated Jurkat T cells (Figure 3-16.B).

To further investigate the role of PKCη in IL-2 production by activated T cells, a PKCη pseudosubstrate (PS) peptide inhibitor was used. This peptide mimics the pseudosubstrate site (negative regulator) on the PKCη enzyme and therefore inhibits its activity (Eichholtz, de Bont et al. 1993). Hence, HuT78, Jurkat T cells and PBL were treated with the PKCη PS inhibitor for 30 minutes prior to cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA for 24 hours and levels of IL-2 secreted in the cell supernatant were compared with the IL-2 levels secreted by untreated control cells (Figure 3-17). Interestingly, pre-treatment with PKCη PS inhibitor significantly decreased the amounts of IL-2 secreted in cells activated with anti-CD3 antibody alone or with anti-CD3/CD28 antibodies in both T cell lines: HuT78 and Jurkat (Figure 3-17 A and B respectively). On the other hand, pre-treatment of PBL with PKCη PS inhibitor did not affect IL-2 secretion under any activation conditions (Figure 3-17.C). The results observed for Jurkat T cells and PBL were in accordance with the previous findings described in the gene knockdown approach, in which the levels of IL-2 produced by activated Jurkat cells transfected with PKCη siRNA were lower than the control whilst the same method resulted in no significant effect in IL-2 production by activated PBL. However, the use of a PS inhibitor to suppress the activity of PKCη significantly reduced IL-2 secretion in HuT78, while knockdown of the PKCη gene in this T cell line affected IL-2 secretion inconsistently. Although knockdown of PKCη in these cells decreased PKCη expression by approximately 75%, it is possible that the residual PKCη activity in HuT78 after knockdown was enough to provide its function while treatment with the PKCη PS inhibitor could have inhibited completely the activity of this enzyme, explaining the distinct results.

Taken together these finding suggest that PKCη regulates IL-2 secretion in response to cell activation in the T cell lines but not in primary cells.

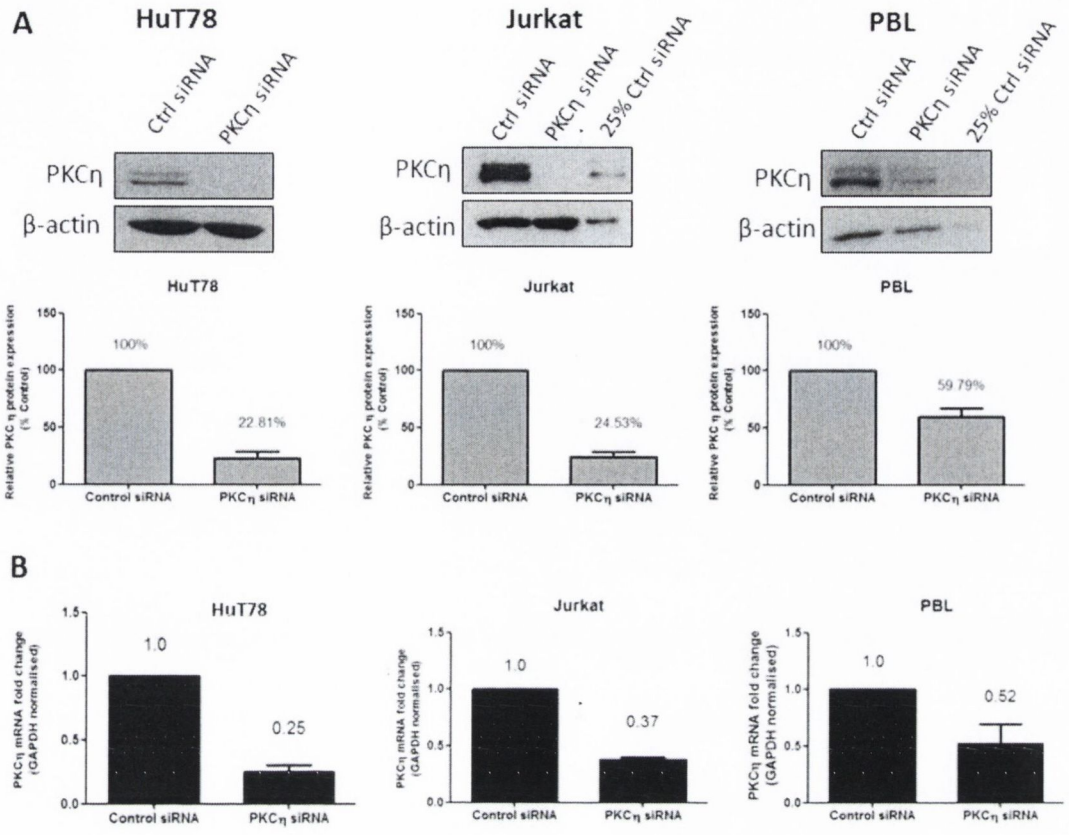


Figure 3-15 Efficiency of PKCη gene knockdown in T cell lines and primary lymphocytes. A) Western blot analysis (upper panels) of PKCη protein levels in HuT78, Jurkat and PBLs at 72 hours post-transfection with Control siRNA and PKCη siRNA. Equal amounts of cell lysates (20 μg for Hut78, 10 μg for Jurkat and 5 μg for PBL) were loaded per lane and β-actin protein levels were used as loading control. Western blots are representative of at least 4 independent experiments. Bar charts in gray (lower panels) show percentage of PKCη protein levels in samples transfected with PKCη siRNA in relation to Control siRNA. Values shown are mean ± S.E.M. based on the densitometric analysis of the western blots obtained in experiments. 25% Control siRNA means 25% of the amount of protein in the Control siRNA which was loaded to represent a reference value for the intensity of the PKCη band. B) Analysis of PKCη mRNA levels by real-time PCR obtained in HuT78, Jurkat and PBLs upon transfection with Control siRNA or PKCη siRNA. The relative fold change in mRNA levels were measured at 72 hours post-transfection. Values shown are mean ± S.E.M. of three experiments

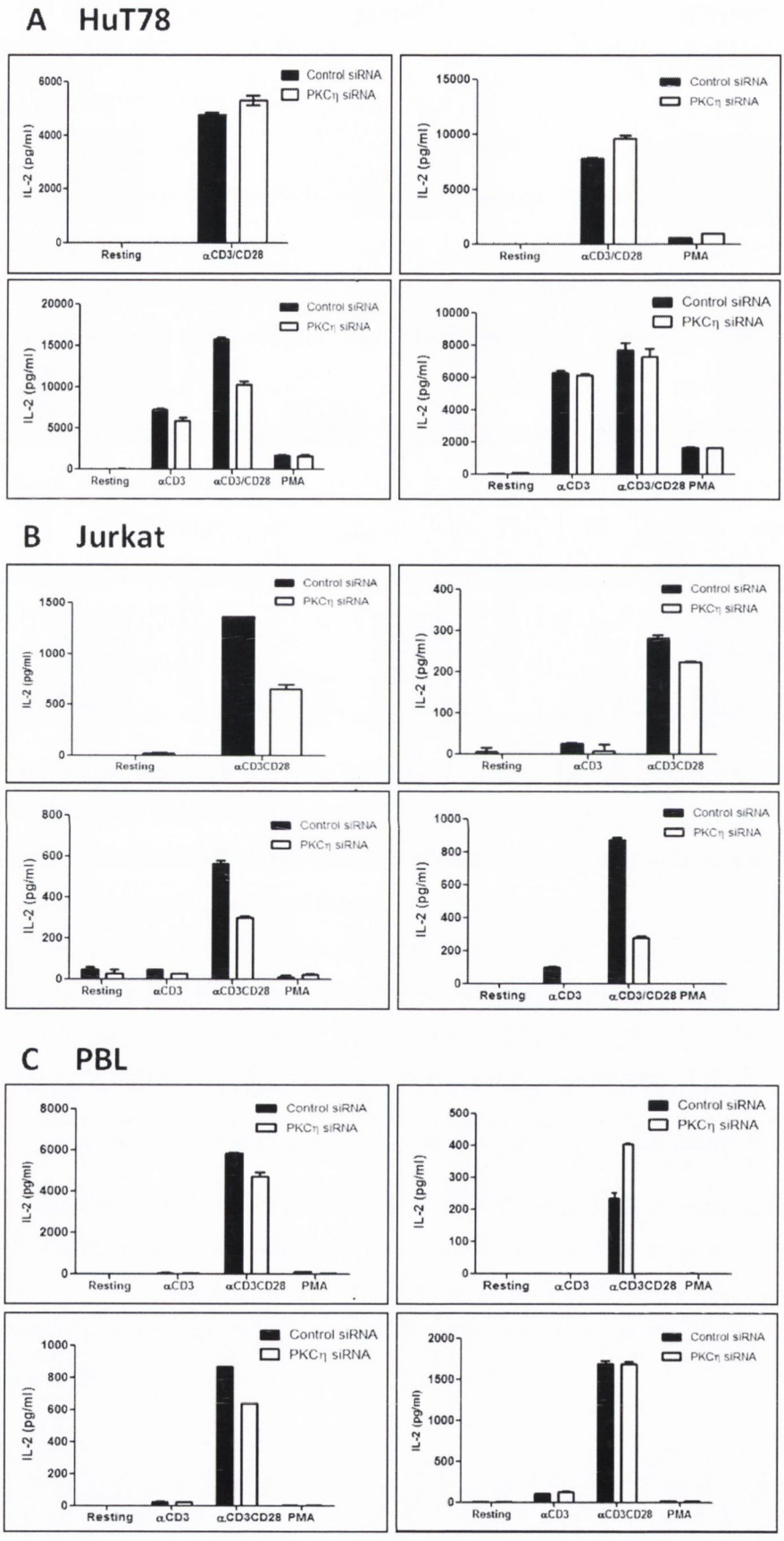

A HuT78
Control siRNA
PKCη siRNA
IL-2 (pg/ml)
Resting
αCD3/CD28
PMA
αCD3
B Jurkat
αCD3CD28
C PBL

Figure 3-16 Effect of PKCη gene knockdown in IL-2 production in activated T cells. HuT78, Jurkat and PBLs were transfected with either Control siRNA or PKCη siRNA and left to recover for 72 hours. The cells were then left unstimulated (resting) or activated with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA for 24 hours and IL-2 production was measured in the cell supernatant. Bar charts show mean ± SD of IL-2 amounts in pg/ml measured by ELISA. A) Concentration of IL-2 found in the cell supernatant of HuT78 cells in four independent experiments. The average efficiency of PKCη gene knockdown for these experiments was approximately 75% as shown in Figure 3-15.A. B) Concentration of IL-2 measured in the cell supernatant of Jurkat cells in four independent experiments. The average efficiency of PKCη gene knockdown for these experiments was approximately 75% as shown in Figure 3-15.A. C) Concentration of IL-2 measured in the cell supernatant of PBLs in four independent experiments. The average efficiency of PKCη gene knockdown for these experiments was approximately 40% as shown in Figure 3-15.A.

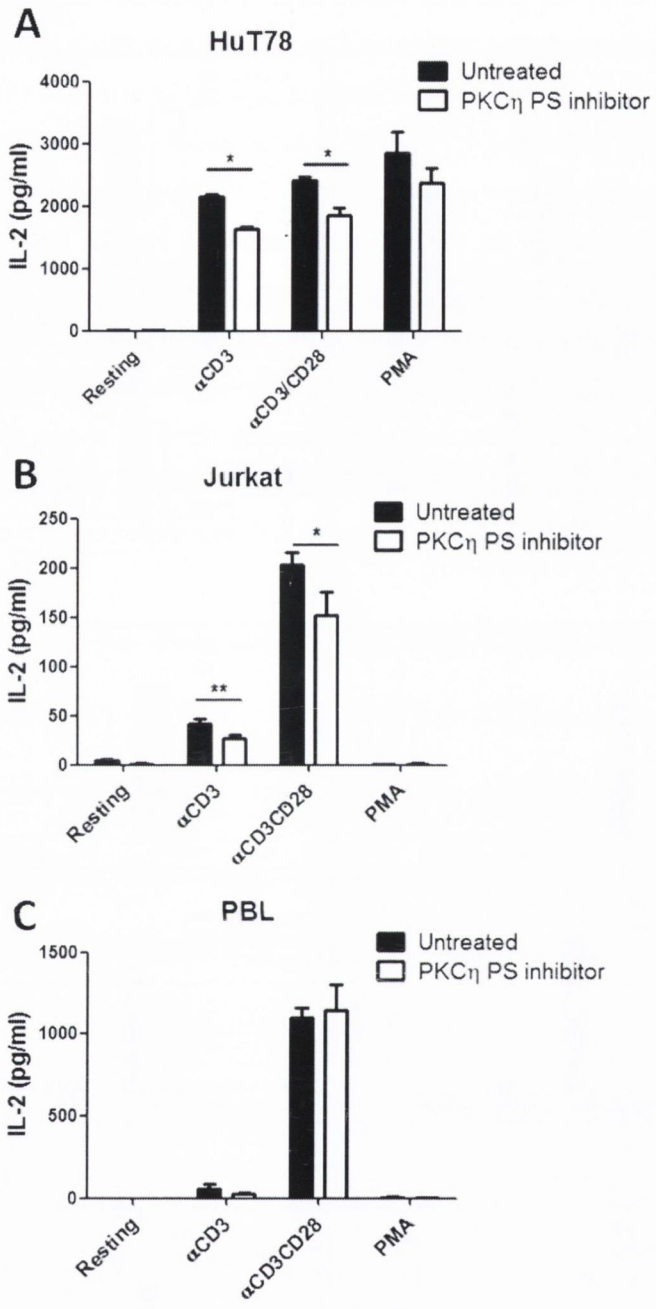


Figure 3-17 Effect of pre-treatment with PKCη pseudosubstrate inhibitor on IL-2 production in activated HuT78, Jurkat T cells and PBL. Cells were treated with 10 μM of PKCη pseudo-substrate (PS) inhibitor for 30 minutes prior cell activation with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA. The cell supernatant was collected at 24 hours upon cell stimulation and amounts of secreted IL-2 (in pg/ml) were measured in A) HuT78, B) Jurkat and C) PBL by ELISA. Bar charts show mean values ± S.E.M of at least three independent experiments.

As the levels of IL-2 secreted by activated Jurkat T cells were consistently decreased as a result of PKCη suppression by either PKCη gene knockdown or pre-treatment with a PKCη PS inhibitor, it was decided to investigate if PKCη was regulating IL-2 production in activated Jurkat T cells also at mRNA levels. The PKCη gene was then knocked-down in Jurkat T cells by nucleofection with PKCη siRNA and IL-2 mRNA levels were analysed by real-time PCR following cell activation with anti-CD3 antibody alone and anti-CD3/CD28 (Figure 3-18). PMA stimulation was not included in these experiments for the reason that it did not induce significant IL-2 secretion in Jurkat T cells (see Figure 3-16.B). Levels of IL-2 mRNA were analysed at 6 hours following cell activation, a time point that has been described as the peak for cytokine mRNA production by activated T cells (Abdalla, Kiaii et al. 2003) as well as at 24 hours following cell activation, which correlates with the increased levels of PKCη mRNA (see Figure 3-2.B).

Activation of Jurkat T cells with anti-CD3 antibody alone resulted in significant lower induction of IL-2 mRNA than activation with both anti-CD3 and anti-CD28 antibodies, consistent with IL-2 protein expression observed before for these two forms of stimulation (see figure 3-16.B). In addition, knockdown of the PKCη gene in Jurkat T cells significantly decreased IL-2 mRNA levels in comparison with control cells in both anti-CD3 antibody only and anti-CD3/CD28 stimulation (Figure 3-18). These results show that production of IL-2 following T cell activation is regulated at the mRNA levels in Jurkat T cells suggesting that reduced IL-2 secretion by PKCη-knockdown Jurkat T cells in comparison to control is a result of a decrease in IL-2 transcription in these cells.

3.4.2 Relevance of CD28 signalling in the regulation of IL-2 production by PKCη

HuT78 T cells responded differently from Jurkat T cells in terms of IL-2 production considering the distinct stimulus used to induce cell activation. In HuT78, activation with either anti-CD3 antibody alone or a mix of anti-CD3/CD28 antibodies induced similar responses in relation to IL-2 production. On the other hand, activation of Jurkat T cells with both anti-CD3/CD28 antibodies showed to be a considerably stronger stimulus than anti-CD3 antibody alone and the amount of IL-2 produced in response to these two

stimuli were significantly different in this T cell line. In addition, HuT78 T cells were the only T cell line to produce detectable levels of IL-2 in response to PMA stimulation. An important characteristic that differs between these two T cell lines regarding IL-2 production is the absence of the co-receptor CD28 in HuT78 T cells (Bartelt, Cruz-Orcutt et al. 2009). Co-stimulation of CD28 is necessary for a full activation of T cells, as it enhances the signal for cytokine production (including IL-2), cell growth, differentiation and proliferation (Acuto and Michel 2003; Riha and Rudd 2010). The absence of CD28 in HuT78 therefore, could explain the distinct responses observed between these two T cell lines in relation to PKCη expression and regulation of IL-2 production by PKCη following cell activation.

In order to investigate the importance of the CD28 co-receptor in this context, a HuT78 clone transfected with a plasmid encoding the CD28 gene and its related wild type (WT) control were used. Firstly, CD28 expression was analysed and compared in the T cell lines used in this study by flow cytometry (Figure 3-19). Absence of CD28 co-receptor was confirmed in the HuT78 and the WT control whereas expression of CD28 was detected in 99% of the $CD28^+$ HuT78 clone and the Jurkat cell line (Figure 3-19.A). To investigate if PKCη is important in the context of CD28 signalling for IL-2 production, WT and $CD28^+$ HuT78 were then transfected with control siRNA or PKCη siRNA followed by activation with anti-CD3 antibody alone, anti-CD3/anti-CD28 or PMA for 24 hours. IL-2 produced by these cells was measured by ELISA. PKCη gene knockdown at 72 hours following transfection with siRNA was confirmed by western blotting (Figure 3-19.B). Interestingly, both WT and $CD28^+$ HuT78 showed higher basal PKCη protein levels than the HuT78 T cells that had been used in the study and therefore presence of CD28 could not be associated with high basal expression levels of PKCη. In relation to IL-2 secretion in response to cell activation, the WT cells showed the same response as observed for the HuT78 T cell line, producing comparable levels of IL-2 in cells activated with either CD3 antibody alone or anti-CD3/anti-CD28 while the $CD28^+$ HuT78 showed similar responses to the Jurkat T cells and PBL, with considerably lower levels of IL-2 being produced in response to anti-CD3 antibody alone or PMA (Figure 3-19.C). IL-2 production in the cells in which the PKCη gene had been knocked down was consistently lower (although not statistically significant) than the control in both WT and $CD28^+$ HuT78 (Figure 3-19.C). These findings support the idea that PKCη also regulates IL-2 production in response to cell activation in HuT78 T cells. Although the results suggest

that regulation of IL-2 production by PKCη following cell activation is independent of CD28 signalling, it was not possible to definitively determine this mechanism.

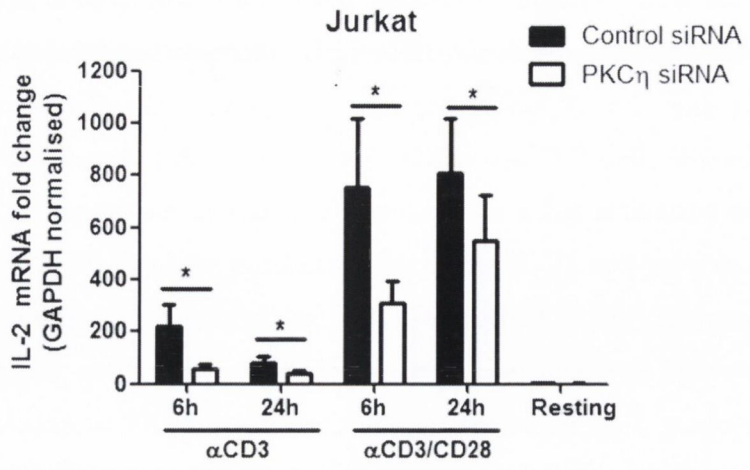


Figure 3-18 Effect of PKCη gene knockdown in activation-induced synthesis of IL-2 mRNA in Jurkat T cells. Analysis of IL-2 mRNA levels in activated Jurkat T cells after PKCη gene knockdown. Cells were transfected with either Control siRNA or PKCη siRNA and left to recover for 72 hours. Cells were then activated with anti-CD3 alone or anti-CD3/CD28 antibodies. Total mRNA was isolated at the indicated time points and IL-2 mRNA levels were analysed by qRT-PCR. Results shown are mean values ± S.E.M of four independent experiments. * $p<0.05$ as values differ significantly between PKCη knockdown and control groups (Student's *t* test).

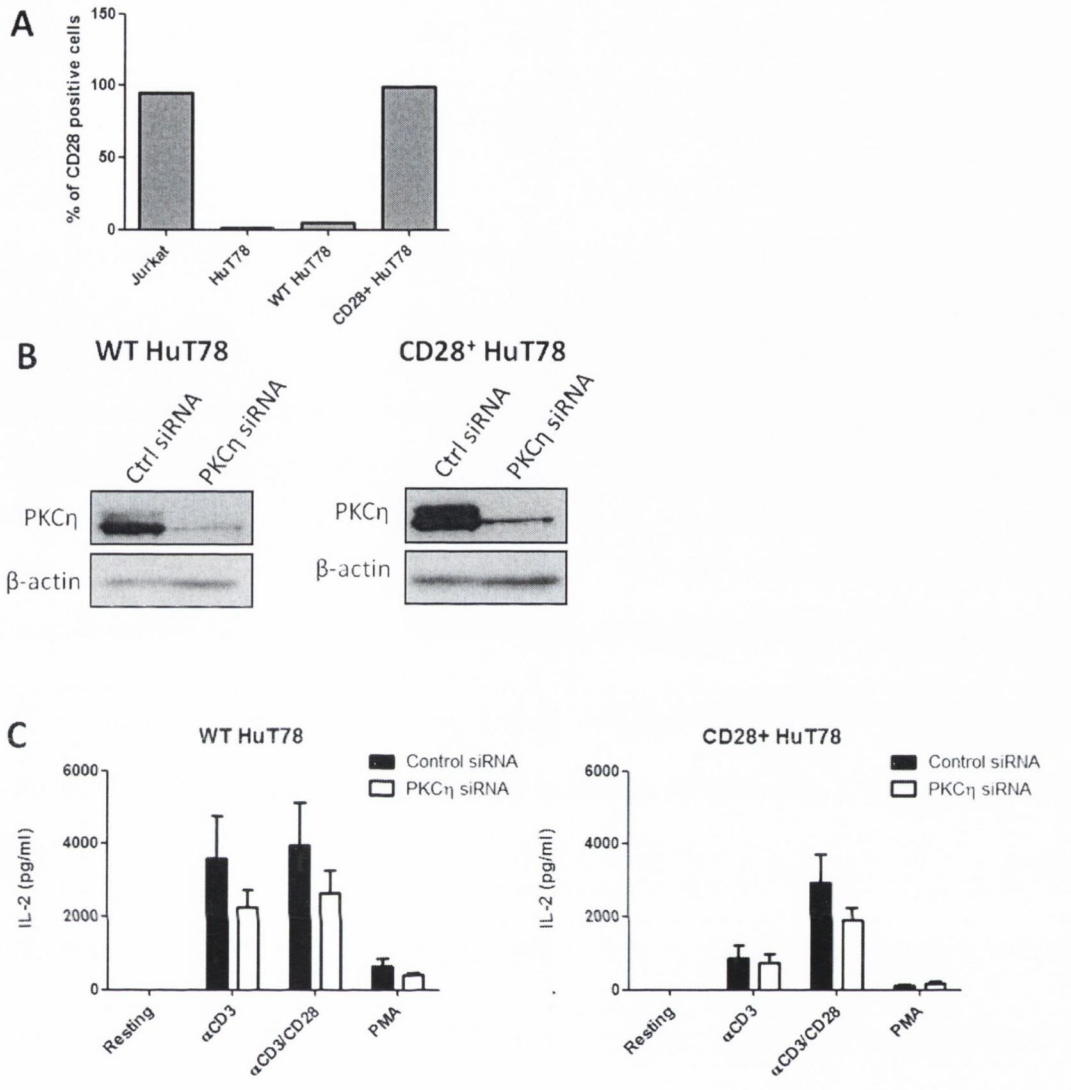


Figure 3-19 Effect of PKCη gene knockdown in IL-2 production by CD28+ HuT78. A) Expression of the CD28 co-receptor in T cell lines. Percentage of $CD28^+$ cells in samples of Jurkat, HuT78, wild-type (WT) HuT78 and $CD28^+$ HuT78 was detected by flow cytometry using fluorescent-labelled anti-CD28 antibody. B) Western blot analysis of PKCη protein levels in WT and $CD28^+$ HuT78 at 72 hours upon transfection with control siRNA or PKCη siRNA. Data is one representative of two independent experiments. C) Bar charts show IL-2 levels measured by ELISA in the cell supernatant of WT HuT78 (left panel) and CD28+ HuT78 (right panel) transfected with control siRNA or PKCη siRNA and activated for 24 hours with anti-CD3 antibody alone, anti-CD3/CD28 antibodies or PMA. Unstimulated (resting) cells were used as negative control. Data shows mean±S.E.M of two independent experiments.

3.5 Summary

In the first part of this chapter I have investigated and compared the expression and the regulation of expression, following T cell activation, of the novel PKCη isoform in T cell lines (HuT78 and Jurkat) and in primary lymphocytes (PBL). HuT78 T cells showed the lowest basal expression levels of PKCη among the three cell types and these levels were up-regulated at both protein and mRNA levels following activation. Jurkat T cells showed high basal expression of PKCη at protein levels that did not show significant up-regulation in response to cell activation. However, PKCη mRNA levels in Jurkat T cells were up-regulated following cell activation and PKCη protein levels were induced in the Jurkat Tet-On clones, suggesting that PKCη expression may also be increased in this T cell line but not clearly visible at protein levels due to the already high basal expression levels of PKCη. PBL in turn, showed relative low basal protein levels of PKCη that were up-regulated in response to cell activation but also in cells in normal growth conditions. Following the same trend, analysis of PKCη at mRNA levels in these primary cells showed that it was also up-regulated following cell activation. Furthermore, the activation-induced up-regulation in response to cell activation was specific for the PKCη isoform in the T cell lines but not in PBL.

I have demonstrated that the PI3K pathway is at least partially involved in regulation of PKCη expression in the context of T cell activation. Pre-treatment with the PI3K inhibitor LY294002 blocked the up-regulation of PKCη induced by activation of HuT78 T cells at both protein and mRNA levels, while it down-regulated PKCη protein, but not PKCη mRNA levels in Jurkat cells following cell activation. In PBL, LY294002 only partially inhibited up-regulation of PKCη protein levels in response to cell activation.

It was also determined that basal and activation-induced expression of PKCη in HuT78, Jurkat T cells and PBL is regulated by PKC activity whereas the MAPK/ERK pathway is not involved in regulation of PKCη following T cell activation. Moreover, PKCη protein levels were down-regulated in both cell lines (HuT78 and Jurkat) and in PBL in response to cell stress caused by amino acid starvation.

In the second part of this chapter, I have investigated if PKCη played a role in IL-2 production in response to cell activation in HuT78, Jurkat T cells and PBL. I have shown that suppression of PKCη activity in Jurkat T cells by either gene silencing or treatment with a PS inhibitor consistently resulted in a decrease in IL-2 production following cell activation. In addition, silencing of the PKCη gene in Jurkat T cells significantly reduced also the production of IL-2 following cell activation at mRNA levels. Finally, I have demonstrated that PKCη does not regulate IL-2 production in activated primary PBL, as knockdown of the PKCη gene did not have any significant effect on IL-2 secretion in these cells in response to cell activation.

3.6 Discussion

In this chapter, I have investigated and compared the regulation of PKCη expression in response to cell activation in two T cell lines (HuT78 and Jurkat) and in human peripheral blood lymphocytes (PBL). In addition, I have also described the effects of the suppression of PKCη expression and activity on IL-2 production following cell activation in all three cell types studied, HuT78, Jurkat and PBL.

PKCη is a member of the novel PKC subgroup primarily expressed in epithelial tissues (Osada, Hashimoto et al. 1993) but also shown to be expressed at high level in T cells (Mischak, Kolch et al. 1991). The basal expression level of PKCη was shown to vary among T cells lines and primary lymphocytes. Resting HuT78 T cells expressed the lowest levels of PKCη, followed by PBL and Jurkat T cells, which expressed PKCη at high levels.

In this study, it has been demonstrated that expression of PKCη at basal and activated levels is regulated in diverse way in different cell systems. In addition to binding of co-factors and phosphorylation, many PKC isorforms, including PKCη, are activated by tumor-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA). However, prolonged treatment with phorbol esters results in down-regulation of expression of most PKC isoforms (Parker, Bosca et al. 1995). Nevertheless, regulation of PKCη has been shown to be unique in this context, as prolonged treatment with phorbol induces membrane translocation of PKCη but not its down-regulation in many cells types (Murakami, Chida et al. 1996; Chen, Wang et al. 1997) or it results in selective up-regulation of PKCη expression in other cells, including EL4 mouse thymoma cells and MCF-7 breast cancer cells (Resnick, Luo et al. 1997; Pal, Outram et al. 2012). Expression of PKCη was also selectively up-regulated in HuT78 T cells not only after prolonged treatment with PMA but also following cell activation with anti-CD3/CD28 for 24 hours, which was a consequence of induction of PKCη also at mRNA level. In Jurkat T cells, although PKCη mRNA expression was also induced following anti-CD3/CD28 activation, the induction of PKCη at protein levels was not so clear, as this T cell line expresses basally high PKCη protein levels. PKCη protein levels were clearly induced in

the Jurkat Tet-On clones in response to activation with anti-CD3/CD28 antibodies and also after treatment with PMA at 24 hours, indicating that PKCη expression can vary among the different Jurkat clones but also supporting the idea that PKCη expression is induced following cell activation in the Jurkat T cell line. In PBL, up-regulation of PKCη protein levels was observed in cells activated with anti-CD3/CD28 antibodies and PMA, and also to a lesser extent in resting cells at 24 hours. Analysis of the PKCη mRNA levels in these cells showed also up-regulation at this level in response to anti-CD3/CD28 stimulation.

Contrasting to the HuT78 and Jurkat cell lines that are pure T cell populations, it is well known that PBL are a mixed cell population. The percentage of lymphocyte subsets comprising the PBL varies among the population, but on average, PBL are comprised in the majority by T cells (61-85%) while B cells comprise 7-23% and NK cells 6-29% of the population (Reichert, DeBruyere et al. 1991). I have determined by flow cytometric analysis of the surface markers CD3 (T cell subset), CD4 and CD8 that the PBL population used in this study was comprised of 78.37% $CD3^+$ cells (SD±6.58%), 57.88% $CD4^+$ cells (SD±9.6%) and 25.6% $CD8^+$ cells (SD±5.01%) (data not shown), which is in accordance with the reference values described. It is known that T cells express PKCη at high basal levels compared to B cells and other hemopoietic cells (Mischak, Kolch et al. 1991). Therefore, the higher levels of PKCη observed at 24 hours following cell activation could be a result of an enrichment of T cells in the PBL population. This fact is also supported by the analysis of protein expression of the other PKC isoforms in PBL following T cell activation in which protein levels of PKCα, PKCδ, PKCζ and PKCθ were also up-regulated after 24 hours of activation of PBL with anti-CD3/CD28 (Figure 3-4). Members of the PKC family have been described to be regulated by phosphorylation following TCR stimulation rather than at transcriptional levels (Szamel, Appel et al. 1998).

Ligation of the T cell receptor (TCR)/CD28 activates several tyrosine kinases and signalling pathways that result in T cell activation, proliferation and differentiation (Smith-Garvin, Koretzky et al. 2009; Brownlie and Zamoyska 2013). Among these, the PI3K pathway is an important pathway, activated primarily downstream of CD28 (Kane and Weiss 2003; Riha and Rudd 2010). PKB/Akt is a central kinase in the PI3K pathway has been shown to regulate cell activation, differentiation and survival (Rudd, Taylor et

al. 2009). I have used pharmacological inhibitors of Akt (wortmannin and LY294002) and of its downstream target mTOR (rapamycin) to investigate the involvement of the PI3K pathway in the regulation of PKCη expression following T cell activation in HuT78 and Jurkat T cell lines and in PBL. Treatment of cells with wortmannin did not have any effect in the activation-induced up-regulation of PKCη in any of the three cell types studied. In turn, treatment with LY294002 resulted in down-regulation of PKCη protein levels after 24 hours of cell activation with anti-CD3/CD28 antibodies in Jurkat T cells and blocked the up-regulation of PKCη induced by anti-CD3/CD28 activation in HuT78. In addition, treatment with LY294002 only partially blocked the up-regulation of PKCη in response to cell activation observed in PBL. Down-regulation of PKCη expression in response to treatment with LY294002 has also been shown in MCF-7 breast cancer cells (Pal, Outram et al. 2013). In this case, the down-regulation of PKCη levels observed was attributed to the indirect inhibition by LY294002 of the phosphoinosidite-dependent kinase-1 (PDK-1), which is a downstream target of Akt and a key regulator of PKC activity. Treatment with the mTOR inhibitor rapamycin also resulted in down-regulation of PKCη expression following cell activation but only in Jurkat cells, suggesting that in this cell line other elements of the PI3K pathway, rather than Akt could also be involved in the regulation of PKCη expression.

These results together suggest that the PI3K pathway at least partially regulates PKCη in response to T cell activation. However, very important facts need to be taken into consideration regarding the differences in the cell models used in the investigation. It is known that T-leukemic cell lines, such as the Jurkat T cell line, are not optimal for studying PI3K function in T cells, as many of them show constitutive activation of this pathway and can lead to misguided results (Astoul, Edmunds et al. 2001). The Hut78 T cells, although lacking the CD28 co-receptor, have been described to have an intact PI3K pathway and normally induced phosphorylation of Akt in response to anti-CD3/CD28 stimulation (Freeburn, Wright et al. 2002). Pharmacological inhibitors also need to be used with caution as often their activity is not specific for the target molecule. Wortmannin for example is considered a potent irreversible inhibitor of PI3K and other members of the PI3K superfamily but it was first described as an inhibitor of the myosin light chain kinase (Nakanishi, Kakita et al. 1992). LY24002 is considered a much less potent inhibitor of PI3K than wortmannin and it has been found to inhibit protein kinases

CK2 and Pim-1 with similar potency as PI3K and in higher doses mTOR and GSK-3β (Davies, Reddy et al. 2000; Gharbi, Zvelebil et al. 2007).

Up-regulation of PKCη observed in HuT78, Jurkat and PBL in response to treatment for 24 hours with the direct PKC activator PMA suggested that this process may be controlled by members of the PKC family. Moreover, engagement of the TCR results in Ca^{2+} influx from outside of the cell and production of DAG, the two natural activators of conventional and novel PKCs (Brownlie and Zamoyska 2013). I have therefore investigated if PKCη expression was regulated by members of the PKC family using three pharmacological inhibitors of PKC activity: bisindolylmaleimide (BIS) I, Gö6976 and LY333531. BIS I is a pan PKC inhibitor, while Gö6976 inhibits conventional PKCs and LY333531 has been characterised as specific for PKCβ (Martiny-Baron, Kazanietz et al. 1993; Ishii, Jirousek et al. 1996). Treatment with BIS I and LY333531 resulted in substantial down-regulation of PKCη protein levels in the T cell lines (HuT78 and Jurkat) and PBL in both resting and activated cells. Treatment with Gö6976 had a similar effect in HuT78 and Jurkat but not in PBL. These findings suggest that PKCη expression is regulated at basal levels and following cell activation by PKC activity in T cells. However, it is not clear if regulation of PKCη expression by members of the PKC family at basal levels and following cell activation are related or regulated by two distinct mechanisms. Studies with MCF-7 breast cancer cells have also described down-regulation of PKCη following treatment with the PKC inhibitors BIS-I and Gö6983 but not with Gö6976 (Pal, Outram et al. 2012). Using a siRNA approach, they have also described that the novel PKCε was responsible for regulating PKCη expression in this context. Transphosphorylation of PKCs by other PKC isoforms has been described before in other contexts (Gould and Newton 2008; Steinberg 2008). Sensitivity to treatment with LY333531 observed in the T cell lines HuT78 and Jurkat and in PBL suggested a possible involvement of PKCβ in regulation of PKCη expression in T cells. However, this fact could not be confirmed, as treatment with Gö6976, which also inhibits PKCβ had no effect in PBL. In addition, it has been described that LY333531 can also affect the kinase activity of PKCα, PIM-1 and PIM-3 (Bain, Plater et al. 2007), suggesting that this inhibitor may be acting on other kinases.

Since its discovery, PKCη has been shown to play an important role in cellular functions including differentiation, cell cycle regulation, secretion and apoptosis (Kashiwagi, Ohba

et al. 2002; Diaz Anel and Malhotra 2005; Rotem-Dai, Oberkovitz et al. 2009; Kamioka, Akahane et al. 2010).

