Modifying T cell differentiation via protein based delivery of signalling attenuators

Thesis

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Modifying T cell differentiation via protein based delivery of signalling attenuators

Submitted by

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Abstract

In recent years, evidence has accumulated that hierarchical thresholds for proliferation and differentiation may determine the generation of various effector T cell modes. It is as yet poorly understood, however, how changes in TCR-mediated signalling and its downstream signalling cascades drive the differentiation of the naive T cell into different response modes. This project aims to study whether manipulation of the signalling pathways directly downstream of the TCR/CD28 receptor can result in distinct functional differentiation of monoclonal $CD4^+$ T cell populations with known antigenic specificity.

To this end, two potential protein transfer mechanisms were tested for their suitability to act as inert delivery vehicles to deliver proteins into *ex vivo* naive T cells. The B subunit pentamer of enterotoxin B induced high levels apoptosis in naive T cells, which precluded its use as an inert delivery vehicle. The protein transduction domain of the HIV-1 protein Tat, however, did not influence T cell physiology and was subsequently successfully tested for its ability to transduce *ex vivo* T cells genetically fused to large proteins.

Two fusion partners, the kinase truncated forms of ZAP-70 and Lck, were tested for their ability to interfere with TCR-mediated signalling. The kinase-truncated form of ZAP-70 showed a concentration dependent effect on calcium mobilisation, which was reliant on the strength of CD3 crosslinking. The kinase-truncated form of Lck also altered calcium mobilisation in a concentration dependent manner, but independent of TCR strength. In addition, it was shown to influence T cell activation, to be capable of altering the signal transduction cascade after T cell priming, and to dramatically change the cytokine profile.

"From the moment I picked up your book until I laid it down, I was convulsed with laughter. Some day I intend to read it." (Groucho Marx)

Table of contents

Figures

Abbreviations

7-AAD	7-Amino-Actinomycin D
Ab	Antibody
AICD	Activation-Induced Cell Death
Antp	Antennapedia
AP-1	Activator Protein-1
APCs	Antigen Presenting Cells
APC	AlloPhycoCyanin
APL	Altered Peptide Ligand
β2m	β2-microglobulin
BCR	B Cell Receptor
Bio	Biotin
BM	Bone Marrow
C	Constant segment
CD	Cluster of Differentiation
CD40L	CD40 ligand
Cdk	Cyclin dependent kinase
CLIP	Class II-associated invariant chain peptide
CLP	Common Lymphoid Precursor
cSMAC	central SupraMolecular Activation Cluster
C-terminal	Carboxy-terminal
CTL	Cytotoxic T cell
CTLA-4	Cytolytic T Lymphocyte associated Antigen 4
D	Diversity segment
DAG	DiAcylGlycerol
DC	Dendritic Cell
DD	Death Domain
DN	Double Negative
DNA	DeoxyriboNucleic Acid
DP	Double Positive
ECL	Enhanced Chemo Luminescence
ELISA	Enzyme Linked Immunosorbent Assay

ER	Endoplasmic Reticulum
Erk	Extracellular signal-regulated kinase
FACS	Fluorescence Activated Cell Sorter
FCS	Fetal Calf Serum
FDC	Follicular Dendritic Cell
FITC	Fluorescein IsoThioCyanate
FLICE	Fas-associating protein with DD-like Il-1β converting enzyme
FLIP	FLICE-like Inhibitory Protein
Fyb	Fyn-binding protein
GAP	GTPase-Activating Protein
GC	Germinal Center
GDP	Guanine DiPhosphate
GEF	Guanine nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
GPI	GlycosylPhosphatidylInositol
GTP	Guanine TriPhosphate
GTPase	Guanine TriPhosphatase
H	Heavy chain
HEV	High Endothelial Venule
HIV	Human Immunodeficiency Virus
HSC	Hematopoietic Stem Cells
HSP	Heat Shock Protein
HSPG	Heparan Sulphate ProteoGlycan
IFN	Interferon
Ig	Immonoglobulin
Ii	Invariant chain
Il-	Interleukine-
IMDM	Iscove's Modified Dulbecco's Medium
IP3	Inositol-1,4,5-triphophate
IPTG	IsoPropyl β-D-ThioGalactopyranoside
IS	Immunological Synapse
ITAM	Immunoregulatory Tyrosine Activation Motif

J	Joining segment
JNK	N-terminal c-Jun kinase
L	Light chain
LAT	Linker of Activation of T cells
LB	Luria Bertani (Broth)
LC	Langerhans Cell
LFA-1	Lymphocyte Function-Associated antigen-1
LPS	LipoPolySaccharide
LT	Lymphotoxin
MACS	Magnetic Activated Cell Sorter
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
MKK	MAP Kinase Kinase
MKKK	MAP Kinase Kinase Kinase
MTOC	MicroTubule Organising Centre
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
Ni-NTA	Nickel-NitriloTriacetic Acid
NK	Natural Killer
N-terminal	Amino-terminal
o/n	over night
p27kip1	27 kD cyclin-dependent kinase inhibitor
PALS	PeriArtiolar Lymphoid Sheath
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PE	PhycoErythrin
PEP	Proline- Glutamic acid- Serine- Threonine-rich domain Phosphatase
PI	PhosphoInositol
PI(4,5)P2	Phosphatidyl inositol-4,5 biphosphate
PI3K	PhosphoInositol-3 Kinase
PKC	Protein Kinase C
PLC	PhosphoLipase C

PRR	Pattern-Recognition Receptor
pSMAC	peripheral Supra Molecular Activation Cluster
PTD	Protein-Tranduction Domain
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RAG	Recombination Activating Genes
RNA	RiboNucleic Acid
RT	Room Temperature
SDS-PAGE	Sodium Dodisyl Sulphate- Protein acrylamide Gel Electrophoresus
SH	Src Homology
SHP-1	SH2 domain containing tyrosine phosphatase
SLAP	SLP-76-Associated Protein
SLP-76	SH2 domain-containing Leukocyte Protein of 76 kDa
SP	Single Positive
SR	Scavenger Receptor
TAP	Transporter Associated with Processing
Tat	Transactivator of transcription
TB	Terrific Broth
TCR	T Cell Receptor
TFA	TriFluoroacetic Acid
TGF	Transforming Growth Factor
Th	T helper cells
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
Treg	T regulatory cells
V	Variable segment
WASP	Wiskott Aldrich Syndrome Protein
Y	Tyrosine
ZAP-70	Zeta-chain Associated Protein of 70 kDa

1. Introduction

1.1 The Immune System

Our bodies are constantly at war, under assault 24-hours a day from infection and toxins. The fact that we survive and most of the times not even give way to infection is due to our immune system, a network of cells and chemicals that protect the body. Two sub-groups of the immune system, the innate and the adaptive immune system, are responsible for protection and elimination of pathogens such as bacteria, viruses, parasites and fungi.

1.1.1 Innate Immunity

The immediate defence of the body against an invasion must be in the hands of preformed molecules present constitutively. Proteins, such as lysozyme, c-Reactive Protein, interferons and the complement system, are present in the body secretions and fluids and attack different microorganisms in a variety of ways, often by dissolving their protective layer. The strategy of innate immunity is based on the detection of constitutive and conserved products of microbil metabolism. Many metabolic pathways and gene products are unique to microorganisms of a certain class and absent from host cells. Therefore, these products are molecular signatures of invaders, and their recognition by the innate immune system signals the presence of infection.

Most of the white blood cells, called granulocytes (including neutrophils, basophils and eosinophils), alongside others called macrophages, mast cells and natural killer (NK) cells, have a role in initial defence. Their action does not depend upon prior exposure to the pathogen and it does not generate immunological memory. Consequently, these cells react the same way when a second encounter with the same antigen takes place.

Because the targets of the innate immune system are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMP). PAMPs are produced by microbes and not by host cells, allowing the innate immune system to discriminate between self and non-self. Since PAMPs are invariant between

microorganisms of a given class, this allows a limited number of receptors to detect the presence of any microbial infection. Importantly, PAMPs are essential for the microorganism and mutation or loss is either lethal or results in greatly reduced fitness. Accordingly, the receptors of the innate immune system that recognise PAMPs are the pattern-recognition receptors (PRR). These receptors recognise for example carbohydrate structures that are not present on host cells, including certain mannose linkages and lipopolysaccharide (LPS) on gram-negative bacteria. Various PRRs are expressed on the cell surface, in intracellular compartments, or secreted into tissue fluids. The principal functions of PRRs include: opsonisation, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways and induction of apoptosis (Janeway Jr, 1989).