Several reports suggest that PKCη plays a special role in the response to stress induced by damaging stimuli, in which expression of PKCη protects the cells against apoptosis (Abu-Ghanem, Oberkovitz et al. 2007; Rotem-Dai, Oberkovitz et al. 2009). In response to stressful stimuli, such as exposure to drugs or toxins, hypoxia and nutrient limitation, the cells usually mount a cellular stress response to ensure survival, (Kultz 2005). One of the key events of the stress response is the phosphorylation of the eukaryotic initiation factor 2 (eIF2α) on Ser51 of the alpha subunit by the endoplasmic reticulum (ER) protein kinase PERK, which results in inhibition of global protein synthesis while genes related to cell survival are preferentially translated (Holcik and Sonenberg 2005). It has been shown that expression of the PKCη gene is specifically regulated at the translational level under normal growth and during stress conditions in MCF-7 breast cancer cells (Raveh-Amit, Maissel et al. 2009). In these cells, the expression of PKCη was induced during stress conditions imposed by amino acid starvation in a time-dependent manner (Raveh-Amit, Maissel et al. 2009). In contrast, amino acid starvation of HuT78, Jurkat cells and PBL was shown to down-regulate PKCη protein levels in these cells even though the phosphorylation levels of eIF2α were increased, confirming the induction of cell stress in response to amino acid starvation. Also, amino acid starvation induced considerable cell death in the Jurkat T cell line. The mTOR pathway is a central regulator of cell metabolism and growth, and amino acids have been shown to directly activate mTOR (Avruch, Hara et al. 2006; Vinod and Venkatesh 2009). This fact suggests that mTOR may be involved in the regulation of PKCη expression under stress conditions in the T cell lines and PBL. Down-regulation of PKCη expression was also observed in relation to inhibition of mTOR activity by rapamycin in activated Jurkat cells, supporting the role for mTOR in regulation of PKCη expression in this cell line. However, as treatment with rapamycin did not affect the protein levels in either HuT78 or PBL, this relationship observed in Jurkat T cells may be related to the constitutively activate PKB/Akt pathway in this cell line, which also activates mTOR. This may also explain why amino acid starvation led to cell death in Jurkat T cells and not in the other cell types.

A role for PKCη in secretory processes has also been suggested. PKCη has been shown to co-localise with the Golgi apparatus and its activity is necessary for protein transport

from the trans-Golgi network to the cell surface in HeLa cells (Diaz Anel and Malhotra 2005; Maissel, Marom et al. 2006). Moreover, recent studies using murine T cells have described that PKCη and PKCε also accumulated in the immunological synapse in response to TCR stimulation and are required for recruitment of PKCθ to the site, in a signalling cascade leading to IL-2 production (Quann, Liu et al. 2011). Fu et al. (2011) have also shown that T cells from the PKCη-deficient mouse produced less IL-2 and interferon (IFN)-γ than T cells from a wild-type mouse, a characteristic shared with $PKC\theta^{-/-}$ T cells. These observations, together with the fact that PKCη expression is up-regulated at 24 hours following cell activation in HuT78, Jurkat and PBL, which corresponds with the time described for the peak of IL-2 secretion (Abdalla, Kiaii et al. 2003), suggest a role for PKCη in IL-2 production also in these cells. Therefore, the involvement of PKCη in IL-2 production was investigated in the T cell lines HuT78 and Jurkat and in PBL using a siRNA approach. The results showed that PKCη activity was necessary for IL-2 production in the T cell lines but not in PBL. In the Jurkat T cell line, either silencing of PKCη expression with siRNA or treatment with the PKCη pseudosubstrate inhibitor resulted in significant decrease in the levels of IL-2 produced by this T cell line in response to anti-CD3 antibody alone or with anti-CD3/CD28 antibodies. Further investigation revealed that silencing of PKCη expression also decreased the levels of IL-2 mRNA induced following cell activation, suggesting that in Jurkat T cells, PKCη regulates IL-2 production at transcriptional levels. In HuT78 T cells, although silencing of PKCη expression inconsistently affected IL-2 secretion in activated cells, neutralisation of PKCη activity with a PKCη PS inhibitor consistently reduced the amounts of IL-2 secreted by these cells in response to cell activation with anti-CD3 antibody alone or with anti-CD3/CD28. These results in HuT78 cells suggest that silencing of PKCη with siRNA may not be enough to completely abrogate PKCη activity in these cells. In contrast, treatment with the PKCη PS inhibitor completely inhibited PKCη activity, showing its importance in IL-2 production in response to cell activation also in HuT78. On the contrary, either silencing of PKCη expression with siRNA or treatment with PKCη pseudosubstrate inhibitor had no effect in IL-2 production in PBL, reflecting a difference between primary cells and cell lines.

As mentioned before, PKCη shows high structural similarity with the PKCε isoform and these two isoforms have been shown to have redundant roles in the context of T cell activation leading to IL-2 production (Quann, Liu et al. 2011). Redundant functions of

PKCη and PKCθ have been also shown in the context of cytokine production by activated murine T cells (Fu, Hu et al. 2011). These facts may explain the absence of a PKCη-related phenotype in relation to production of IL-2 in PBL, in which PKCε or PKCθ might be compensating for the absence of PKCη. However, it may also be that the requirement for PKCη in cytokine production is exclusive for the T cell subpopulation and the other type of lymphocytes present on the PBL population are interfering with the results. Therefore, the investigation of a functional role for PKCη in IL-2 production in the context of cell activation should be continued in a more pure population of T cells.

Even though it was not possible to confirm the requirement for PKCη activity in the mixed population of PBL, it was clear that this isoform plays an important role in IL-2 secretion by leukemic T cell lines. Also, PKCη expression is specifically up-regulated following activation in both HuT78 and Jurkat, although each cell line presented different patterns of PKCη expression and regulation. It was also possible to show that the involvement of the PI3K pathway in regulation of the activation-induced expression of PKCη was more pronounced in the T cell lines than in PBL. These findings together confirmed PKCη as a potential player in T cell activation and the differences observed between the immortalised T cell lines and primary lymphocytes may provide new insights into the biology of these cells.

4 Chapter 4 – Investigation of a functional role for PKCη in primary human lymphocytes

4.1 Introduction

Protein kinase C (PKC) is a family of serine/threonine kinases involved in signal transduction and regulation of a variety of cell functions (reviewed by Steinberg 2008; Newton 2010; Pfeifhofer-Obermair, Thuille et al. 2012). The PKC family is comprised of ten isoforms divided in three subgroups according to their domain composition, which in turn dictates the second messenger requirements for their activation. The conventional PKCs (cPKCs) are comprised of PKCα, βI, βII and γ and they require both diacylglycerol (DAG) and Ca^{2+} for activation. In contrast, the novel PKCs (nPKCs) are comprised of δ, ε, η and θ and are activated only by DAG. Finally, the atypical PKCs (aPKCs) are comprised of the ζ and λ/ι isoforms and both are regulated independently of DAG and Ca^{2+} (Newton 2010; Pfeifhofer-Obermair, Thuille et al. 2012).

T lymphocytes or T cells are key players in the cell-mediated immune response to pathogens. Naive, unstimulated T cells circulate in the bloodstream until their T cell receptor (TCR) recognises a peptide antigen bound to a major histocompatibility complex (MHC) molecule presented on the surface of antigen presenting cells (APC). The ligation of TCR with peptide:MHC complex activates the T cell and triggers a series of processes involving cell proliferation and differentiation of the naive cells into effector cells that actively secrete immunoregulatory cytokines or display cytotoxic activity, ultimately leading to an effective immune response (Nel and Slaughter 2002; Sundberg, Deng et al. 2007). Once the immune stimulus has been cleared, the response is resolved and most of the activated T cells die by apoptosis. However, a proportion of these cells survive and become memory T cells. In the event of a secondary infection, the response produced by these memory T cells will be typically faster and stronger than the primary response (Berard and Tough 2002).

Stimulation of the TCR results in activation of the phospholipase Cγ1 (PLCγ1), which hydrolises phosphatidylinositol 4,5 biphosphate (PIP_2) to form inositol triphosphate (IP_3) and DAG. IP_3 production, in turn, results in release of Ca^{2+} from intracellular stores and its accumulation in the cytosol (Smith-Garvin, Koretzky et al. 2009). These two co-factors DAG and Ca^{2+} are essential for activation of members of the PKC family.

In T cells, PKC isoforms have shown to be involved in activation, differentiation, migration and cellular survival (Pfeifhofer-Obermair, Thuille et al. 2012). T cells express nine out of the ten different PKC isotypes, making it difficult to distinguish the specific cellular functions of each individual isoform (Baier 2003). PKCθ, a member of the novel PKCs, was originally described by Baier et al. (1993) and demonstrated a relatively restricted expression pattern, being expressed in skeletal muscle, lymphoid organs and hematopoietic cell lines, in particular T cells. PKCθ is the most studied isoform in T cells. PKCθ is activated downstream the TCR and is selectively recruited to the centre of the immunological synapse (IS), which is the site of contact between the T cell and APC (Monks, Kupfer et al. 1997). Recruitment of PKCθ to the IS is essential for activation of downstream signalling pathways, including the transcription factors NF-AT, NF-kB and AP-1, which are critical for T cell activation, cytokine production, proliferation and differentiation (Isakov and Altman 2012; Pfeifhofer-Obermair, Thuille et al. 2012). Moreover, PKCθ has recently shown to be involved in T cell differentiation into the Th17 subset through up-regulation of the transcription factor STAT-3 (Kwon, Ma et al. 2012).

PKCη is another member of the novel PKC subfamily described for the first time by Osada et al. (1990) and shows high similarity with the PKCε isoform (Littler, Walker et al. 2006). PKCη is predominantly expressed in epithelial tissues such as lung and skin (Osada, Mizuno et al. 1990) but it has also been described to be highly expressed in T cells (Mischak, Kolch et al. 1991). PKCη has been shown to be involved in proliferation, differentiation and cell cycle regulation in several cell types. In keratinocytes, for example, PKCη has been reported to promote cell cycle arrest and switching from proliferation to terminal differentiation (Cabodi, Calautti et al. 2000; Hara, Miyazaki et al. 2011). On the other hand, PKCη activity has been shown to induce proliferation in glioblastoma cell lines (Aeder, Martin et al. 2004; Uht, Amos et al. 2007). In the MCF-7 breast cancer cell line, induction of PKCη expression enhanced cell growth and resulted in increased expression of cell cycle regulators cyclins D and E (Fima, Shtutman et al. 2001). In addition, studies in this same breast cancer cell line as well as in fibroblasts have reported that PKCη associates with cyclin E in quiescent cells and that these proteins dissociate once the cells enter the cell cycle (Shtutman, Hershko et al. 2003).

PKCη has been shown to be involved in secretory processes. In HeLa cells, PKCη activity is required for protein transport from the trans-Golgi network (TGN) to the cell

surface (Diaz Anel and Malhotra 2005). In addition, PKCη has been described to co-localise with the Golgi apparatus in HCT116 colon carcinoma cells and mediate its fragmentation after treatment with bile acids (Byrne, Foran et al. 2010).

Despite the fact that it has been more than 20 years since PKCη was reported to be highly expressed in T cells (Mischak, Kolch et al. 1991), very little work has been carried out to investigate a role of PKCη in these cells until recently. Working with mouse models, Quann et al. (2011) showed that PKCη, together with PKCε, were also translocated to the immunological synapse almost immediately upon TCR stimulation, and that the expression of both kinases were required for PKCθ recruitment to the same site. They also showed that knocking down of the PKCη and the PKCε genes together significantly reduced IL-2 production by activated T cells. Hence, this study demonstrates that PKCη, together with PKCε, regulates T cell polarity and effector functions (Quann, Liu et al. 2011). Following the same concept, Fu et al. (2011) found a pivotal role of PKCη in T cell activation and homeostatic proliferation. Comparing the phenotypes of $PKC\eta^{-/-}$, $PKC\theta^{-/-}$, and double-knockout mice, they were able to show that both isoforms shared some redundant roles in T cell biology, although only PKCη deficient T cells showed defective homeostatic proliferation. T cell homeostatic proliferation occurs in response to a lymphopenic environment and is driven by TCR signalling. Selective engagement of the TCR by self-peptide:MHC complexes promotes proliferation of T cells under severe lymphopenic conditions, and this division is associated with and activation marker phenotype that is different from that induced by antigenic stimulation(Clarke and Rudensky 2000; Sprent and Surh 2011).

PKCη has been reported to be involved in the cell secretory machinery, as well as to participate in cell cycle regulation, cell proliferation and differentiation, all processes that are directly related to the cellular response following activation of the T cell receptor. Although some information about the role of PKCη in T cell biology has been gathered lately, much is yet to be explored. To the best of my knowledge, studies involving PKCη and human T cells have been carried out mainly in T cell lines and no functional role of PKCη has been reported in primary human lymphocytes. For this reason, the aim of this study was to investigate a functional role for PKCη in the context of T cell activation using primary human lymphocytes.

4.2 Aims and objectives

The overall objective of this chapter was to investigate a role for PKCη in primary human lymphocytes in the context of T cell activation, emphasising possible differences between a mixed population of lymphocytes and an expanded population comprising mainly T lymphocytes. For this purpose, I have used freshly isolated peripheral blood lymphocytes (PBL), which is a mixture of T, B and NK cells; PHA/IL-2 stimulated peripheral blood T lymphocytes (PBTL), which is comprised of T cells; and human invariant natural killer T cells (iNKT cells). Therefore the aims of this chapter were to:

- determine the involvement of PKCη in cellular functions triggered by T cell activation in PBL, including cytokine production, cell cycle progression and cell differentiation into effector T cell subsets;
- determine the involvement of PKCη in cytokine production and cell proliferation in stimulated PBTL;
- investigate the role of PKCη in cytokine production and secretion by iNKT cells, following cell activation.

4.3 Investigation of a role for PKCη in the activation of primary human lymphocytes.

4.3.1 Silencing of PKCη expression by siRNA does not affect cytokine production by primary T cells following cell activation.

As described in the previous chapter, expression of the novel PKCη isoform was shown to be regulated following activation of freshly isolated PBL. Activation of these cells with plate-bound anti-CD3/anti-CD28 antibodies, as well as PMA for 24 hours led to an increase in total PKCη protein levels in these cells (Figure 3-1.C). These observations brought attention to the importance of investigating a possible role for PKCη in the regulation of events connected to T cell activation. I started the investigation by determining if PKCη had a role in IL-2 secretion in response to activation of PBLs, since previous reports with murine T cells had shown that PKCη accumulated in the immunological synapse and was required for recruitment of PKCθ to the site, in pathway leading to IL-2 production (Quann, Liu et al. 2011). However, the results showed that silencing of PKCη expression with siRNA, or suppressing its activity with a PKCη-specific pseudosubstrate inhibitor, did not affect IL-2 production by PBL after 24 hours of cell activation (Figures 3-16.C and 3-17.C).

As PBL is a mixed lymphocyte population, it was decided to continue the investigation of a functional role for PKCη in cytokine production following cell activation in individual T cell subpopulations. The flow cytometry technology enables us to distinguish the different subpopulations of lymphocytes in the main PBL population by using fluorescent labelled specific antibodies against the CD3 complex (T cell marker), and co-receptors CD4 and CD8 expressed on the surface of T cells. Therefore, IL-2 and interferon (IFN)-γ production were analysed by intracellular staining separately in the $CD4^+$ T cell subset and in the $CD8^+$ T cell subset using flow cytometry. IFN-γ was chosen in addition to IL-2 since it is another important cytokine produced by T cells, especially by Th1 cells in response to cell activation and has been described to be crucial for macrophage activation, inflammation and tumour surveillance (Hu and Ivashkiv 2009).

For this purpose, a siRNA approach for silencing PKCη expression was used to investigate if PKCη was required for the production of IL-2 and IFN-γ by PBL in response to cell activation with anti-CD3/anti-CD28 antibodies or PMA/Ionomycin (Io) in $CD4^+$ T cells and in $CD8^+$ T cells (Figure 4-1.A and 4-2.A). To note, nucleofection of PBL with PKCη siRNA resulted on average in a decrease of approximately 50% of the total PKCη protein levels expressed by these cells (see Figure 3-15).

Activation of PBL with anti-CD3/anti-CD28 or PMA/Io for 4 hours induced production of both cytokines (IL-2 and IFN-γ) in the two T cell subpopulations analysed. Approximately 25% of the $CD4^+$ T cells activated with either anti-CD3/anti-CD28 antibodies or PMA/Io were positive for IL-2 production (Figure 4-1.B). However, silencing of the PKCη expression with siRNA did not affect the number of IL-2 positive $CD4^+$ T cells following anti-CD3/anti-CD28 activation, as the number of IL-2-positive cells following transfection with PKCη siRNA were comparable to the number of IL-2-positive cells in PBL transfected with control siRNA. Although not statistically significant, a small decrease in IL-2 positive cells was observed for the cells transfected with PKCη siRNA and activated with PMA/Io (Figure 4-1.B). Production of IFN-γ was detected in approximately 10% of the $CD4^+$ T cells upon cell stimulation for 4 hours with anti-CD3/anti-CD28 antibodies or PMA/Io. Again, however, no differences were observed in the number of IFN-γ positive cells between activated PBL in which the PKCη gene had been silenced and control cells (Figure 4-1.C).

In the $CD8^+$ T cell subset, IL-2 production was detected in approximately 25% of the cells in response to activation with anti-CD3/anti-CD28 or PMA/Io for 4 hours (Figure 4-2.B) while 30% of the $CD8^+$ T cells were producers of IFN-γ in the same conditions (Figure 4-2.C). Nonetheless, there were no differences in number of cells positive for either IL-2 or IFN-γ production between PBL transfected with PKCη siRNA and control siRNA (Figures 4-2.B and 4-2.C).

These results therefore demonstrate that production of IL-2 and IFN-γ is not regulated by PKCη following T cell activation in either the $CD4^+$ or the $CD8^+$ T cell subsets.

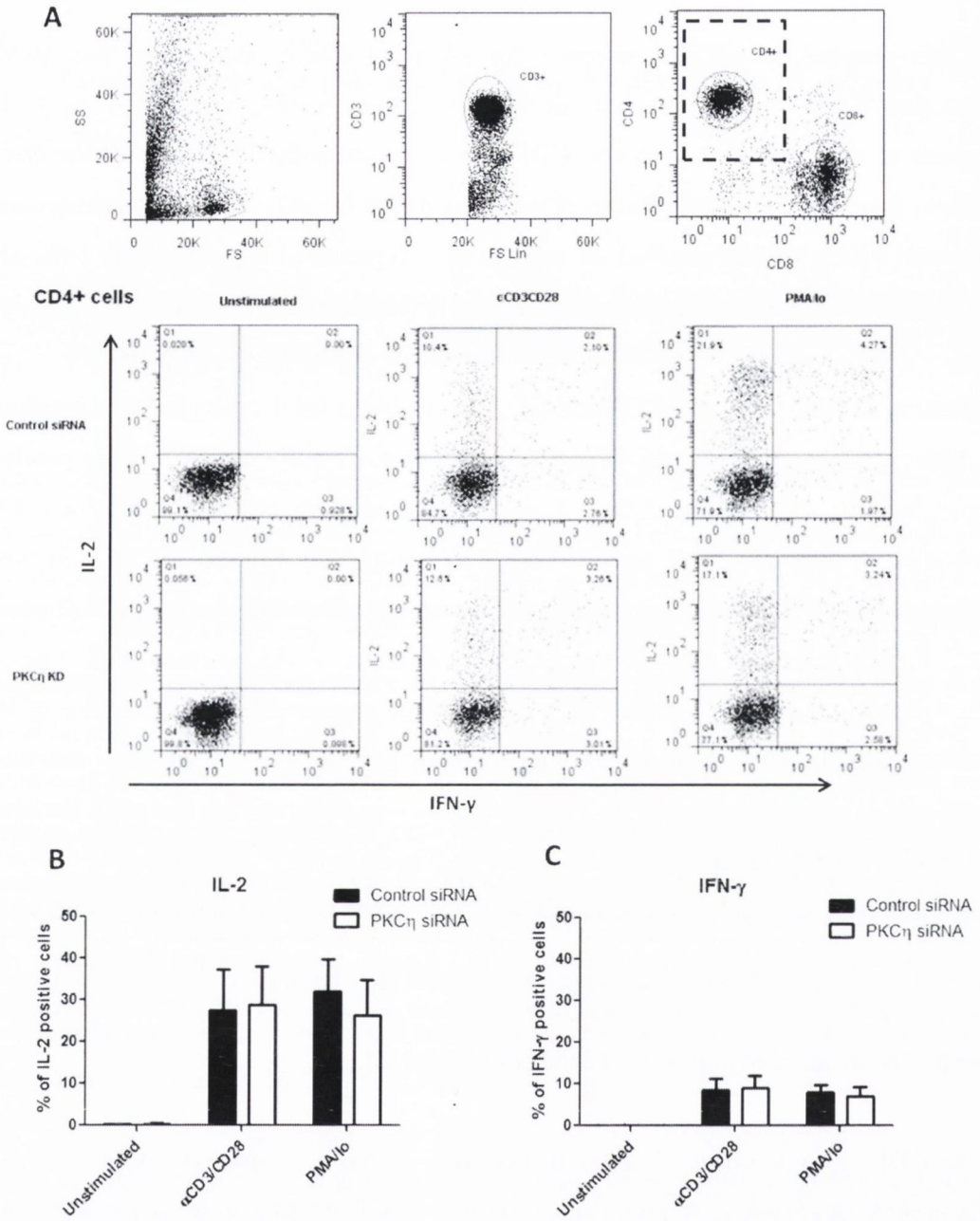


Figure 4-1 IL-2 and IFN-γ production in the CD4+ T cell subset is not affected by knockdown of the PKCη gene. PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene silencing efficiency is shown in Figure 3.15) and then were left unstimulated or activated with anti-CD3/CD28 antibodies or with 50 ng/ml PMA+ 1 μg/ml Ionomycin (Io) for 4h. Production of the cytokines IL-2 and IFN-γ was determined by intracellular cytokine detection by flow cytometry (section 2.15.2). A) Representative flow cytometry plots showing the gating strategy used for identifying $CD4^+$ T cells (upper panels – hatched box) and IL-2 and IFN- γ production in this subset following cell activation (lower panels). Percentage of IL-2 (B) and IFN- γ (C)- producing cells did not differ between PKCη siRNA and control siRNA transfected cells. Charts show mean percentage ± S.E.M (n=3 donors).

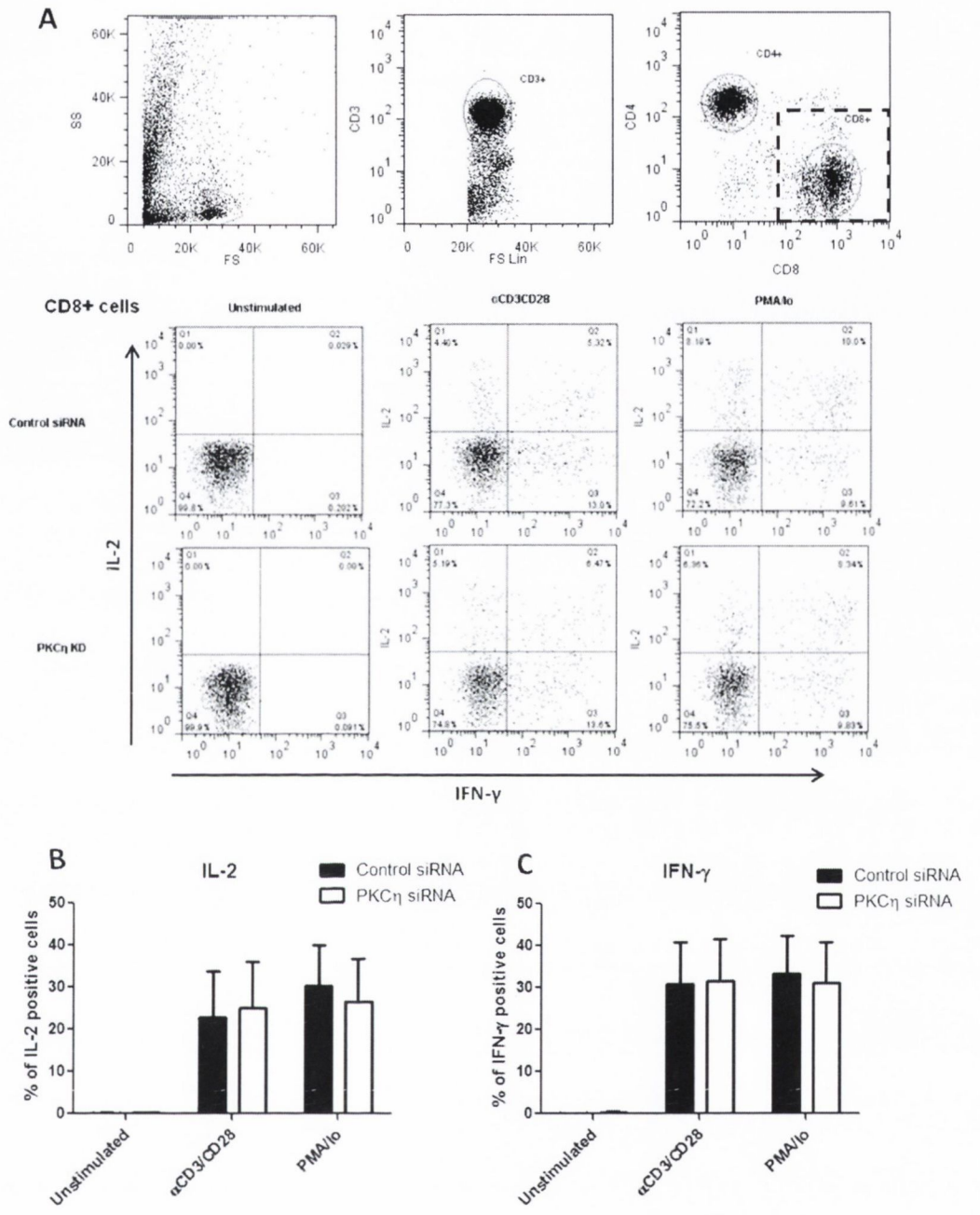


Figure 4-2 IL-2 and IFN-γ production in the CD8+ T cell subset is not affected by knockdown of the PKCη gene. PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene knockdown efficiency is shown in Figure 3.15) and then were left unstimulated or activated with anti-CD3/CD28 antibodies or with 50 ng/ml PMA+ 1 μg/ml Ionomycin (Io) for 4h. Production of the cytokines IL-2 and IFN-γ was determined by intracellular cytokine detection by flow cytometry. A) Representative flow cytometry plots showing the gating strategy used for identifying $CD8^+$ T cells (upper panels – hatched box) and IL-2 and IFN- γ production in this subset following cell activation (lower panels). Percentage of IL-2 (B) and IFN- γ (C)- producing cells did not differ between PKCη siRNA and control siRNA transfected cells. Charts show mean percentage ± S.E.M (n=3 donors).

4.3.2 PKCη does not regulate cell cycle progression in peripheral blood lymphocytes (PBL) in response to TCR stimulation.

Up-regulation of PKCη expression at 24 hours following PBL activation with anti-CD3/CD28 stimulation suggests that PKCη may play a role in events in the late phase of cell activation. Activation of T lymphocytes ultimately results in cell cycle progression, cell proliferation and differentiation into effector T cells. Several reports have shown PKCη to be involved in cell proliferation in different cell types (Uht, Amos et al. 2007; Shahaf, Rotem-Dai et al. 2012). In addition, PKCη has been described to associate with Cyclin E (Kashiwagi, Ohba et al. 2000; Shtutman, Hershko et al. 2003), which is a cell cycle regulator required for the transition from G1 to the S phase of the cell cycle.

Consequently, the next step in this investigation was to determine whether PKCη was implicated in cell cycle progression in T cells in response to cell activation. To do so, the expression of PKCη was silenced with siRNAs in freshly isolated PBL prior to cell activation with anti-CD3/anti-CD28 antibodies for 72 hours. The cells were then fixed and stained with propidium iodide (PI), a fluorescent DNA-binding dye that permits the DNA content of the cell to be quantified and analysed by flow cytometry (see section 2.15.5). In this way, the percentage of cells in each phase of the cell cycle can be determined as follows: cells that are in the G2 and mitosis phase (G2/M) will have approximately twice the amount of the DNA found in cells in the G0/G1 phase, while cells in the S phase will have more DNA than the cells in G1 and less than the cells in G2/M phase (Figure 2-3). Apoptotic cells can also be distinguished by this methodology as they contain less DNA than the cells in G1 phase caused by DNA fragmentation.

As a result of cell activation with anti-CD3/anti-CD28 antibodies for 72 hours, approximately 45% of the activated PBL entered the cell cycle, with approximately 25% of the cells in the S phase and 20% in the G2/M phase (Figure 4-3.B). PBL that were transfected with control siRNA but left unstimulated did not progress into the cell cycle as expected and the great majority of the cells (approximately 90%) remained in the G0/G1 phase. When comparing the percentage of cells in each phase of the cell cycle following anti-CD3/CD28 stimulation in PKCη siRNA-transfected PBL with the PBL

transfected with control siRNA, no differences were found, suggesting that PKCη does not regulate cell cycle progression in primary human lymphocytes following T cell activation.

4.3.3 PKCη does not regulate T cell differentiation *in vitro*.

Using the rationale described in section 4.3.2 above, it was also investigated if PKCη had a role in T cell differentiation into the Th1, Th17 and Treg subsets *in vitro*. For this purpose, freshly isolated PBL which had been transfected with PKCη siRNA or control siRNA were stimulated for 4 days with plate-bound anti-CD3/anti-CD28 antibodies. In addition to the antibody stimulation, the cells were exposed to a cocktail of specific cytokines to induce Th1 (IL-12), Th17 (IL-23, IL-6 and IL-1β) or Treg (IL-2 and TGF- β) polarization (Hsieh, Macatonia et al. 1993; Zheng, Wang et al. 2004; Chung, Chang et al. 2009). Following the differentiation process, the cells were analysed by flow cytometry. Firstly, a gate was drawn to select for analysis only the cells that were positive for the CD4 co-receptor, as only $CD4^+$ T cells differentiate into the Th effector subsets. After that, the gated $CD4^+$ T cells were analysed for detection of intracellular IFN-γ, IL-17 and FoxP3 to identify Th1, Th17 and Treg cells, respectively. As it is known that T cell activation causes T cell differentiation into the various T cell subsets, PBL activated with anti-CD3/anti-CD28 antibodies for the same period of time but in the absence of the polarization cytokines (Th0) were used as control for the polarization conditions.

Th1 polarization conditions resulted in an increase in the percentage of IFN-γ producing cells compared to the cells activated in the absence of the Th1 polarization cytokines (Figure 4-4.B). The amount of IFN-γ produced by Th1 polarized cells was also slightly higher than the Th0 control cells, as determined by the mean fluorescence intensity (MFI) values of the selected cell population, (Figure 4-4.C) suggesting that the cytokines added to the cells successfully induced Th1 differentiation *in vitro*. Silencing of the PKCη expression showed no effect in Th1 polarization and the number of Th1 cells, as the amount of IFN-γ produced by these cells were comparable for both PKCη siRNA and control siRNA transfected cells, suggesting that PKCη does not regulate Th1 cell differentiation *in vitro*.

In regard to the differentiation into Th17 cells, the mix of cytokines used to induce Th17 polarization did not increase the number of IL-17 producing cells in comparison to the Th0 control cells (Figure 4-5). For this reason, even though silencing of PKCη did not affect the number of IL-17 producing cells, no conclusions could be made concerning the involvement of this kinase in Th17 cells differentiation, since the polarization conditions used in this experiment did not induce the expected numbers of Th17 cells *in vitro*.

The mix of cytokines (IL-2 and TGF-β) used to polarize Treg cells *in vitro* resulted in a higher, although not significant, number of FoxP3 expressing cells compared to the Th0 control group. However, there were no differences in the number of Treg cells induced by these cytokines between the cells transfected with PKCη siRNA and control siRNA (Figure 4-6) suggesting that PKCη does not regulate Treg cells differentiation *in vitro*.

Taken together, the findings described above in freshly isolated peripheral blood lymphocytes (PBL) suggest that PKCη is not involved in cytokine production (IL-2 and IFN-γ), cell cycle progression or Th1 and Treg differentiation *in vitro* following stimulation of these cells. However, the lack of a PKCη-associated phenotype observed could be due in part to the technique used for knocking down the PKCη gene in PBL. It is important to take into account that transfection of PBL with a PKCη siRNA resulted in reduction of approximately 50% in the amount of total PKCη in these cells, which may not be enough to cause a significant response in the cells.

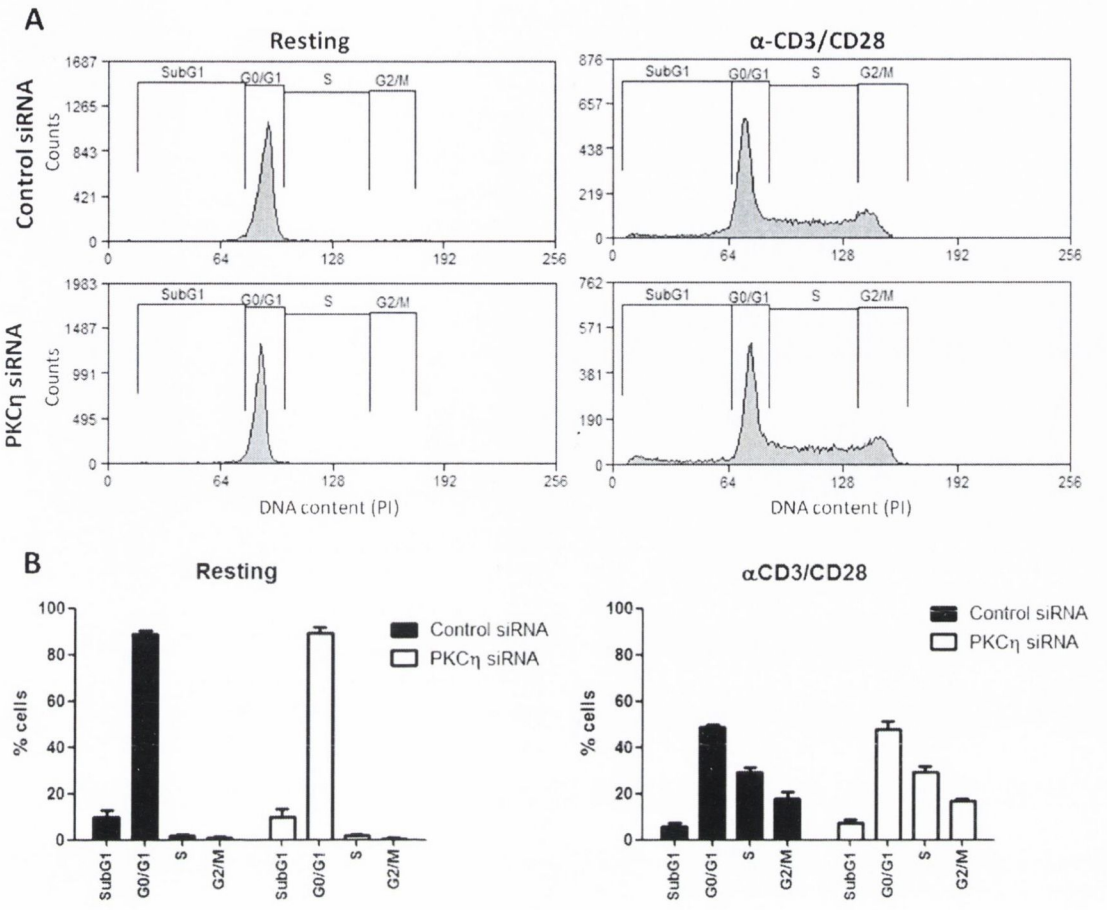


Figure 4-3 Silencing of PKCη expression with siRNA does not impair cell cycle progression in PBL in response to T cell activation. PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene knockdown efficiency is shown in Figure 3.15) and then activated by crosslinking pre-bound antibodies against CD3 and CD28 for 72 h. Resting PBL were used as control cells. The cells were then subjected to cell cycle progression analysis by flow cytometry analysis of DNA content after PI staining (section 2.15.5). A) Representative flow cytometry plots showing the gating strategy used to determine number of cells in each phase of cell cycle (SubG1 phase shows apoptotic cells, G0/G1 phase shows 2n/diploid cells, S shows cells undergoing DNA synthesis and G2/M phase shows double diploid cells). Activation with anti-CD3/CD28 antibodies induced cell cycle progression in PBL but no differences were observed between PKCη siRNA transfected cells and control siRNA transfected cells. B) Percentage of cells in each phase of cell cycle observed for the cells analysed as described in A. Data shown are mean values ± S.E.M (n=3 donors).

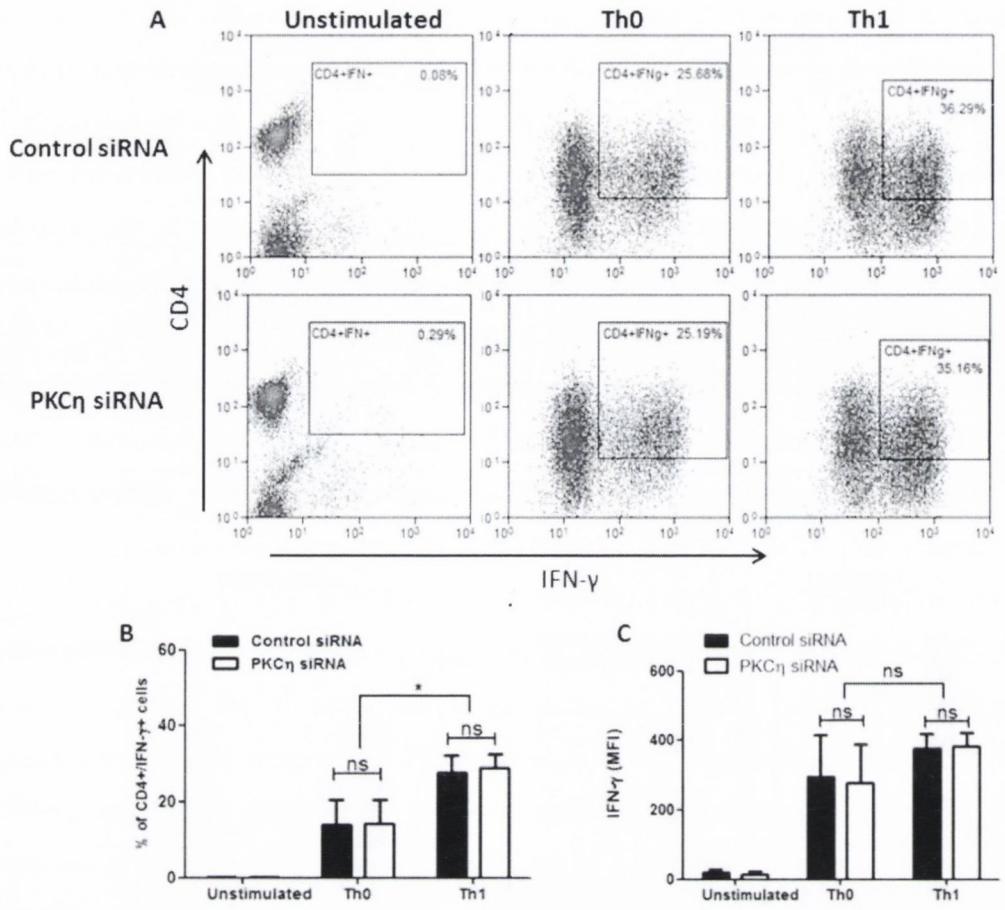


Figure 4-4 Silencing of PKCη expression with siRNA does not affect Th1 polarization. Freshly isolated PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene knockdown efficiency is shown in Figure 3.15) and left to recover for 72h. The cells were then left unstimulated or activated with cross-linking anti-CD3/CD28 antibodies for 4 days in the presence (Th1) or not (Th0) of the Th1 polarization cytokine IL-12 (50 ng/ml). Presence of the co-receptor CD4 and intracellular IFN-γ was assessed by flow cytometric analysis and $CD4^+$/IFN-γ+ cells were considered Th1 cells. A) Representative flow cytometry plots showing IFN-γ production in response to the different conditions (Unstimulated, Th0 and Th1) by cells transfected with control siRNA or PKCη siRNA. B) Number of Th1 cells (CD4+ and IFN-γ+) were significantly increased as a result of the Th1 polarization but no differences were found between control and PKCη siRNA transfected cells. Data show mean percentage ± S.E.M (n=3 donors). C) Amounts of IFN-γ produced by Th1 cells were slightly higher compared to Th0 but knockdown of the PKCη gene did not have an effect on the levels of IFN-γ produced by these cells. Data show mean values ± S.E.M (n=3 donors). (*) $p<0.05$ calculated using the Student's t test.

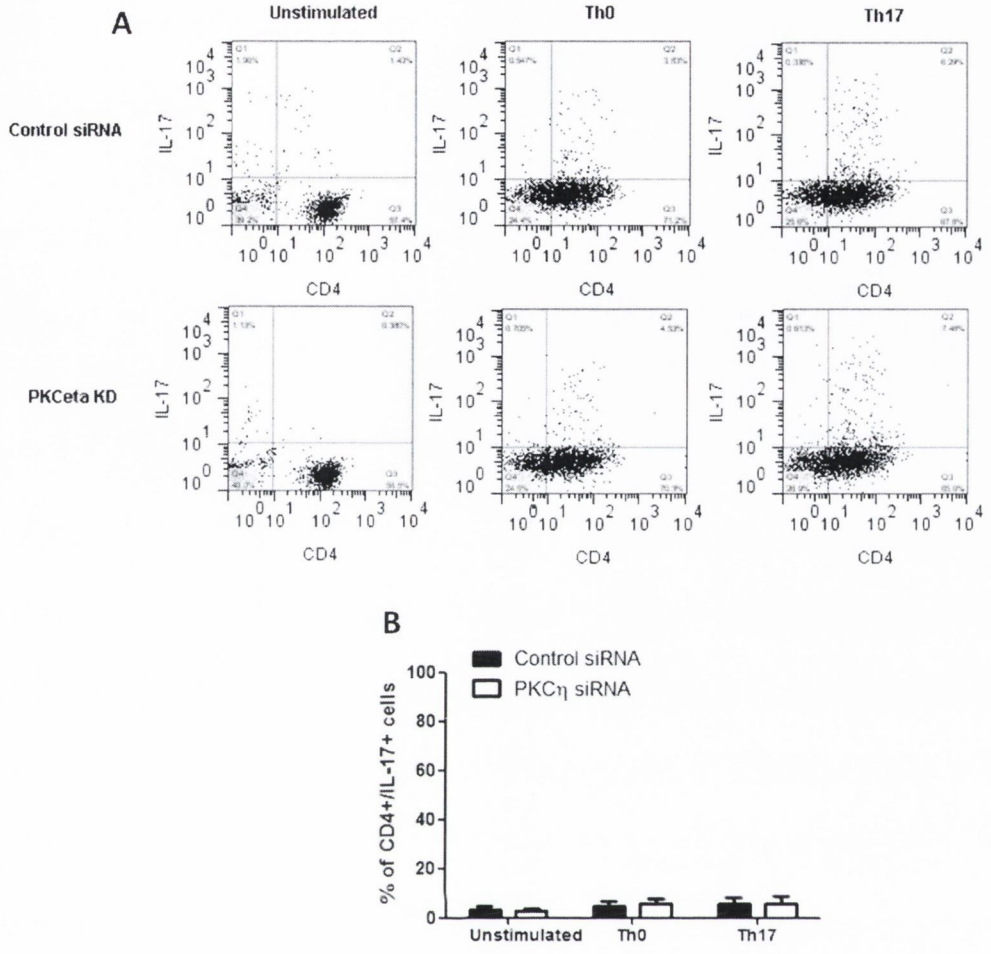


Figure 4-5 Th17 polarization conditions used in this study failed to produce Th17 cells. Freshly isolated PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene knockdown efficiency is shown in Figure 3-15) and left to recover for 72h. The cells were then left unstimulated or activated with cross linked anti-CD3/CD28 antibodies for 4 days in the presence (Th17) or not (Th0) of Th17 polarization conditions (50 ng/ml IL-23, 30 ng/ml IL-6, and 50 ng/ml IL-1β). Presence of the co-receptor CD4 and intracellular IL-17 were assessed by flow cytometric analysis and $CD4^{+}$/IL-17+ cells were considered Th17 cells. A) Representative flow cytometry plots showing IL-17 production in response to the different conditions (Unstimulated, Th0 and Th17) by cells transfected with control siRNA or PKCη siRNA. B) Th17 polarization used did not result in higher numbers of Th17 cells and no differences were found between control and PKCη siRNA transfected cells. Data show mean percentage ± S.E.M (n=3 donors).

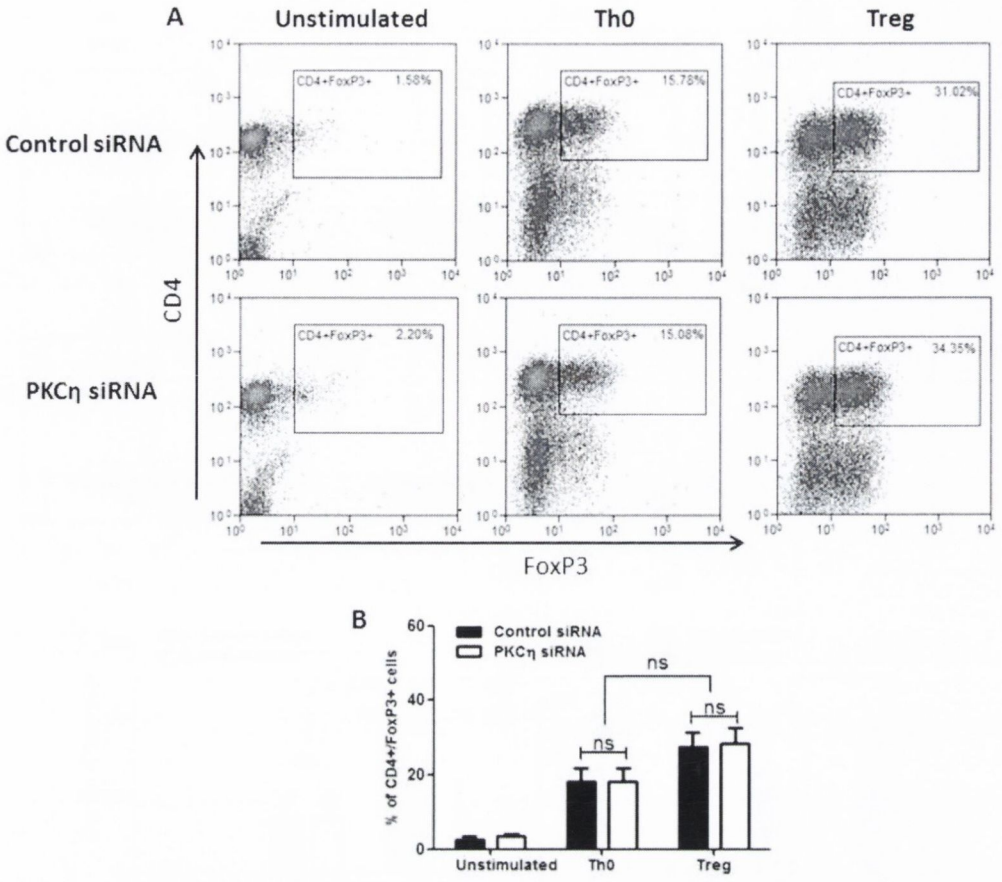


Figure 4-6 Silencing of PKCη expression with siRNA does not affect Treg polarization. Freshly isolated PBL were transfected with 1000 nM of control siRNA or PKCη siRNA (gene knockdown efficiency is shown in Figure 3.15) and left to recover for 72h. The cells were then left unstimulated or activated with cross linked anti-CD3/CD28 antibodies for 4 days in the presence (Treg) or not (Th0) of Treg polarization conditions (20 ng/ml IL-2 and 5 ng/ml TGF-β). Presence of the co-receptor CD4 and expression of the transcription factor FoxP3 were assessed by flow cytometric analysis and $CD4^{+}$/FoxP3+ cells were considered Treg cells. A) Representative flow cytometry plots showing FoxP3 expresssion in the different conditions (Unstimulated, Th0 and Treg) in cells transfected with control siRNA or PKCη siRNA. B) Number of Treg cells were increased, although not significantly, as a result of the Treg polarization but no differences were found between control and PKCη siRNA transfected cells. Data show mean percentage ± S.E.M (n=3 donors).

4.4 Investigation of a role for PKCη in stimulated primary human T cells.

4.4.1 Regulation of PKCη expression in stimulated Peripheral Blood T lymphocytes (PBTL).

Experiments using freshly isolated PBL did not show any clear function for the PKCη isoform in the context of T cell activation, in relation to cytokine secretion, cell cycle progression or T cell differentiation. PBL is a mixed cell population comprised mainly of T cells, but containing a significant number of B cells and NK cells that can respond differently to the stimulus and interfere with the results of the investigation. It was then decided to continue the investigation of a functional role for PKCη in the context of T cell activation in stimulated peripheral blood T lymphocytes (PBTL), which is a cell population comprised almost in its totality by T cells. These cells are derived from PBL by a process of expansion in response to phytohaemagglutinin (PHA) for 3 days followed by further stimulation with IL-2 for a period of 5 days resulting in a cell population comprised of >98% of T cell ($CD3^+$) phenotype (Lefort and Kim 2010). However, as a result of the stimulation with PHA and IL-2, these cells present characteristics of experienced/effector cells which is confirmed by the expression of activation markers on their surface (Bartelt, Cruz-Orcutt et al. 2009). At the same time, a new methodology was developed in our laboratory for siRNA delivery to this type of stimulated primary human T cells, which consisted of a 'two-hit' delivery of siRNA by nucleofection at 0 and 72 hours and further cell recovery for an additional 48 hours (Freeley and Long 2013). This methodology resulted in a much improved efficiency in gene silencing compared to the one hit used in freshly isolated primary lymphocytes. For these reasons stimulated PBTL were considered a potential T cell model to study more effectively the function of PKCη in T cells.

Firstly, the expression profile of PKCη was determined during the expansion period used to obtain PBTL from PBL. To generate stimulated PBTL, PBL were first treated with PHA for 3 days followed by IL-2 for a further 5 days (see section 2.4.2). Levels of PKCη

were analysed by Western blot in the cells stimulated for 3 days with PHA only and then at days 2 and 5 of the 5-day period of IL-2 stimulation. Interestingly, protein levels of PKCη significantly increased at day 2 and persisted at day 5 of the IL-2 stimulation period compared to levels in cells stimulated for 3 days with PHA only (Figure 4-7.A). These findings suggested that the observed up-regulation of PKCη was a result of the addition of recombinant IL-2 to the cell culture. However, it has been described that addition of exogenous recombinant IL-2 to the cell culture medium of EL4 thymoma cells did not change PKCη protein levels, while treatment of these cells with phorbol esters resulted in up-regulation of PKCη protein (Resnick, Luo et al. 1997). Also, the T cell population has been shown to express PKCη at high level in comparison to other hematopoietic cells (Mischak, Kolch et al. 1991). During the expansion process with PHA/IL-2 stimulation, the percentage of T cells in the population increases from approximately 70% in PBL (Bisset, Lung et al. 2004) to > 98% in PBTL (Lefort and Kim 2010) and therefore this increase in PKCη highly expressed cells in the population may be reflecting the levels of total PKCη protein observed in increased numbers of T cells in the Western blot analysis.

Following from this, the PBTL were activated for a further 24 hours with anti-CD3/anti-CD28 antibodies or PMA to investigate the regulation of PKCη expression in response to cell activation with these two factors (Figure 4-7.B). The results showed no apparent changes in the PKCη protein levels in response to either anti-CD3/CD28 stimulation or PMA, suggesting that PKCη expression was not regulated in response to cell activation in PBTL.

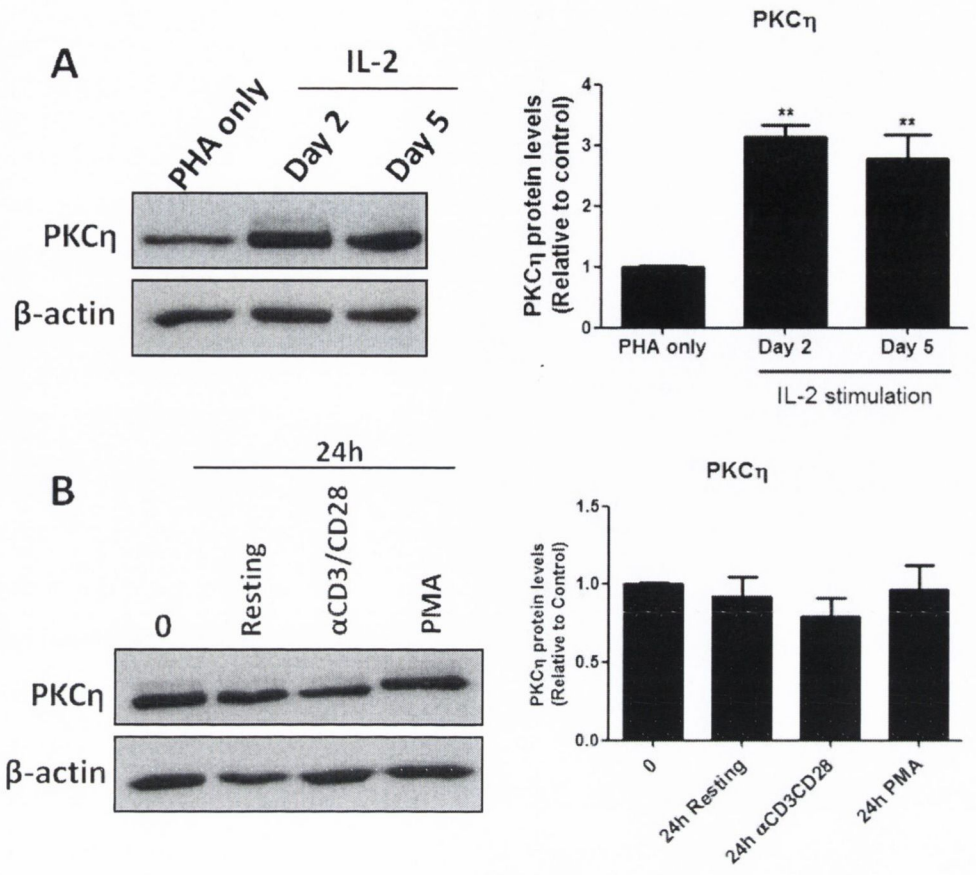


Figure 4-7 Regulation of PKCη expression in PBTL. A) Freshly isolated PBL were stimulated with 2 μg/ml phytohaemagglutin (PHA) for 72 hours followed by stimulation with 20 ng/ml of recombinant IL-2 for another 5 days. Amounts of PKCη in the whole-cell extracts were analysed by Western blotting at 72 hours of stimulation with PHA only and at days 2 and 5 of IL-2 stimulation (left). Densitometric analysis of the blots (right) confirmed that PKCη protein levels were significantly increased from day 2 of IL-2 stimulation compared to PHA only stimulated cells. Western blots are representative and bar chart show mean values ± S.E.M (n=3 donors) of PKCη levels relative to PHA only stimulated cells. (**) $p<0.01$ calculated by Student's t test as samples significantly differ from PHA only group. B) After the PHA/IL-2 stimulation period, the cells now named PBTL were incubated in culture medium without FCS and IL-2 for 2 hours and then left unstimulated (resting) or activated with anti-CD3/CD28 antibodies or with 50 ng/ml PMA for 24 h in the absence of exogenous IL-2. Levels of PKCη in the whole-cell lysates were analysed by Western blotting at the beginning (0) and at 24h upon activation. Densitometric analysis of the blots (bar chart on the right) showed no changes in PKCη protein levels in activated cells. Western blots are representative and bar chart show mean values ± S.E.M (n=3 donors) of PKCη levels relative to 0h.

4.4.1.1 Silencing of PKCη expression with siRNA does not affect cytokine production by stimulated PBTL following cell activation.

As shown in the previous section, expression of PKCη did not change in stimulated PBTL following activation with either anti-CD3/anti-CD28 antibodies or PMA for 24 hours. However, this observation does not suggest the absence of a role for PKCη in this context, as activity of PKC isoforms can also be regulated by phosphorylation (Newton 2010; Freeley, Kelleher et al. 2011). In fact, the high PKCη protein levels observed in PBTL (comprised of mainly T cells) in comparison to protein levels of PKCη observed in resting PBL supports the concept of PKCη as being a potential player in the biology of these stimulated PBTL. For this reason, it was decided to investigate a functional role for PKCη in stimulated PBTL, starting with the possible involvement of PKCη in regulation of cytokine production (IL-2 and IFN-γ) in response to cell activation. The two-hit delivery of siRNA described in section 2.11.3 was used in order to silence PKCη expression in stimulated PBTL. As a result, PKCη protein levels were decreased on average by 80% in the PBTL transfected with the PKCη siRNA compared to cells transfected with control siRNA (Figure 4-8).

To determine if PKCη had an effect on cytokine production in stimulated PBTL, PKCη expression was silenced with siRNA as described before (section 2.11.3). The cells were then activated with anti-CD3/anti-CD28 antibodies or PMA/Io for 4 hours and production of IL-2 and IFN-γ was detected by intracellular staining and analysed by flow cytometry (section 2.15.2). The CD4+ and CD8+ T cell subsets were analysed separately (Figure 4-9.A and 4-10.A).

In the $CD4^+$ T cell population, approximately 60% of the cells produced IL-2 in response to cell activation with either anti-CD3/anti-CD28 antibodies or PMA/Io (Figure 4.9.B) while approximately 30% of this $CD4^+$ T cell population produced IFN-γ upon cell activation (Figure 4-9.C). However, the percentage of IL-2 and IFN-γ producing cells were the same in the PKCη siRNA-transfected compared to the control siRNA-transfected PBTL.

In the $CD8^+$ T cell subset, activation of cells with anti-CD3/anti-CD28 or PMA/Io resulted in IL-2 production by 60% of the cells (Figure 4-10.B) while 50% were producing IFN-γ (Figure 4-10.C). Again, the number of cells producing IL-2 and IFN-γ were comparable between the stimulated PBTL in which the expression of PKCη had been silenced and the control cells.

Altogether, these results showed that silencing of PKCη expression with siRNA does not affect cytokine production (IL-2 and IFN-γ) in stimulated PBTL in response to cell activation, in either $CD4^+$ T cells or $CD8^+$ T cells.

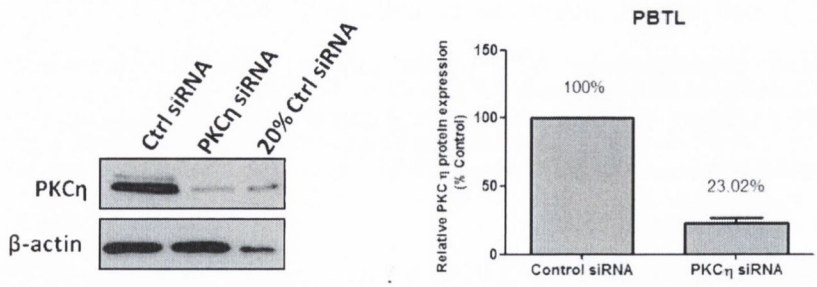


Figure 4-8 Efficiency of silencing of PKCη expression with siRNA in stimulated PBTL. Cells were transfected with control siRNA or PKCη siRNA (at 1000 nM each) and then re-electroporated following the same conditions 72h later. Whole cell lysates were prepared 48h after the second transfection and PKCη protein levels were determined by Western blotting (left). Densitometric analysis of the blots (right) showed an average of 75% of down –regulation in PKCη expression in cells transfected with PKCη siRNA compared to control siRNA. Western blots are representative and bar chart show mean values ± S.E.M (n=5 donors) of PKCη levels in PKCη siRNA-transfected cells relative to control.

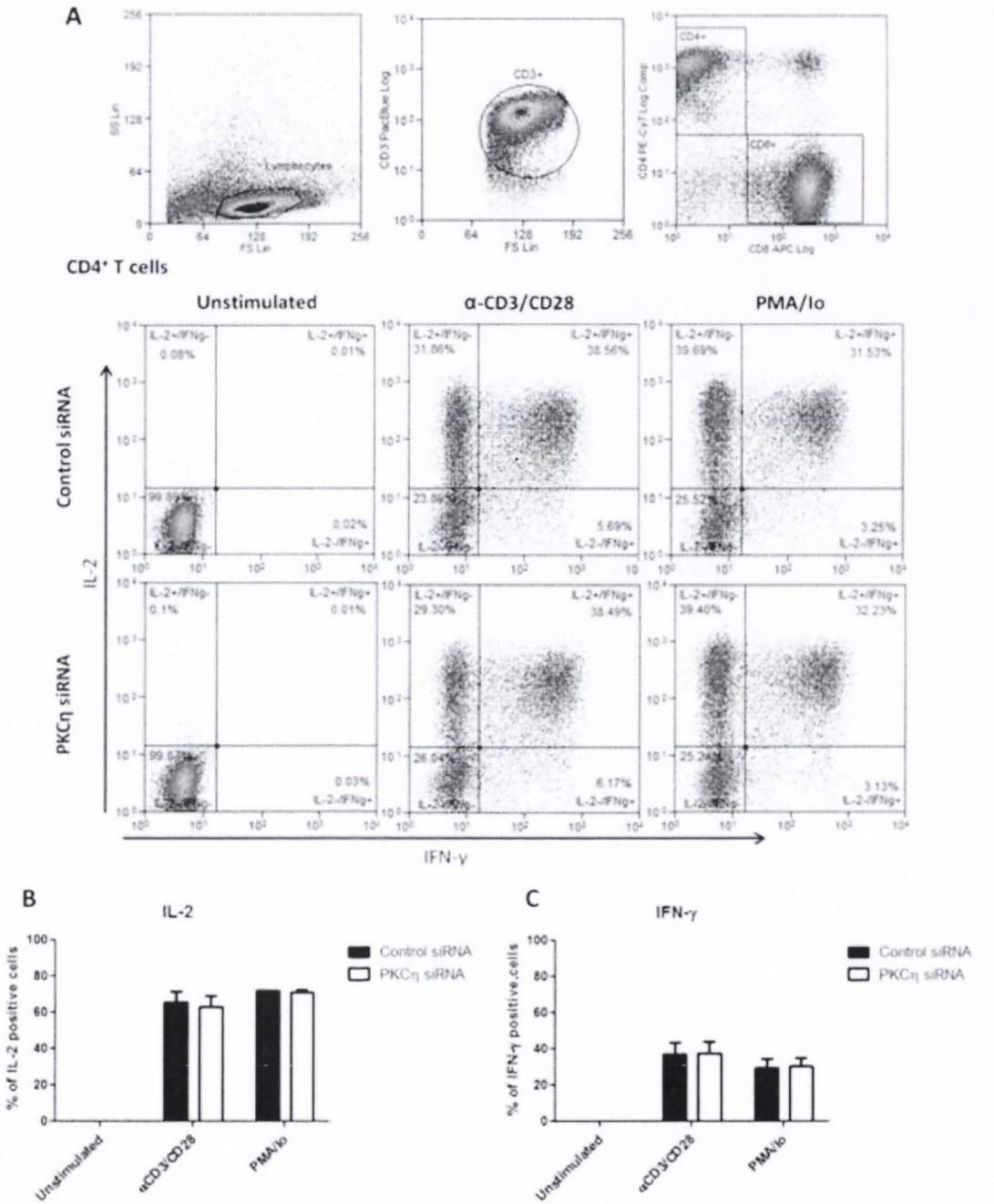


Figure 4-9 IL-2 and IFN-γ production in the CD4$^+$ T cell subset of stimulated PBTL is not affected by silencing of PKCη expression with siRNA. Stimulated PBTL were transfected with 1000 nM of control siRNA or PKCη siRNA following the two-hit protocol described in section 2.11.3 (gene knockdown efficiency is shown in Figure 4.8). Cells were then serum and IL-2 starved for 2 hours and left unstimulated or activated with anti-CD3/CD28 antibodies or with 50 ng/ml PMA+ 1 μg/ml Ionomycin (Io) for 4h, all in the absence of exogenous IL-2. Production of the cytokines IL-2 and IFN-γ were determined by intracellular cytokine detection by flow cytometry (section 2.15.2). A) Representative flow cytometry plots showing the gating strategy used for identifying CD4$^+$ T cells (upper panels) and IL-2 and IFN- γ production in this subset

following cell activation (lower panels). Percentage of IL-2 (B) and IFN- γ (C)- producing cells did not differ between PKCη siRNA and control siRNA transfected cells. Charts show mean percentage ± S.E.M (n=2 donors).

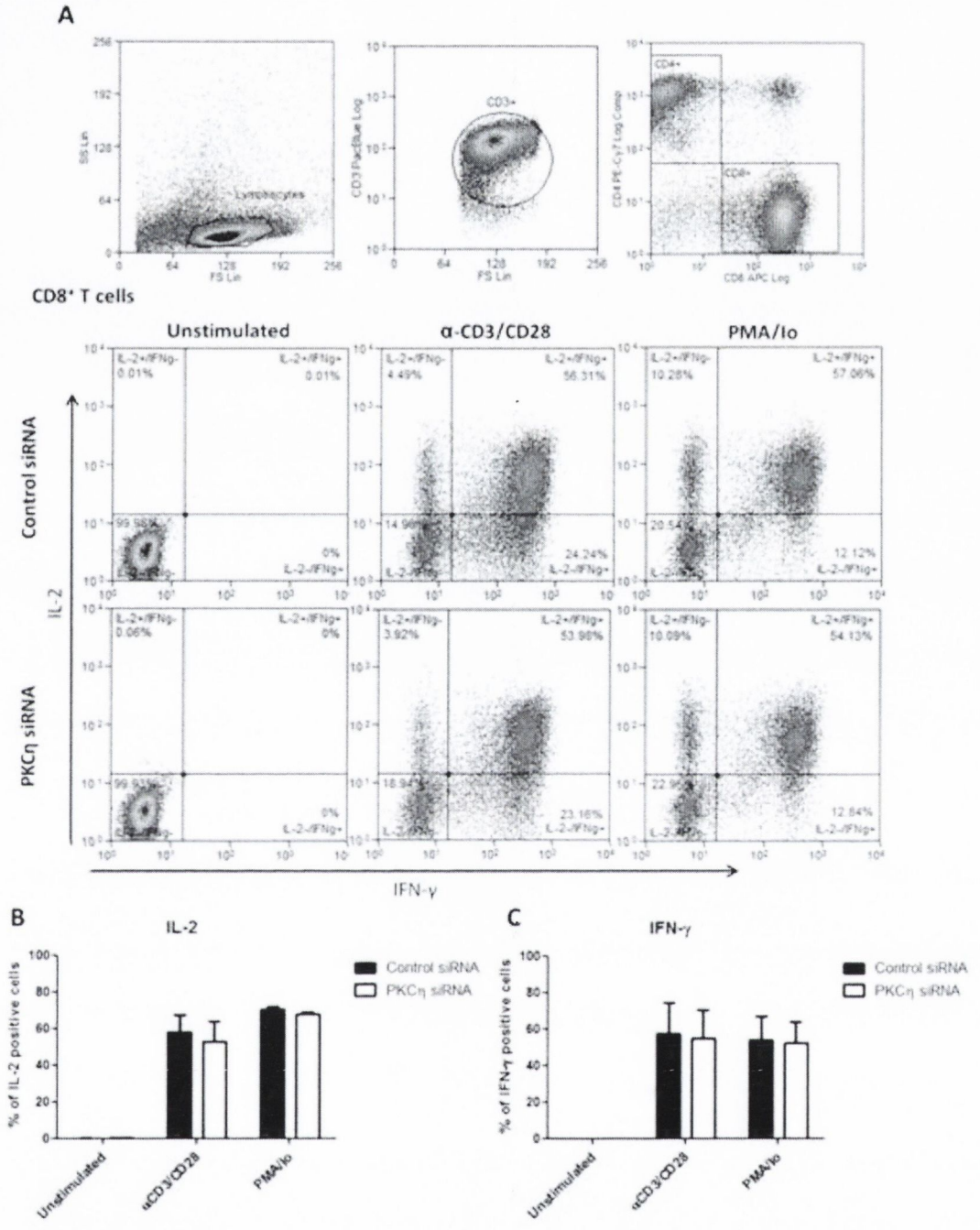


Figure 4-10 IL-2 and IFN-γ production in the CD8$^+$ T cell subset of stimulated PBTL is not affected by silencing of PKCη expression by siRNA. Stimulated PBTL were transfected with 1000 nM of control siRNA or PKCη siRNA following the two-hit protocol described in section 2.11.3 (gene knockdown efficiency is shown in Figure 4.8). Cells were then serum and IL-2 starved for 2 hours and left unstimulated or activated with anti-CD3/CD28 antibodies or with 50 ng/ml PMA+ 1 μg/ml Ionomycin (Io) for 4h, all in the absence of exogenous IL-2. Production of the cytokines IL-2 and IFN-γ were determined by intracellular cytokine detection by flow cytometry (section 2.15.2). A) Representative flow cytometry plots showing the gating strategy used for identifying CD8$^+$ T cells (upper panels) and IL-2 and IFN- γ production in this subset

following cell activation (lower panels). Percentage of IL-2 (B) and IFN- γ (C)- producing cells did not differ between PKCη siRNA and control siRNA transfected cells. Charts show mean percentage ± S.E.M (n=2 donors).

4.4.1.2 Silencing of PKCη expression with siRNA does not impair stimulated PBTL proliferation in response to cell activation with anti-CD3/anti-CD28 antibodies.

A function for PKCη in the context of T cell proliferation following cell activation was also investigated. To do this, following silencing of PKCη expression with siRNA the PBTL were serum starved for 4 hours and labelled with CFSE (carboxyfluorescein diacetate, succinimidyl ester). CFSE is a fluorescent cell staining dye which passively diffuses into cells and it is inherited by daughter cells after each cell division. Due to the progressive halving of CFSE fluorescence within daughter cells following each cell division, this technique allows cell proliferation to be monitored by flow cytometric analysis of the CFSE dilution (Lyons 2000). Serum starvation is recommended before cell proliferation assays to synchronize all cells in the G0 phase and also to switch off any signalling pathways that could have been activated by the IL-2 added to the cell culture. Following serum starvation, cells were activated with anti-CD3/anti-CD28 antibodies for 5 days in the absence of IL-2 and analysed by flow cytometry (Figure 4-11.A).

Cell proliferation analysis showed that the great majority of the cells activated with anti-CD3/anti-CD28 underwent more than one cell division during the 5-day period of cell stimulation. Unexpectedly, cells that were transfected with either PKCη or siRNA but left unstimulated also proliferated to a certain extent, as most of these cells underwent one cycle of cell division. This fact indicates that the period of 4 hours for serum starvation prior to the assay was not enough to synchronize all the cells in the G0 phase.

As regards the effect of PKCη knockdown, the cell proliferation profile of the cells transfected with PKCη siRNA was identical to the cells transfected with the control siRNA (Figure 4-11.B). These findings suggest that PKCη does not regulate proliferation of stimulated PBTL in response to anti-CD3/anti-CD28 activation.

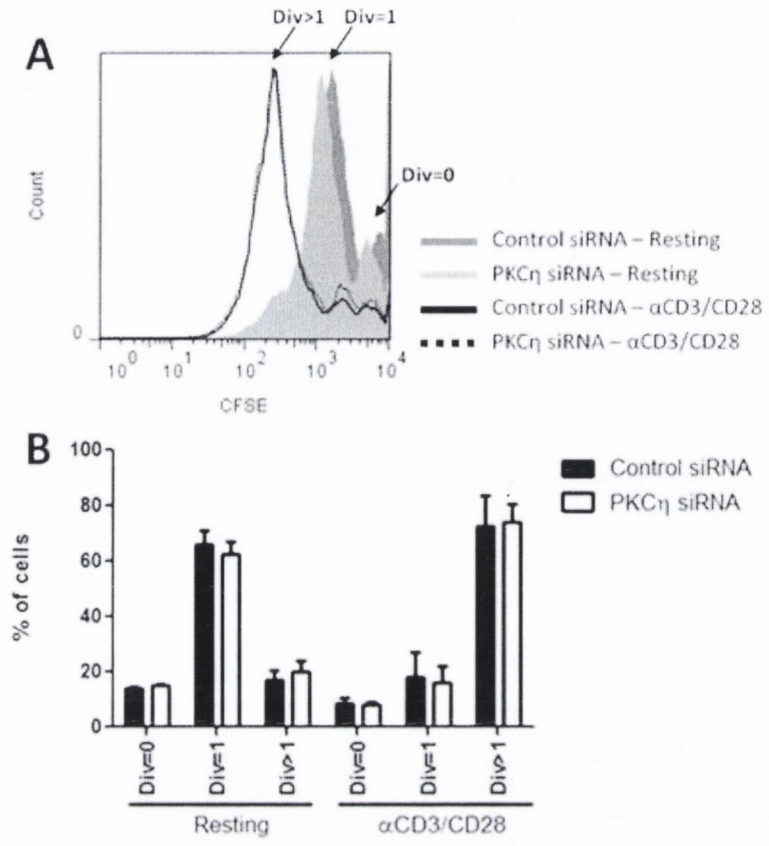


Figure 4-11 Silencing of PKCη expression with siRNA does not affect stimulated PBTL proliferation in response to cell activation with anti-CD3/CD28 antibodies. Stimulated PBTL were transfected with control siRNA or PKCη siRNA following the two-hit protocol described in section 2.11.3 (gene knockdown efficiency is shown in Figure 4-8). The cells were then starved of serum and IL-2 for 4 h and then labelled with 10 μM CFSE. No IL-2 was added to the cell culture after this step. The cells were then assessed for their ability to proliferate when stimulated with cross-linked anti-CD3/CD28 antibodies for 96 hours. Resting cells were used as unstimulated control. Cell division was measured by flow cytometric analysis of the CFSE dilution profile (section 2.15.4). A) Representative overlay histograms showing profile of proliferation in resting and anti-CD3/CD28 activated PBTL. The first peak on the right of the graph represents non-dividing cells (Div=0) and each of the other peaks to the left represent cells that divided only once (Div=1) or more than once (Div>1). Resting cells divided less than activated cells during the 5-day period. There were no differences in the cell proliferation profiles between PKCη siRNA and control siRNA transfected cells. B) Bar chart shows percentage of cells in each division categories considered for the cells analysed in A. Data shown is mean ± S.E.M (n=2 donors).

4.4.2 A role for PKCη in human invariant natural killer T cells (iNKT cells)

4.4.2.1 Silencing of PKCη expression with siRNA does not affect cytokine production and secretion by activated iNKT cells.

To continue with the investigation of a functional role for PKCη in primary human T cells, I had the opportunity to also investigate the requirement for PKCη in cytokine production in the context of cell activation in a very specific subset of T cells, the iNKT cells. iNKT cells are a subset of T cells comprising approximately 0.1% of all peripheral blood T cells that express simultaneously an invariant αβ T cell receptor and surface markers associated with NK cells, such as NK1.1 (Godfrey, MacDonald et al. 2004). These cells are described to present with a memory-activated phenotype, which is acquired before birth (van Der Vliet, Nishi et al. 2000). iNKT cells recognise glycolipid antigens presented by the major histocompatibility complex class I-like molecule CD1d and can be activated *in vitro* by the marine sponge-derived glycolipid α-galactosylceramide, also known as α-GalCer, coupled to a CD1d molecule (Kawano, Cui et al. 1997).