PRRs that have a unique and essential function in immunity are the Toll-like receptors (TLR). TLRs comprise a family of at least ten receptors in mammalian species, each one with a distinct function in innate immune recognition. The TLR ligands are PAMPs, and many TLRs can recognise several structurally unrelated ligands; some TLRs, however, require accessory proteins to recognise their ligand. TLR4 was the first characterised mammalian Toll receptor (Medzhitov et al., 1997). It is expressed in a variety of cell types, predominantly macrophages and Dendritic cells (DC). It functions as a signal transduction receptor for LPS, but requires accessory molecules to do so. TLR2 is involved in the recognition of an unusually broad range of microbial products, including cell wall products of gram-positive bacteria and yeast, and is only found expressed by antigen-presenting cells (APCs) and endothelial cells.

The secondary effect of recognition of microbial pathogens via either the PRRs or the complement receptors is to activate cells to synthesise cytokines. In particular, inflammatory cytokines and chemokines such as Tumour Necrosis Factor (TNF)-α, Interleukin (Il)-12, Il-6 and Il-1β play an important role in the second phase response. In addition, interferon (IFN)α and IFNβ are produced by a variety of cells in response to viral infection. They have an important role in limiting viral infection in the early phase. They act on a wide variety of cell types to induce the synthesis of a series of proteins that interfere with viral replication both by degrading ribonucleic acid (RNA) and by inhibiting protein synthesis. They also potently activate NK cells.

NK cells are another component of the innate immune response and have the ability to both lyse target cells and provide an early source of cytokines. NK receptors are crucial for distinguishing normal cells from transformed or foreign cells, thereby eliminating the latter. In more recent years several lines of evidence have shown that besides γδ T cells and αβ T cells a separate lineage of NK T cells exists, which co-express NK receptors such as NK1.1 and the αβ T cell receptor (TCR). NK T cells recognise a glycosphingolipid presented by cluster of differentiation (CD)1d with a highly skewed TCR repertoire (Bendelac et al., 1997). The precise origin of the NK T cell remains unknown, but many gene defects influencing NK T cell development also affect NK cells, suggesting a common precursor. Several studies have indicated a thymic dependency for the development of NK T cells (Bendelac et al., 1994; Bendelac, 1995; Coles and Raulet, 2000), but it is as yet poorly understood which role these cells play in immune responses.

1.1.2 Adaptive Immunity

Protection against the enormous variety of microorganisms, continuously changing and evolving, cannot be achieved by the innate system alone. Adaptive immune recognition relies on the generation of a random and highly diverse repertoire of antigen receptors, followed by clonal selection and expansion of receptors with relevant specificities. This mechanism accounts for the generation of immunological memory, which provides a significant adaptive fitness. However, these randomly generated receptors are unable to determine the source and biological context of the antigen for which they are specific. The clonal distribution of antigen receptors also requires that specific clones expand and differentiate into effector cells before they can contribute to host defence. This requires time, typically 4-7 days, which is too much of a delay to combat quickly replicating microbial invaders. This illustrates that the adaptive immune system operates in the context of the innate immune system. Almost every aspect of the adaptive immune system is controlled by a combination of permissive and instructive signals, provided by the evolutionarily ancient and more universal innate system. Thus, the innate system detects the presence and the nature of infection, provides the first line of defence and controls the initiation of the effector cells of the adaptive immune response. Conversely, the adaptive system is able to change and adapt, resulting in a more swift and efficient response upon subsequent encounters with the same antigen.

1.2 Lymphocytes

The two major types of lymphocytes, B and T cells, generated in the bone marrow and thymus respectively, are both capable of producing an almost unlimited number of receptors. Both B and T cells are derived from a common hematopoietic stem cell. The B cell receptor (BCR) and the TCR are encoded by a finite number of genes, each of which encodes a part of the antigen receptor (Tonegawa, 1976).

1.2.1 B lymphocytes

The differentiation of precursors along the pathway of B cell development has been well characterised. The BCR, an immonoglobulin (Ig), is composed of four polypeptide chains; two identical light (L) chains, associated with two identical heavy (H) chains. The genes encoding for the L chain are organised in multiple variable (V), joining (J) and constant (C) segments; the H chain genes have diversity (D) segments in addition. During B cell development any V gene segment can be joined to any J or DJ gene segment by somatic recombination, resulting in an enormous diversity of receptor specificities. Different gene segments are joined in different B cells, generating a variety of Ig chains, during a process known as rearrangement, driven by enzymes encoded by recombination activating genes (RAG). In this way a few hundred gene segments can combine in different ways to form thousands of different receptor chains. Finally, the light and heavy chain are assembled together, to form a functional receptor increasing variety even more.

The recognition of proteins by the BCR and its secreted counterpart, the antibody (Ab), involves direct binding to the native protein structure. As a result of the gene rearrangements generating the broad repertoire of BCRs, B cells will also be capable of recognising self-antigen. Those immature B cells that express autoreactive receptors have a chance to survive by undergoing secondary L chain gene rearrangements (Gay et al., 1993). As soon as a non-autoreactive receptor is expressed the recombination mechanisms are turned off ensuring that each B cell produces only one type of receptor. This mechanism is known as allelic exclusion. Remaining autoreactive cells are deleted by accelerated apoptosis.

Mature B cells may be stimulated by foreign antigen to enter the cell cycle, differentiate to Ig-secreting plasma cells or become long-lived memory B cells. T cell-independent antigen will not induce longevity and memory. With the help of T cells, however, B cells can become long-lived, can switch the expression of the H chain they express and can hypermutate the V regions of their IgH and IgL chain genes. A germinal center (GC)-specific activator-induced cytidine deaminase, a potential RNA editing enzyme, is a key factor controlling the two GC-specific events, somatic hypermutation and class switch recombination of immunoglobulins (Muramatsu et al., 2000).

Antigen is bound by B cells, internalised, processed and returned to the cell surface in association with a molecule known as major histocompatibility complex (MHC) class II. The MHC/peptide complex is then recognised by activated T cells carrying a specific TCR (Lanzavecchia, 1985). The T cells express additional cell surface molecules that interact with receptors on B cells providing important activation signals. The immediate effect of activation by T cells is to trigger B cell proliferation. However, another important effect can be observed when CD40 ligand (CD40L), a molecule expressed by activated T cells (Armitage et al., 1992), binds CD40. CD40L/CD40 interactions, in synergy with the effects of cytokines such as Il-4, Il-5 and Il-6 that are secreted by the T cell, induce B cell differentiation into plasma cells and isotype switching (Jabara et al., 1990).

High-affinity Igs are produced through a process of maturation and selection in the GCs. The germline antibody repertoire is selected during evolution and permits the low-affinity recognition of antigens. It would be difficult and inefficient to select for specialised high-affinity receptors at that stage. However, to control invaders and provide better protection in the future, a more fitted repertoire is generated during maturation of an immune response by a specific mechanism that introduces single point mutations within specific V genes. This process of hypermutation permits selection of high-affinity clones in a locally controlled microenvironment (Liu et al., 1992). The antigen is trapped on the surface of specialised follicular dendritic cells (FDC), and the BCRs of the surrounding B cells compete for it. Consequently, antigen can select the better binding mutant B cells in GCs and expand them selectively over other mutants that have not improved their specificity for the antigen.

After contact with the specific antigen, B cells differentiate into plasma cells producing and secreting large amounts of the soluble form of their Igs called antibodies. These circulating antibodies can bind to the pathogen protecting the host in three major ways. They facilitate antigen recognition and uptake by phagocytes, they activate the complement system and they neutralise pathogenic effects by coating the microbial surface. Phagocytes in their turn process and present the endocytosed pathogen to T cells.

1.2.2 T lymphocytes

T lymphocytes are the other pillar of the adaptive immune response. Although the thymus is the major anatomical site of T cell development, a distinct lineage of extrathymically-derived T cells is present in the adult gut mucosa. However, the majority of T cells arise from a lymphoid progenitor that develops in the thymus by cell-intrinsic and lineage-specific differentiation programs, guided by cell-cell interactions and soluble factors.

T and B cells share many developmental properties, including rearrangement of their receptor genes. In contrast to B cells, however, most T cells need their antigen to be presented in the context of MHC molecules (Zinkernagel and Doherty, 1979). These are glycoproteins, of which the two main classes, I and II, bind largely non-overlapping sets of peptides that are generated in distinct intracellular locations. Their function is to collect peptides inside the cell and transport them to the cell surface, where the peptide/MHC complex can then be recognised by the TCR. MHC class I molecules consist of a membrane-inserted heavy chain and a non-covalently attached light chain, also known as β_2-microglobulin (β_2m). The structure of the class I molecule has been resolved, and resembles a linear groove surrounded by a wall on each side (Stern and Wiley, 1994). Class II molecules resemble class I molecules in structure, but both α and β chains are membrane inserted. Intriguingly, MHC genes are characterised by their polymorphism; dozens or hundreds of alleles can be present in a species.

The TCR itself is also a glycoprotein, formed by two polypeptide chains, α and β or alternatively γ and δ. Each chain consists of two Ig-like domains, a transmembrane part and a short cytoplasmic tail. $\gamma\delta$ T cells and $\alpha\beta$ T cells are not just

similar lymphocytes with subtly different receptors. The cell types differ in the type of antigen they recognise, the mechanism of antigen presentation and recognition and the mechanism and kinetics of downstream signalling events. Antigen recognition by γδ T cells, which represent about 5% of peripheral blood T cells, resembles recognition by B cells, because they recognise intact protein antigens and small phosphate- or amine-containing compounds (Hayday, 2000). Although MHC-presentation of these antigens is not required, cell-cell contact seems to be crucial for stimulation, suggesting that co-stimulation is required or that non-MHC molecules play a role in presentation (Morita et al., 1995). γδ T cells have been implicated in several immunological roles, including immediate responses to pathogenic invasion and long-term modulation of inflammation (Carding and Egan, 2000).

The two main populations of αβ T cells, named according to the expression of the co-receptor molecules CD4 and CD8, are restricted to one class of MHC. CD4 and CD8 are non-clonally distributed proteins that, because they recognise the ligands of the TCR, are often referred to as co-receptors. The CD4 subset, also known as helper T cells (Th) recognises antigen in context of MHC class II, expressed constitutively by professional APCs like DCs and B cells. The CD8 subset, also known as cytotoxic T cells (CTL), recognise antigen in the context of the MHC class I molecule, generally expressed in every nucleated cell.

1.2.2.1 CD4 T cells

CD4 T cells have a central role in the immune system, and their activation is often a prerequisite for responses by other types of cells, including CD8 T cells and B cells. CD4 T cells themselves, however, are phenotypically heterogeneous, and the different subsets are involved in markedly diverse immunological functions. They are primarily responsible for providing help to other immune cells through direct cell–cell interactions or the secretion of cytokines. CD4 T cells recognise their specific antigen in the context of MHC class II, and differentiate into different subsets of effector cells most noticeably Th1, Th2 and possibly T regulatory (T_{reg}) cells, characterised by their cytokine profile.

MHC class II molecules can be expressed by DCs, macrophages and B cells, the so-called APCs. They are generally involved in initiating the adaptive immune response by presenting peptides mostly derived from extracellular origin, such as

pathogens and bacterial toxins. These APCs have an array of receptors enabling them to engulf pathogens and their products to classify the invader. Once internalised, the antigen ends up first in endosomal vesicles, and later in lysosomes. It is between these compartments where a progressively decreasing pH activates proteases, specifically targeted to the endosomal-lysosomal compartments from the endoplasmic reticulum (ER) and Golgi system, leading to degradation of the antigens. Class II molecules also start their existence in the ER. The two chains assemble and are bound by a chaperone-like molecule, the invariant chain (Ii) (Cresswell, 1994). Ii serves two functions; it directs the class II molecule to the class II loading compartment, a specialised vesicle characterised by the presence of high amounts of MHC II. In addition, it prevents premature peptide occupancy of the groove of the class II molecules. A stretch of the Ii binds into the groove and thereby, competitively prevents the binding of peptides. This class II-associated invariant chain peptide (CLIP) is removed from the heterodimer in the class II loading compartment, giving the opportunity to fill the groove with peptide fragments present there. Finally, the peptide-loaded class II proteins are translocated to the cell surface. The protein loaded onto MHC II is under normal circumstances from endogenous origin due to a constant process of endocytosis. These include especially membrane-bound proteins, which co-localise in the coated vesicles together with proteins from serum and the extracellular matrix, but in the case of an ongoing infection, exogenous protein is presented this way to CD4 T cells.

As described, an essentially common pathway achieves MHC class II-restricted antigen presentation to CD4 T cells. This is, however, subject to variation with regard to the location and extent of degradation of protein antigens and the site of peptide binding to MHC class II molecules (Griffin et al., 1997; Fernandes et al., 2000). These subtle variations reveal flexibility in the ways a diverse peptide repertoire is displayed on the APC surface. This diversity has profound consequences for the induction of immunity, and leads to the differentiation of CD4 T cells into subsets of T helper cells, Th1 and Th2, and possibly T_{reg}. Th1 cells are important for the eradication of intracellular pathogens like some bacteria and parasites, and viruses, Th2 cells are targeting extracellular parasites and soluble toxins, while T_{reg} are reported to inhibit immune responses.

1.2.2.2 CD8 T cells

In contrast to CD4 T cells, CD8 T cells recognise antigen in the context of class I MHC molecules (Townsend et al., 1986), which are expressed not only on APCs but also on every nucleated cell. Class I molecules are synthesised in the ER, but unlike class II molecules this is also where they get loaded with peptide. Cytosolic proteins are degraded by the proteasome, a multiunit structure with several activities located in the cytosol (Rock et al., 1994). The endopeptidase specificity of the proteasome is such that a protein is in principle cut after a hydrophobic residue. At least two proteasome subunits, LMP2 and LMP7 influence the fine-tuning of the specificity and endopeptidase activity, and are regulated by IFNs. Protected from complete degradation by the chaperone Heat Shock Protein (HSP)70, the resulting peptides are transported into the lumen of the ER by the Transporter Associated with Processing (TAP) (Neefjes et al., 1993). Once inside the ER, and with the help of several other molecules, peptide fragments are loaded onto class I molecules. Upon peptide loading, the MHC class I is released by calnexin for transport to the cell surface.

Thus, peptides loaded onto class I molecules and presented to CD8 T cells are derived from cytosolic proteins, under normal circumstances produced by the cell itself. When a cell is infected, however, the pathogen's deoxyribonucleic acid (DNA) is transcribed, RNA translated, and the protein synthesised in the host cytosol. Fragments derived from this invader are presented via the described MHC class I, resulting in presentation to CD8 T cells. Upon recognition by CD8 T cells, the T cells differentiate into effector cells and are able to specifically kill infected cells, presenting the specific peptide, by apoptosis. As a result, the apoptotic bodies left behind are internalised by phagocytes.

The described loading of class I and class II MHC molecules suggests a strict separation of the processing pathways. However, there is strong evidence for considerable cross-talk between the two pathways. Peptides derived from cytosolic proteins can be loaded onto class II molecules (Pinet et al., 1994). On the other hand, exogenous antigens can also be presented by class I MHC molecules by a process called cross-presentation (Bevan, 1976). This pathway is less well defined but can overlap those pathways operating in classical MHC class I presentation. Protein antigens that are synthesised in one cell can be captured as exogenous antigens by

APCs and processed into the MHC class I presentation pathway for priming CTL immunity. This may, however, not be a common property, and limited to a subset of DCs (den Haan et al., 2000; Pooley et al., 2001).

Concurrent with the initiation of proliferation is the start of a program of gene expression that arms the CD8 T cell with an arsenal of effector mechanisms to combat the infection. The capacity to mobilize these effector mechanisms, which include cytolysis of infected cells and the production of cytokines, chemokines, or microbicidal molecules, develops over time after the initial stimulation of naive CD8 T cells and manifests upon subsequent encounter of the effector CD8 T cells with infected cells. Activated CD8 T cells are able to induce cytolysis of infected cells by two distinct molecular pathways: the granule exocytosis pathway, dependent on the pore-forming molecule perforin, or by the upregulation of FasL (CD95L), which can initiate programmed cell death by aggregation of Fas (CD95) on target cells (Berke, 1995).

1.3 T Cell development

Like other cells of the blood-forming system, T lymphocytes are derived from pluripotent hematopoietic stem cells (HSC) present in the fetal liver or adult bone marrow (BM). T lymphocyte development depends on interactions between differentiating progenitor cells and a complexity of supportive stromal cells in primary lymphoid organs. Although the thymus is the major site of T cell development, a distinct lineage of extrathymically-derived T cells is present in the adult gut mucosa. These T cells are derived from precursors present in specialised structures in the lamina propria (Saito et al., 1998).

HSCs give rise to a common lymphoid precursor (CLP), which has lost the erythroid and myeloid potential but is capable of giving rise to lymphocytes (B, T and NK cells) and possibly thymic DCs (Ardavin et al., 1993). Since B and T cells share a remarkable number of developmental properties, it has been suggested that they originate from a common precursor. However, more recent results challenge this view, and indicate that commitment to T cell and B cell lineages occurs instead through myeloid/T and myeloid/B bipotential stages, respectively (Kawamoto et al., 2000). Notch-1, a transmembrane receptor, is thought to play an important role in this

lineage decision between B and T cell (Pui et al., 1999; Radtke et al., 1999). CLPs committed to a T cell fate thereafter migrate from the BM to the thymus or gut.