The work in iNKT cells was done in collaboration with Dr. Derek Doherty and his PhD student Yasmeen Ghnewa and it was investigated if silencing of PKCη expression with siRNA had an effect on cytokine production and secretion by iNKT cells upon cell activation. Hence, iNKT cells isolated from 4 different donors were transiently transfected with siRNA (PKCη or control) using the two-hit protocol described in section 2.11.3. These cells were then activated with α-GalCer (see section 2.6.3) or PMA/Io overnight and cytokine production was detected by flow cytometric analysis of intracellular staining (section 2.15.2) and secreted cytokines were measured in the cell supernatant by ELISA (section 2.13.).

Immunoblot analysis showed that iNKT cells express high basal PKCη protein levels, comparable to the ones found for PBTL. Moreover, silencing of PKCη expression with

siRNA in these cells using the two-hit protocol successfully decreased PKCη expression by 85% at the protein level in the cells transfected with PKCη siRNA in comparison to control siRNA (Figure 4-12).

Production of the cytokines IL-2, IL-4, IFN-γ and TNF-α were analysed separately for each $CD4^+$, $CD8^+$ and $CD4^-/CD8^-$ (double negative - DN) iNKT cell subsets by flow cytometric analysis. Cell activation with both α-GalCer and PMA/Io induced production of all the cytokines considered. However, silencing of PKCη did not affect the number of cytokine-producing iNKT cells for any of the four cytokines studied (Figure 4-13).

Additionally, levels of secreted IFN-γ and IL-10 were measured by ELISA in the cell supernatant of the same cells described above. Activation of iNKT cells with PMA/Io induced considerably lower levels of both cytokines compared to activation with α-GalCer. However, no differences were observed in the levels of IFN-γ and IL-10 secreted by iNKT cells when the expression of PKCη was silenced with siRNA (Figure 4-14).

Taken together these findings suggest that PKCη is not involved in cytokine production by iNKT cells in response to cell activation.

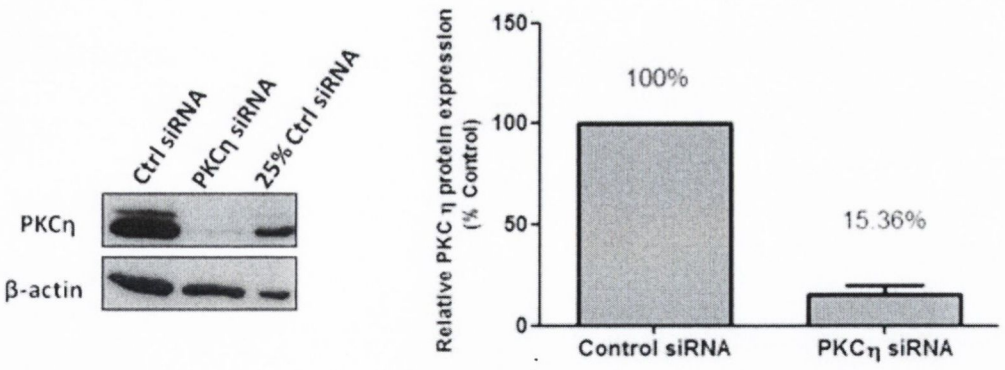


Figure 4-12 Efficiency of silencing of PKCη expression with siRNA in iNKT cells. iNKT cells were transfected with control siRNA or PKCη siRNA (at 1000 nM each) and then re-electroporated under the same conditions 72h later. Whole cells lysates were prepared 48h after the second transfection and PKCη protein levels were determined by Western blotting (left). Densitometric analysis of the blots (right) showed an average of 85% of down–regulation in PKCη expression in cells transfected with PKCη siRNA compared to control siRNA. Western blots are representative and bar chart show mean values ± S.E.M (n=4 donors) of PKCη levels in PKCη siRNA-transfected cells relative to control.

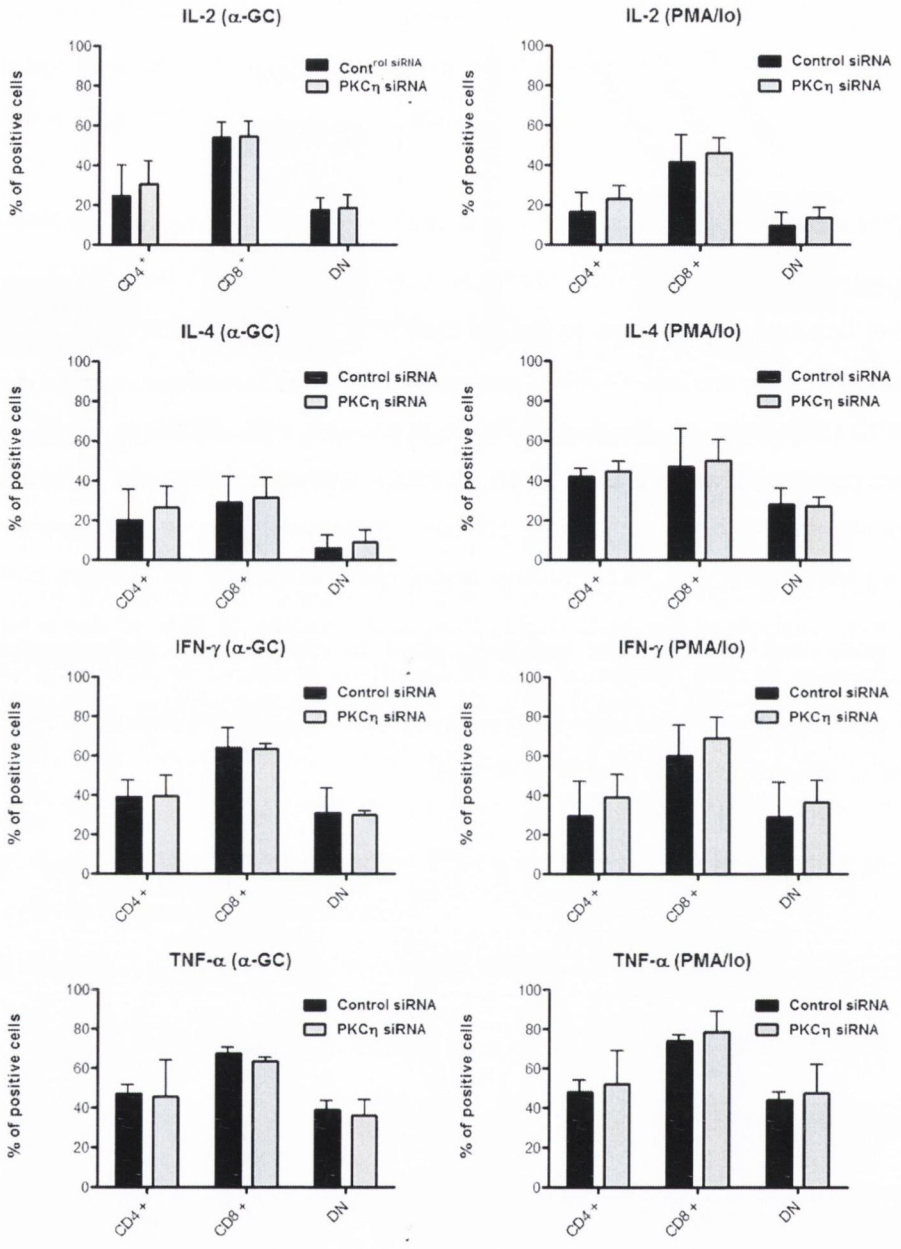


Figure 4-13 IL-2, IL-4, IFN-γ and TNF-α production is not affected in activated iNKT cells following silencing of PKCη expression. iNKT cells transfected with control siRNA or PKCη siRNA following the two-hit protocol described in section 2.11.3 (gene knockdown efficiency is shown in Figure 4-12). The cells were then stimulated overnight with alpha-galactosylceramide(α-GG) pulsed HeLa-CD1d cells or with 50 ng/ml PMA+ 1 ug/ml Ionomycin (Io). Production of the cytokines IL-2, IL-2, TNF-α and IFN-γ was determined by intracellular cytokine detection by flow cytometry separately in the $CD4^+$, $CD8^+$ and double-negative (DN) subsets of NKT cells. Data shown are mean percentages ± S.E.M (n=4 donors).

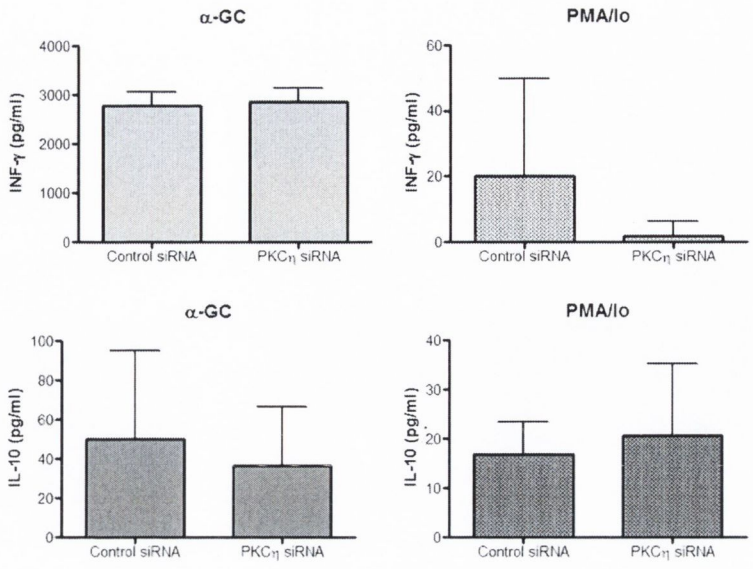


Figure 4-14 IFN-γ and IL-10 secretion is not impaired in activated iNKT cells following silencing of PKCη expression with siRNA. The culture supernatant of the cells analysed in Figure 4.13 were collected and IFN-γ and IL-10 concentration were measured by ELISA. Bar charts show mean ± S.E.M of IL-10 and IFN-γ concentration (n=4 donors).

4.5 Summary

In this chapter, I have further investigated a role for PKCη in cytokine production following cell activation of freshly-isolated PBL. In this study, it was taken into consideration the $CD4^+$ and $CD8^+$ T cell subsets separately and IL-2 and IFN-γ production were analysed in these cells by intracellular cytokine staining following stimulation with anti-CD3/CD28 antibodies or PMA. The results confirmed the observations of the last chapter that silencing of PKCη expression with siRNA does not affect cytokine production in response to cell activation by any of the subsets analysed.

In addition, I have determined by using a siRNA approach, that expression of PKCη is not required in either cell cycle progression or cell differentiation into the effector T cell subsets Th1 or Treg in freshly-isolated PBL following cell activation.

I have also investigated a role for PKCη in a PHA/IL-2 stimulated cell population comprising a majority of T cells, named here stimulated peripheral blood T cells (PBTL). I have shown that PBTL express high basal levels of PKCη, which are not regulated following cell activation with either anti-CD3/CD28 antibodies or PMA. As regards to the function of PKCη in these cells, I have shown that the PKCη isoform is not involved in cytokine production (IL-2 and IFN-γ) or cell proliferation in response to cell activation.

A role for PKCη in the context of cytokine production following cell activation was also investigated in iNKT cells. Here again, knockdown of the PKCη gene in these cells did not affect production of IL-2, IL-4, IL-10, IFN-γ or TNF-α by activated iNKT cells.

4.6 Discussion

PKCη is a novel PKC isoform primarily expressed in epithelial tissues such as lung and skin (Osada, Hashimoto et al. 1993) but also expressed at high levels in T cells (Baier 2003). This isoform has been shown to be involved in cell differentiation, proliferation, secretion, and apoptosis in different cell types (Kashiwagi, Ohba et al. 2002; Hara, Miyazaki et al. 2011; Pal, Outram et al. 2013). PKCη was shown to co-localise with the Golgi apparatus in HeLa cells and PKCη activity is required for protein transport from the trans-Golgi network (TGN) to the cell surface (Diaz Anel and Malhotra 2005). In keratinocytes, PKCη has been shown to be a key regulator of cell differentiation (Cabodi, Calautti et al. 2000; Kashiwagi, Ohba et al. 2002). Moreover, association of PKCη with cell cycle regulators was well as involvement of PKCη in cell proliferation have been described in several MCF-7, glioblastoma and NIH-3T3 cells (Fima, Shtutman et al. 2001; Shtutman, Hershko et al. 2003; Aeder, Martin et al. 2004; Uht, Amos et al. 2007).

Activation of T cells through stimulation of their TCR/CD28 by an antigen peptide bound to a MHC molecule presented on the surface of antigen presenting cells results in activation of a complex signalling cascade that results in cytokine secretion, cell proliferation, and differentiation into the effector T cell subsets (Smith-Garvin, Koretzky et al. 2009; Brownlie and Zamoyska 2013). Differentiation of activated T cells into the various subsets of effector cells is critical to an effective cell-mediated immune response. Depending on the nature of the antigenic stimulus, as well as the cytokine milieu present in the microenvironment, the activated T cells can differentiate, among other recently described subsets, into Th1 cells (developed mainly following intracellular infection by bacteria and some viruses); Th2 cells (predominantly formed to support humoral and allergic responses); Th17 cells (induced in response to extracellular bacteria and fungal infections) or regulatory T cells (Treg), responsible for immunomodulation (Luckheeram, Zhou et al. 2012). The requirement of PKCη expression for differentiation of human T cells into the effector subsets was investigated in the present study.

T cell differentiation can be achieved *in vitro* from naive $CD4^+$ T cells by addition of specific polarization cytokines to cells activated with anti-CD3/CD28 antibodies (Zhu, Yamane et al. 2010). It has been shown that neutralization of IL-4 and addition of IL-12

to the culture are essential to Th1 differentiation in vitro (Hsieh, Macatonia et al. 1993). In this study, addition of IL-12 only to the culture was used to induce Th1 cells and it was shown to effectively induce Th1 polarization. In Treg cell polarization, IL-2 and TGF-β have been shown to be essential in the differentiation process (Chen, Jin et al. 2003; Zheng, Wang et al. 2004) and addition of these two cytokines to the cell culture resulted in an increase, although not significant, in the number of Treg cells. However, silencing of PKCη expression with siRNA did not affect T cell differentiation into either the Th1 or Treg subsets. In turn, differentiation of human T cells into Th17 cells was shown to require the cytokines IL-1, IL-6 and IL-23 (Chen and O'Shea 2008; Chung, Chang et al. 2009). Although all these cytokines described were used in this study to induce Th17 cell polarization, no significant increase in the number of Th17 cells was observed in comparison to the control group. For this reason it was not possible to make any conclusions regarding the involvement of PKCη in the differentiation of Th17 cells. It is important to note that in this study, PBL were used as the starting cell population in the T cell differentiation assays. PBL is a mixed population consisting of approximately 60% of $CD4^+$ T cells (data not shown) and therefore could explain the sub-optimal results obtained in the experiments described here.

In T cells, PKCη has been shown to be involved in signal transduction in a pathway leading to cytokine production in a mouse model. However, a redundant role for PKCη and PKCε was suggested in this context, as only knockdown of both isoforms together reduced the production of IL-2 and IFN-γ in response to T cell activation (Quann, Liu et al. 2011). In the present study, PKCη was not involved in cytokine production in PBL, stimulated PBTL or iNKT cells following cell activation. These results corroborate the idea of a redundant role of PKCη and PKCε in cytokine production in T cells, which were described to act together in the recruitment of PKCθ to the immunological synapse, an event necessary for translocation of transcription factors and consequent cytokine production (Quann, Liu et al. 2011).

Fu et al. (2011) have presented some redundant and specific functions for PKCη and PKCθ in T cell proliferation. $PKC\eta^{-/-}$ T cells showed poor proliferation in response to antigen stimulation, a characteristic shared with $PKC\theta^{-/-}$ T cells. However, only $PKC\eta^{-/-}$ T cells showed abnormal homeostatic proliferation, which is dependent on interaction with self-antigens. In the present study, however, silencing of PKCη expression with

siRNA did not affect cell cycle progression in resting PBL or cell proliferation in stimulated PBTL in response to activation with anti-CD3/CD28 antibodies.

In addition to the functional studies of PKCη, the expression and regulation of PKCη expression in response to cell activation in the stimulated PBTL population was investigated. The process to obtain stimulated PBTL involves stimulating freshly-isolated PBL with PHA followed by a period of IL-2 stimulation. The resulting cell population consist of >98% of T ($CD3^+$) cells (Lefort and Kim 2010). Western blot analysis showed that the total protein levels of PKCη are higher in the PBTL than the original PBL population. It has been shown that levels of PKCη expression vary among the hemopoietic cells and T cells express this PKC isoform at very high levels compared to B cells (Mischak, Kolch et al. 1991). Therefore, these observations suggest that the increased protein levels of PKCη observed in PBTL is likely to be a result of a selective enrichment of T cells in this population, which express high basal levels of PKCη. Moreover, it was described in Chapter 3 that activation of HuT78, Jurkat and PBL with anti-CD3/CD28 antibodies or with PMA resulted in up-regulation of PKCη expression in these cells. Up-regulation of PKCη was also described in EL4 thymoma cells and breast cancer cells following stimulation with phorbol esters (Resnick, Luo et al. 1997; Pal, Outram et al. 2013). In contrast, PKCη protein levels remained constant in PBTL following cell activation with anti-CD3/CD28 antibodies or PMA for 24 hours, showing that PKCη expression was distinctly regulated between PBTL and the other cell types studied in the previous chapter (HuT78, Jurkat, PBL).

It was not possible to identify a function for PKCη in the context of T cell activation in the models of primary human lymphocytes used in this study. The technique chosen to investigate a functional role for PKCη comprised of gene silencing through transfection of the cells with a small interfering RNA (siRNA) targeting the PKCη gene. Unstimulated primary T cells are known to be a hard-to-transfect type of cell and the average of 50% decrease in PKCη expression achieved by the method for PBL may have not been sufficient in the functional study. On the other hand, the two-hit technique used for PKCη silencing in the stimulated T cells proved to be more efficient and yet no differences in cytokine production were observed for stimulated PBTL and iNKT cells.

Although primary human lymphocytes are the ideal model to study T cell biology *in vitro*, the use of these cells in experimentation has some limitations. PBL are a mixed cell population and do not survive for long periods in culture. Also, the low efficiency in silencing of gene expression in these cells can mask real results in functional assays. Stimulated PBTL, in turn, are easier cells to work with. They are bigger cells than PBL, are more amenable to transfection with siRNA and thus exhibit higher transfection efficiencies. However, these cells are derived using an expansion process of stimulation with PHA and IL-2, which results in activation of several signalling pathways and induction of activation markers that need to be taken into account when using these cells for experimentation (Cooper and Pellis 1998; Bartelt, Cruz-Orcutt et al. 2009; Malek and Castro 2010). Furthermore, the evidence of redundant roles with the PKCε and PKCθ isoforms, increases the difficulty in determining a specific function for PKCη in T cells.

Apart from the iNKT cells that are a more homogeneous cell population, in which PKCη was shown not to be involved in cytokine secretion following activation, PBL and PBTL comprise of a mixed T cell population that have different functions in the immune system. The distinct results in relation to expression levels of PKCη and its regulation following cell activation in PBL and PBTL indicate that different cell subpopulations may be interfering with the results and a more thorough investigation of the role of PKCη should be carried out in the isolated subsets of T cells.

5 Chapter 5 - Regulation of expression and function of PKCη in $CD4^+$ T cells

5.1 Introduction

Protein kinase Cη (PKCη) is a member of the novel PKC subfamily that shows high structural similarity with PKCε, another member of the novel PKCs. PKCη is activated by dialcylglycerol (DAG) but is insensitive to Ca^{2+} signalling (Baier 2003; Newton 2010). PKCη has been shown to be involved in cell differentiation, secretion, proliferation and cell survival in many different cell types, including keratinocytes, glioblastoma and breast cancer cells (Diaz Anel and Malhotra 2005; Hara, Miyazaki et al. 2011; Pal, Outram et al. 2013).

As mentioned previously, PKCη has recently emerged as a key player on the biology of T cells, which have been shown to also express PKCη at high levels (Baier 2003; Fu and Gascoigne 2012).

Studies using mouse models have shown PKCη to translocate (together with PKCε isoforms) to the immunological synapse following cell activation with both isoforms operating redundantly to promote the recruitment of PKCθ to the synapse (Quann, Liu et al. 2011). This localisation and activity of PKCθ is required for the activation of downstream pathways leading, in turn, to activation of transcription factors core to T cell activity and function (reviewed in Pfeifhofer-Obermair, Thuille et al. 2012). Moreover, a detailed investigation of PKCη in T cells using a PKCη-knockout mouse model suggested that PKCη is required for T cell activation and proliferation (Fu, Hu et al. 2011). In this study, it was demonstrated that there is functional redundancy for PKCη and PKCθ in T cell activation, cytokine production and cell proliferation in response to antigen stimulation, but only PKCη was critical for homeostatic proliferation, as $PKC\eta^{-/-}$ T cells showed impaired proliferation in lymphopenic animals.

Even with this recent evidence suggesting PKCη as a key player in T cell biology, much work has yet to be done to elucidate the specific role of PKCη in this context. Also, little is known about the regulation of PKCη gene expression in T cells and therefore it is important to investigate the mechanisms involved in this process. Moreover, the majority of studies on PKCη in T cells have been carried out in mouse models or T cell lines and

no information about the role of PKCη has been described for primary human T cells. In the previous chapters of this study, I have shown that the expression of PKCη is distinctly regulated in T cell lines and primary T cells. Furthermore, the different levels of PKCη protein expression in freshly-isolated PBL and stimulated PBTL described in Chapter 4 suggest that PKCη may be distinctly regulated and may even play distinct functions within different subpopulations of T cells. For this reason, in this part of the study it was decided to continue the investigation of a role for PKCη in the context of T cell activation focused on the $CD4^+$ subset of T cells.

$CD4^+$ T cells are also known as T helper cells and play a central role in the function of the immune system, mediating their function by production a wide variety of cytokines. They help B cells to produce antibodies, regulate $CD8^+$ T cells responses and macrophage functions, orchestrate immune responses against a wide variety of pathogenic microorganisms, and regulate/suppress immune responses to control autoimmunity and to adjust the magnitude and duration of responses (reviewed by Zhu, Yamane et al.2010; Luckheeram, Zhou et al. 2012). $CD4^+$ T cells mediate their function in the immune system by production of a wide variety of cytokines, including IL-2. IL-2 is a central player for T cell-dependent immune responses, at the same time inducing T cell growth and proliferation but also regulating development and peripheral expansion of $CD4^+CD25^+$ regulatory T cells (reviewed by Nelson 2004; Malek 2008). The study using $CD4^+$ T cells from PKCη-deficient mice (Fu, Hu et al. 2011) showed that expression of PKCη was required for IL-2 production, which was confirmed in my previous experiments with T cell lines (HuT78 and Jurkat). However, silencing of PKCη expression did not have an effect in IL-2 production in PBL. This suggests other lymphocytes rather than $CD4^+$ T cells may be masking the requirement for PKCη in IL-2 production in this subset and therefore a more specific investigation in the $CD4^+$ T cells would be necessary. In addition, PKCη expression was shown to be distinctly regulated following activation across all cell types studied, emphasising the importance to investigate the regulation of PKCη expression also in $CD4^+$ T cells.

5.2 Aims and objectives

The overall aims of this chapter were to investigate the regulation of PKCη expression and a role for PKCη in response to activation of primary human $CD4^+$ T cells. To achieve this goal, two approaches have been taken. Firstly, the mechanisms involved in regulation of PKCη expression in $CD4^+$ T cells have been characterised and secondly, a functional role for this PKC isoform in $CD4^+$ T cells in response to T cell activation has been investigated.

To investigate the regulation of PKCη expression in response to T cell activation in $CD4^+$ T cells the objectives were to:

- determine the expression of PKCη at protein and mRNA levels in $CD4^+$ T cells in the resting state and following T cell activation;
- use a panel of pharmacological inhibitors to determine regulators of PKCη protein expression in resting cells and in response to T cell activation;

To investigate the function of PKCη in $CD4^+$ T cells in response to cell activation the objective to was to:

- use two different approaches to identify a PKCη-related phenotype in relation to cell cycle progression, proliferation, survival and IL-2 production in $CD4^+$ T cells following cell activation. These approaches were to: (i) over-express PKCη in $CD4^+$ T cells through the delivery of a recombinant plasmid encoding PKCη and (ii) to silence the expression of PKCη using siRNA.

5.3 Regulation of PKCη expression in $CD4^+$ T cells following cell activation.

Following the decision to investigate a role for PKCη in the context of T cell activation in the different subsets of T cells, $CD4^+$ T cells and $CD8^+$ T cells were purified from the peripheral blood lymphocytes (PBL) population as described in section 2.4.3 and 2.4.4 respectively. Expression of PKCη in the resting state and following cell activation for 24 hours with anti-CD3/CD28 antibodies was analysed separately in these subsets and compared to PBL by Western blot (Figure 5-1). Both $CD4^+$ and $CD8^+$ T cell subsets expressed higher basal levels of PKCη protein compared to the mixed population of PBL. Interestingly, PKCη protein levels were distinctly regulated across the three different cell populations following cell activation with anti-CD3/CD28 antibodies for 24 hours. PKCη levels were up-regulated following activation in PBL (Figure 5-1). However, stimulation of $CD4^+$ T cells with anti-CD3/CD28 antibodies resulted in down-regulation of PKCη protein levels in $CD4^+$ T cells, while the same stimulation had no substantial effect on the expression levels of PKCη in $CD8^+$ T cells. Since basal expression levels of PKCη was higher in the $CD4^+$ T cells in addition to the fact that activation of these cells with anti-CD3/CD28 antibodies resulted in down-regulation of the PKCη protein levels, it was decided to focus the investigation of a role for PKCη in this $CD4^+$ T cell subset in further experiments.

5.3.1 PKCη is expressed at high levels in $CD4^+$ T cells

The $CD4^+$ T cells used in this part of the study were isolated as described in section 2.4.3. The resultant enriched population consisted on average of 97.7% (S.E.M=±1.28% - data not shown) $CD4^+$ T cells.

To understand the role of PKCη in activated $CD4^+$ T cells the expression of this protein was assessed in this T cell subpopulation and compared to the levels of PKCη in resting T cell lines HuT78 and Jurkat as well as in peripheral blood lymphocytes (PBL). PKCη was most highly expressed in $CD4^+$ T cells, while lower levels of expression were

observed in the Jurkat T cell line and in PBL (Figure 5-2). The expression levels of PKCη were lower and/or below the limit of detection in the HuT78 T cell line. Interestingly, PKCη migrated on Western blots as a doublet in $CD4^+$ T cells and Jurkat T cells (Figure 5-2). This may be due to the fact that the PKCη gene has been described to contain two major transcription initiation sites (Bacher, Zisman et al. 1991), or alternatively, it may represent differently phosphorylated forms of the protein.

5.3.2 PKCη expression is down-regulated at protein levels but not at mRNA levels in $CD4^+$ T cells in response to cell activation.

As described above, PKCη protein levels were down-regulated in $CD4^+$ T cells response to cell activation with anti-CD3/CD28 antibodies (Figure 5-1). Following on from this, it was investigated if PKCη down-regulation occurred also at the mRNA level. Hence, PKCη mRNA was monitored in anti-CD3/CD28 activated $CD4^+$ T cells by real-time PCR as described in section 2.12. The analysis showed that activation of $CD4^+$ T cells did not alter PKCη mRNA levels, which did not significantly change from 6 to 24 hours following anti-CD3/CD28 activation (Figure 5-3). These results suggest that PKCη level is regulated at the post-transcriptional level following T cell activation.

Additional analysis showed that activation-induced down-regulation of PKCη protein levels in $CD4^+$ T cells was specific for the PKCη isoform, since none of the other PKC isoforms expressed in T cells (PKC α, β, δ, ε, θ and ζ) showed a decrease in protein levels in response to anti-CD3/CD28 activation after 24 hours (Figure 5.4).

Increasing evidence has demonstrated that the strength of the stimulus by which T cells are activated through their T cell receptor (TCR) is crucial to determine the outcome of the T cell response. For example, a strong stimulus is necessary for intracellular calcium signalling, which is critical in signal transduction (Wulfing, Rabinowitz et al. 1997) and also in the induction of apoptosis (Kishimoto and Sprent 1999; Yu, Martin et al. 2003). In contrast, a low TCR stimulus promotes T cell differentiation into effector T cell subsets, such as Th1, Th2, Treg or Th17. (Tao, Constant et al. 1997; Purvis, Stoop et al. 2010; Molinero, Miller et al. 2011). For this reason, it was investigated if the strength of the stimulus influences the down-regulation of PKCη in response to anti-CD3/CD28

stimulation. $CD4^+$ T cells were therefore stimulated with the original antibody mix consisting of 10 μg/ml of anti-CD3 and 0.1 μg/ml of anti-CD28, and 2-fold serial dilutions thereafter down to a 1:16 dilution (Figure 5-5). After 24 hours of cell activation, the levels of PKCη were equally down-regulated across all the different stimuli (Figure 5-5.A). Also, the levels of secreted IL-2 were measured in the supernatant of the activated $CD4^+$ T cells with different stimulus strength and confirmed that dilution of the anti-CD3/CD28 antibody mix was efficient and delivered weaker signals for T cell activation (Figure 5-5.B). The $CD4^+$ T cells produced gradually less amounts of IL-2 in response to lower stimulus induced by dilution of the anti-CD3/CD28 antibodies (Figure 5-5.B). These findings suggest that PKCη is down-regulated even in response to stimulus that did not result in fully activation of the $CD4^+$ T cells.

To further investigate the regulation of PKCη at the protein level observed, a time-course analysis with time points earlier than 24 hours was performed to determine at which point following cell activation the down-regulation of PKCη at protein levels started. Western blot analysis showed that PKCη protein levels began to decrease at 4 hours following anti-CD3/CD28 activation in $CD4^+$ T cells (Figure 5-6.A). In addition, it was also investigated if the low protein levels of PKCη were maintained for more than 24 hours following cell activation. The results showed that the low PKCη protein levels were maintained at 72 hours following anti-CD3/CD28 stimulation (Figure 5-6.B). Moreover, activation of $CD4^+$ T cells with anti-CD3 antibody alone also resulted in down-regulation of PKCη protein levels at 24, 48 and 72 hours (Figure 5-6.B).

In summary, these results show that PKCη is specifically down-regulated in $CD4^+$ T cells in response to cell activation at the protein level. In addition, the decrease in PKCη is independent of the intensity of the stimulus by which the cells were activated. Furthermore, protein levels of PKCη are gradually decreased from as early as 4 hours upon anti-CD3/CD28 stimulation of $CD4^+$ T cells and the low PKCη protein levels are maintained for at least 72 hours. Also, the fact that PKCη is also down-regulated in $CD4^+$ T cells in response to stimulation with anti-CD3 antibody alone implies that the co-stimulatory signal of T cell activation - in this case delivered by the CD28 antibody - is not necessary in the regulation of PKCη expression.

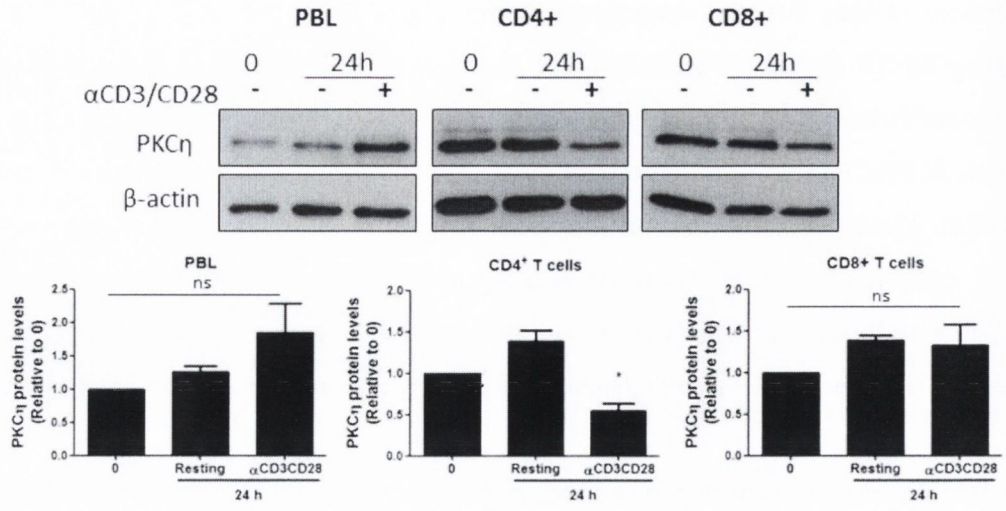


Figure 5-1 Expression of PKCη in resting and activated PBL, $CD4^+$, and $CD8^+$ T cells. Western blot analysis and corresponding densitometric analysis of PKCη protein levels at 0 and 24h in resting (-) and anti-CD3/CD28-activated (+) PBL, CD4+ T cells and CD8+ T cells. 5 μg of total protein extracts were loaded per lane and resolved by SDS-PAGE. Immunoblotting was performed with anti-PKCη antibody and β-actin as a loading control. Western blots are one representative and graphs show mean ± S.E.M. of three experiments with different donors. *p<0.05 using Student's t test as sample significantly differ from 0h time point.

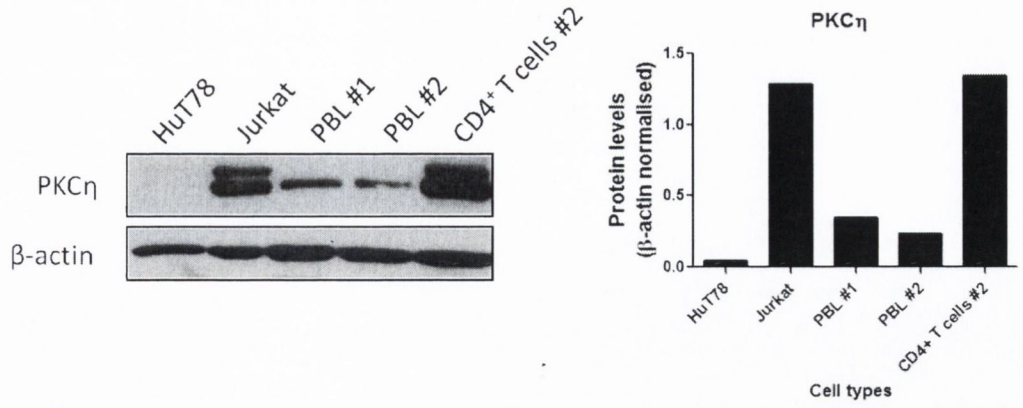


Figure 5-2 PKCη is expressed at high levels in CD4$^+$ T cells. Western blot analysis and corresponding densitometric analysis of PKCη protein levels in HuT78 T cells, Jurkat T cells, PBL from two different donors (#1 and #2) and CD4$^+$ T cells from donor #2. 10 μg of total protein extracts were loaded per lane and resolved by SDS-PAGE. Immunoblotting was performed with an anti-PKCη, while β-actin levels were used as a loading control. Data are representative of two experiments.

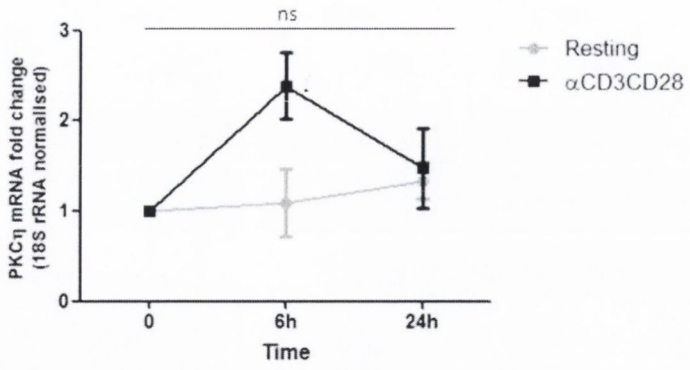


Figure 5-3 Regulation of PKCη expression in CD4$^+$ T cells following cell activation. CD4$^+$ T cells were left unstimulated (resting) or activated with anti-CD3/CD28 antibodies for the given time points and then processed for analysis of PKCη mRNA expression by real-time PCR. The graph shows PKCη mRNA fold change at 6 and 24 hours in resting and anti-CD3/CD28 activated CD4$^+$ T cells in relation to 0 time point. Ns=not significant as samples do not differ significantly from the 0 time point (Student's *t* test). Data show mean values ± S.E.M of three experiments with different donors.