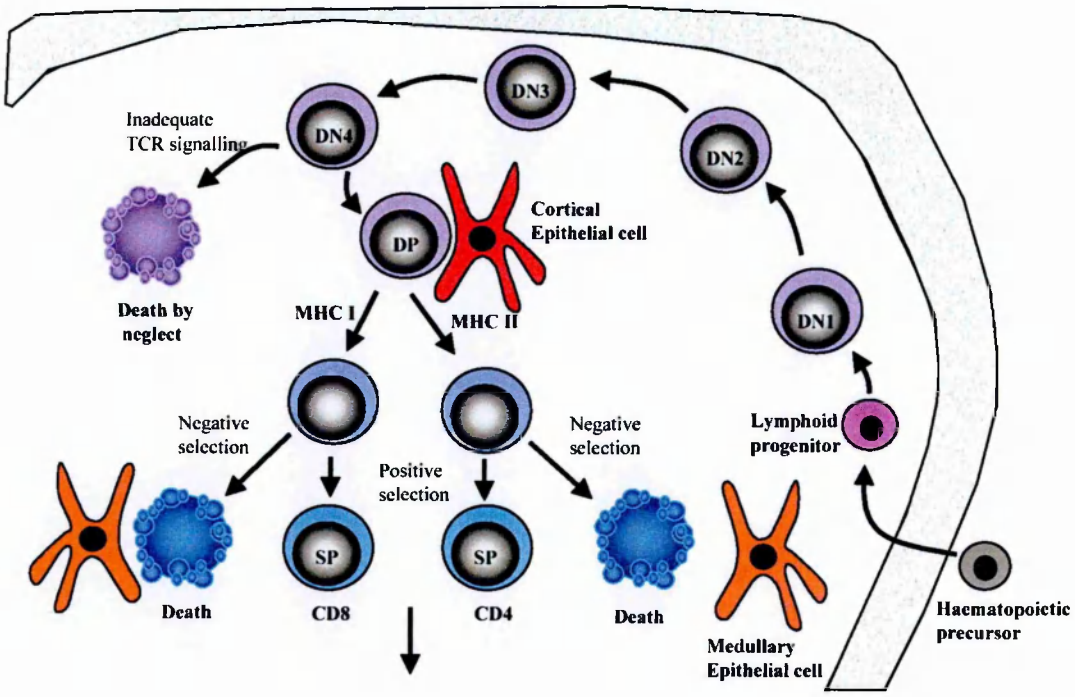


Figure 1. Overall scheme of T cell development in the thymus. Committed lymphoid progenitors in the bone marrow migrate to the thymus. Early committed T cells lack expression of CD4, CD8 and TCR, termed double negative (DN) thymocytes. As cells progress through the four DN stages, they express the pre-TCR. Successful expression results in proliferation during the DN4 to double positive (DP) transition, and the mature TCR is expressed. Interactions with cortical epithelial cells, expressing a high density of MHC molecules, associated with self-antigens, results in positive and negative selection. Thymocytes that express TCRs that interact with MHC class I molecules become CD8 single positive (SP) T cells, whereas those that express TCRs that bind MHC class II become CD4 SP T cells, ready to be exported to the peripheral lymphoid organs. Derived from Germain, 2002.

Following definitive commitment to the T cell lineage, intrathymic precursors express CD25 and begin to rearrange and express their TCR β, γ and δ genes. Cells that successfully rearrange their γ and δ genes express a γδ TCR and can proceed along the γδ lineage. Similarly, successful β rearrangement leads to cells expressing a pre-TCR, formed together with the invariant preTα chain. It is currently ill understood what leads to αβ/γδ lineage commitment. αβ/γδ lineage decision seems to occur independently of the TCR, as shown by analysis of the developmental potential of pro-T cells. These T cells are characterised by a very limited β, γ and δ gene rearrangement and heterogeneous expression of the surface marker CD127. The CD127lo subset was found biased towards αβ T cell development, whereas the CD127hi subset gave rise preferentially to γδ T cells (Kang et al., 2001).

Mature αβ T cells are comprised of two lineages, CD4 and CD8 T cells, the antigen recognition of which is focused on either MHC class II- or class I-associated peptides and whose functions are tuned to these recognition biases.

1.3.1 Selection events

Following successful TCR production and surface expression, CD4 and CD8 double positive (DP) thymocytes are subject to a rigorous selection process. They are tested for recognition of peptide/MHC complexes through the mechanisms known as positive and negative selection. The procedure of removing overt self-reactivity while imparting self-MHC restriction to the mature TCR repertoire is thought to operate via selective processes regulated primarily by the affinity/avidity of the TCR for thymic ligands (self-MHC and self-peptide) and the resulting TCR-mediated signals.

1.3.1.1 Positive selection

After the rearrangement of the α and β chains, most DP thymocytes express TCRs that interact with available self-peptide-MHC ligands at very low affinity, or do not interact at all. Intracellular signals that are required to sustain viability in these circumstances are thought not to be generated, leading to death by neglect and ensuring that the T cells that survive the process are able to interact with self-MHC-peptide complexes (Huesmann et al., 1991). Selected cells then down-regulate expression of RAG and proceed to the next stage of development in the medulla.

How cortical epithelial cells drive efficient positive selection is currently unknown. It is clear however that thymic-epithelial cells play a key role by providing peptide-MHC ligands for the αβTCR. Several studies have shown that the ability of the epithelium to promote positive selection is neither due to provision of a specialised repertoire of peptide-MHC complexes (Bevan, 1997), nor merely to maintenance of thymocyte viability by the production of survival promoting signals (Anderson et al., 1997). It is likely that thymic epithelial cells express unique, or a unique combination of, cell surface molecules that provide accessory signals during selection (McGargill et al., 2000). This idea is supported by the observation that under normal circumstances thymic epithelial cells lack the ability to provide co-stimulation for peripheral T cells (Jenkinson et al., 1994).

1.3.2.2 Negative selection

A generally accepted model of thymocyte selection is the "strength of signal" hypothesis. In this quantitative model, "strong" activation signals result in cell death (negative selection) and "moderate" activation signals result in survival (positive selection). Extremely low or no signal at all subsequently leads to death by neglect. The fate of a developing T cell is thus critically determined by the interaction between the TCR and its ligands (Grossman and Singer, 1996). Therefore, in contrast to thymocytes with lower affinity TCRs, if at this stage of development the thymocyte expressed a TCR with high affinity, it was thought that the cell would be deleted (Kisielow et al., 1988). However, more recent data suggest that thymocytes with high affinity TCRs get a second chance and are allowed to continue to rearrange the α-locus until a TCR is displayed that is successful in interacting with self-MHC with moderate affinity (McGargill et al., 2000).

It was also thought that the most efficient mediators of negative selection in the thymus were BM-derived DCs, residing at the corticomedullary junction. However, lately the medullary epithelium has also been implicated in the process. It has been shown to express many proteins that were previously thought to be tissue specific, suggesting a role in tolerance (Klein et al., 1998; Klein et al., 2000). Additional results suggest that the origin of the APC that is encountered by the thymocyte might play an important role. If a strong interaction takes place with a BM-derived APC, the thymocyte is eventually deleted when failing to express a proper TCR, but when the encounter is with an epithelial cell the thymocyte might be

diverted to a subset of immunoregulatory CD4+CD25+ T cells (Jordan et al., 2001). The end result however is that the TCR repertoire expressed by mature T cells is largely devoid of autoreactivity, due to negative selection and is restricted to recognising foreign peptides in the context of self-MHC molecules, due to positive selection. In addition, and the focus of recent interest, expression of self-antigens by thymic epithelium may encourage peripheral tolerance by the promotion of T_{reg}, which might more actively prevent autoimmunity.

1.3.1.3 Lineage commitment

The final stage of differentiation is the down-regulation of one of the co-receptors, so that single positive (SP) T cells, either CD4 or CD8 can leave the thymus as mature cells. Successful maturation of DP thymocytes seems to be restricted to those that maintain expression of the co-receptor molecule that has a MHC class specificity that matches the TCR. How the commitment to one or the other lineage is made is still poorly understood. The debate on the mechanism responsible focuses on whether the commitment decision is the result of instructive or stochastic signals (Jameson et al., 1995; von Boehmer, 1996).

In brief, the instructive model proposes that the interaction of a TCR and co-receptor with a MHC ligand instructs the cell, by a unique biochemical event, as to which path to take and which co-receptor to down-regulate and stop transcribing. The stochastic model proposes random down-regulation of one of the co-receptors, after which the survival of the thymocyte depends on the signal of a matching TCR and co-receptor. Silencing the transcription of one co-receptor locus is accompanied by other genetic events that determine the effector potential of the mature T cell.

DP thymocytes do not simply extinguish transcription of one co-receptor; instead it was shown that TCR signalling first leads to some loss of both co-receptors irrespective of the recognised MHC class (Lucas and Germain, 1996). This loss seems to correlate with the strength of TCR signalling whereby stronger TCR signals result in more down-regulation of both co-receptors. Thereafter, first CD4 and shortly after CD8 are re-expressed, followed by the loss of expression of CD4 for cells committed to the CD8 lineage and loss of CD8 for cells committed to the CD4 lineage.

Although not all available data is interpreted in the same way, and elements of both hypotheses have been found to contribute to the final result, currently an instructive model for lineage commitment is favoured (Itano and Robey, 2000). Exchanging cytoplasmic tails of CD4 and CD8 indicated that lineage fate was largely dictated by the signalling part of the co-receptors, rather than by the class of MHC recognised (Seong et al., 1992; Itano et al., 1994; Itano et al., 1996). In addition it was demonstrated that MHC class II-restricted thymocytes became CD8 SP T cells in the absence of CD4, suggesting that a weak signal leads to the CD8 fate (Matechak et al., 1996).

The above observations also led to a 'strength of signalling' hypothesis whereby strong signals drive the lineage fate to CD4 and weak signals to CD8 SP T cells. The hypothesis was later reinforced by showing that varying the concentration and duration of exposure of pre-selection DP thymocytes to phorbol ester and ionomycin led to the selective production of either CD4 or CD8 SP T cells (Ohoka et al., 1997). In addition, crosslinking TCR and the co-receptor together gave rise to CD4 T cells, and crosslinking the TCR alone resulted in CD8 SP T cells (Bommhardt et al., 1997; Basson et al., 1998). A key signalling component proposed is the tyrosine kinase Lck, engaged with co-receptors, both CD4 and CD8. CD4 molecules isolated from DP thymocytes are associated with more Lck than CD8 molecules (Veillette et al., 1989). Therefore, co-engagement of CD4 and TCR with MHC class II promotes the recruitment of more Lck into the signalling complex than CD8 and TCR engagement with MHC class I.

The strength of signal hypothesis, however, could not fully explain why duration of a stimulus influences the lineage fate, and was accordingly adjusted to a signal duration concept (Ohoka et al., 1997; Basson et al., 1998). Experiments in mature T cells have shown that Lck coupling to the co-receptors influences the quality of receptor signalling, consequently effecting desensitisation and duration of signalling (Madrenas et al., 1997). Thus, strong sustained activation of Lck is correlated with CD4 commitment, and weaker more transient Lck activation with CD8 commitment (Legname et al., 2000).

1.3.2 SP thymocytes

After positive and negative selection and following lineage commitment, DP thymocytes become SP thymocytes, either $CD4^+CD8^-$ or $CD4^-CD8^+$. However, development does not stop there, and functional competence still needs to be acquired. SP thymocytes have been shown to reside in medullary areas for up to two weeks, during which they undergo changes in expression of a variety of cell-surface molecules (Gabor et al., 1997), and several rounds of cell division (Ernst et al., 1995), before they are exported to the periphery. The acquisition of full T cell functions is believed to take place during these later cell divisions, reflected by the change of cell-surface molecules, and in close contact with thymic epithelial cells.

Despite at least six rounds of cell division, the number of SP thymocytes is very low (about 2% of total thymocytes). This could be attributed predominantly to the low efficiency of successful rearrangements of the α-locus and to a lesser extent the frequency of DP cells expressing self-reactive TCRs, which would then be negatively selected (Surh and Sprent, 1994). However, even in TCR transgenic mice where most DP thymocytes express a pre-selected TCR only 20% of the DP thymocytes cells become mature SP thymocytes (Huesmann et al., 1991). This is thought to be due to limiting stromal cell microenvironments that can sustain the DP to SP transition, at least in a TCR transgenic scenario (Merkenschlager et al., 1994).

1.4 Peripheral T cells

The capacity of the peripheral immune system to deal with a broad range of antigens in an adequate way relies on the ability of the system as a whole to recognise virtually all antigens. The thymus serves its function in creating a vast array of TCRs and exporting naive T cells displaying unique TCRs. Mature thymocytes leaving the thymus contribute to a heterogeneous pool of peripheral lymphocytes adding their unique receptor to an existing collection. However, with such a vast array of TCRs, the frequency of T cells capable of responding to each antigen is very low. To deal efficiently with antigenic challenges, naive cells recognising their respective antigen have to be expanded to overcome the numeric disadvantage at the start of a response. On the other hand, naive T cell numbers need to be maintained since they form as a collective the basis of specific antigen recognition. In order to deal more efficiently

with any subsequent encounters with the same antigen, T cells that have been proven useful must also be maintained. Thus, the peripheral pool of T cells consists of different subsets at various stages of activation and differentiation generally divided into three simplified sub-populations: naive cells, effector cells and memory cells.

1.4.1 Naive T cells

Naive T cells are the cells that have never encountered their specific antigen, and continuously recirculate through the body. Their mobility enables them to access the secondary lymphoid tissues (lymph nodes and spleen) where they scan the present APCs for their specific antigen, thus surveying the body for the presence of intruders. Despite its complexity and the variety of challenges that it is subject to, the immune system maintains a dynamic equilibrium in terms of size and subset composition throughout much of normal adult life. For the purpose of maintaining cell numbers the T cell population seems to be divided into two major compartments: a naive pool consisting of recent thymic emigrants and T cells that have yet to encounter their antigen, and a memory pool consisting of antigen-experienced T cells (Freitas and Rocha, 2000). This separation may form the basis of ensuring that the TCR diversity in the naive pool is not compromised by proliferation of cells in the memory compartment, which has a much-reduced diversity (Arstila et al., 1999). Vice versa it prevents the displacement of memory cells by new thymic emigrants, thus protecting T cells essential for a fast reactivation of the immune system.

Maintaining numbers consists of two aspects, compensating for newly derived thymic emigrants and the loss of cells from the naive pool. The constant arrival of thymic emigrants suggests that space has to be created to accommodate them in the naive T cell pool, and subsequently that naive T cells have a limited life span. Upon recognition of an antigen naive T cells are activated and differentiate into effector cells, thus disappearing from the pool of naive T cells. The loss of cells is primarily counterbalanced by the new thymic output, but it is thought that this is insufficient to replenish the loss of T cells in immune-compromised situations as in HIV infection or old age. An important contribution toward restoring T cell numbers is made by the proliferation of peripheral T cells (Rocha et al., 1989; Dummer et al., 2001), known as homeostatic proliferation. Expansion of peripheral T cells cannot regenerate the TCR diversity found in the full naive compartment, but it creates more diversity than expansion of the memory pool.

The survival of peripheral T cells and the mechanism behind homeostatic proliferation has been investigated in many different systems. Several groups have now shown that the spontaneous proliferation of naive cells as well as survival of mature naive T cells seems to depend on interactions between TCR and peptide-MHC in the periphery (Takeda et al., 1996; Kirberg et al., 1997; Freitas and Rocha, 2000); these results are however still disputed and a molecular mechanism has yet to be found (Dorfman et al., 2000). In addition to T cell homeostasis regulated by cell-cell contact, cytokines are also known to regulate the T cell compartment. Among the many cytokines that influence T cell survival and proliferation, Il-7 has been shown to be an important factor (Maraskovsky et al., 1996; Schluns et al., 2000).

1.4.2 Effector T cells

After naive T cells have encountered their antigen, they become activated and differentiate into effector cells, ready to neutralise invading pathogens. Initially the activated cells go through many rounds of cell division, generating a pool of effector cells. However, following the peak of expansion, there is a rapid decline in the number of antigen-specific cells. Although effector cells are generally thought not to belong to either the naive or the memory compartment, and thus not to comply with mechanisms of T cell homeostasis in either of these pools, numbers of T cells need to be controlled (Freitas and Rocha, 2000). The death of effector T cells ensures T cell homeostasis, preventing an accumulation of effector cells with a certain specificity that would compromise the capacity to respond properly to new pathogens, and reducing the metabolic costs to the organism. If the immune response unfolds in the absence of inflammatory stimuli the extent of T cell death is so profound that the animal contains fewer antigen-specific cells than before the response occurred (Chen et al., 1995; Mondino et al., 1996). As a result the organism is tolerised for future responses against the same antigen. If the response is accompanied by inflammatory stimuli, such as provided by infectious agents, the immune response results in more antigen-specific T cells than before the encounter occurred, although less than present at the peak of the response.