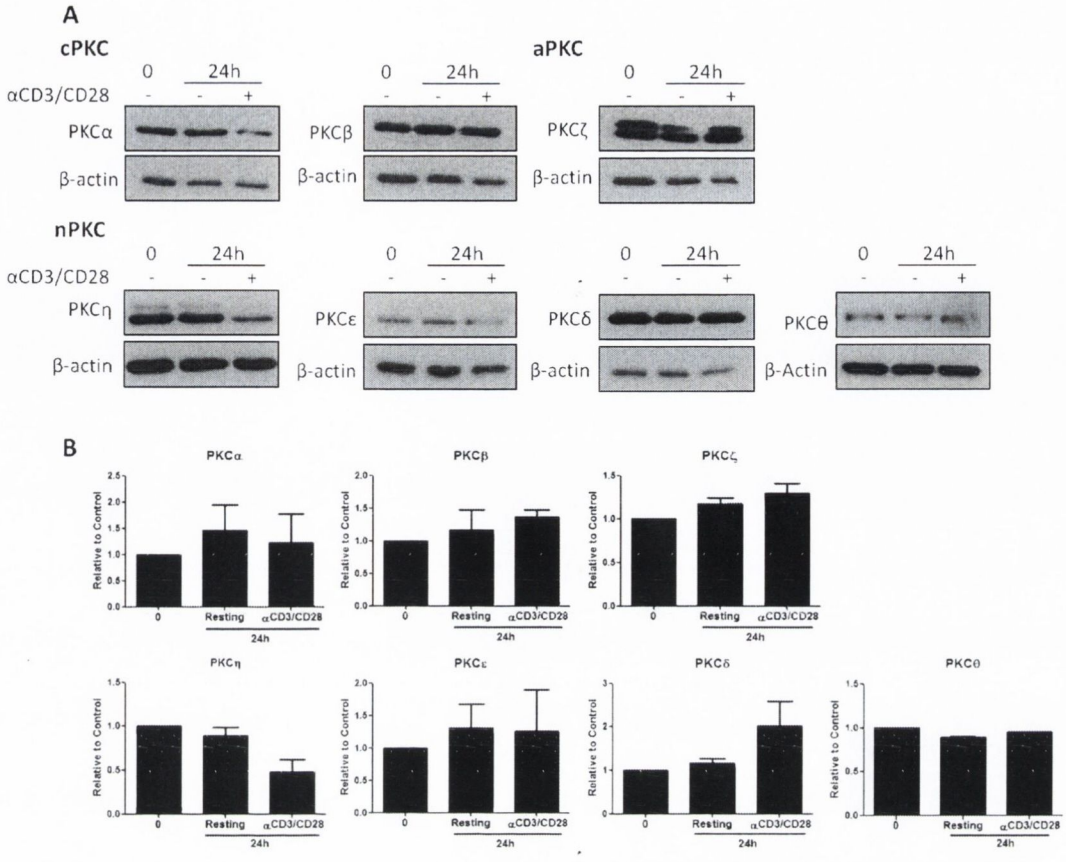


Figure 5-4 PKCη protein levels are specifically down-regulated in CD4+ T cells following activation. A) Western blot analysis of protein levels for conventional PKC (α and β), atypical PKC (ζ) and novel PKC (η,ε,δ and θ) isoforms at 0 and 24 hours in resting and anti-CD3/CD28 activated cells. 5ug of total protein lysates were loaded per lane and β-actin levels were used as loading control. Immunoblots are representative of three experiments with different donors. B) Bar charts show mean±SEM of protein levels of the PKC isoforms analysed in relation to 0 time point based on the densitometric analysis of the Western blots (n= 3 donors).

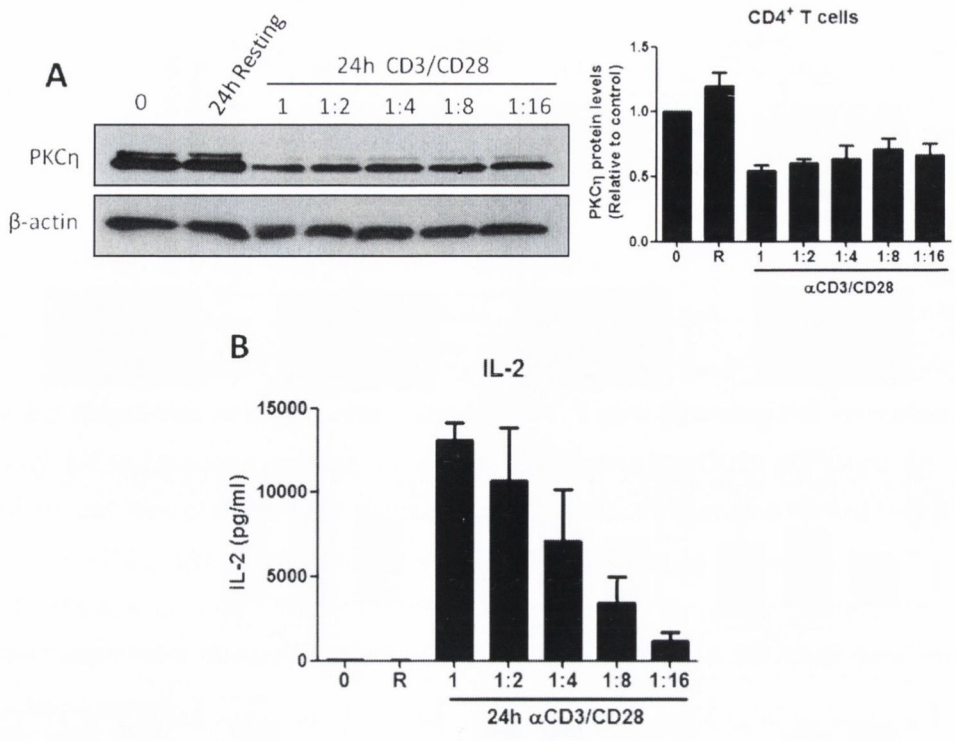


Figure 5-5 Activation-induced down-regulation of PKCη is independent of strength of the stimulus for T cell activation. $CD4^+$ T cells were left unstimulated (resting) or stimulated with 2-fold serial dilutions (starting with 10 μg/ml CD3 and 0.1 μg/ml CD28 down to 1:16 dilution) of a mix of anti-CD3/CD28 antibodies for 24h. A) Western blot analysis (left) of PKCη protein levels in the $CD4^+$ T cells following activation. 5μg of total protein was loaded per lane and β-actin protein levels were used as loading control. Western blot is representative of three experiments with different donors. Bar chart (right) shows mean±SEM of PKCη protein levels in relation to 0 time point based on the densitometric analysis of the Western blots (n= 3). B) Concentration of IL-2 measured in the supernatant of the same cells by ELISA (section 2.13). Data are mean±SEM of experiments from three different donors.

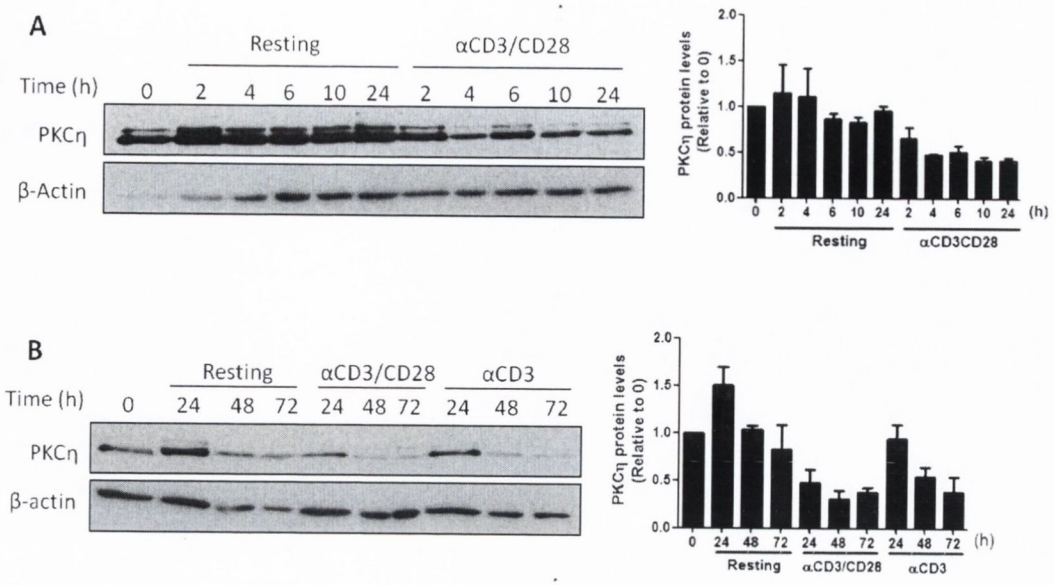


Figure 5-6 Time-course analysis of PKCη protein expression in CD4+ T cells following cell activation. $CD4^+$ T cells were left unstimulated (resting) or were activated with anti-CD3/CD28 antibodies for the given time points. Whole cell lysates were then prepared and PKCη protein levels were analysed in the samples by Western blotting. 5 μg of total protein was loaded per lane and β-actin levels were used as a loading control. A) Immunoblot of PKCη protein levels up to 24 hours following cell activation (left). Representative of three experiments with different donors. Bar chart (right) shows mean±SEM of PKCη protein levels in relation to 0 time point based on the densitometric analysis of the western blots (n= 3). B) Immunoblot of PKCη protein levels at 0, 24, 48 and 72 hours following cell activation (left). Representative of three experiments with different donors. Bar chart (right) shows mean±SEM of PKCη protein levels in relation to 0 time point based on the densitometric analysis of the Western blots (n= 3).

5.3.3 Full-length PKCη protein is cleaved following cell activation in $CD4^+$ T cells.

During the investigation of PKCη down-regulation in response to T cell activation with anti-CD3/CD28 antibodies at the protein level from 24 to 72 hours, it was observed that the decrease in the 78 kDa full-length PKCη protein corresponded with the appearance of a lower molecular weight fragment of approximately 35 kDa (Figure 5-7). The 35 kDa fragment was observed from 48 hours following activation of $CD4^+$ T cells with anti-CD3/CD28 antibodies and was still present at 72 hours. A second fragment of approximately 50 kDa was also observed, although inconsistently, in both resting and activated cells and its levels remained constant across all samples.

The presence of a fragment with smaller molecular weight in addition to the observation that PKCη mRNA levels were not altered following cell activation supported the idea that PKCη was being processed and down-regulated at the protein level. For this reason it was decided to investigate the mechanisms behind the degradation of PKCη in this context.

In eukaryotic cells, the majority of the proteins are degraded via the ubiquitin-proteasome pathway. In this pathway, the proteins are targeted for degradation by addition of ubiquitin markers which in turn are recognized by a large protease complex named the proteasome, responsible for digesting the protein back into amino acids (Lecker, Goldberg et al. 2006). Also, protein degradation by the action of caspases has been described. Caspases are a family of aspartate-specific proteases which mediate their effects through cleavage of their protein target. Usually, the action of caspases is related to the induction and transduction of apoptotic signals, but they have also been involved in many other aspects of cell biology, such as cell proliferation and differentiation (Launay, Hermine et al. 2005; Pop and Salvesen 2009).

To investigate whether any of these mechanisms described for protein degradation are involved in the activation-induced down-regulation of PKCη at the protein level, $CD4^+$ T cells were treated with a proteasome inhibitor (MG132) or a pan-caspase inhibitor (Z-

VAD-FMK) prior to cell activation with anti-CD3/CD28 and the effect of these inhibitors in regulation of PKCη protein levels was analysed.

The proteasome inhibitor MG132 is known to be toxic to cells and causes cell death at high concentrations or after prolonged treatment. Therefore, the effect of pre-treatment with MG132 on PKCη protein levels was assessed at 24 hours following $CD4^+$ T cell activation and not at further time points. Western blot analysis showed that the decrease in PKCη protein levels was insensitive to treatment with the proteasome inhibitor (Figure 5-8.A) and implied that PKCη was not degraded via the ubiquitin-proteasome pathway.

On the other hand, activation-induced down-regulation of PKCη protein levels in $CD4^+$ T cells was blocked following treatment with the caspase inhibitor Z-VAD-FMK (Figure 5-8.B). The Western blot analysis showed that the PKCη protein levels in the samples treated with Z-VAD-FMK remained constant following activation of CD4+ T cells with anti-CD3/C28 for up to 72 hours while PKCη levels were decreased in the activated but untreated samples. Moreover, the 35 kDa fragment of PKCη observed in the activated samples was not present when the activated $CD4^+$ T cells were pre-treated with the caspase inhibitor (Figure 5-8.B).

Taken together, these results suggest that down-regulation of PKCη protein levels following activation in $CD4^+$ T cells is independent of the ubiquitin-proteasome pathway but it is a result of cleavage by caspase(s).

As mentioned before, caspase activity is mainly related to cell death pathways leading to apoptosis. It was thus necessary to investigate if activation of $CD4^+$ T cells with plate-bound anti-CD3/CD28 antibodies was inducing cell death. To do so, induction of apoptosis was examined through binding of annexin V and propidium iodide staining in resting $CD4^+$ T cells and in cells that had been activated with anti-CD3/CD28 for 24, 48 and 72 hours. Activation of $CD4^+$ T cells slightly increased the percentage of cells in early apoptosis (AnV+/PI-) over time, but the overall number of dead cells did not differ from resting cells. (Figure 5-9).

Caspases typically recognise a four amino acid long motif $P_4P_3P_2P_1$ in their target sequences in which the last amino acid P_1 is usually an Aspartic acid (D) (Talanian,

Quinlan et al. 1997). There are several tools available to predict caspase substrate cleavage sites based on the amino acid sequence of the target protein. One of the most used and reliable tools is the CASVM web server (Wee, Tan et al. 2007). Analysis of the amino acid sequence of the PKCη full-length protein using this database in its default settings predicts a single cleavage site at the sequence NVTD ending at amino acid 95 of the protein (Figure 5-10.A). The full-length human PKCη is a 683 amino acid-long protein and is divided structurally into two parts: an amino-terminal regulatory domain comprising the C1 domain that binds DAG, and a C2 domain that in PKCη lacks the binding site for Ca^{2+} and a C-terminus catalytic domain, which is connected to the regulatory domain by a hinge region called V3 (Figure 5-10.B). Caspase typically cleave PKC isoforms at the V3 hinge region, releasing the auto-inhibitory amino-terminal regulatory domain from the catalytic domain (Steinberg 2008). Hence, the catalytic domain is constitutively active and can signal in the absence of lipid co-factors. The structure of the PKCη C2 domain has been solved and consists of residues 1 to 138 of the protein (Littler, Walker et al. 2006). Taken together, these data suggest that PKCη is a substrate for caspase(s) and has a predicted cleavage site within the C2 domain. Considering that the full-length PKCη has a molecular weight of 78 kDa, cleavage of the molecule at residue 95 would generate a fragment of approximately 10 kDa in the amino-terminal domain. Since the anti-PKCη antibody used in this study recognises an epitope at the C-terminus of PKCη, the analysis of cleaved PKCη by Western blotting would be predicted to detect a product of approximately 68 kDa.

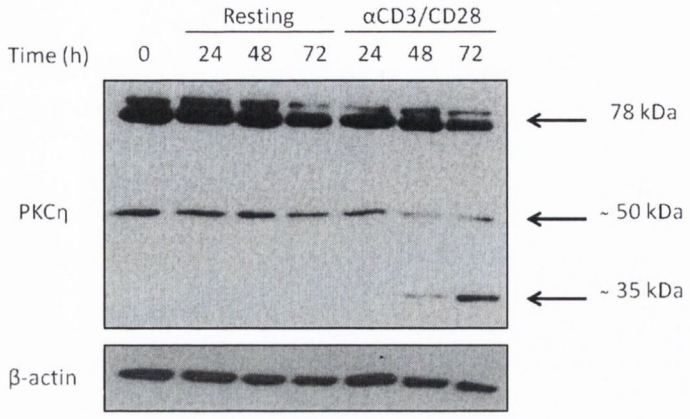


Figure 5-7 Cleavage of the full-length PKCη protein following activation in CD4+ T cells. Western blot analysis of PKCη protein levels at 0, 24, 48 and 72h in resting and anti-CD3/CD28 activated $CD4^+$ T cells. 5 μg of total protein was loaded per lane and β-actin levels were used as a loading control. Western blot is representative of three experiments with different donors.

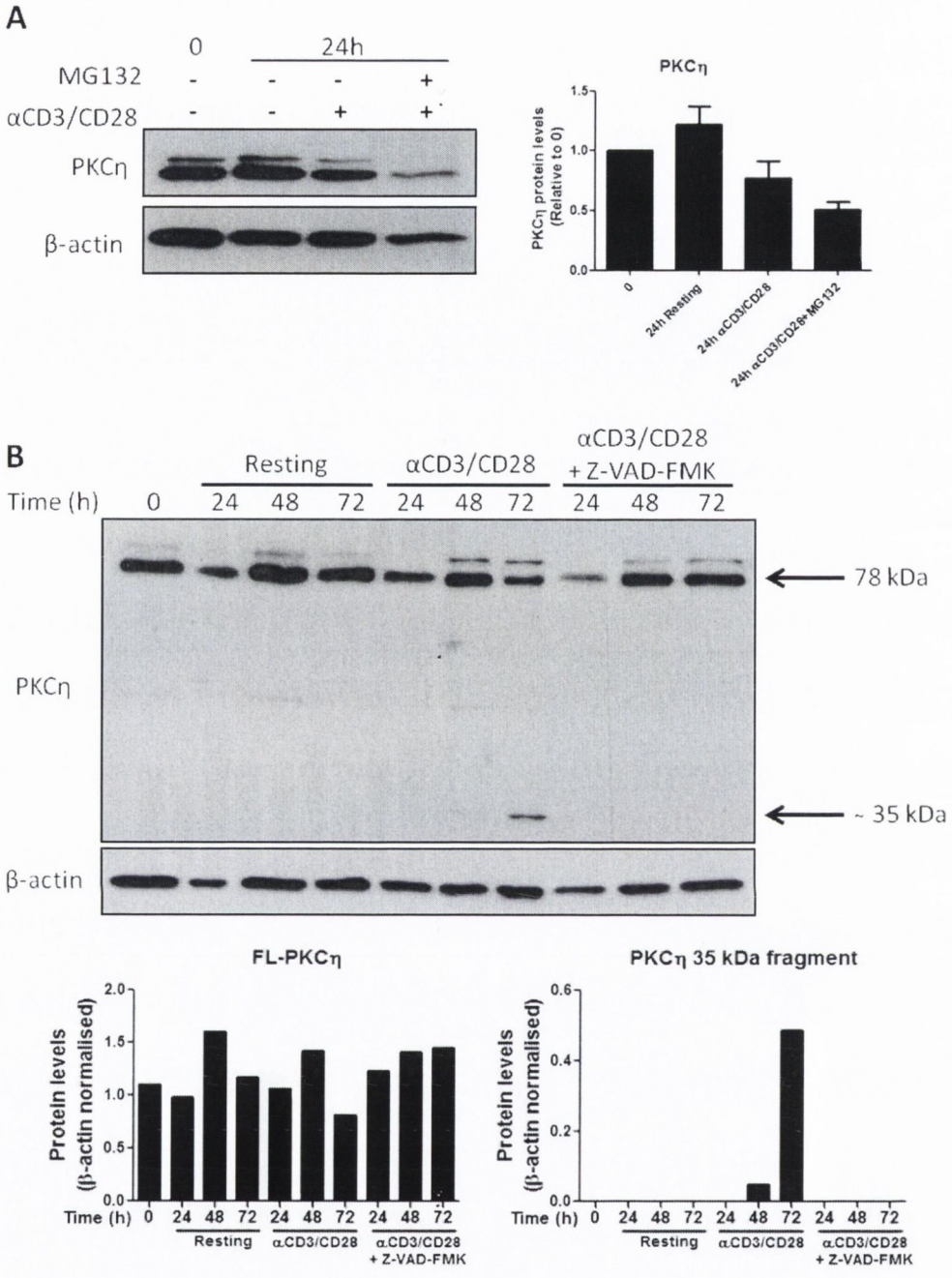


Figure 5-8 Activation-induced cleavage of PKCη in CD4+ T cells is insensitive to treatment with a proteasome inhibitor but is blocked with an inhibitor of caspases. A) Western blot analysis (left) of PKCη protein levels after pre-treatment with the proteasome inhibitor MG132. $CD4^+$ T cells were pre-treated for 1 hour with 10μM MG132 and then left unstimulated (resting) or were activated with anti-CD3/CD28 antibodies for 24 hours. DMSO was used as vehicle control in the resting and activated samples. PKCη protein levels were analysed by Western blotting. 5 μg of total protein was loaded per lane and β-actin levels were used as loading control. Bar chart (right) shows mean±SEM of PKCη protein levels in relation to 0 time point based on the densitometric analysis of the western blots (n= 2 donors). B) Western blot analysis and

corresponding densitometric analysis of PKCη protein levels in $CD4^+$ T cells following pre-treatment with the caspase inhibitor Z-VAD-FMK (50 μM for 3 hours). The cells were then left unstimulated (resting) or activated with anti-CD3/CD28 antibodies and PKCη protein levels were analysed at the given time points. DMSO was used as vehicle control in the resting and activated only samples. 5 μg of total protein was loaded per lane and β-actin levels were used as loading control. Immunoblots are representative of 2 experiments with different donors.

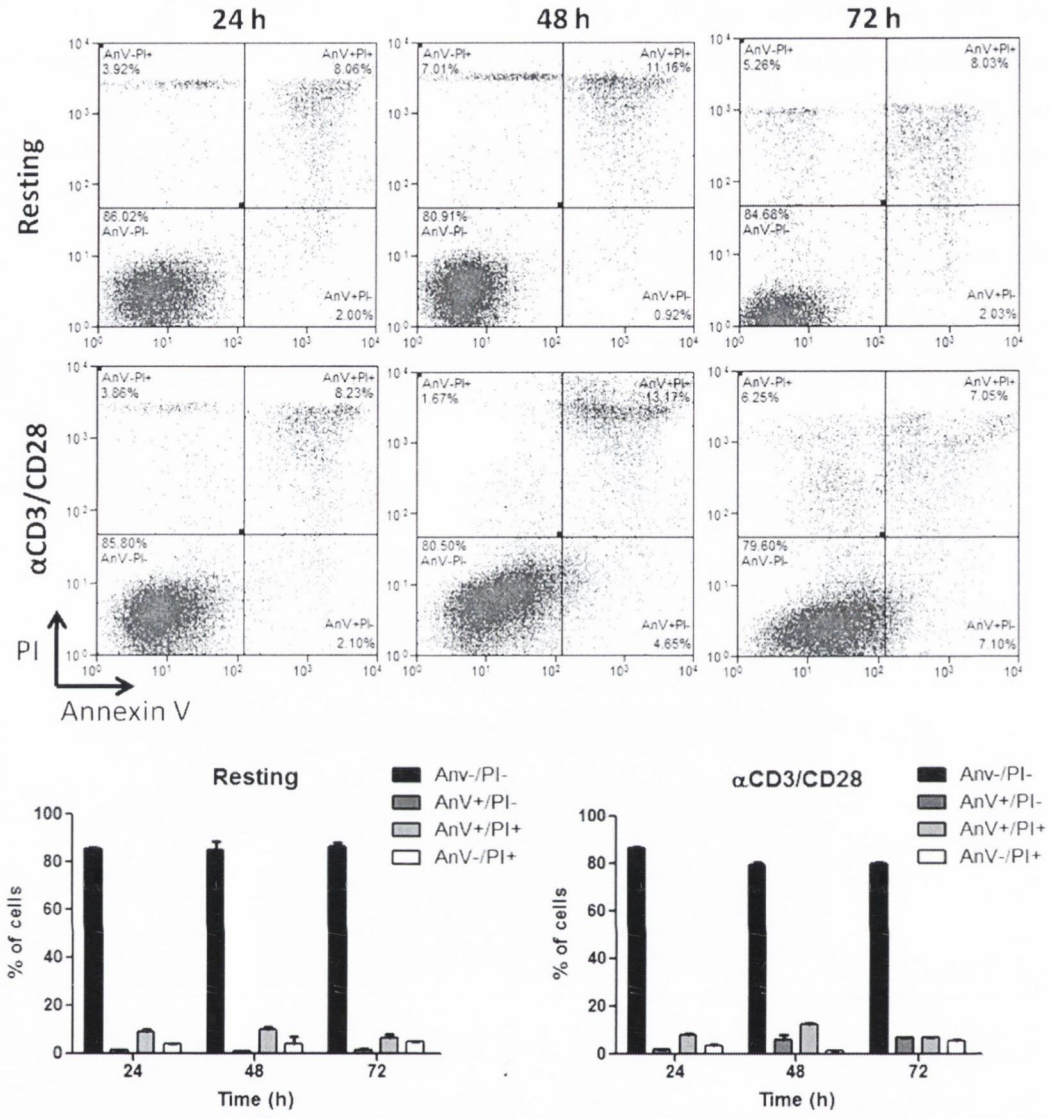


Figure 5-9 Activation of CD4$^+$ T cells with anti-CD3/CD28 antibodies does not induce cell death. Flow cytometric analysis of annexin V (AnV)/propidium iodide (PI) staining of CD4$^+$ T cells that were left resting or were activated with anti-CD3/CD28 antibodies at 24, 48 and 72 hours. Results are representative of a single experiment carried out with CD4$^+$ T cells isolated from two independent donors. The graphs show average percentage ±SEM of viable cells (AnV-/PI-), early apoptotic cells (AnV+/PI-), late apoptotic cells (AnV+/PI+) and necrotic cells (AnV-/PI+) in resting and anti-CD3/CD28 activated CD4$^+$ T cells.

A Protein kinase C eta [Homo sapiens] amino acid sequence

>gi|28557781|ref|NP_006246.2| protein kinase C eta type [Homo sapiens]
MSSGTMKFNGYLRVRIGEAVGLQPTRWSLRHSLFKKGHQLLDPYLTVSVDQVRVGQTSTKQKTNKPTYNE
EFCA**NVTD**GGHLELAVFHETPLGYDHFVANCTLQFQELLRTTGASDTFEGWVDLEPEGKVFVVITLTGSF
TEATLQRDRIFKHFTRKRQRAMRRRVHQINGHKFMATYLRQPTYCSHCREFIWGVFGKQGYQCQVCTCVV
HKRCHHLIVTACTCQNNINKVDSKIAEQRFGINIPHKFSIHNYKVPTFCDHCGSLLWGIMRQGLQCKICK
MNVHIRCQANVAPNCGVNAVELAKTLAGMGLQPGNISPTSKLVSRSTLRRQGKESSKEGNGIGVNSSNRL
GIDNFEFIRVLGKGSFGKVMLARVKETGDLYAVKVLKKDVILQDDDVECTMTEKRILSLARNHPFLTQLF
CCFQTPDRLFFVMEFVNGGDLMFHIQKSRRFDEARARFYAAEIISALMFLHDKGIIYRDLKLDNVLLDHE
GHCKLADFGMCKEGICNGVTTATFCGTPDYIAPEILQEMLYGPAVDWWAMGVLLYEMLCGHAPFEAENED
DLFEAILNDEVVYPTWLHEDATGILKSFMTKNPTMRLGSLTQGGEHAILRHPFFKEIDWAQLNHRQIEPP
FRPRIKSREDVSNFDPDFIKEEPVLTPIDEGHLPMINQDEFRNFSYVSPELQP

B PKCη structure

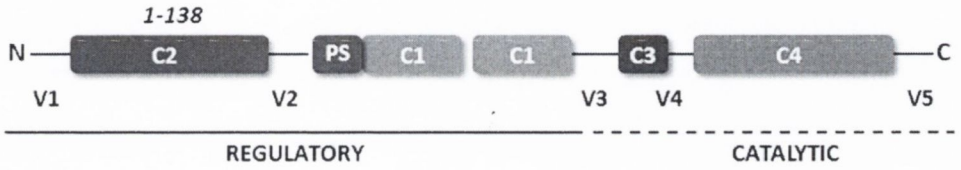


Figure 5-10 Amino acid sequence and structure of the human PKCη protein. A) Amino acid sequence of the PKCη protein in FASTA format obtained in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/protein/28557781?report=fasta). The predicted caspase cleavage site using the CASVM web server is highlighted in bold. B) Schematic representation of the PKCη structure. The numbers above the C2 domain represent the residues that form this domain.

5.3.4 Basal expression of PKCη requires PKC activity while activation-induced down-regulation of PKCη is blocked by the GSK-3 inhibitor BIO-Acetoxime/X in $CD4^+$ T cells.

Stimulation of the T cell receptor (TCR) by an antigen initiates an intricate signalling cascade which results in cell activation and differentiation into effector cells which results in an effective immune response (Salmond, Filby et al. 2009). Multiple protein kinases and phosphatases are activated during this process and are essential for transduction of intracellular signals which culminate in facilitating the T cells to become fully capable of exerting their functions. Three major signalling pathways and their respective main kinases are activated downstream of TCR/CD28 co-receptor: the PI3K pathway, which activates protein kinase B(PKB)/Akt , that in turn is key activator of the the mammalian target of rapamycin (mTOR); the MAPK pathway, which relies on the extracellular signal-regulated kinase (ERK) for signal transduction and the PKC pathway (Smith-Garvin, Koretzky et al. 2009). The glycogen synthase kinase-3 (GSK-3) is also an important kinase activated in T cells following cell activation, where it functions in the inhibition of the nuclear export of the transcription factor NFAT and enhancement of gene transcription (Diehn, Alizadeh et al. 2002; Lovatt and Bijlmakers 2010). Moreover, accumulation of intracellular Ca^{2+} following T cell activation results in activation of calcineurin, a serine/threonine protein phosphatase that is essential for dephosphorylation of NFAT and consequent activation of gene transcription (Macian, Garcia-Cozar et al. 2002). Considering that expression of the PKCη isoform is regulated in response to T cell activation in $CD4^+$ T cells, it was investigated whether any of these effector molecules activated during T cell activation were also involved in regulation of PKCη protein expression.

Hence, $CD4^+$ T cells were pre-treated for a period ranging from 30 minutes to 2 hours (see Table 2-1) with a panel of inhibitors covering the main protein kinases and phosphatases involved in T cell activation. Their effect on PKCη expression was analysed by Western blotting in resting and anti-CD3/CD28 activated cells (Figure 5-11). The panel consisted of the PI3K/AKT inhibitors LY294002 and Wortmannin; the mTOR inhibitor Rapamycin; the MAPK/ERK inhibitor PD98059; the PKC inhibitors

Bisindolylmaleimide (BIS) I, Gö6976 and LY333531; the Glycogen synthase kinase 3 (GSK-3) inhibitors Lithium Chloride (LiCl) and BIO-Acetoxime/X; and the calcineurin inhibitor Cyclosporin A. Because the PKCη gene has been shown to be regulated at the translational level both under normal growth conditions and during cellular stress (Raveh-Amit, Maissel et al. 2009), I also investigated whether Salubrinal, an inhibitor of the eIF2α initiation factor involved in protein synthesis and cell stress (Holcik and Sonenberg 2005), influenced the levels of PKCη expression.

Interestingly, pre-treatment of $CD4^+$ T cells with the PKC inhibitors BIS I and LY333531, but not Gö6976, decreased the PKCη protein levels in both resting and anti-CD3/CD28 activated cells, suggesting that basal expression of PKCη requires PKC activity. BIS I is a broad-spectrum inhibitor of most PKC isoforms whereas Gö6976 is described to inhibit only the conventional PKC isoforms PKCα and PKCβ. LY333531, in turn, is described to selectively inhibit PKCβ. Therefore, with these findings it is not possible to suggest which PKC isoform or group of PKC are regulating PKCη expression, as treatment with Gö6976, which should also inhibit PKCβ did not affect PKCη expression. These findings are in accordance to the results observed in HuT78 and Jurkat T cells and PBL described earlier (Figure 3-12), in which BIS I and LY333531 down-regulated PKCη protein expression in the three cell types while Gö6976 had an effect only on the T cell lines but not in PBL. Moreover, the activation-induced down-regulation of PKCη was blocked by pre-treatment of $CD4^+$ T cells with the GSK-3 inhibitor BIO-Acetoxime/X but not with LiCl (Figure 5-11.A). These observations are not related to cell death because none of the inhibitors tested affected cell viability of the $CD4^+$ T cells after 24 hours of treatment (Figure 5-11.B).

Of the two GSK-3 inhibitors used in the panel, only treatment with BIO-Acetoxime/X had an effect on PKCη protein levels following activation in $CD4^+$ T cells and therefore this inhibitor was the focus of further investigation. Western blot analysis revealed that the effect of Bio-Acetoxime/X treatment in blocking activation-induced down-regulation of PKCη was maintained for up to 72 hours following anti-CD3/CD28 activation of $CD4^+$ T cells (Figure 5-12.A). In addition, this experiment also showed that GSK-3 was inhibited following T cell activation with increased levels of phosphorylation of GSK-3β at Ser9. This is consistent with previous studies demonstrating inhibition of GSK-3 activity following T cell activation (Diehn, Alizadeh et al. 2002; Lovatt and Bijlmakers

2010) (Figure 5-12.A). Phosphorylation of GSK-3β at Ser9 was not observed in the samples treated with the inhibitor suggesting that the mechanism by which BIO-Acetoxime/X inhibits GSK-3 is not reflected in the phosphorylation of Ser9 of this kinase or alternatively, BIO-Acetoxime/X does not inhibit GSK-3 in this model (Figure 5-12.A). The effect of the inhibitor Bio-acetoxime/X in the activation of $CD4^+$ T cells was also analysed both by measuring the amount of secreted IL-2 in the cell supernatant by ELISA and by microscopic analysis of the cells at 72 hours following anti-CD3/CD28 stimulation. While resting $CD4^+$ T cells did not produce detectable levels of IL-2, the anti-CD3/CD28 activated cells secreted high levels of IL-2, which were sustained from 24 to 48 hours and then started to decrease after 72 hours of cell activation (Figure 5-12.B). Interestingly, activated $CD4^+$ T cells pre-treated with Bio-Acetoxime/X did not produce any IL-2 over the 72-hour period, similar to what was observed for resting cells (Figure 5-12.B). In addition, microscopic analysis of the cells at 72 hours following cell activation, demonstrated that while the anti-CD3/CD28- activated $CD4^+$ T were bigger and formed activation-induced clusters, the resting and activated $CD4^+$ T cells that had been treated with Bio-acetoxime/X exhibited comparable morphology, with small, single cells in the whole cell culture (Figure 5-12.C). Taken together, these findings suggest that the GSK-3 inhibitor Bio-Acetoxime/X may be inhibiting T cell activation and not inducing it as expected with inactivation of GSK-3. Consequently, the blockage in the activation-induced down-regulation of PKCη protein levels observed in $CD4^+$ T cells after treatment with Bio-Acetoxime/X could be a non-specific effect of treatment with this inhibitor.

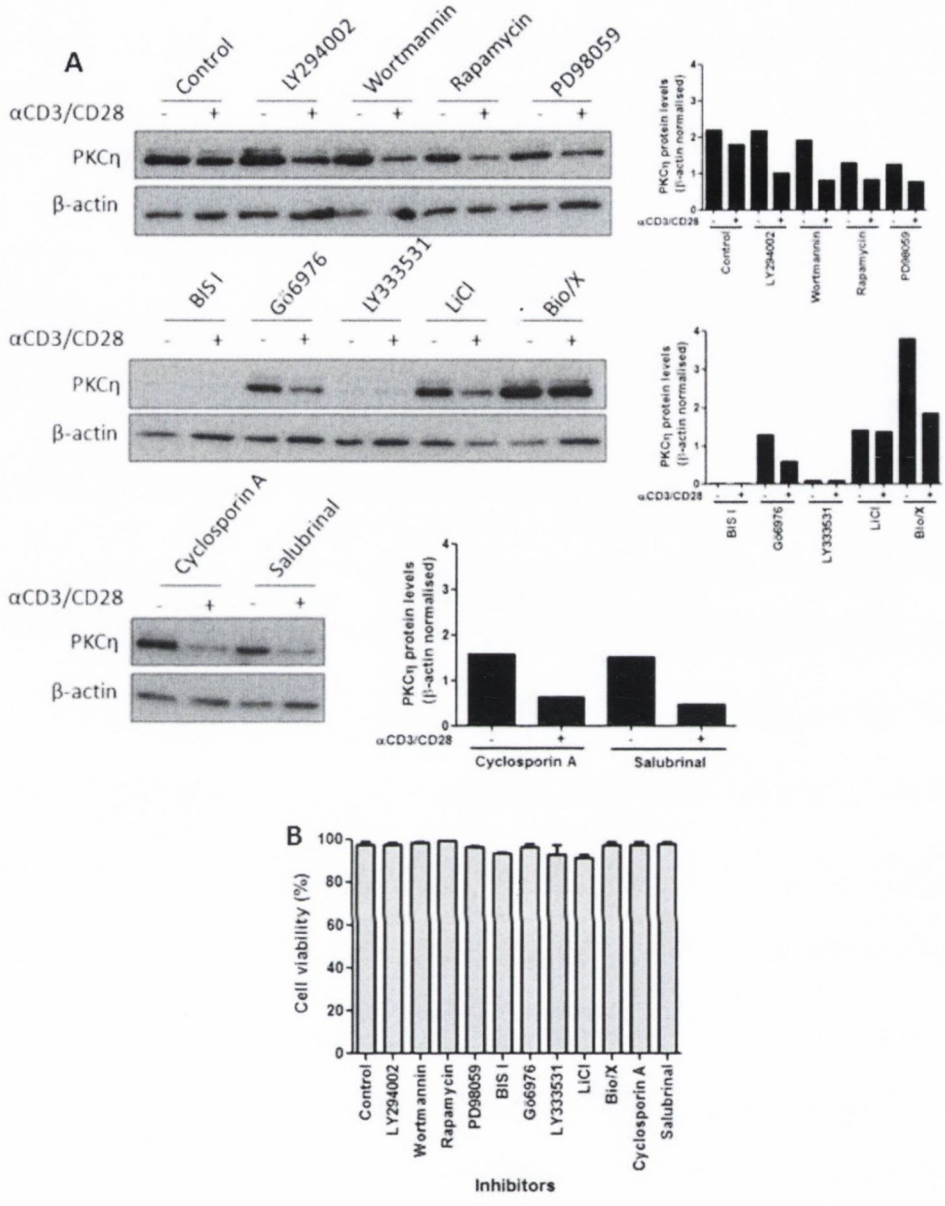


Figure 5-11 Basal expression of PKCη is regulated by PKC activity while pre-treatment with the GSK-3 inhibitor Bio/X blocked activation-induced down-regulation of PKCη. A) Western blot analysis (left) and corresponding densitometric analysis of PKCη protein levels at 24h in unstimulated and anti-CD3/CD28 activated CD4+ T cells after treatment with a panel of inhibitors. The period of pre-treatment and concentrations used for each inhibitor were as follows: LY294002: 1h, 10μM; Wortmannin: 1h, 100nM; Rapamycin: 2h, 100nM; BIM 1: 30min, 10 μM; Gö6976: 30min, 5μM; LY333531: 30min, 5μM; PD98059: 30min, 25 μM; LiCl: 30min, 20mM, Bio/X: 30min, 5μM; Cyclosporin A: 30min, 1μM and Salubrinal: 30min, 1μM. 5μg of total protein was loaded per lane and β-actin protein levels were used as loading control. Immunoblots are representative of five experiments with different donors. B) Percentage of viable $CD4^+$ T cells at 24h following treatment with a panel of inhibitors measured by trypan blue dye exclusion. Data are mean±SEM (n=5 donors).