At least two separate types of cell death control the number of effector T cells; activation-induced cell death (AICD) and cytokine withdrawal. There is some confusion with the term AICD, and under different circumstances, for example *in vivo* and *in vitro*, the underlying mechanism is likely to differ. AICD was first used in the

context of T cell hybridomas and thymocytes shown to die by apoptosis following CD3 stimulation *in vitro* (Shi et al., 1989). It was thereafter shown that Fas (CD95), and possibly TNFα receptors drive the *in vitro* death after CD3/TCR stimulation (Lenardo et al., 1999). These death receptors when engaged activate signalling pathways by recruiting adapter molecules through their death domain (DD), which lead to caspase activation and eventually to cell death by apoptosis. In contrast, primary T cells are resistant to AICD because they express high levels of Fas-associating protein with death domain-like interleukin-1β converting enzyme (FLICE)-like inhibitory protein (FLIP), an enzymatic caspase-8 homologue and inhibitor of the Fas signalling pathway (Algeciras-Schimnich et al., 1999; Himeji et al., 2002). However, upon activation, T cells produce the cytokine Il-2, and this is responsible for a decrease in FLIP levels and subsequent sensitisation of primary T cells to AICD (Algeciras-Schimnich et al., 1999). Low levels of TGF-β may also increase AICD susceptibility because T cells deficient in TGF-β have increased sensitivity to Fas mediated death, possibly due to a failure in elevating FLIP levels (Chen et al., 2001).

There are additional mechanisms to those driven by death receptors, illustrated by showing that activated T cells die *in vivo* in the absence of death receptors (Lohman et al., 1996; Van Parijs et al., 1998). An alternative pathway leading to death by apoptosis was found earlier and described as cytokine withdrawal (Duke and Cohen, 1986). Despite its importance in maintaining T cell numbers, the molecular mechanism behind cytokine withdrawal is poorly understood. Cytokine withdrawal has been shown to involve members of the Bcl-2 family, and to be responsible for the majority of activated T cell death *in vivo*. At the peak of T cell activation Bcl-2 levels within activated T cells are decreased compared with resting T cells (Marrack et al., 1999).

Several studies have shown that Bcl-2, a proto-oncogene, is involved in a separate pathway compared with Fas, controlling death in activated T cells (Strasser et al., 1995; Van Parijs et al., 1998). Overexpression of Bcl-2 does not protect against Fas-driven cell death nor does the absence of Fas protect cells from death otherwise prevented by Bcl-2 overexpression. Since the discovery of Bcl-2, a whole family of related proteins has been found. These molecules have either anti- or pro-apoptotic activity and it is generally thought that anti-apoptotic proteins like Bcl-2 form

heterodimers with pro-apoptotic proteins thereby preventing homodimerisation, which is essential for their pro-apoptotic function (Cheng et al., 2001).

1.4.3 Memory T cells

An important aspect of the immune system is that upon reencounter of a pathogen, the immune response is faster and more vigorous compared with the primary response. This implies that after initial contact with antigen, for B as well as T cells, some of the cells involved form memory cells (Ahmed and Gray, 1996). As stated above, most activated T cells participating in a primary response die by apoptosis, and it is largely unresolved how a small proportion of activated cells survive and become long-lived memory cells. It seems that at least for CD8+ T cells, the memory cells are established at the end of a primary response (Sourdive et al., 1998) and that there is a correlation between the size of the initial pool of effector cells and the size of the resulting memory pool (Hou et al., 1994). This suggested that memory cells are derived from fully differentiated effector cells, but did not exclude that they arise from partially differentiated cells. It is well established now that naive T cells need to undergo several rounds of division before long-term memory arises (Swain, 1994; Garcia et al., 1999; Opferman et al., 1999). More evidence that memory cells are directly derived from effector cells came with the observations that they descend from cells which synthesise Il-2 and express perforin (Opferman et al., 1999; Saparov et al., 1999).

Although it appears that T cell-dependent immune responses culminate in memory cell generation, it has been shown that in cases where T cells undergo proliferation after encountering high doses of antigen or a superantigen, although there is a marked expansion of specific T cells, this is subsequently followed by their disappearance (Webb et al., 1990; Moskophidis et al., 1993). The end result is clonal deletion of the specific TCRs from the repertoire and tolerance by exhaustion. It implies that prolonged contact with antigen drives all effector cells to programmed cell death. Accordingly, memory cells may be derived from effector cells expressing a full range of effector functions, but which have not engaged their antigen at a high frequency or for a period of time long enough to drive them into apoptosis. In accordance, it has been shown that death and survival are greatly determined by the particular conditions encountered during the interactions with APCs. Many co-stimulatory molecules play a role in priming T cell responses, and influence the size

of the effector and ultimately the memory population (Whitmire and Ahmed, 2000). Either directly or indirectly these co-stimulatory molecules induce the up- and down-regulation of pro- and anti-apoptitic molecules like the previously mentioned Bcl-2 family (Garcia et al., 1999; Grayson et al., 2000).

Memory cells differ generally from naive cells in terms of function and surface markers; for example they display a $CD44^{hi}$ phenotype. They are hyper-responsive to antigen and synthesise large quantities of cytokines (Lanzavecchia and Sallusto, 2000). The particular cytokines secreted are determined by the conditions encountered during T cell priming. On basis of their activation status memory T cells have been divided into two categories. Resting or central memory cells closely resemble naive T cells, they express the lymph-node homing receptors CD62L and CCR7 (Sallusto et al., 1999), but they have a faster turnover rate than their naive counterparts (Tough and Sprent, 1994). Activated or effector memory cells display many features of effector cells and lack CD62L and CCR7 expression which results in exclusion from the lymph-nodes. They are thus mainly found in spleen and gut mucosa and in non-lympoid tissues like liver, lung and kidneys (Kim et al., 1999; Masopust et al., 2001). There is continuing debate on the importance of resting and activated memory cells and on whether they represent subsets with a different role in immunity or whether effector memory cells reflect an intermediate state of activation (Ochsenbein et al., 1999; Tough and Sprent, 1999; Appay et al., 2002).

Immunological memory depends on the maintenance of T cells proven to possess a useful TCR, in a higher frequency than in the naive pool, which can respond quicker and stronger upon subsequent encounter of their antigen. In comparison to naive T cells, the long-term survival of memory T cells does not seem to be a passive process, but memory cells divide slowly even in a full T cell compartment. In contrast to naive T cells, where most, but not all, available data indicate a requirement of MHC molecules, memory cells seem to survive in the absence of MHC molecules (Murali-Krishna et al., 1999; Swain et al., 1999; Kassiotis et al., 2002). In accordance, loss of contact with MHC ligands does not interfere with the high rate of memory T cell proliferation (Murali-Krishna et al., 1999). Although most results implicate that neither survival nor homeostatic proliferation depends on TCR ligation, the functional ability of at least CD4+ memory T cells to adequately respond to their specific antigen is impaired when denied access to MHC class II molecules (Kassiotis et al.,

2002). Accordingly, MHC may not be an absolute necessity for survival, but is essential for maintenance of the functional quality of the secondary immune response.

These findings suggest that other non-MHC ligands provide the stimulus for cell division. Currently the emphasis of memory T cell survival and proliferation is on cytokines, but although Il-7 plays an important role in naive T cell homeostasis, only a partial requirement was demonstrated for memory cells in lymphopenic hosts (Schluns et al., 2000). Most data is, however, obtained using CD8+ memory T cells, and consequently much less is know about the factors controlling CD4+ memory T cells. There is now strong evidence that the cytokine Il-15 acts directly on CD8+ memory cells (Kennedy et al., 2000; Nishimura et al., 2000); it is, however, only a poor stimulator of CD4+ memory cells (Zhang et al., 1999; Tan et al., 2002). The explanation probably lies in the selective expression of the Il-15 receptor on CD8+ memory cells, it being very low on CD4+ memory cells and absent on naive T cells (Ku et al., 2000).

Over the course of a lifetime, the impact of infections on existing memory cell populations constantly reshapes the memory pool. Reoccurring infections quantitatively delete and qualitatively alter the memory pool of T cells specific to a previously encountered virus (Selin et al., 1999; Appay et al., 2002), ultimately providing the organism with an up to date and highly effective system to fight off pathogens.

1.5 Antigen presentation

The sophisticated adaptive immune system depends on instructions by cells capable of trapping, processing and efficiently presenting antigens. The first step towards an adaptive immune response is undertaken by professional APCs such as macrophages, B lymphocytes and DCs. These cells have the potential to sense foreign antigens and non-specific inflammatory tissue damage and acquire the cellular specialisation to select and activate naive antigen-specific T cells. Following recognition and uptake of antigen, an array of antigen-derived peptides is displayed on the surface on MHC molecules. Subsequently, T lymphocytes, the executioners of the adaptive immune response, are activated through direct contact with APCs.

The central role for APCs in immune responses is also illustrated by their capacity to recognise conserved pathogen-associated molecular patterns by way of their TLRs, identifying the species of pathogen. Efficient priming of T cells requires not only the presentation of antigen in the context of a MHC molecule, but also the induction of accessory molecules on APCs and the local production of cytokines (Fearon and Locksley, 1996; Ito et al., 2002). Depending which TLRs are triggered, APCs secrete appropriate cytokines and thus determine the milieu in which the T cells are primed (Schnare et al., 2001).

Each of the APC groups varies in their location in the organism, in the mechanism of antigen uptake, the expression of MHC molecules and the availability of accessory molecules. This equips the organism with optimal detection methods for invading pathogens and their subsequent processing and presentation to T lymphocytes.

1.5.1 Macrophages

Macrophages originate in the bone marrow and migrate to body tissues via the blood stream. In adult life, the bone marrow releases macrophage precursors (immature myeloid cells) and monocytes into peripheral blood, but only upon entering the various tissues through the endothelial lining of blood vessels do precursor cells undergo their final differentiation. Myeloid cells become residential macrophages through stimulation with macrophage differentiation or growth factors, such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), produced *in situ* and/or supplied humorally. Monocytes only migrate into tissues in response to inflammatory stimuli and differentiate there into macrophages. Like monocytes, the monocyte-derived macrophages have no proliferative potential and are short-lived, whereas the resident macrophages are long-lived in tissue, possess proliferative capacity and can be sustained by self-renewal (Naito et al., 1996). In order to become activated and fully functional, macrophages need to interact with either the cytokine IFNγ, released by activated T lymphocytes, or microorganism-derived components (Celada and Nathan, 1994).

Antigen uptake is achieved mainly by phagocytosis and is mediated through specific receptors expressed on the surface, which among others include Fcγ-receptors (Ravetch and Bolland, 2001), complement receptors and scavenger receptors (SR) (Hughes et al., 1995). SRs play an important role in uptake and clearance not only of pathogens but also of modified host molecules and apoptotic cells. The complexity of endocytosis, in which many receptors and signalling molecules are involved, was shown when purified macrophage phagosomes where found to contain more than 140 proteins (Garin et al., 2001).

A critical feature for an effective immune response is the production of cytokines and chemokines during phagocytosis. Some phagocytic receptors alone trigger the production of chemokines and cytokines, but often engagement of TLRs is required. Several TLRs are actively recruited to the phagosomes during internalisation of microbes where they sample the phagosomes content to determine the nature of the ingested pathogen (Underhill et al., 1999).

1.5.2 B cells

While macrophages are the scavengers of the immune system, B cells are non-phagocytic. However, B cells are unique in binding, internalising and presenting specific soluble antigens. This is due to their unique Ig receptor, assembled after a process of rearrangements and expressed at the cell surface. After binding to the BCR, the antigen is internalised and processed in intracellular vesicles. These are the same vesicles where newly synthesised MHC class II molecules are directed, and subsequently loaded with internalised peptides (Lanzavecchia, 1985). Due to constitutive expression of MHC class II molecules, they are capable of displaying peptides derived from specific antigens at high levels and with high density on the cell surface.

Like macrophages, triggering of the BCR also up-regulates the expression of accessory molecules (Lenschow et al., 1994), which can also be induced by microbial-specific products like LPS (Hathcock et al., 1994), allowing full activation of T lymphocytes. Although activation of T lymphocytes by B lymphocytes is clearly demonstrated *in vitro* (Lanzavecchia, 1985) and is shown to be vital for the production of antibodies, for T cell expansion and for systemic responses to low

antigen concentrations (Rivera et al., 2001), it appears to be less important in initiating T cell responses.

Studies of early events in T cell activation *in vivo* have shown that naive CD4 T cells are found primarily, if not exclusively, in secondary lymphoid tissues (spleen, lymph nodes and Peyer's patches) (Reinhardt et al., 2001). Naive T cells express a unique set of receptors, which bind ligands only expressed on the high endothelial venules (HEV) in secondary lymphoid organs. Once inside, the T cells are restricted to T cell-rich areas known as the paracortex in the lymph nodes and Peyer's patches and the periartiolar lymphoid sheath (PALS) in the spleen. B cells reside in specialised B cell follicles and are thus anatomically separated from naive T cells in the secondary lymphoid organs. Accordingly it was shown that labelled adoptively transferred T cells stay in the paracortex of the lymph nodes without the presence of macrophages or B cells (Ingulli et al., 1997). Only after initial activation and proliferation do T cells migrate to the border region to make contact with B cells (Gulbranson-Judge and MacLennan, 1996; Garside et al., 1998; Reinhardt et al., 2001). This suggests that the importance of B cells as APCs *in vivo* is small, and that the presentation of peptides in the context of MHC class II molecules on antigen specific B cells mainly serves to obtain help from primed CD4 T cells for antibody production.

1.5.3 Dendritic cells

Dendritic cells represent a heterogeneous cell population, distributed throughout the body in all tissues, particularly at sites of contact with the surrounding environment, like skin and mucosae. This indicates their central role in antigen capture and initiation of immune responses (Steinman et al., 1997). In the absence of ongoing inflammatory and immune responses, immature DCs, representing 1-2% of the total cell numbers, constitutively patrol the tissues (Banchereau and Steinman, 1998). They are potent in antigen uptake and efficiently capture invading pathogens. This is achieved by sampling the surrounding tissue by macro-pinocytosis (Sallusto et al., 1995), internalising self and non-self antigens, which are subsequently processed and loaded onto MHC molecules. Immature DCs express low amounts of accessory molecules and MHC class II; as a result they are inefficient in activating naive T cells.

After antigen uptake, immature DCs rapidly cross the endothelium of lymphatic vessels and migrate to the draining secondary lymphoid organs. During this migration, DCs undergo the maturation process that is characterised by downregulation of the capacity to capture antigens and upregulation of antigen processing and presentation, of expression of costimulatory molecules and of dendritic morphology. Mature DCs no longer capture antigens, but are powerful initiators of the adaptive immune system by their antigen processing and presenting capacity. The signal for DCs to mature is often referred to as a danger signal. In this respect, DCs form a bridge between innate and adaptive immunity, since the danger signal often results from the innate arm of the immune system.

DCs sense danger with a wide variety of receptors constitutively expressed on their surface. They express a subset of TLRs, allowing them to respond to various conserved pathogenic compounds (Visintin et al., 2001). When these compounds have triggered TLR on other cell types, DCs sense danger indirectly through inflammatory mediators such as TNFα, IL-1β and prostaglandin E2 (Banchereau and Steinman, 1998). Ongoing immune responses are sensed by the presence of antibodies, which bind to their Fc-receptors (Regnault et al., 1999), or by triggering of their complement receptors (Reis e Sousa et al., 1993). Activated CD4 T cells can also induce DC maturation, potentially for initiating cytotoxic T cells, by triggering of CD40, OX40 and CD95 (Ohshima et al., 1997; Rescigno et al., 2000; Schuurhuis et al., 2000). Although the nature of the activating compound is still unclear, cell death is also sensed by DCs and results in enhanced T cell responses (Shi et al., 2000).

The T cell areas are occupied by at least two subsets of DCs (Shortman et al., 1998), possibly originating from different precursor cells. For example, precursors in the skin, Langerhans cells (LC) and myeloid monocytes, both migrate into T cell areas of draining lymph nodes (Silberberg-Sinakin et al., 1976; Randolph et al., 1999), where LCs display DEC205 while myeloid monocytes lack DEC205 and CD8α expression (Inaba et al., 1995; Randolph et al., 1999). This led to the division of DC populations in lympoid DCs ($CD11c^+CD8\alpha^+DEC205^+CD11b^-$), derived from migrating LCs, and myeloid DCs ($CD11c^+CD8\alpha^-DEC205^-CD11b^+$), derived from migrating monocytes (Shortman and Wu, 2001). However, demonstrating that both common myeloid and lymphoid precursors can give rise to both DC subsets recently challenged this distinction of origin (Manz et al., 2001).