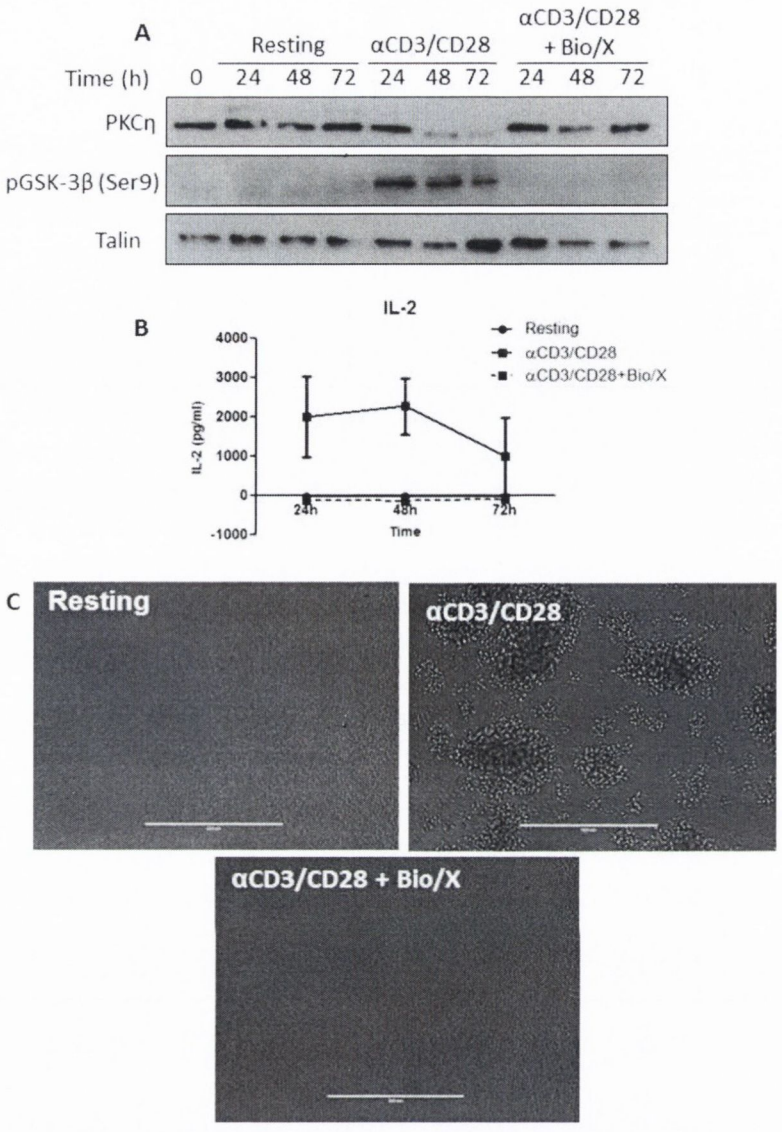


Figure 5-12 Pre-treatment with the GSK-3 inhibitor Bio-acetoxime/X (Bio/X) blocks activation-induced down-regulation of PKCη in CD4$^+$T cells but not in a GSK-3-dependent manner. A) CD4$^+$ T cells were pre-treated with either 5uM of Bio/X or the vehicle control DMSO for 30 minutes and then left resting or were activated with anti-CD3/CD28 antibodies. Total cell lysates were prepared at the given time points and levels of PKCη and phosphorylated GSK-3β (Ser9) were analysed by western blotting. 5ug of total protein was loaded per lane and Talin levels were used as loading control. Immunoblots are representative of experiments performed with cells from three different donors. B) IL-2 concentration measured by ELISA in the supernatant of the CD4$^+$ T cells described in A. Graph shows mean±SEM of IL-2 concentration in pg/ml (n=3 donors). C) Microscopic analysis (10X objective) at 72h of resting and anti-CD3/CD28 activated CD4$^+$ T cells pre-treated or not with 5uM Bio/X. Images are representative of three experiments from different donors.

5.4 Investigation of the role of PKCη in $CD4^+$ T cells in response to TCR stimulation

The second part of this chapter focused on the investigation of a functional role for PKCη in activation of $CD4^+$ T cells. PKCη has already been shown to be involved in several mechanisms of the cell biology of cell types other than T cells that are nonetheless central to the function/regulation of activated T cells. For example, PKCη has been described to be involved in secretory processes (Diaz Anel and Malhotra 2005; Byrne, Foran et al. 2010), regulation of cell cycle progression and cell proliferation (Fima, Shtutman et al. 2001; Shtutman, Hershko et al. 2003; Uht, Amos et al. 2007) as well as in promoting cell survival (Akkaraju and Basu 2000; Rotem-Dai, Oberkovitz et al. 2009). For this reason, a role for PKCη was investigated in IL-2 secretion, cell cycle progression, proliferation and survival of $CD4^+$ T cells in response to cell activation.

Since protein levels of PKCη were down-regulated following anti-CD3/CD28 stimulation in $CD4^+$ T cells, it was decided to over-express PKCη in these cells prior to cell activation. If regulation of PKCη expression is important (i.e. activation-induced decrease of PKCη) then over-expression of PKCη may inhibit T cell activation. $CD4^+$ T cells were therefore transfected with a plasmid DNA encoding the human PKCη gene linked to a FLAG-tag (FLAG-PKCη) to over-express the protein. In this assay, control cells were transfected with the empty vector pcDNA at the same concentration used for the FLAG-PKCη plasmid. The transfection efficiency was measured by Western blotting in which the total levels of PKCη were compared between the FLAG-PKCη and pcDNA transfected $CD4^+$ T cells (Figure 5-13.A). As the FLAG-tag has a very low molecular weight (1 kDa), the exogenously expressed PKCη appeared on Western blots at the same position as the endogenous PKCη. The analysis showed that total levels of PKCη in $CD4^+$ T cells transfected with FLAG-PKCη were on average three times higher (318.75%) than those transfected with the pcDNA control (Figure 5-13.B). Cell viability at 24 hours post-transfection was an average of 50% for both FLAG-PKCη and pcDNA transfected $CD4^+$ T cells (Figure 5-13.C).

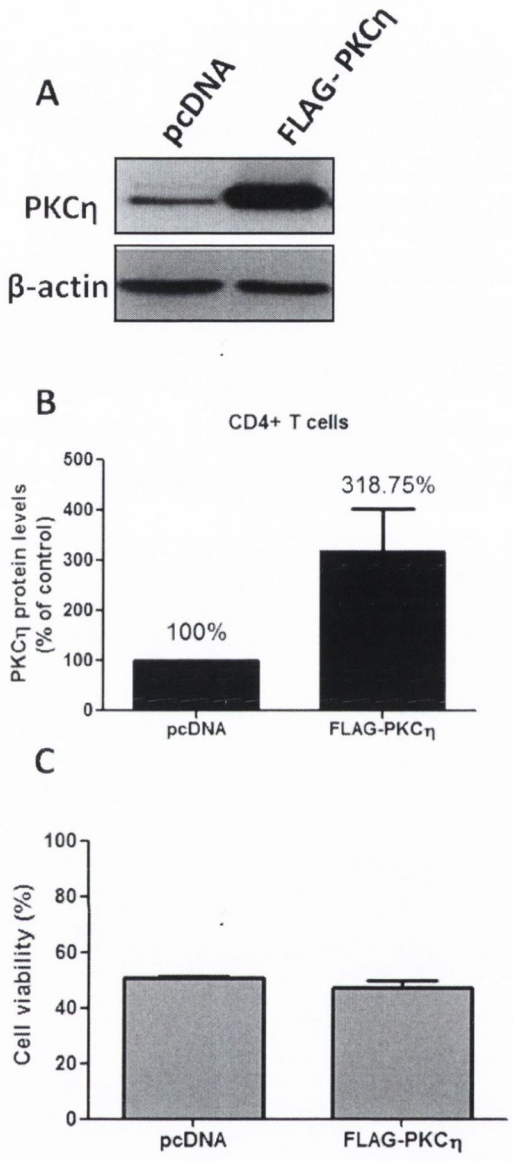


Figure 5-13 Efficiency of PKCη overexpression in CD4$^+$ T cells. Freshly isolated CD4$^+$T cells were transfected with either the empty vector pcDNA or the plasmid FLAG-PKCη (at 5μg each) and left to recover for 24h. Whole cell lysates were prepared and PKCη protein levels were determined by Western blotting (left). Densitometric analysis of the blots (black bars) showed an average of 318.75% overexpression of PKCη protein in cells transfected with FLAG-PKCη in relation to the pcDNA transfected cells. Also, the percentage of viable cells at 24h after transfection was assessed in these cells by Trypan Blue dye exclusion (gray bars). Western blots are representative and bar chart show mean values ± S.E.M (n=5 donors).

5.4.1 Delivery of plasmid DNA by electroporation affects activation of $CD4^+$ T cells.

The first aspect of T cell activation in relation to PKCη to be investigated was IL-2 production by these cells in response to anti-CD3/CD28 stimulation Secretion of IL-2 by activated T cells is essential for cell growth, proliferation and differentiation into effector T subsets (Boyman and Sprent 2012). $CD4^+$ T cells that had been transfected with either pcDNA or FLAG-PKCη as well as untransfected cells were activated for 24 hours with anti-CD3/CD28 antibodies and the concentration of IL-2 secreted was measured in the cell supernatant by ELISA. The results showed that $CD4^+$ T cells expressing FLAG-PKCη produced significantly less IL-2 in comparison to $CD4^+$ T cells transfected with pcDNA following stimulation with anti-CD3/CD28. However, these two groups together secreted considerably less amounts of IL-2 in comparison to the untransfected cells (Figure 5-14) Although these results suggest that over-expression of PKCη impairs IL-2 production following anti-CD3/CD28 in $CD4^+$ T cells, the difference in IL-2 production observed between untransfected and transfected cells (particularly pcDNA-transfected control cells) suggest that these latter cells were not completely activated at 24 hours following anti-CD3/CD28 stimulation (Figure 5-14).

The expression of the activation markers CD25, CD69 and CD71 were also analysed in the context of PKCη over-expression in $CD4^+$ T cells at 24 hours following anti-CD3/CD28 activation by flow cytometry (Figure 5-15). Expression of CD25 was clearly up-regulated in the untransfected cells following activation with anti-CD3/CD28 antibodies with an average of 96.6% positive cells for this marker. However, only around 30% of the transfected $CD4^+$ T cells were expressing CD25. The average percentage of CD25-positive FLAG-PKCη transfected $CD4^+$ T cells was slightly decreased in comparison to pcDNA control (28% against 37% of CD25 positive cells) but when the levels of CD25 expression were considered, by looking at the mean fluorescence intensity (MFI) of the fluorochrome used in the experiment, both cell groups (pcDNA and FLAG-PKCη transfected) showed values that were only slightly increased in comparison to the basal levels found for resting $CD4^+$ T cells (Figure 5-15.A). Regarding expression of CD69 following activation with anti-CD3/CD28, the great majority of all $CD4^+$ T cells

analysed were CD69-positive following activation in all cell groups (untransfected, pcDNA and FLAG-PKCη transfected cells) presenting more than 96% of the cells positive for expression of CD69 (Figure 5-15.B). Analysis of the MFI values for this marker showed that the $CD4^+$ T cells over-expressing PKCη had the smallest CD69 expression levels of all groups (FLAG-PKCη MFI=1347.36; pcDNA MFI=1741.74 and untransfected MFI=2382.69), although the difference among them was not statistically significant (Figure 5-15.B). Considering expression of the CD71 marker, 68.25% of the activated untransfected $CD4^+$ T cells were positive for CD71, against 24.5% and 18.83% of positive cells for pcDNA control and FLAG-PKCη transfected respectively (Figure 5-15.C). However, once again the expression levels of the marker comparing the MFI values for CD71 fluorescence showed that pcDNA and FLAG-PKCη transfected $CD4^+$ T cells were just slightly higher than the MFI values found for resting $CD4^+$ T cells (Figure 5-15.C). Altogether, these results suggest that over-expression of PKCη in $CD4^+$ T cells lessens to some extent the expression of the activation of the markers CD25, CD69 and CD71 in response to stimulation with anti-CD3/CD28 antibodies. However, it is important to note the fact that expression levels of CD25 and CD71 were very similar to the levels found in resting $CD4^+$ T cells. This supports the idea that both pcDNA and FLAG-PKCη transfected cells were not responding normally to anti-CD3/CD28 stimulation. More importantly, the pcDNA transfected control cells should show equivalent levels relatively of markers to the untransfected cells to allow comparison to the FLAG-PKCη transfected $CD4^+$ T cells.

Another aspect of T cell activation investigated was the involvement of PKCη in cell cycle progression in $CD4^+$ T cells in response to anti-CD3/CD28. Under normal conditions, quiescent T cells enter the cell cycle following TCR activation by its cognate antigen, which results in clonal expansion of the activated T cells. The cell cycle is divided into four phases named G1 (preparation for DNA synthesis), S phase (synthesis of DNA), G2 (preparation for division) and M phase or mitosis (cell division). Passage through each of these phases is tightly regulated by four major classes of cyclins A, B, D and E, in association with specific cyclin-dependent kinases (cdks) (Satyanarayana and Kaldis 2009). Entry into the cell cycle and transit through early G1 is regulated by type D cyclins. Among the three D-type cyclins, T cells express only cyclins D2 and D3. Expression of cyclin D2 is rapidly up-regulated in response to TCR stimulation and is described to be a key player in T cells (White, Shore et al. 2006). Cyclin E regulates

transit through late G1 phase and progression into S phase; while cyclins A and B regulate S phase transit and early G2; and progression into M phase respectively. PKCη has been described as a cell cycle regulator in breast cancer cells and keratinocytes where it has been shown to associate with the cyclin E/cdk2/p21 complex (Kashiwagi, Ohba et al. 2000; Fima, Shtutman et al. 2001; Shtutman, Hershko et al. 2003). For this reason, it was also investigated if PKCη was involved in regulation of cell cycle in response to anti-CD3/CD28 activation in $CD4^+$ T cells. It was first examined if over-expression of PKCη had an effect on cell cycle progression following $CD4^+$ T cell activation. To do so, $CD4^+$ T cells transfected with FLAG-PKCη or pcDNA were activated with anti-CD3/CD28 antibodies for 48 hours and DNA content of the cells was stained with propidium iodide and analysed by flow cytometry (Figure 5-16). The analysis showed that neither pcDNA nor FLAG-PKCη transfected cells entered the cell cycle at 48 hours following cell activation. While the untransfected $CD4^+$ T cells showed an average of 20% of the cells in the S phase and 6% in the G2/M phase in anti-CD3/CD28 activated cells, the vast majority of $CD4^+$ T cells that were transfected with pcDNA or FLAG-PKCη remained in the G0/G1 phase of the cell cycle (Figure 5-16). Interestingly, the percentage of apoptotic cells, represented by cells in the Sub G1 phase was slightly increased in the $CD4^+$ T cells over-expressing PKCη in comparison to the pcDNA control in both resting and activated cells (Figure 5-16). Due to the fact that the $CD4^+$ T cells that were transfected with the plasmid DNA did not go into cell cycle, it was not possible to determine the involvement of the PKCη isoform in this context in $CD4^+$ T cells.

Additionally to the cell cycle progression analysis, the role of PKCη in regulation of expression of the cyclins D2 and E in $CD4^+$ T cells in response to cell activation was investigated. Expression of cyclins D2 and E were analysed by Western blotting at 24 hours following anti-CD3/CD28 activation of $CD4^+$ T cells transfected with either pcDNA or FLAG-PKCη and in untransfected cells (Figure 5.17). Unfortunately, expression of cyclin E by these cells, which reportedly migrates on Western blots as a 53 kDa protein, was below the levels of detection using Western blotting and the conditions used. It was only possible to observe very low levels of cyclin E expression at 24 hours following cell activation in the non-transfected cells although the levels observed were sometimes difficult to distinguish from the background (Figure 5-17). In relation to detection of cyclin D2 in these cells, up-regulation of cyclin D2 at 24 hours following anti-CD3/CD28 stimulation was only observed in the non-transfected $CD4^+$ T cells

(Figure 5-17). The $CD4^+$ T cells transfected with FLAG-PKCη and pcDNA did not express detectable levels of cyclin D2 in response to cell activation and therefore no conclusions could be made.

Subsequent to investigation of a role for PKCη in cell cycle regulation, it was examined if over-expression of PKCη had an effect on the proliferation of activated $CD4^+$ T cells. Cell proliferation was analysed by flow cytometry at 96 hours following activation of $CD4^+$ T cells with anti-CD3/CD28 antibodies in cells transfected with FLAG-PKCη or pcDNA and in non-transfected cells (Figure 5-18.A). The analysis showed anti-CD3/CD28 activation induced cell proliferation in untransfected $CD4^+$ T cells. However, no cell division was observed for the FLAG-PKCη or the pcDNA transfected cells (Figure 5-18.A). In fact, all the $CD4^+$ T cells that were expressing FLAG-PKCη were dead at the time point tested as determined by Propidium iodide staining (data not shown). When the morphology of these three groups of $CD4^+$ T cells were examined under a microscope, it was observed that unlike the non-transfected $CD4^+$ T cells that were bigger and formed activation-induced clusters, the cells transfected with plasmid DNA were small, round and resembled resting cells (Figure 5-18.B).

Taken together, the results obtained following transfection of $CD4^+$ T cells with plasmid DNA suggested that the technique employed to deliver the plasmids into these cells affected the cell biology and impaired the response to anti-CD3/CD28 stimulation. Approximately 50% of the $CD4^+$ T cells were viable (as determined by Trypan Blue dye exclusion) after the transfection and within those there were cells successfully expressing the FLAG-PKCη plasmid. However, these cells failed to respond properly to activation with anti-CD3/CD28 antibodies and died after 96 hours in culture even under stimulation conditions. As a result, it was not possible to determine a functional role for PKCη in the context of $CD4^+$ T cell activation using this approach. Even though primary T cells are considered difficult to transfect, the use of plasmid DNA to study gene function in human primary $CD4^+$ T cells has been successfully described before (Tahvanainen, Pykalainen et al. 2006). However, it has been shown recently that transfection of primary T cells with green fluorescent protein (GFP)-tagged plasmids affects T cell activation and cytokine production following *in vitro* stimulation (Koelsch, Wang et al. 2013).

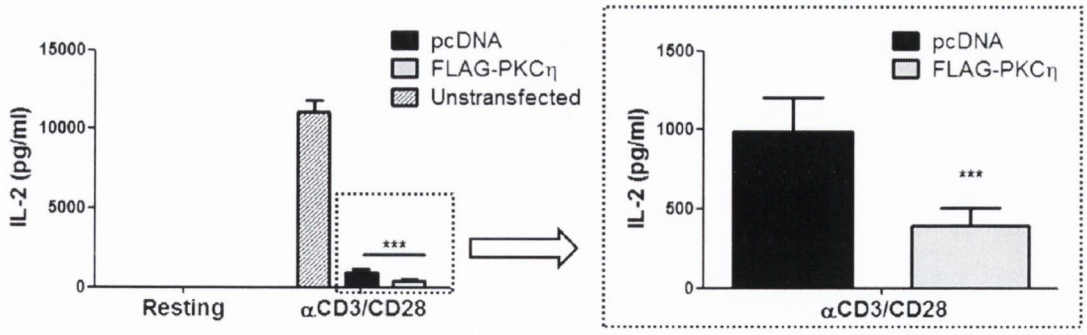


Figure 5-14 IL-2 secretion following anti-CD3/CD28 in $CD4^+$ T cells over-expressing PKCη. IL-2 concentration (pg/ml) was measured in the cell supernatant of $CD4^+$ T cells previously transfected with either pcDNA or FLAG-PKCη at 24 hours following cell activation with anti-CD3/CD28 antibodies and in resting cells. Untransfected $CD4^+$ T cells were used as control for the transfection procedure. Data show mean concentration ± S.E.M of five experiments with different donors. ***=$p<0.0001$ (Student *t* test). Bar chart on the right show the same information as the highlighted part of the graph on the left.

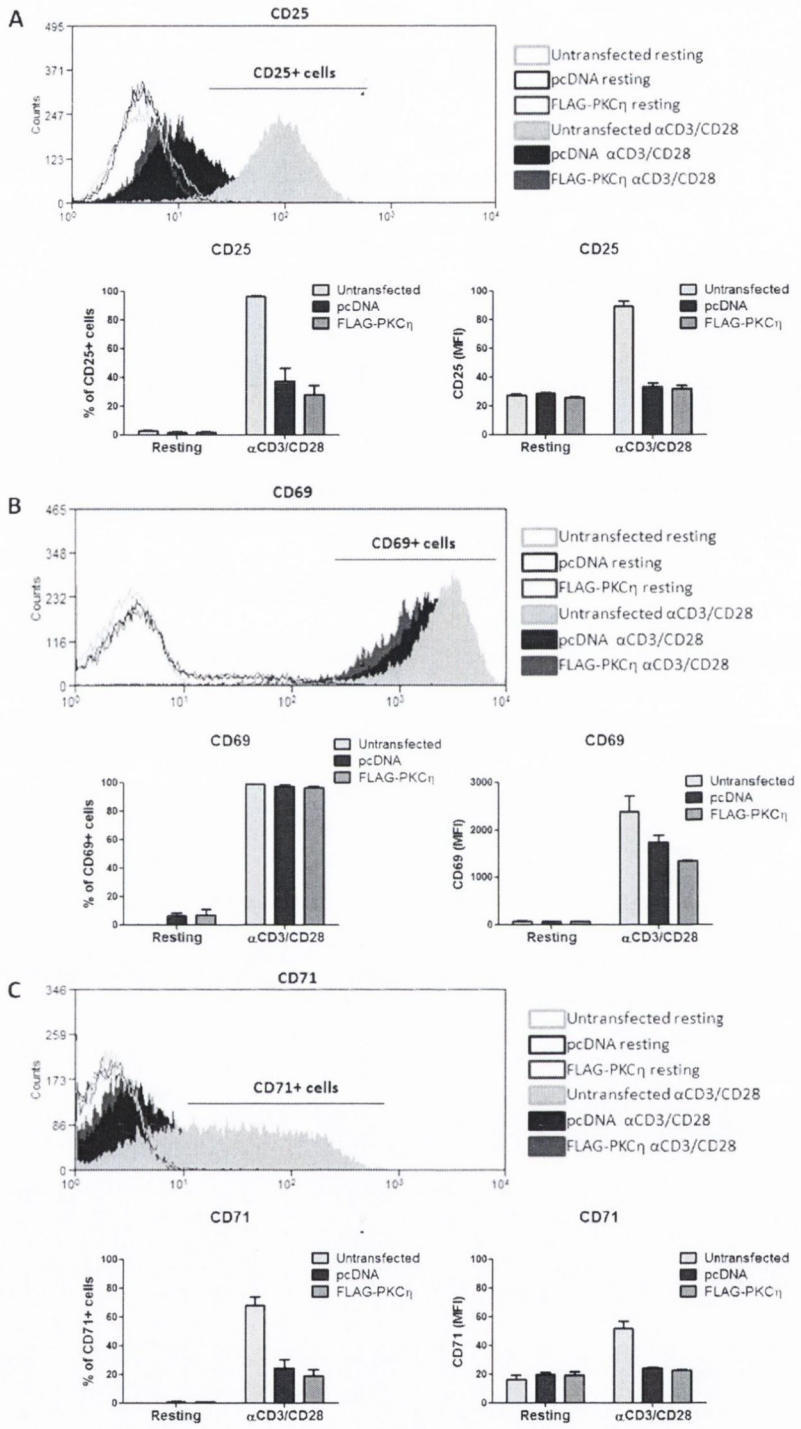


Figure 5-15 T cell activation markers CD25, CD69 and CD71 in CD4$^+$ T cells after overexpression of PKCη. Flow cytometric analysis of CD4$^+$ T cells transfected or not with empty vector pcDNA or FLAG-PKCη (both at 5ug) at 24 hours in resting cells and after anti-CD3/CD28 stimulation. CD4+ T cells were stained for the activation markers A) CD25, B) CD69 and C) CD71. Bar charts show percentage of positive cells for the given activation marker (left) and mean fluorescence intensity (MFI) of the fluorochrome used for detection of the marker (right). Data are mean±SEM of experiments with three different donors.

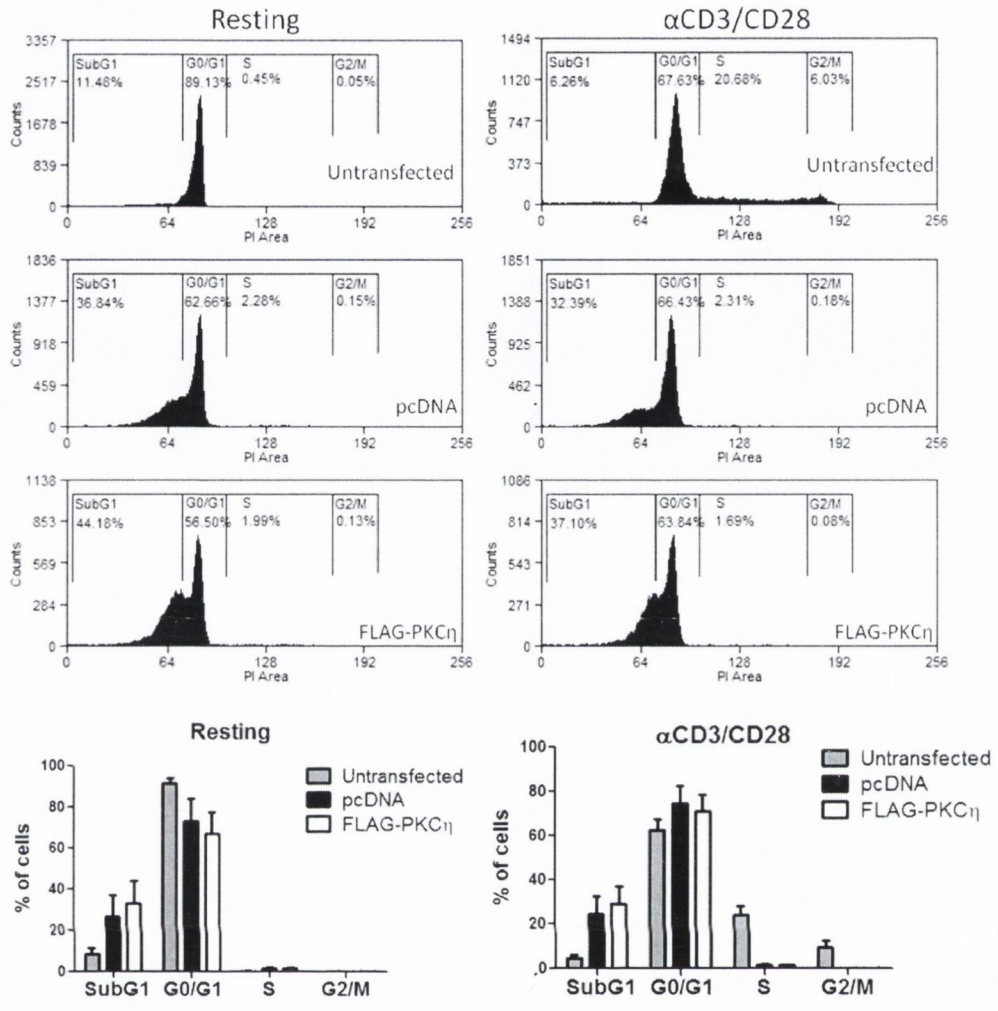


Figure 5-16 Effect of PKCη overexpression in cell cycle progression in CD4$^+$ T cells following cell activation. Flow cytometric analysis of cell cycle progression by propidium iodide (PI) staining at 48 hours in resting and anti-CD3/CD28 activated CD4$^+$ T cells that had been previously transfected with pcDNA or FLAG-PKCη plasmids (at 5μg each). Resting cells were used as unstimulated control while untransfected cells were used as a control for the transfection process. Flow cytometry plots are representative and show gating strategy used to determine percentage of cells in each phase of the cell cycle (SubG1 phase represents apoptotic cells, G0/G1 phase shows 2n/diploid cells, S represents cells undergoing DNA synthesis and G2/M phase shows double diploid cells. Bar charts show mean percentage±SEM of cells in each phase of the cell cycle (n= 2 donors).

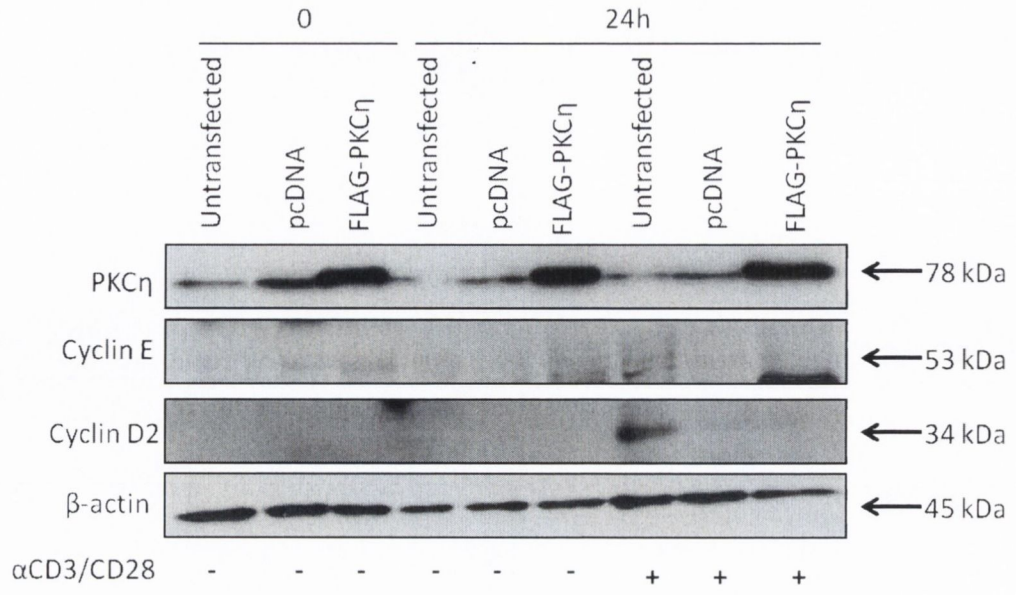


Figure 5-17 Effect of PKCη over-expression in up-regulation of cyclins D2 and E in response to CD4$^+$ T cell activation. CD4$^+$ T cells were transfected with empty pcDNA or FLAG-PKCη plasmids (at 5μg each) and then were left unstimulated (-) or were activated with cross-linked anti-CD3/CD28 antibodies for 24 hours. Untransfected cells were run alongside as a control for the transfection procedure. Whole cell lysates were prepared with RIPA buffer at 0 and 24h and 5 μg of total protein was loaded per lane. Protein levels of PKCη, Cyclin D2 and Cyclin E were analysed in these samples by Western blotting. β-actin protein levels were used as loading control. Data are representative of five experiments with different donors.

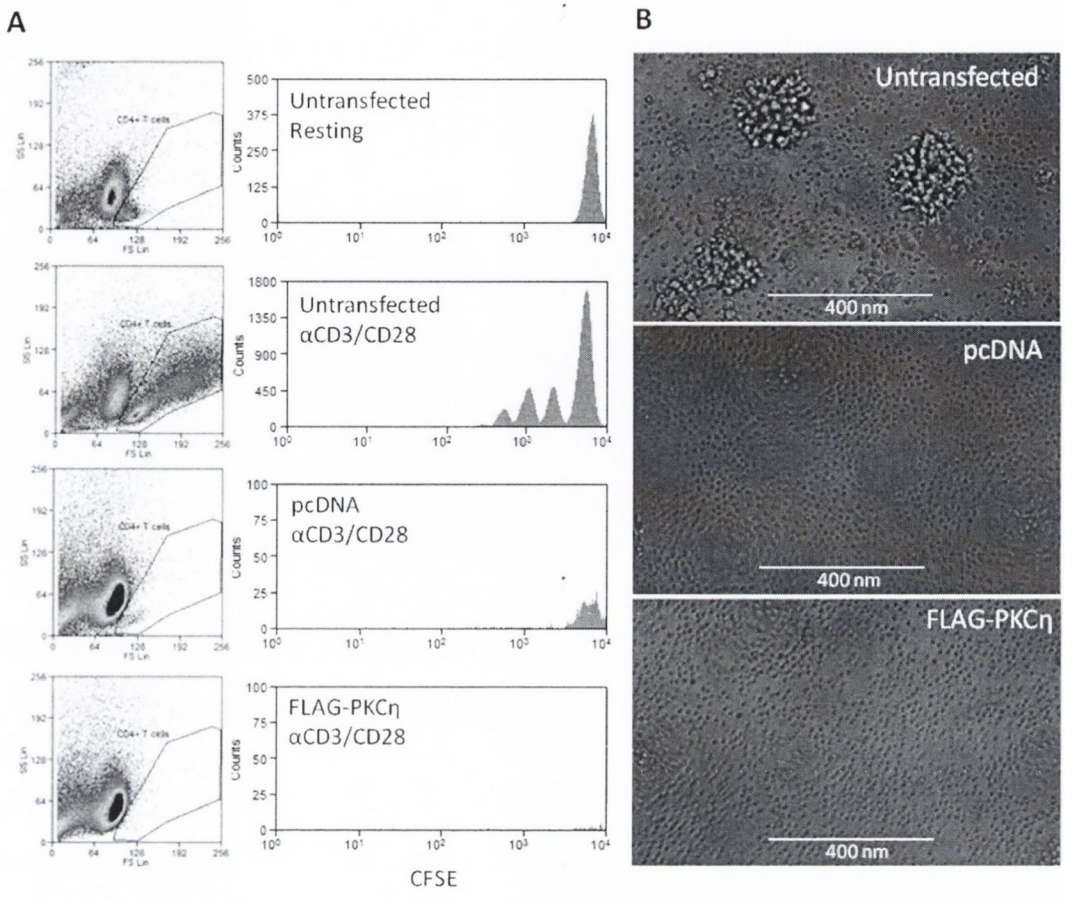


Figure 5-18 Effect of PKCη over-expression on CD4$^+$ T cells proliferation in response to cell activation. CD4$^+$ T cells were transfected with 5μg of either pcDNA or FLAG-PKCη and left to recover for 24h. These cells were then labelled with 10μM CFSE and then assessed for their ability to proliferate when activated with cross-linked anti-CD3/CD28 antibodies for 4 days. Cell division was measured by flow cytometric analysis of the CFSE dilution and Propidium Iodide (PI) staining was used to exclude dead cells. Resting cells were used as non-dividing cells and untransfected CD4$^+$ T cells were run alongside as a control for the transfection process. A) Flow cytometric plots showing the gating strategy used to exclude apoptotic cells (first column) and then showing CFSE intensity in cells that are PI negative (second column). While untransfected CD4$^+$ T cells underwent several cell divisions in response to anti-CD3/CD28 stimulation, the cells that were transfected with pcDNA or FLAG-PKCη that did not survive to the 4-day period. B) Microscopic analysis in bright field (10X objective) in CD4$^+$ T cells at day 4 of cell activation with anti-CD3/CD28 antibodies.

5.4.2 Investigation of the role of PKCη in activated $CD4^+$ T cells by delivery of PKCη small interfering RNA (siRNA)

In this study, delivery of plasmid DNA into the $CD4^+$ T cells was performed by nucleofection, which is proven to be the most effective method for transfection of this type of cells (Gresch, Engel et al. 2004; Maurisse, De Semir et al. 2010). However, to induce exogenous expression of the target gene, it is necessary for the plasmid DNA to be delivered to the cell nuclei and therefore nucleofection needs to provoke the plasmid to pass through the plasma and nuclear membranes. This event is thus often associated with massive cell death (Ovcharenko, Jarvis et al. 2005). An alternative approach to study gene function is to induce gene silencing by using short interfering RNA (siRNA). siRNAs are smaller than plasmid DNA and only need to be delivered into the cellular cytoplasm to be active (Ovcharenko, Jarvis et al. 2005). This technique has been already employed in this study, being the method of choice to study the function of PKCη in T cell lines and other primary lymphocytes. As the results obtained in these experiments showed full functionality of the cells after the transfection procedure, it was decided to also employ this methodology to study the function of the PKCη gene in $CD4^+$ T cells.

To induce PKCη gene silencing, the $CD4^+$ T cells were transfected by nucleofection with a siRNA specific for the PKCη gene. A non-targeting siRNA was used to transfect control cells. The transfection efficiency was measured by Western blot analysis of the total PKCη protein expression in $CD4^+$ T cells transfected with PKCη siRNA in comparison with control siRNA (Figure 5-19). On average, the $CD4^+$ T cells in which the PKCη gene had been silenced expressed 55.48% of the PKCη levels expressed by the control cells (Figure 5-19). Cell viability at 72 hours post-transfection was on average 63% for control cells and 54% for PKCη siRNA-transfected $CD4^+$ T cells (Figure 5-19).

Following on from this, these cells were tested for the different aspects of T cell activation that were examined in the previous section using PKCη plasmid DNA. In this case, a role for PKCη was investigated in the context of IL-2 secretion, regulation of cell cycle and cell proliferation following $CD4^+$ T cell stimulation with anti-CD3/CD28 antibodies when PKCη expression was silenced using a siRNA approach.

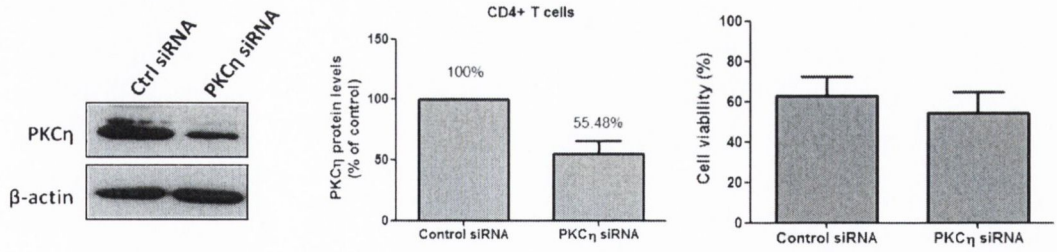


Figure 5-19 Efficiency of the PKCη gene silencing in CD4$^+$ T cells. Freshly isolated CD4$^+$ T cells were transfected with control siRNA or PKCη siRNA (at 1000 nM each) and left to recover for 72 hours. Whole cell lysates were prepared and PKCη protein levels were determined by Western blotting (left). Densitometric analysis of the blots (light gray bars) showed an average of 45% down-regulation in PKCη protein expression in cells transfected with PKCη siRNA compared to control siRNA. The percentage of viable cells at 72h after transfection was assessed in these cells by Trypan Blue dye exclusion (dark gray bars). Western blots are representative and bar chart show mean percentage values ± S.E.M (n=5 donors).

5.4.2.1 Silencing of PKCη expression with siRNA does not affect IL-2 secretion by CD4+ T cells in response to cell activation.

The effect of silencing PKCη expression on IL-2 production was examined in $CD4^+$ T cells following activation. For this purpose, $CD4^+$ T cells were transfected with PKCη siRNA or control siRNA and then activated with anti-CD3/CD28 antibodies for 24 hours. The concentration of IL-2 was measured in the supernatant of these cells by ELISA. The results showed that both PKCη siRNA- and control siRNA-transfected cells produced considerable amounts of IL-2 following cell activation (approximately 10μg/ml). However, no significant differences in the amount of IL-2 produced were observed when comparing PKCη siRNA transfected cells and control cells (Figure 5-20), suggesting that PKCη is not involved in secretion/production of IL-2 by $CD4^+$ T cells in response to anti-CD3/CD28 stimulation.

5.4.2.2 PKCη is not involved in regulation of cell cycle progression in response to cell activation in CD4+ T cells.

The next context to be investigated was the role of PKCη in cell cycle progression of $CD4^+$ T cells in response to anti-CD3/CD28 stimulation. As mentioned earlier in this chapter, PKCη has been described to regulate cell cycle progression in several cell types. Moreover, regulation of cell cycle by PKCη happens through its association with the cyclin E/cdk2p21 complex, which causes cell cycle arrest in the G1 phase (Kashiwagi, Ohba et al. 2000; Shtutman, Hershko et al. 2003). It was first examined if silencing of the PKCη gene in $CD4^+$ T cells had an effect on cell cycle progression following activation with anti-CD3/CD28 antibodies. The DNA content of resting and anti-CD3/CD28-activated $CD4^+$ T cells that had been transfected with either control or PKCη siRNA was analysed by propidium iodide (PI) staining and flow cytometry (Figure 5-21). The analysis showed that stimulation of $CD4^+$ T cells with anti-CD3/CD28 antibodies for 48 hours induced cells to enter the cell cycle. While in resting cells more than 91% of the cells in both groups were arrested in the G0/G1 phase at 48 hours, activated cells presented less cells in this cell cycle phase and a considerable percentage of cells in the S

and G2/M phases (Figure 5.21). Nonetheless, the percentages of $CD4^+$ T cells found in each of the phases of the cell cycle were comparable between cells transfected with control siRNA and cells transfected with PKCη siRNA (Figure 5-21), suggesting that lack of PKCη expression did not impair cell cycle progression in response to anti-CD3/CD28 stimulation.

In addition, to fully characterise any putative role for PKCη in cell cycle progression in $CD4^+$ T cells, it was also determined if PKCη regulated expression of the cyclins D2 and E following anti-CD3/CD28 stimulation in these cells. The reasons why these two cyclins were chosen for investigation were described previously in this chapter. Briefly, cyclin D2 is a major player in T cell activation and controls entry of cells into the S phase while cyclin E has been described to associate with PKCη in different cell types (Kashiwagi, Ohba et al. 2000; Shtutman, Hershko et al. 2003; White, Shore et al. 2006). The expression of these two cyclins was analysed at 24 and 48 hours following anti-CD3/CD28 stimulation in $CD4^+$ T cells transfected with control siRNA and PKCη-specific siRNA (Figure 5-22). Expression of cyclin E was detected successfully at 48 hours following cell activation in $CD4^+$ T cells transfected with either PKCη siRNA or control siRNA (Figure 5-22). However, the protein levels of cyclin E observed were similar between $CD4^+$ T cells transfected with control siRNA and PKCη-specific (Figure 5-22). In turn, expression of cyclin D2 was detected at high levels in $CD4^+$ T cells already at 24 hours following activation with anti-CD3/CD28 antibodies and was maintained at 48 hours. However, no significant differences were observed in the protein levels of cyclin D2 in the cells which the PKCη gene had been silenced in comparison to cells transfected with control siRNA (Figure 5-22), suggesting that PKCη is not directly related to regulation of expression of any of these cyclins in response to cell activation.

These last findings corroborate the results observed for analysis of cell cycle progression in activated $CD4^+$ T cells following PKCη gene silencing where no differences were found in comparison to control siRNA. The fact that silencing of PKCη expression had no effect on anti-CD3/CD28-induced up-regulation of cyclins D2 and E in $CD4^+$ T cells, in addition to normal cell cycle progression in $CD4^+$ T cells transfected with PKCη siRNA following cell activation, suggests that PKCη is not involved in regulation of cell cycle in activated T cells.

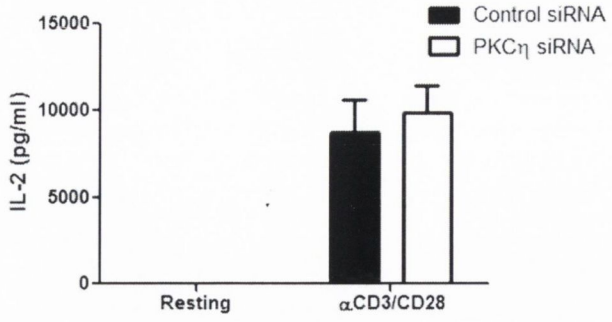


Figure 5-20 Silencing of PKCη expression does not affect IL-2 secretion by $CD4^+$ T cells in response to cell activation. Concentrations of IL-2 (pg/ml) were measured at 24h following cell activation with anti-CD3/CD28 antibodies in the cell supernatant of $CD4^+$ T cells previously transfected with either control siRNA or PKCη siRNA. Resting cells transfected with either control siRNA or PKCη siRNA were used as a control. Data show mean concentration ± S.E.M of three experiments with different donors.

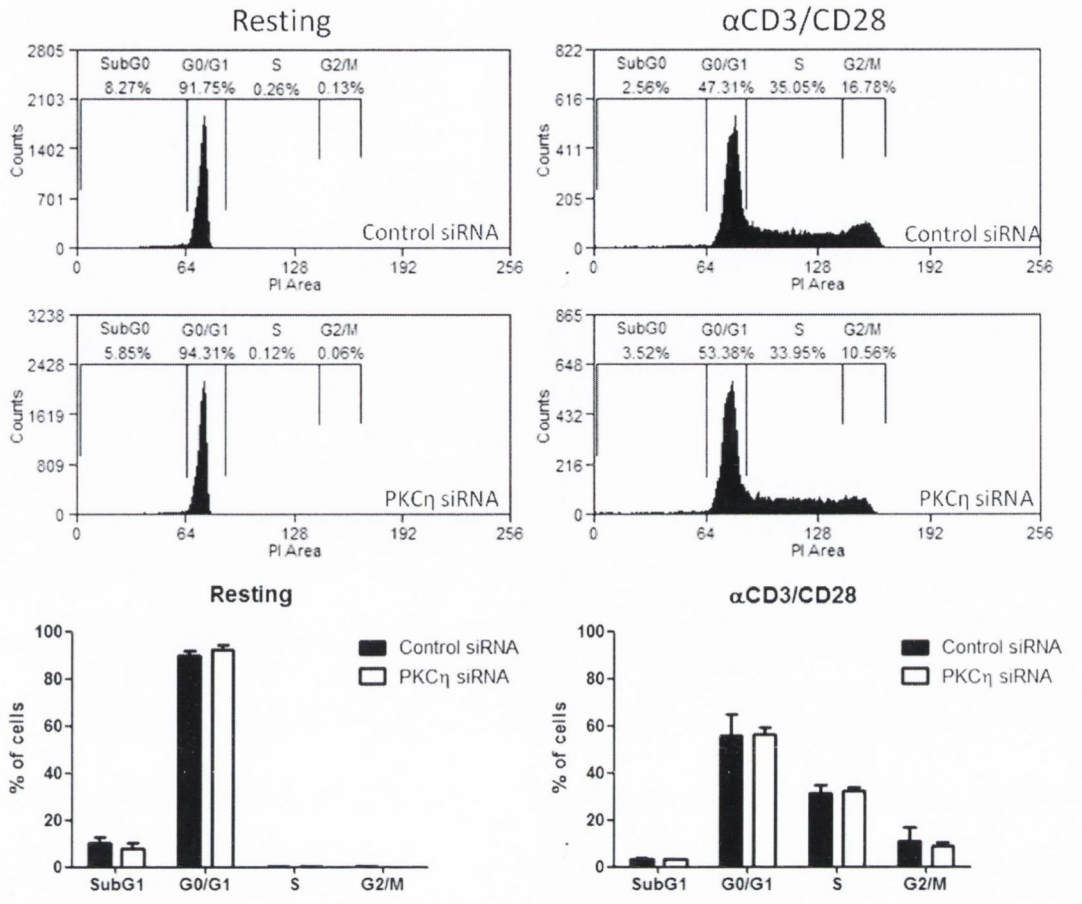


Figure 5-21 Silencing of PKCη expression does not affect cell cycle progression in response to cell activation in CD4$^+$ T cells. Flow cytometry analysis of cell cycle progression by propidium iodide (PI) staining at 48 hours in resting and anti-CD3/CD28 activated CD4$^+$ T cells that had been previously transfected with control siRNA or PKCη siRNA (at 1000 nM each). Resting cells were used as unstimulated control. Flow cytometry plots are representative and show the gating strategy used to determine percentage of cells in each phase of the cell cycle (SubG1 phase represents apoptotic cells, G0/G1 phase shows 2n/diploid cells, S represents cells undergoing DNA synthesis and G2/M phase shows double diploid cells. Bar charts show mean percentage±SEM of cells in each phase of the cell cycle (n= 2 donors).

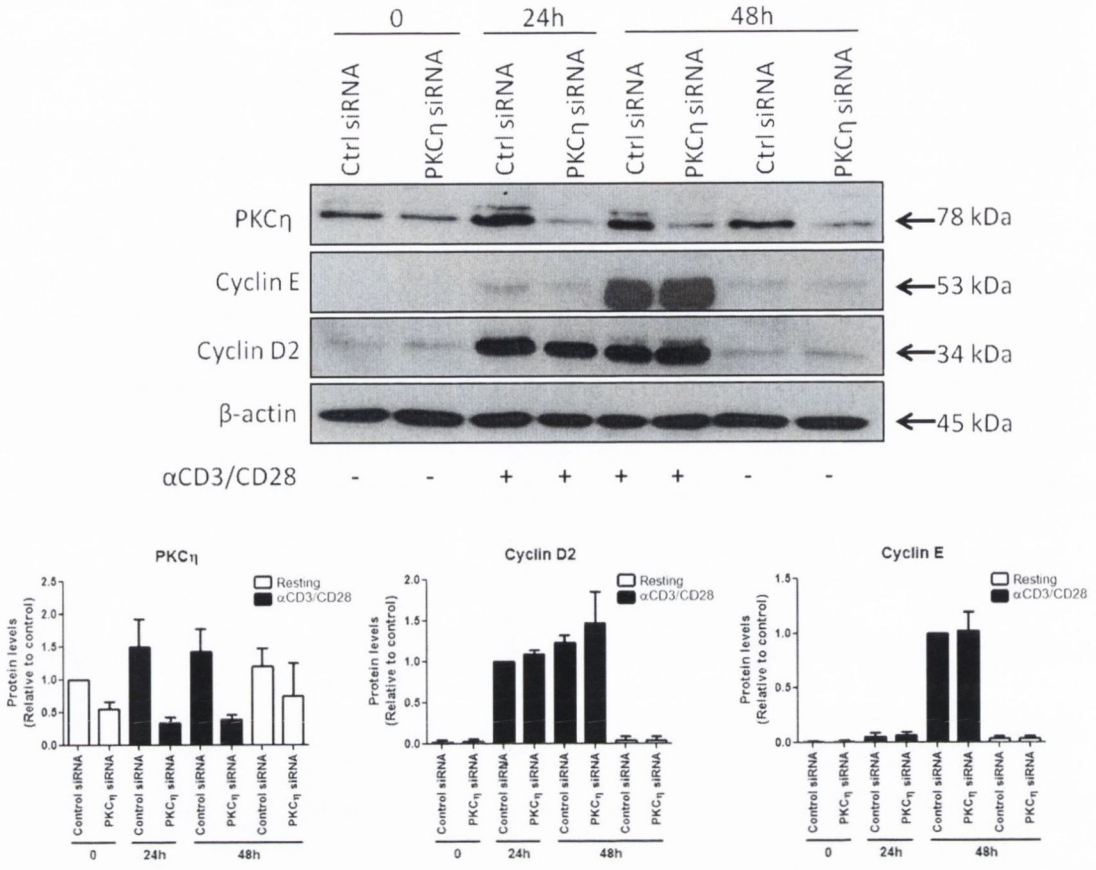


Figure 5-22 Silencing of PKCη expression does not affect up-regulation of Cyclins D2 and E in CD4$^+$ T cells in response to cell activation. CD4$^+$ T cells that had been previously transfected with either control siRNA or PKCη siRNA (at 1000 nM each) were left unstimulated (-) or were activated with cross-linked anti-CD3/CD28 antibodies for the given time points. Whole cell lysates were then prepared with RIPA buffer and protein levels of PKCη, Cyclin D2 and Cyclin E were analysed in these samples by Western blotting. β-actin protein levels were used as loading control. Bar charts show relative levels of PKCη, Cyclin D2 and Cyclin E based on the densitometric analysis performed on the Western blots. Data are mean±S.E.M of three experiments from different donors.

5.4.2.3 Silencing of PKCη expression impairs CD4+ T cell proliferation in response to cell activation while cell survival is not affected.

The involvement of PKCη in $CD4^+$ T cell proliferation following cell activation was also investigated. $CD4^+$ T cells that had been previously transfected with control or PKCη siRNA were labelled with CFSE and then allowed to proliferate for 96 hours in response to activation with anti-CD3/CD28 antibodies. Cell proliferation was measured in these cells by analysis of the CFSE dilution by flow cytometry (Figure 5-23). Interestingly, $CD4^+$ T cells in which PKCη expression had been silenced did not proliferate as efficiently as cells transfected with control siRNA following anti-CD3/CD28 stimulation (Figure 5-23). In fact, the great majority of cells in the PKCη-silenced population did not go through any cell division during this period. It was then investigated if the proliferative defect observed in $CD4^+$ T cells in which PKCη had been knocked-down could be related to induction of cell death. Hence, binding of annexin V (AnV) and presence of propidium iodide (PI) positive cells was analysed by flow cytometry at 24 hours in resting and anti-CD3/CD28 activated $CD4^+$ T cells transfected with either PKCη siRNA or control siRNA (Figure 5-24.A). The results show that silencing of PKCη expression did not induce apoptosis in either resting or activated $CD4^+$ T cells. Moreover, analysis of the effect of PKCη gene silencing on cell survival was extended to up 72 hours following cell activation. Resting and anti-CD3/CD28 activated $CD4^+$ T cells transfected with either PKCη siRNA or control siRNA were stained with PI and analysed by flow cytometry. The percentage of viable (PI negative) cells was calculated at 24, 48 and 72 hours following cell stimulation (Figure 5-24.B). This extended analysis confirmed that silencing of PKCη expression had no effect on cell survival in either resting or activated $CD4^+$ T cells.

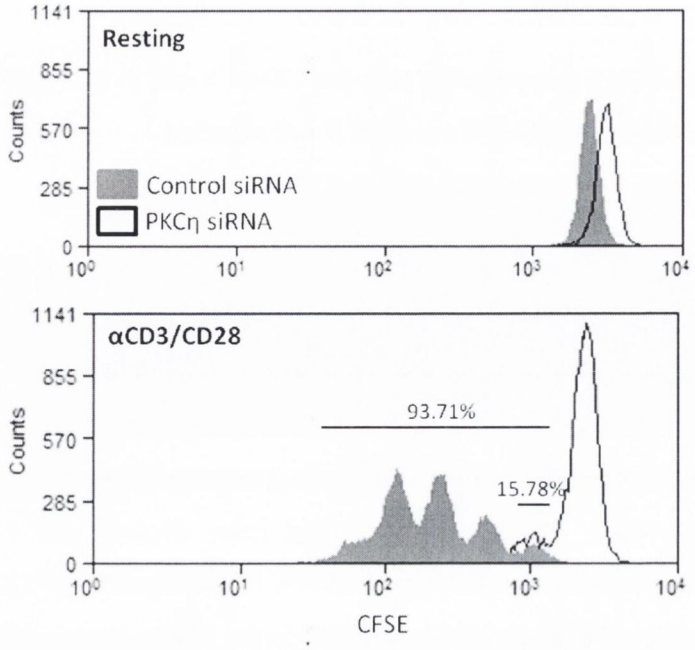


Figure 5-23 Silencing of PKCη expression impairs proliferation of CD4$^+$ T cells in response to cell activation. CFSE-labelled CD4$^+$ T cells that were transfected with control siRNA or PKCη siRNA were left unstimulated (resting) or were activated with anti-CD3/CD28 antibodies for 96 hours. The cells were then analysed by flow cytometry for CFSE fluorescence as an indicator of cell division. Number show percentages of CD4$^+$ T cells that underwent cell division in the population analysed. The overlay histograms are representative of two experiments with different donors.

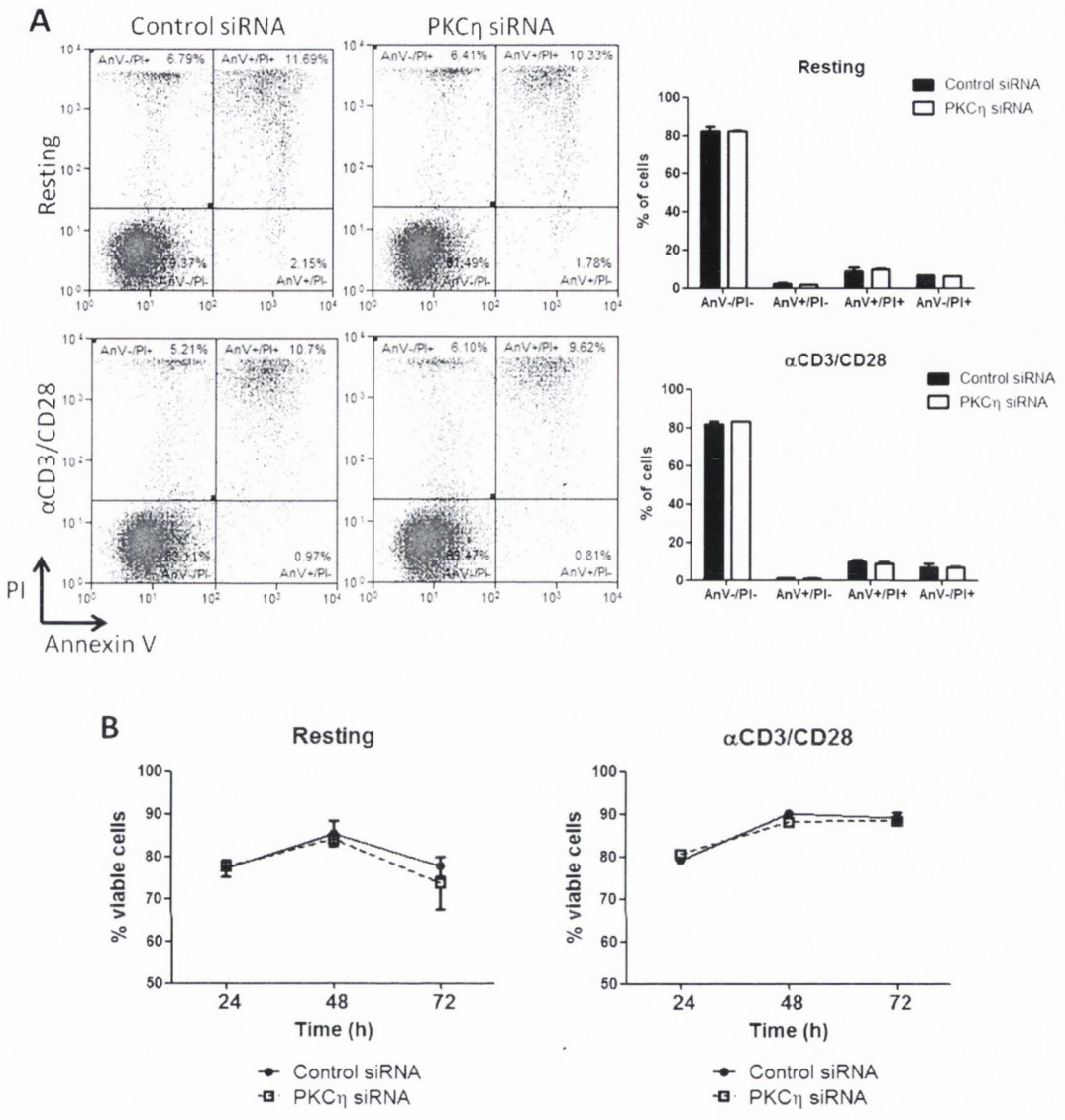


Figure 5-24 Silencing of PKCη expression does not affect survival of CD4$^+$ T cells. CD4+ T cells were transfected with control siRNA or PKCη siRNA and then left unstimulated (resting) or were activated with anti-CD3/CD28 antibodies for the given time points and presence of apoptosis as well as cell viability were analysed. A) Flow cytometric analysis of apoptosis with annexin V (AnV)/propidium iodide (PI) staining at 24 hours following cell activation. Flow cytometry plots are representative of two experiments with different donors. Bar charts show average percentage ±SEM of viable cells (AnV-/PI-), early apoptotic cells (AnV+/PI-), late apoptotic cells (AnV+/PI+) and necrotic cells (AnV-/PI+) in resting and anti-CD3/CD28 activated cells. B) Percentage of viable cells in PKCη-silenced CD4$^+$ T cells and cells transfected with control siRNA at 24, 48 and 72 hours following anti-CD3/CD28 activation and in resting cells. Cells were stained with PI at the given time points and analysed by flow cytometry. Percentage of viable cells (PI negative) are shown. Values are mean±SEM (n=2 donors).

5.5 Summary

In this chapter I have described that the novel PKCη isoform is expressed at high levels in primary human $CD4^+$ T cells and is specifically down-regulated at the protein level in response to anti-CD3/CD28 stimulation. Moreover, I have shown that the full-length PKCη protein is being cleaved potentially by the action of caspases and that a fragment of approximately 35 kDa is accumulated from 48 hours following cell activation.

I have shown that expression of PKCη in $CD4^+$ T cells at basal levels requires PKC activity. In addition, I have described that the GSK-3 inhibitor BIO-Acetoxime/X blocks activation-induced down-regulation of PKCη in $CD4^+$ T cells, but this is probably an off-target effect of the inhibitor and not due to inhibition of GSK-3.

In order to determine a function for PKCη in the context of $CD4^+$ T cell activation, I have used two approaches to study function of this protein; (i) over-expression of the PKCη gene by delivery of a plasmid DNA coding for the PKCη gene and (ii) silencing of PKCη by nucleofection with siRNA. I have demonstrated that the use of plasmid DNA in primary T cells needs to be handled with caution, as the procedure can affect the biology of the cells. On the other hand, nucleofection of $CD4^+$ T cells with siRNA in order to silence the PKCη gene has been proven to be effective in generating a gene-specific phenotype. Using this technique, I have demonstrated that PKCη is not required for many aspects of T cell activation including IL-2 secretion, cell cycle progression and regulation of expression of cyclins D2 and E, but this isoform plays a critical role in proliferation of $CD4^+$ T cells in response to anti-CD3/CD28 stimulation.

5.6 Discussion

In this chapter, I have investigated the mechanisms of regulation of the novel PKCη in human primary $CD4^+$ T cells in response to cell activation and a functional role for PKCη in this context. The PKCη isoform was first described to be expressed at high level in epithelial tissues and it has since been proven to be a key player in differentiation of keratinocytes (Osada, Hashimoto et al. 1993; Kashiwagi, Ohba et al. 2002; Shirai, Morioka et al. 2011). Interestingly, PKCη has also been shown to be highly expressed in T cells (Mischak, Kolch et al. 1991). Moreover, mRNA microarray analysis of cells from transgenic mice has characterised the presence of PKCη among the genes that were up-regulated in double positive thymocytes that have gone through positive selection (Mick, Starr et al. 2004). These facts suggested a potential role for PKCη in T cells, which was only investigated more meticulously very recently by Fu et al. (2011). Using a PKCη knockout mouse model they have also described, among other findings that will be discussed later, that PKCη mRNA was increased during positive selection of thymocytes and that $CD4^+$ T cells express high levels of PKCη mRNA. In accordance with this idea, I have shown by Western blotting that PKCη is also expressed at high levels in human $CD4^+$ T cells, and that its levels are regulated post-transcriptionally in response to activation with anti-CD3/CD28 antibodies.

Members of the PKC family are usually regulated by phosphorylation following T cell activation rather than at the transcriptional level. Indeed, it was observed in this study that PKCη levels were down-regulated in reponse to T cell activation at the protein level but not at the mRNA level. In addition, the down-regulation at protein level was specific for the PKCη isoform, as no other PKC isoforms expressed in T cells displayed a similar result. Regulation of PKCη appears to be quite distinct from that of other PKC isoforms. Expression of many PKC isoforms is down-regulated in response to prolonged activation by long-term treatment with phorbol ester tumour promoters primarily as a result of proteolysis of activated PKC isoforms rather than diminished synthesis (Parker, Bosca et al. 1995). Conversely, prolonged treatment with phorbol esters does not cause down-regulation of PKCη in normal human keratinocytes (Murakami, Chida et al. 1996) and results in up-regulation of PKCη in EL4 mouse thymoma cells and breast cancer cells (Resnick, Luo et al. 1997; Pal, Outram et al. 2012).

In human $CD4^+$ T cells, down-regulation of the full-length PKCη (78 kDa) in response to cell activation corresponded with the appearance of a lower molecular weight band of approximately 35 kDa from 48 hours following cell activation. Studies using baby hamster kidney (BHK) cells have shown that transient expression of a constitutively active form of PKCη resulted in its rapid degradation with the appearance of several low molecular weight bands that shared the PKCη C-terminal epitope, suggesting that degradation of PKCη requires the active PKCη conformation as well as its kinase activity (Kang, French et al. 2000). Degradation of the full-length PKCη to generate fragments of approximately 45-50 kDa has also been observed in EL4 mouse thymoma cells following virus-based transient expression of a constitutively active form of PKCη (Resnick, Kang et al. 1998). It has been suggested that the ubiquitin-proteasome pathway mediates degradation of PKCη, as treatment of BHK cells with proteasome inhibitors (LLnL and lactacystin) resulted in greater accumulation of the full-length PKCη (Kang, French et al. 2000). However, pre-treatment of human $CD4^+$ T cells with the proteasome inhibitor MG132 did not have this effect, suggesting that the down-regulation of PKCη in $CD4^+$ T cells following cell activation is independent of proteasomal degradation in this case.

On the other hand, treatment of the $CD4^+$ T cells with the pan caspase inhibitor Z-VAD-FMK prior to cell activation resulted in accumulation of the full-length PKCη and disappearance of the lower molecular band fragment, suggesting that degradation of PKCη is a result of caspase activity. Caspases play a central role in most cell death pathways leading to apoptosis but growing evidences implicates these enzymes also in non-apoptotic functions (Launay, Hermine et al. 2005). For this reason, I first hypothesised that the methodology used in this study to activate the $CD4^+$ T cells was leading to cell death and the down-regulation of PKCη observed in $CD4^+$ T cells following cell activation was due to induction of apoptosis and cell death. However, this idea was not substantiated as no significant increase in apoptotic markers or dead cells was observed in $CD4^+$ T cells following activation with anti-CD3/CD28. Thus, the caspase activity involved in down-regulation of PKCη in response to $CD4^+$ T cell activation was not related to cell death. Studies with murine B-cells have described PKCη as a substrate for caspases and the resultant fragment of 50 kDa as constitutively active and pro-apoptotic (Morrow, Muljo et al. 1999). It was also shown that cleavage of PKCη in these apoptotic B cells was inhibited by treatment with Z-VAD-FMK and Z-

DEVD-FMK (inhibitors of caspases 3,6,7 and 10) and also with high doses of the caspase-1 inhibitor Ac-YVAD-CHO.

Activation of capases has been described to be required for T cell function, as treatment with the inhibitor Z-VAD-FMK inhibits proliferation and IL-2 production in T cells following anti-CD3 stimulation (Kennedy, Kataoka et al. 1999). Caspase-8 has been characterised as an essential player in this context (Salmena, Lemmers et al. 2003). Deficiency of caspase-8 in humans has been associated with defective activation of T and B lymphocytes and natural killer cells leading to immunodeficiency (Chun, Zheng et al. 2002). Moreover, cleavage of caspase-8 substrates, and not those of caspase-3, was observed in effector $CD4^+$ T cells (Misra, Jelley-Gibbs et al. 2005).

It is known that caspases cleave substrates at specific tetrapeptide sites (denoted P_4-P_3-P_2-P_1) with a highly conserved aspartate (D) at the P_1 position (Talanian, Quinlan et al. 1997). Group I caspases (caspases-1, -4 and -5) usually recognise the sequence (W/L)EHD; group II caspases (-2, -3 and -7) prefer the sequence DEXD; while group III caspases (-6, -8, -9 and -10) cleave protein with the sequence (L/V)E(T/H)D (Wee, Tan et al. 2007). Nevertheless, no rigid rules can be applied to define D-containing peptides as substrates or non-substrates and rather the definition of "bad", "intermediate" and "good" caspase substrates is more acceptable (Timmer and Salvesen 2007). The specific cleavage site for PKCη is unknown but it is suggested to be in or upstream of the V3 region (Morrow, Muljo et al. 1999). Several tools have been developed to predict caspase cleavage sites in proteins through searching for potential sites using examples of known cleavage sites, such as the web server CASVM (Wee, Tan et al. 2007). Analysis of the amino acid sequence of the PKCη protein using CASVM permitted the identification of the tetrapeptide NVTD (with the P1 at residue 95 of the sequence) as a potential cleavage site for caspases. The regulatory C2 domain of human PKCη has been resolved and comprises residues 1-138 of the protein, including the N-terminal (Littler, Walker et al. 2006). Altogether, these data suggest that the cleavage site for PKCη is within the C2 domain, supporting the idea of a cleavage site upstream of the V3 region. Moreover, considering the similarity of the tetrapeptide sequence; it is likely that PKCη is a substrate for group III caspases, which includes caspase 8.

Cleavage of the full-length PKCη protein in the predicted NVTD site would generate a fragment of approximately 10 kDa. Considering that the anti- PKCη antibody used in this study detects an epitope in the C-terminus, it would be expected to observe a fragment of around 68 kDa in the western blot analysis and not the 35 kDa fragment observed in $CD4^+$ T cells in response to cell activation. This fact indicates that the PKCη fragment may undergo further processing following cleavage by caspases, although further investigation is necessary in this subject.

In addition to the use of a pan caspase inhibitor, I have also investigated the existence of other regulators of PKCη expression using a panel of pharmacological inhibitors against several key molecules that have been involved in signal transduction in the event of T cell activation. Treatment of $CD4^+$ T cells with the PKC inhibitors bisindolylmaleimide-1 (BIS I) and LY333531, but not Gö6976 considerably decreased PKCη protein levels. This finding suggests that expression of PKCη is cross-regulated at basal levels by members of the PKC family. Bis-1 is a general PKC inhibitor whereas Gö6976 is described to inhibit only the Ca^{2+}-dependent PKCα and PKCβ isoforms (Martiny-Baron, Kazanietz et al. 1993). In turn, LY333531 is characterised as a PKCβ inhibitor although it was also shown to strongly inhibit other kinases such as PKCα, PIM1 and PIM3 (Bain, Plater et al. 2007). Studies with breast cancer cells have also reported down-regulation of PKCη protein levels upon treatment with the PKC inhibitors Gö6983 and BIS I but not with Gö6976 (Pal, Outram et al. 2012). Moreover, it was also shown in the mentioned study that PKCη levels are altered at the post-transcriptional level, as treatment with the PKC inhibitors did not change PKCη mRNA expression. Furthermore, using a gene silencing approach, the authors reported that the PKCε isoform regulates PKCη expression in these cells.

Another interesting finding obtained with the panel of inhibitors was related to the glycogen synthase kinase-3 (GSK-3) inhibitor BIO-Acetoxime/X. Treatment of $CD4^+$ T cells with this inhibitor prior to cell activation with anti-CD3/CD28 antibodies blocked the activation- induced down-regulation of PKCη at the protein level. However, following activation, the cells treated with BIO-Acetoxime/X failed to produce IL-2 and resembled resting cells even after 72 hours of cell stimulation. BIO-acetoxime/A (6'-Bromoindirubin-3'-acetoxime) is a derivative indirubin inhibitor of GSK-3 that has been also described to inhibit cyclin-dependent kinases (Cdk) and other kinases (Bain,

McLauchlan et al. 2003). More interestingly, derivatives of indirubin have been used for a long time in traditional Chinese medicine to treat various inflammatory diseases and have been described to inhibit lymphocyte proliferation by blocking cell cycle progression due to its ability to inhibit Cdks (Kagialis-Girard, Mialou et al. 2007; Chebel, Kagialis-Girard et al. 2009). Taken together, these data suggest that the effect of BIO/X observed in preventing down-regulation of PKCη protein levels in $CD4^+$ T cells in response to cell activation was a secondary effect of inhibition of T cell function and not due to specific GSK-3 inhibition. This fact is supported by the absence of effect in down-regulation of PKCη in $CD4^+$ T cells pre-treated with Lithium Chloride, an irreversible GSK-3 inhibitor that does not inhibit activity of Cdks (Bain, Plater et al. 2007).

Comparing to the amount of information gathered about PKCθ, the most studied PKC isoform in T cells, there is still a lot to be investigated about the functional role of PKCη in T cell biology. Resnick et al. (1998) suggested a role for PKCη in cell morphology and cytoskeletal changes in EL4 mouse thymoma cells. Using a virus-based transient expression system they have observed that expression of a constitutively active form of PKCη resulted in dramatic changes in the cell morphology as well as cytoskeletal organization in EL4 cells. Studies with cisplatin-induced apoptotic Jurkat T cells showed a different lipid raft localization pattern of PKCα, PKCθ and PKCη in these cells (Solstad, Bjorgo et al. 2010). They have described a selective up-regulation of PKCα in these microdomains upon apoptosis induction whereas the levels of PKCθ and PKCη were significantly reduced.

In relation to the role of PKCη in the context of T cell activation, Quann et al. (2011) showed for the first time that PKCθ was not the only PKC isoform to localise in the immunological synapse (IS) following TCR stimulation. They have reported that PKCη and PKCε arrived first at the IS and accumulated in a broad region of the plasma membrane and were necessary for recruitment of PKCθ to the site. These data suggested a redundant function for PKCη and PKCε, which was confirmed by requirement of both PKCη and PKCε gene knockdown for the inhibition of the microtubule-organizing centre (MTOC) polarization and cytokine production (IL-2 and IFN-γ). During my investigation of a functional role for PKCη in primary human $CD4^+$ T cells, in which I used nucleofection with siRNA to silence the PKCη gene in these cells, I did not observe a decrease in IL-2 production in response to T cell activation as described by the other

reports. However, this fact could be explained by the redundant roles of PKCη and the other novel isoforms, PKCθ and PKCε in this context.

Using a PKCη-deficient ($PKC\eta^{-/-}$) mouse model, Fu et al. (2011) have described that expression of PKCη is necessary for T cell activation and proliferation in response to antigen stimulation. However, these characteristics were shared in some extent by T cells from PKCθ-deficient mice, suggesting redundant roles for these two PKC isoforms in this context. In the present study I was able to confirm the requirement of PKCη for a normal proliferation response in $CD4^+$ T cells following anti-CD3/CD28 stimulation for 96 hours in human cells. Interestingly, these same cells did not present a defective cell cycle progression at 48 hours following cell activation, suggesting that PKCη activity is crucial in the period between 48 and 96 hours following cell activation. I have shown previously that the full-length PKCη is cleaved following activation in $CD4^+$ T cells and the accumulation of a lower molecular weight fragment was detected by Western blotting after 48 hours of cell activation. Previous studies showing a lower molecular weight fragment of PKCη have described that the fragment observed has kinase activity and is then functional. (Morrow, Muljo et al. 1999; Kang, French et al. 2000). Therefore, I hypothesise that following activation of $CD4^+$ T cells with anti-CD3/CD28 antibodies, the full-length PKCη is cleaved to originate a constitutively active fragment that is necessary for cell proliferation.

In addition to the redundancy for the PKCη and PKCθ isoforms observed in the study with PKCη-deficient mice (Fu, Hu et al. 2011), the authors have also described a specific role for PKCη in homeostatic proliferation. $PKC\eta^{-/-}$ $CD8^+$ T cells proliferated less well than did wild-type cells after they were injected in a irradiated recipient mouse. Homeostatic proliferation of T cell occurs in response in response to a lymphopenic environment in order to restore the normal pool of peripheral of T cells. This mechanism also ensures the viability of memory T cells by inducing cell proliferation in the absence of cell activation (Sprent and Surh 2011). Such cell proliferation has been described to be regulated by low-affinity interactions of the TCR with self-peptide/MHC molecules (which do not cause cell activation) (Boyman, Letourneau et al. 2009). This fact may be linked to the observation that protein levels of PKCη were also down-regulated in response to very weak stimuli cause by the dilution of the anti-CD3/CD28 antibodies used

to activate the primary human $CD4^+$T cells, which induced down-regulation of PKCη but did not fully activate the cells.

Recent studies have contributed to deepening the understanding of the role of the novel PKCη isoform in the biology of T cells. However, most of the work done in this regard was carried out in mouse models or T cell lines. With this study, I have characterised the regulation of expression and the role of PKCη in primary human T cells, more specifically in the $CD4^+$ T cell subset. I have described that PKCη is the only PKC isoform to be down-regulated at the protein level following cell activation in $CD4^+$ T cells and that the full-length PKCη protein is being cleaved probably by the action of caspases. Finally, despite of all the difficulties in studying gene function in a cell type that is known as "hard to transfect", I was able to confirm, using a siRNA approach, the importance of PKCη in proliferation of activated T cells.

6 Chapter 6 – General Discussion

Protein kinase C (PKC) η is a member of the novel PKC isoforms that shows a high sequence similarity to PKCε (Osada, Mizuno et al. 1990). This isoform has a unique tissue distribution and is predominantly expressed in epithelial tissues including skin, tongue, esophagus and lungs (Hashimoto, Osada et al. 1992).

T cells, the key mediators of cellular immunity, have also been shown to express PKCη at a high level (Mischak, Kolch et al. 1991). However, little is known about the mechanistic role of PKCη in T cells, particularly in the human context. Here, I have investigated the expression and regulation of expression of PKCη following cell activation in two different T cell lines, HuT78 and Jurkat, and in primary human T cells. Moreover, I have investigated a functional role for PKCη in these cell models in the context of T cell activation.

Experiments with HuT78 T cells showed that this T cell line expressess very low basal protein level of PKCη compared to Jurkat T cells. As regards primary human cells, the mixed population of freshly-isolated peripheral blood lymphocytes (PBL, comprised of T cells, B cells and NK cells) showed low basal PKCη protein level compared to enriched T cell populations such as PHA/IL-2-stimulated peripheral blood T lymphocytes (PBTL) and $CD4^+$ T cells or $CD8^+$ T cells. It has been described that PKCη mRNA is increased during positive selection of thymocytes and single positive $CD4^+$ T cells and $CD8^+$ T cells express higher levels of PKCη in comparison to the original cell population (Mick, Starr et al. 2004; Fu, Hu et al. 2011). The reason HuT78 T cells express such low levels of PKCη protein is unknown, as these cells express normal levels of the T cell receptor (TCR). However, it might be related to the fact that this T cell line does not express the co-receptor CD28 (Bartelt, Cruz-Orcutt et al. 2009). Indeed, experiments with HuT78 transiently expressing CD28 ($CD28^+$HuT78) showed higher basal protein levels of PKCη in comparison to control HuT78 cells but further investigation is necessary to elucidate this relationship.

Activity of PKC members is usually regulated by phosphorylation, translocation to the membrane (where the enzyme can associate with lipid co-factors) and down-regulation by

proteolysis (Newton 2010). However, PKCη has been shown to be uniquely regulated compared to other PKC isoforms. Phorbol esters are natural products derived from plants of the family Euphorbiaceae that initially became the object of intense research on the basis of their potent activity as mouse skin tumour promoters. (Boutwell 1974). Following on from this, it was described that phorbol esters had also a profound effect on a wide variety of biological systems through their ability to mimic the action of diacylglycerol (DAG) and activate PKC isoforms that were sensitive to this second messenger (Blumberg 1988; Goel, Makkar et al. 2007). However, prolonged treatment with phorbol esters results in down-regulation of several PKC isoforms causing termination of the PKC signalling (Parker, Bosca et al. 1995). PKCη levels do not change in response to prolonged treatment with phorbol esters in keratinocytes (Murakami, Chida et al. 1996) and are up-regulated following the same treatment in EL4 thymoma cells, MCF-7 cells, among others (Resnick, Luo et al. 1997; Pal, Outram et al. 2012). Here, I have shown that PKCη is also up-regulated following 24-hour treatment with PMA in the HuT78 and Jurkat T cell lines and in PBL, but not in stimulated PBTL. Because of their ability to activate PKC signalling, phorbol esters have been intensively used to study the mechanisms of T cell activation *in vitro*. Although treatment of T cells with phorbol esters alone enhances cell responsiveness, it is not enough to induce a full response and promote cytokine production and cell proliferation. A second stimulus is thus necessary and can be provided by a Ca^{2+} ionophore, such as Ionomycin (Davis and Lipsky 1986; Nobrega, Maldonado et al. 1986). It has been proposed that phorbol esters convert PKC into a constitutively active form that is irreversibly inserted into the membrane resulting in hyperactivity of the PKC signalling which in turn, induces gene transcription leading to induction of cell proliferation (Mosior and Newton 1995; Goel, Makkar et al. 2007). As the PKCη isoform was shown to be responsive to treatment with phorbol esters, which induces PKCη expression in the T cell lines studied, it is possible that constitutive activation of this isoform may play a role in cell survival and proliferation (important in the context of later experiments). It has been shown that constitutively active-mutants of PKCθ and PKCε provide a T cell survival signal by protecting the cells from CD95-induced apoptosis (Bertolotto, Maulon et al. 2000). In turn, PKCη over-expression has been associated with advanced stages of non-small cell lung cancer and human breast cancer tumours (Masso-Welch, Winston et al. 2001; Krasnitsky, Baumfeld et al. 2012). Moreover, PKCη expression provides protection against apoptosis induced by

chemotherapic drugs in Hodgkin's lymphoma cell lines and breast adenocarcinoma MCF-7 cells (Abu-Ghanem, Oberkovitz et al. 2007; Rotem-Dai, Oberkovitz et al. 2009).

PKCη was also up-regulated at protein and mRNA levels in response to cell activation with plate-bound anti-CD3/anti-CD28 antibodies in both T cell lines (HuT78 and Jurkat) and in PBL. On the other hand, anti-CD3/CD28 stimulation of primary human $CD4^+$ T cells resulted in down-regulation of PKCη at the protein level, while PKCη mRNA levels did not change significantly. This fact suggests that in $CD4^+$ T cells, PKCη is regulated at the post-transcriptional level following T cell activation. Interestingly, the decrease in levels of the full-length PKCη (78 kDa) in response to cell activation of $CD4^+$ T cells corresponded with the appearance of a lower molecular weight band of approximately 35 kDa from 48 hours following cell activation. Treatment of the $CD4^+$ T cells with the pan-caspase inhibitor Z-VAD-FMK prior to cell activation blocked the generation of the 35-kDa PKCη fragment, suggesting that PKCη might serve as a caspase substrate in the context of T cell activation. Caspase activity plays a central role in most cell death pathways but increasing evidence has also associated these proteases in non-apoptotic functions (Launay, Hermine et al. 2005). In this study, it was shown by analysis of annexin V binding and propidium iodide staining that activation of $CD4^+$ T cells with plate-bound anti-CD3/CD28 antibodies was not leading to cell death. Activation of caspases has been shown to be necessary for cell proliferation and IL-2 production in T cells following TCR stimulation, with capase-8 playing a key role in this context (Kennedy, Kataoka et al. 1999; Salmena, Lemmers et al. 2003; Misra, Jelley-Gibbs et al. 2005).

Degradation of full-length PKCη with the appearance of lower molecular weight products has been described before. Kang et al. (2000) showed that transient expression of a constitutively active form of PKCη in baby hamster kidney (BHK) cells resulted in its rapid degradation into several lower molecular weight fragments suggesting that active conformation of PKCη is necessary for its down-regulation. Similar results were also observed in EL4 mouse thymoma cells, in which the degradation of a constitutively active PKCη protein transiently expressed was much faster when compared to cells overexpressing normal PKCη (Resnick, Kang et al. 1998). Studies with pro-B-cells have described cleavage of the full-length PKCη into approximately 50 kDa fragments in

response to various pro-apoptotic stimuli. It was also shown that the fragments generated were kinase-active and had pro-apoptotic functional activity (Morrow, Muljo et al. 1999).

Other PKC isoforms have also been described to be substrates for caspases to generate catalytically active carboxy-terminal fragments in response to apoptotic stimuli. Novel PKCθ and PKCδ isoforms have been shown to be cleaved by caspase-3 to generate carboxy-terminal catalytically active fragments that were directly associated with cell death (Emoto, Manome et al. 1995; Datta, Kojima et al. 1997). PKCε and the atypical PKCζ isoform have also been shown to be cleaved following apoptotic stimuli. Atypical PKCζ has been shown to serve *in vitro* as a substrate for several caspases including capase-3, -7 and -8, and cleavage of PKCζ results in catalytically active fragments suggested to promote cell survival (Smith, Wang et al. 2003). In turn, PKCε has been also shown to be cleaved by caspases during apoptosis, but proteolytic cleavage of PKCε prevented its anti-apoptotic signal (Basu, Lu et al. 2002).

Phosphorylation is a key event that regulates PKC activity, stability and intracellular localisation (Newton 2003; Freeley, Kelleher et al. 2011). Both autophosphorylation and transphosphorylation have been described to regulate PKC isoforms (Steinberg 2008). The phosphoinositide-dependent kinase-1 (PDK-1) is known as a key kinase that has been shown to phosphorylate several members of the PKC family (Balendran, Hare et al. 2000). PDK-1 has also been implicated in phosphorylation of PKCη at the activation loop (Lachmann, Bar et al. 2008). More recently, studies with MCF-7 breast cancer cells showed that knockdown of PDK-1 by siRNA decreased basal PKCη expression in these cells (Pal, Outram et al. 2013). Cross-regulation of PKC isoforms by other members of the PKC family has been suggested by several studies (Rybin, Sabri et al. 2003; Basu, Sridharan et al. 2009). Here, I found that expression of PKCη in all the cell models tested (HuT78, Jurkat, PBL and $CD4^+$T cells) was down-regulated upon treatment with the PKC inhibitors bisindolylmaleimide-1 (BIS-1) and LY333531 suggesting that basal and activation induced expression of PKCη in human lymphocytes requires PKC activity. Interestingly, the inhibitor of conventional PKC (PKCα and PKCβ) Gö6976 down-regulated PKCη protein levels in both T cell lines (HuT78 and Jurkat) but had no effect in primary lymphocytes. LY333531 is characterised as a PKCβ inhibitor and would suggest this isoform as the regulator of PKCη expression. However, the absence of an effect of treatment with Gö6976 in primary lymphocytes does not confirm this assumption. In

MCF-7 breast cancer cells, treatment with the pan PKC inhibitors BIS-1 and Gö6983 also down-regulated PKCη expression while no effect was observed upon treatment with Gö6976 (Pal, Outram et al. 2012). In the same context, it was shown that knockdown of the novel PKCε substantially decreased the up-regulation of PKCη expression induced by phorbol ester treatment, suggesting that transphosphorylation of PKCη by PKCε may be responsible for PKCη up-regulation. PKC isoforms have been described to modulate the expression and/or activity of several transcription factors, including c-Fos, Jun, NF-κB, STATs and p53 (Satoh, Gukovskaya et al. 2004; Yoshida, Liu et al. 2006; Aziz, Manoharan et al. 2007; Abou-Kandil, Chamias et al. 2012). More recently, a study based in differentially PKCα, PKCε and PKCδ-regulated genes in response to treatment with phorbol esters in prostate cancer cells identified c-Rel as a key transcription factor in the control of PKC-regulated genes, in particular downstream of PKCδ (Garg, Caino et al. 2013). Although there was no mention of the PKCη gene in the original study (Caino, von Burstin et al. 2011), c-Rel has a well characterised role in the immune response and lymphoid malignancies (reviewed by Visekruna, Volkov et al. 2012) and may be a candidate in regulation of PKCη expression. Therefore, PKC isoforms may regulate PKCη expression indirectly via their regulation of transcription factor activity.

In response to stressful stimuli, (e.g. exposure to drugs or toxins, UV irradiation, nutrient limitations) cells usually mount a cellular stress response to ensure survival (Kultz 2005). Several studies have suggested a particular role for PKCη in the response to cell stress and regulation of apoptosis (Akkaraju and Basu 2000; Matsumura, Tanaka et al. 2003). Expression of PKCη has been described to provide protection against apoptotic stimuli in Hodgkin's lymphoma (HL)-derived cell lines and MCF-7 breast cancer cells (Abu-Ghanem, Oberkovitz et al. 2007; Rotem-Dai, Oberkovitz et al. 2009). Moreover, expression of PKCη has been shown to be regulated at a translational level both under normal growth conditions and during cellular stress caused by amino acid starvation in MCF-7 cells (Raveh-Amit, Maissel et al. 2009). In these cells, PKCη expression was specifically induced upon prolonged amino acid starvation in a time-dependent manner. In contrast, amino acid starvation resulted in down-regulation of PKCη protein levels in HuT78, Jurkat T cells and PBLs. Down-regulation of PKCη was also observed following activation of $CD4^+$ T cells with anti-CD3/CD28 antibodies, a signal that also promotes cell survival. Further investigation is necessary to verify if the down-regulation of PKCη induced by amino acid starvation in human lymphocytes is also accompanied by cleavage

of the full-length PKCη protein into lower molecular weight fragments, which could support a functional role of the fragment in cell survival processes. It has been shown that amino acid starvation of mouse C2C12 muscle cells progressively reduced multiple pro-apoptotic proteins including caspase-3 but protein levels of caspase-8 were up-regulated in these cells following amino acid starvation (Martinet, De Meyer et al. 2005). In this context, it will be interesting to investigate if PKCη is a substrate for capase-8 in the context of T cell survival signals (in response to either amino acid starvation of T cell activation).

During the time this study was being undertaken, two important reports were published in mouse models suggesting PKCη as an important player in the T cell biology. The first one showed for the first time that PKCη together with PKCε also accumulate at the immunological synapse (IS) following stimulation of the T cell receptor and are required to recruit PKCθ to the IS. The redundant role between PKCη and PKCε in this context was further confirmed as simultaneous knockdown of both isoforms was necessary for inhibition of the microtubule-organizing centre (MTOC) polarization and production of IL-2 and IFN-γ (Quann, Liu et al. 2011). The other study, using mouse models of $PKC\eta^{-/-}$, $PKC\theta^{-/-}$, and double knockout contributed to deepening the understanding of specific and redundant roles for PKCη in T cell biology (Fu, Hu et al. 2011). They have shown that lack of PKCη in T cells resulted in impairment of several aspects of T cell activation, including cell proliferation, IL-2 production and Ca^{2+} and NFκB signalling. However, all these characteristics were shared to some extent by $PKC\theta^{-/-}$ T cells, showing redundant roles for these PKC isoforms. In contrast, they have shown that PKCη rather than PKCθ plays an important role in T cell homeostatic proliferation, which involves self-antigen recognition.

The requirement for PKCη activity in IL-2 production following cell activation in human T cells was shown to vary considerably among the cell models used in this study. In Jurkat T cells, IL-2 secretion was significantly impaired following PKCη knockdown or treatment with a PKCη pseudosubstrate (PS) inhibitor. However, in HuT78 T cells only treatment with the PKCη PS inhibitor decreased the levels of IL-2 produced following cell activation while silencing of PKCη expression with siRNA did not result in the same effect. It is possible that silencing of PKCη expression in HuT78 T cells was not enough to suppress PKCη activity in these cells, while treatment with the PKCη inhibitor may

have blocked completely its activity. On the other hand, production of IL-2 following cell activation was not affected by silencing of PKCη expression in any of the primary lymphocytes studied. The findings with primary human T cells support the evidence described above of a redundant role for PKCη and PKCε in IL-2 production in murine T cells. Although both T cell lines also express the PKCε isoform (Long and Kelleher 1993; Tsutsumi, Kubo et al. 1993), it is not known why the requirement for PKCη in IL-2 production was more pronounced in these cells than in primary T cells. For this reason, it is important to take into consideration expression levels and redundancy between PKCη and PKCε isoforms in further investigations using T cells.

A redundant role in T cell proliferation was described for PKCη using T cells from a PKCη-deficient mouse, in which PKCη$^{-/-}$ T cells proliferated less well in response to antigen stimulation than cells from wild-type mice, a characteristic that was shared with T cells from PKCθ-deficient mice (Fu, Hu et al. 2011). Silencing of PKCη expression with siRNA in primary human CD4^{+} T cells also impaired cell proliferation in response to anti-CD3/CD28 stimulation and confirmed the importance of this PKC isoform in T cell proliferation in human cells.

Based on the evidence that cleavage of PKC isoforms by caspases generates catalytically active fragments when freed of its regulatory domain I hypothesise that the 35 kDa PKCη fragment observed may be functional in T cells. Both amino acid starvation and T cell activation are stimuli that signal to promote cell survival and cause down-regulation of PKCη protein levels. Also, I showed that silencing of PKCη expression with siRNA does not affect cell cycle progression at 48 hours following anti-CD3/CD28 stimulation in CD4^{+} T cells but impaired cell proliferation, which was analysed at 96 hours following cell activation. This fact suggested that an essential PKCη-dependent event to promote cell proliferation was happening from 48 to 96 hours after TCR stimulation. Timing of this event corresponds with the accumulation of the 35 kDa PKCη fragment, which was only detected by Western blot from 48 hours following cell stimulation. Therefore, these observations suggest that T cell activation results in down-regulation of PKCη and generation of a 35 kDa fragment that may be catalytically active and required to promote cell proliferation.

Apart from the redundant role for the PKCη and PKCθ isoforms in T cell proliferation in response to antigenic stimulation reported in murine T cells, a specific role for PKCη was described in homeostatic proliferation (Fu, Hu et al. 2011). PKCη$^{-/-}$ CD8^{+} T cells that underwent homeostatic proliferation proliferated less well than did wild-type cells. T cell homeostatic proliferation occurs in response to a lymphopenic environment, which can be caused by either cytotoxic drugs, irradiation, or certain viruses, in order to restore the normal peripheral pool of T cells (reviewed in Boyman, Letourneau et al. 2009). Such expansion is regulated by low-affinity interactions of the TCR with self-peptide/MHC molecules (which do not trigger cell activation) plus contact with cytokines, especially IL-7 (Boyman, Purton et al. 2007). This mechanism also ensures the longevity of central memory T cells by inducing cell proliferation in the absence of cellular differentiation or activation (Sprent and Surh 2011). Reactivity of the TCR only changes when these naive cells encounter a foreign antigen presented by an APC and in the presence of co-stimulation via CD28, which leads to cell activation and proliferation. Consistent with this, it was observed that activation of human CD4^{+} T cells with very low stimulus strength, obtained by serial dilution of anti-CD3/CD28 antibodies used to stimulate the cells also resulted in down-regulation of PKCη protein levels, showing that PKCη is also responsive to low TCR stimulus in primary human CD4^{+} T cells.

Understanding key players in the biology of T cells is of high importance, once these cells are the centre of the cell-mediated immune response and have been involved in the etiology of several autoimmune diseases such as multiple sclerosis and systematic lupus erythematosus (SLE) (Lowther, Chong et al. 2013). Although PKCη is highly expressed in T cells (Mischak, Kolch et al. 1991) and its expression is up-regulated during thymocyte positive selection, (Mick, Starr et al. 2004), little is known about its role in the immune system. Since the report showing that PKCθ was the only isoform recruited to the immunological synapse (Monks, Kupfer et al. 1997), PKCθ has been the centre of attention of most studies regarding T cell activation and signalling networks (Isakov and Altman 2012). Only very recently has PKCη emerged as an important player in T cell biology (Fu and Gascoigne 2012) and to the best of my knowledge, all the functional work was carried out in mouse models. With this study I have confirmed the importance of PKCη in human T cell proliferation in response to TCR stimulation and have contributed more information about the role of PKCη in primary human T cells. Another important aspect of this study was to highlight distinct responses between immortalised

cell lines and primary T cells. Biological experiments are most often performed with cell lines because they are readily available and can be expanded without limitation. However, cell lines can differ from the *in vivo* situation in many important aspects.

In spite of the current belief that inhibition of PKCθ is sufficient to achieve complete immunosuppressive effects, increasing evidence of redundant roles of distinct PKC isoforms in T cells suggests that targeting multiple isoforms would provide a more successful approach for treatment of autoimmune diseases (Pfeifhofer-Obermair, Thuille et al. 2012). For this reason, it is crucial to elucidate the biological roles of individual PKC isoforms and the molecular mechanisms controlling their expression in T cells. As observed, PKCη has an important role in T cell proliferation in response to TCR/CD28 stimulation and could function as a target for anti-inflammatory treatments. Moreover, in regards to its role observed in mouse models in homeostatic proliferation, regulation of PKCη expression may have a positive effect in patients recovering from T cell ablative therapies and HIV patients. Furthermore, indentifying distinct mechanisms in immortalised and primary cells especially in the context of cell survival and proliferation can provide insights about new targets for anti-cancer treatments. As showed in this study, expression of PKCη in response to cell activation is distinctly regulated in T cell lines and primary $CD4^+$ T cells, which characterise PKCη as a potential target in this context. Inhibiting the action of specific molecules necessary for tumour growth and progression has been proven to be more precise and cause fewer side effects than the traditional cytotoxic chemotherapies in cancer therapies (Vanneman and Dranoff 2012). In this context, protein kinases have become one of the most intensively pursued classes of drug target due to their essential role in mediating signal transduction (Zhang, Yang et al. 2009). Both inactive and active conformation of the kinase can be target for small molecules inhibitors and therefore the specific roles of the full-length and the PKCη fragment under different circumstances need to be considered for the decision of the best approach to be taken considering PKCη as a potential target for intervention.

There are still many important questions to be answered regarding the mechanisms by which PKCη acts in T cells and the functional consequences. It is essential to investigate if the mechanism inducing up-regulation of PKCη expression in T cell lines and at the same time down-regulating PKCη levels in $CD4^+$ T cells is related to cell survival. This relationship if confirmed may give rise to new insights into sensitivity of the lymphoma T

cells lines to apoptosis. It is also important to study if the mechanism triggering down-regulation of PKCη in response to activation in $CD4^+$ T cells is the same in PKCη down-regulation observed following amino acid starvation in the T cell lines and PBL.

In addition, it is necessary to determine the specific regulators PKCη expression, as to determine which PKC isoform(s) is regulating basal and activation-induced expression of PKCη in T cells and by which caspase member(s) PKCη is being cleaved in response to anti-CD3/CD28 activation in $CD4^+$ T cells. As the results obtained using pharmacological inhibitors of PKC were not conclusive in this matter, an optional approach that can be carried out is silencing the expression of each different PKC isoforms using specific siRNAs and analysing their effect on PKCη expression. Further experiments with more specific caspase inhibtors such as Z-YVAD-FMK for caspase-1, Z-DEVD-FMK for caspases-3 and 7 and Z-IETD-FMK for caspase-8 will be necessary to determine for which caspase member(s) PKCη serves as substrate. Also in relation to this regards, a PKCη construct can be generated with a cleavage-site mutation, which cannot be cleaved by caspases. Expression of this cleavage-site mutant of PKCη by the T cells will serve as a powerful tool to confirm the proteolytic degradation of PKCη by caspases and to further investigate the functional role of the full-length PKCη in activated cells. Taken together, these results will describe a novel mechanism of regulation of PKCη expression in the context of T cell activation.

Previous reports using mouse cells have described that PKCη is involved in recruitment of PKCθ to the immunological synapse and consequent polarization of the T cell microtubule-organising centre (MTOC) in response to T cell activation (Quann, Liu et al. 2011). Re-orientation of the MTOC is essential to stablish an axis of polarity which enables the activated T cell to secrete cytokines and cytolitic factors in a directional manner toward the target cell (Huse, Quann et al. 2008). For this reason it is also important to consider the connection of PKCη in MTOC reorganisation following activation in primary human T cells. This can be achieved by confocal microscopy analysis of the the immunological synapse area with the aid of markers such as tubulin and actin in activated T cells following silencing of the PKCη expression with siRNA.

Moreover, the observation that PKCη is required for $CD4^+$ T cell proliferation needs to be confirmed and further investigated in a more physiological model of T cell activation.

Rather than being activated artificially by the ligation of antibodies directed against the TCR and CD28 co-receptor, T cells can be naturally activated *in vitro* when incubated with antigen presenting cells (APCs). With this purpose, the ability of the PKCη-knockdown $CD4^+$ T cells to proliferate can be measured following co-culture with APCs specially pulsed with a variety of antigen-peptides such as tetanus toxoid or OVA peptide. Alternatively, activation and proliferation of $CD4^+$ T cells can be triggered in a mixed leukocyte reaction (MLR), in which the T cells are incubated with cells from genetically different individuals that serve as APCs, as they bear foreign peptides.

Finally, it is essential to investigate the functionality of the 35 kDa PKCη fragment observed in reponse to cell activation in primary $CD4^+$ T cells and its requirement for cell proliferation in order to determine the real effector molecule in the the context of PKCη activity and function.

7 Appendix

7.1 Reagents

Reagent	Source	Cat. No.
2-Mercapto-Ethanol	Sigma	M6250
2-propanol	Sigma	I9516
4-Iodophenylboronic Acid (4-IPBA)	Sigma	471933
5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE)	Sigma	21888
5X siRNA buffer	Dharmacon	SH3A1946.01
Acrylamide/bis acrylamide 30% solution	Sigma	A3699
Albumin, from bovine serum (BSA)	Sigma	A9647
Amaxa Nucleofector Kit Human T cell	Lonza	VCA-1002
Ammonium Persulfate (APS)	Sigma	A3678
BioTrace PVDF membrane	VWR	66543
Bovine Serum Albumin (BSA)	Sigma	A2153
Brefeldin A	Sigma	B6542
Bromophenol Blue	Sigma	B0126
CL-Xposure Film	Thermo Scientific	34090
Dimethyl Sulphoxide (DMSO)	Sigma	D5879
Dithiothreitol (DTT)	Bio-Rad	161-0611
Ethanol 96%	Merck	1.00971.2500
Foetal Bovine Serum (FBS)	Gibco	10270
FOXP3 Fix/Perm Buffer Set	BioLegend	421403
Glycine	Fisher Scientific	BP381
Hank's Balanced Salt Solution (HBSS)	Sigma	H6648
Hydrogen Peroxide	Sigma	516813
Hygromycin B	Invitrogen	10687-010
Igepal (Nonidet P-40)	Sigma	CA-630
IL-2	Peprotech	200-02
Ingenio® Electroporation Solution	Mirus Bio	MIR 50117
Ionomycin	Sigma	I0634
Leupeptin	Sigma	L8511
Luminol	Sigma	A8511
Lymphoprep™	Axis-Shield	1114547
Magnesium Chloride ($MgCl_2$)	Sigma	7786-30-3

Methanol	Sigma	34860
N,N,N,N-Tetramethylethylenediamine (TEMED)	Sigma	T9281
PageRuler Broad Range Unstained Protein Ladder	Thermo Scientific	26630
Paraformaldehyde (PFA)	Sigma	P6148
Penicillin-Streptomycin Solution (PenStrep)	Lonza	DE17-603E
Phenylmethylsulfonylfluoride (PMSF)	Sigma	P7626
Phorbol 12-myristate 13-acetate (PMA)	Sigma	P8139
Phosphate Buffered Saline (PBS) tablets	Gibco	18912-014
Phytohaemagglutin (PHA)	Sigma	L2769
PI/RNAse Staining Buffer	BDPharmingen	550825
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
Propidium Iodide	Sigma	P4170
Restore Plus Western Blot Stripping Buffer	Thermo Scientific	46428
RPMI 1640	Lonza	BE12-115F/U1
Saponin	Sigma	S7900
Sodium Azide	BDH	10369
Sodium Chloride (NaCl)	Sigma	S-7653
Sodium dodecyl sulfate (SDS)	Fisher Scientific	BP166
Sodium Fluoride (NaF)	Sigma	S-7920
Sodium Orthovanadate (Na_3VO_4)	Sigma	S-6508
Sulphuric Acid	M&B Chemicals	I840
TaqMan Gene Expression Master Mix	Applied Biosystems	4369016
TMB, soluble	Calbiochem	613544
Tris Base	Fisher Scientific	BP152
Triton X-100	Sigma	X100
Tween-20	Sigma	P5927

7.2 Inhibitors

Inhibitor	Source	Cat. No.
Bisindolymaleimide I	Calbiochem	203290
Cyclosporin A	Sigma	C3662
GSK-3 Inhibitor X	Calbiochem	361551
Gö6976	Calbiochem	365250
Lithium Chloried (LiCl)	Sigma	L7026
LY294002	Calbiochem	440204
LY333531	Sigma	SML0693
MG132	Calbiochem	474791
PD98059	Calbiochem	513000
PKCη Pseudosubstrate Inhibitor, Myristoylated	Calbiochem	539604
Rapamycin	Sigma	R0395
Salubrinal	Calbiochem	324895
Wortmannin	Calbiochem	681675
Z-VAD(OMe)-FMK	Bachem	N-1560

7.3 Antibodies

7.3.1 Antibodies used in T cell activation

Antibody	Source	Cat. No.	Working dilution
Purified NA/LE mouse anti-human CD3	BD Pharmingen	555336	1:100
Anti-mouse IgG developed in goat	Sigma	M8642	1:200
Anti-human CD28	AnCell	177-020	1:1000

7.3.2 Antibodies used in Western blotting

Antibody	Source	Cat. no.	Working dilution
Goat anti-mouse IgG polyclonal antibody HRP conjugated	Cell Signalling	7074	1:5000
Goat anti-rabbit IgG polyclonal antibody HRP conjugated	Cell Signalling	7076	1:5000
Phospho-p44/42 MAP kinase (Thr202/Tyr204)	Cell Signalling	9101S	1:1000
44/42 MAP kinase	Cell Signalling	9102	1:1000
Phospho-GSK-3 beta (Ser9)	Cell Signalling	9336	1:1000
Anti-β-Actin	Sigma	A2066	1:500
Anti-Talin	Sigma	T3287	1:2000
Anti-α-Tubulin	Sigma	T6199	1:5000
Anti-PKCη (C-15)	Santa Cruz	Sc-215	1:500
Anti-PKCα (H-7)	Santa Cruz	Sc-8393	1:1000
Anti-PKCδ (C-20)	Santa Cruz	Sc-937	1:1000
Anti-PKCβ (E-3)	Santa Cruz	Sc-8049	1:1000
Anti-PKCθ (C-18)	Santa Cruz	Sc-212	1:500
Anti-PKCζ (C-20)	Santa Cruz	Sc-216	1:1000
Anti-PTEN	Cell Signalling	9552	1:1000
Phospho-Akt (Ser473)	Cell Signalling	9271	1:1000
Phospho-eIF2α (S51)	Cell Signalling	9721L	1:1000
eIF2α	Cell Signalling	9722	1:1000
Cyclin E (M-20)	Santa Cruz	sc-481	1:500
Cyclin D2	BD Pharmingen	554201	1:1000

7.3.3 Antibodies used in Flow Cytometry

Antibody	Source	Cat. No.
IL-17 AlexaFluor488	Biolegend	512307
FoxP3 AlexaFluor488	Biolegend	320111
Anti-CD4 PE	Immunotools	21270044
PE rat anti-human IL-2	BD BioSciences	559334
APC anti-human IFN-γ	Biolegend	502512
Pacific Blue anti-human CD3	Biolegend	300330
PECy7 anti-human CD4	Biolegend	317414
FITC anti-human CD8	Biolegend	300906
APC anti-human IL-10	Biolegend	501409
FITC anti-human IFN-γ	Biolegend	506506
PE anti-human IL-4	Biolegend	500703
FITC anti-human TNF-α	Biolegend	502906

Sources:

AnCell, USA

Applied Biosystems, Carlsbad, CA, USA

Axon Medchem, The Netherlands

BD Biosciences, New Jersey, USA

B.D.H Chemicals, UK

BioLegend, USA

Calbiochem, USA

Lonza, Cologne, Germany

Merck Chemicals, Germany

Mirus Bio, Madison, WA, USA

R&D Systems, Minneapolis, MN, USA

Santa Cruz Biotechnology Inc, USA

Sigma-Aldrich, St. Louis, MO, USA

Thermo Fischer Scientific, Waltham, MA, USA

7.4 Buffers and stock solutions

7.4.1 Western blotting required solutions:

10% Acrylamide resolving gel

dH_20	7.9 ml
30% Acrylamide	6.7 ml
1.5M Tris-HCl (pH8.8)	5.0 ml
10% w/v SDS	200 µL
10% w/v APS	200 µL
TEMED	8 µL

12% Acrylamide resolving gel

dH_20	4.9 ml
30% Acrylamide	6.0 ml
1.5M Tris-HCl (pH8.8)	3.8 ml
10% w/v SDS	150 µL
10% w/v APS	150 µL
TEMED	8 µL

Stacking gel

dH_20	3.4 ml
30% Acrylamide	0.83 ml
1M Tris-HCl (pH6.8)	0.63 ml
10% w/v SDS	50 µL
10% w/v APS	50 µL
TEMED	8 µL

5X SDS-PAGE running buffer

Tris Base	15.1 g
Glycine	94 g
10% w/v SDS	50 ml
dH_20 to 1000 ml	

This solution should be diluted with dH_20 to a final concentration of 1X before running the SDS-PAGE gels.

Western blot transfer buffer

Tris Base	5.8 g
Glycine	2.9 g
10% w/v SDS	8 ml
Methanol	200 ml

dH_20 to 1000 ml.

10X Tris-Buffered Saline (TBS)

Tris Base	24.2 g
NaCl	80 g

dH_20 to 1000 ml

Adjust pH with HCl to 7.6.

Western blot wash buffer (TBS-T)

1X TBS

0.1% v/v Tween-20

Western blot blocking buffer

5% w/v non-fat milk powder

0.1% v/v Tween-20

1X TBS

1X SDS-PAGE sample buffer

Tris Base	1.51 g
Glycerol	20 ml
dH_2O	35ml

Adjust pH to 6.8 with HCl

SDS powder	4 g
Bromophenol Blue	0.002 g

dH_2O to 180ml

Store at room temperature.

Add fresh DTT to 100mM final concentration immediately before use.

HRP development solution

100 mM Tris-HCl (pH 8.5)	12 ml
H_2O_2	7.2 µl

250 mM Luminol	60 µl
90 mM 4-IPBA	266 µl

Comassie stain solution

Comassie brilliant blue-G250	2.5g
dH_2O	450 ml
Methanol	450 ml
Glacial Acetic Acid	100 ml

Comassie destain solution

dH_2O	450 ml
Methanol	450 ml
Glacial Acetic Acid	100 ml

7.4.2 Cell Lysis Buffers

NP40 Cell Lysis Buffer

1% v/v Nonidet P 40

20 mM Tris-HCl (pH 7.4)

150 mM NaCl

5 mM NaF

5mM Sodium pyrophosphate

5 mM EDTA

5 mM EGTA

Add protease inhibitors immediately before use:

1 mM PMSF, 10 µg/ml leupeptin and 1 mM Na_3VO_4

Radioimmunoprecipitation assay (RIPA) buffer

1% v/v Triton-X

50 mM Tris-HCl (pH 7.4)

150 mM NaCl

2 mM EDTA

0.5% w/v Sodium deoxycholate (DCA)

0.1% w/v SDS

Add protease inhibitors immediately before use:

1 mM PMSF, 10 µg/ml leupeptin and 1 mM Na_3VO_4

7.4.3 Buffers required for ELISA

Wash Buffer

0.05% Tween-20 in PBS, pH7.2-7.4.

Block Buffer

1% w/v BSA in PBS.

Reagent Diluent

0.1% w/v BSA, 0.05% Tween-20 in 1X TBS pH 7.2-7.4, 0.2 μm filtered.

Stop Solution

0.5 M H_2SO_4 in distilled water.

Capture Antibody

The mouse anti-human IL-2 antibody was reconstituted with PBS to a concentration of 720 μg/ml and aliquoted and stored at -20°C. This stock solution was diluted to a final concentration of 4 μg/ml prior to use.

Detection Antibody

A 90 μg/ml stock solution of biotinylated goat-human IL-2 was prepared in Reagent Diluent and aliquoted and stored at -20°C. This stock solution was diluted to a final concentration of 500ng/ml prior to use.

IL-2 Standard

A 60 ng/ml stock solution was prepared of recombinant IL-2 in distilled water and aliquoted and stored at -20°C. A seven point standard curve using a 2-fold serial dilution (0 pg/ml - 1000 pg/ml) of this solution was prepared prior to use.

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