As well as their stimulatory functions, there is growing evidence that DCs also maintain and regulate T cell tolerance in the periphery (Jonuleit et al., 2001). They capture antigen from dying cells during normal cell turnover; however, this does not result in an immune response against self-antigens. It was shown that damaged cells are handled differently by DCs, depending on how they die. Necrotic cells promote the maturation of DCs and strong CD4 and CD8 T cell stimulatory activity, whereas apoptotic cells fail to activate DCs (Gallucci et al., 1999; Sauter et al., 2000). In addition, it was demonstrated that the absence of an immune response is not due to lack of presentation, since DCs constitutively transport apoptotic cells to the T cell areas of lymph nodes and present the self-peptides there (Huang et al., 2000). The mechanism behind the induction of tolerance is poorly understood; however, there is increasing evidence that a subpopulation of T cells exert regulatory functions (T_{reg}). DCs might present certain exogenous self-antigens that are recognised by T_{reg} cells, which subsequently block the activation activity of the DC (Kurts et al., 1997; Adler et al., 1998; McGuirk et al., 2002).

1.6 Cytokines

Another key role in the multi-factorial network of an immune response is played by cytokines. They are initially produced by the innate immune response (inflammatory cytokines) and can profoundly influence subsequent adaptive immunity. Their effects are pleiotropic and influenced by dose as well as presence or absence of other cytokines. Once produced, the factors, individually or in combination, can act directly on infected cells, activate cellular constituents of the innate system, and promote T and B cell adaptive responses to mediate defence.

T lymphocytes are one of the richest sources of protein mediators. Many of their effects, such as T cell help, growth stimulation, macrophage activation or suppression are caused by the release of these molecular mediators, which include the interleukins Il-2, Il-3, Il-4, Il-5, Il-6, IFNα and β, TNF, Lymphotoxin (LT), GM-CSF and transforming growth factor (TGF) β. These molecules are only produced transiently after activation of the cells, and the CD4+ cells are the richest sources of cytokines. Il-2, secreted principally by activated helper T cells, plays a pivotal role in the generation and regulation of the immune response. It supports the growth and effector function of a wide array of immunologically relevant cells, including

macrophages, B cells and NK cells, as well as a variety of different T cell subpopulations. It has a short half-life and is secreted in apposition to the cell with which the T cell interacts.

Induced after antigen activation of resting T-cells, Il-2 plays an important role in regulating effector cell numbers. The triggering of the TCR and additional accessory molecules not only results in Il-2 secretion, but also in the expression of its high affinity receptor (CD25). Il-2 serves as an autocrine growth factor, and is responsible, although not solely, for clonal expansion (Schorle et al., 1991). However, Il-2 serves a dual role, since it is also responsible for the elimination of activated T cells by AICD. As previously mentioned cytokines, like Il-7 and Il-15, play an important role in the maintenance of peripheral T cell numbers.

Studies of infections have shown a polarisation of CD4 T cell cytokine responses as either Th1 or Th2 (Heinzel et al., 1989). Each subset produces a wide variety of cytokines of which Il-2, IL-3, TNF-α and GM-CSF are found in common. Th1 cells specifically synthesise cytokines like, IFN-γ and TNF-β, which are not produced by Th2 cells. On the other hand, IL-4, IL-5, and IL-6 are examples of cytokines exclusively made by Th2 cells. The different cytokine profiles reflect the differentiation in function between both subsets, and their specialisation for different pathogens. Th1 cells, targeted to peripheral sides of infection, interact mainly with macrophages by stimulating their microbicidal activity. Their repertoire of cytokines is optimised for dealing with pathogens that have gained access to intracellular compartments, such as viruses and intracellular bacteria. Th2 cells on the other hand, mostly targeted to B cell-rich areas, secrete cytokines that help B cells to differentiate and produce antibodies, thus offering protection against extracellular antigens, like toxins and parasites.

There is accumulating evidence that the development of protective Th1 or Th2 cells is driven through the effects of microbes on DCs (d'Ostiani et al., 2000; Dabbagh et al., 2002; de Jong et al., 2002). Upon activation by signals released from the microorganisms or from infected tissues, DCs undergo maturation and migrate towards the T cell areas of draining lymphoid organs. Here, DCs activate naive CD4 T cells with pathogen-specific (MHC-peptide complexes) and accessory signals. In addition, it seems that a third signal determines the polarisation of naive CD4 T cells into Th1 or Th2 (Kalinski et al., 1999). This third signal is heterogeneous and is

mediated by soluble as well as membrane-bound molecules, including Il-4, Il-12 , Il-18, IFNα and OX40L (Flynn et al., 1998; de Jong et al., 2002; MacDonald and Pearce, 2002). Importantly, the exact combination and expression levels of these polarising molecules by mature DCs strongly depend on the conditions during their initial activation. For example, tissue-derived factors such as IFNγ and prostagladin E2, present during DC activation, promote respectively the generation of DCs that produce high amounts of Il-12 upon subsequent engagement with naive T cells, or the generation of DCs that are Il-12-deficient and drive the development of Th2 (Kalinski et al., 1998; Vieira et al., 2000).

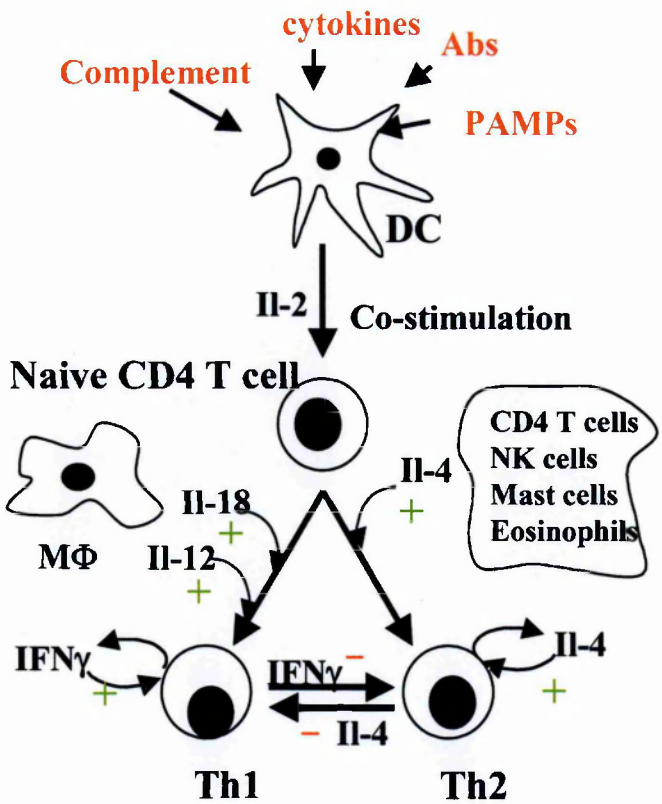


Figure 2. Model of CD4 T cell differentiation. After encountering pathogens in peripheral organs, DCs migrate to the T cell-rich zones in lymphoid organs. The interactions with the pathogen, its products (PAMPs) and the local inflammatory milieu result in differential expression of cytokines, chemokines and co-stimulatory molecules. As a result, naive CD4 T cells differentiate into Th1 effector cells under the influence of Th1 cytokines like Il-12, or Th2 effector cells under the influence of Il-4. Autocrine secretion of IFNγ expands and stabilises the Th1 response while inhibiting a Th2 response and, vice versa, Il-4 expands and stabilises the Th2 response while inhibiting the generation of Th1 cells.

In addition to their distinct roles in disease, Th1 and Th2 cells cross-regulate each other's expansion and functions with their respective repertoire of cytokines (Figure 2). Besides polarising and maintaining the Th2 response, Il-4 is involved in silencing the expression of the Th1 cytokine IFNγ (Tanaka et al., 1993). IFNγ on the other hand, enhances the effect of Il-12, a dominant factor inducing the development of Th1 cells, by upregulating the Il-12R, while inhibiting the growth of Th2 cells (O'Garra, 1998). Il-18 has also been shown to synergise with Il-12 to stimulate the production of IFNγ by Th1 cells, and thus indirectly suppressing the generation of Th2 cells (Okamura et al., 1995).

The T_{reg} cytokine profile is distinct from that of classical Th1 or Th2 cells, but heterogeneous, depending on the tolerance protocol or the site of isolation. Besides Il-10, some produce TGFβ, IFNγ, Il-5, but no or only low levels of Il-2 and no to variable amounts of Il-4 are reported (Chen et al., 1994; Groux and Powrie, 1999). Although they cannot produce it, *in vitro* and *in vivo* these cells seem to be critically dependent on Il-2 for differentiation and survival (Papiernik et al., 1998). The distinct T cell sub-populations that secrete Il-10 and/or TGFβ are considered to play a role in the maintenance of self-tolerance.

1.7 T cell activation

Engagement of the TCR on mature peripheral T cells initiates multiple signals that can lead to cellular proliferation and the differentiation into specialised effector cells. The basis for this activation is formed by the recognition through the TCR of a ligand loaded on a MHC molecule.

1.7.1 The TCR

The complexity of molecular associations involved in TCR signalling begins with the TCR itself. It is composed of six different polypeptide chains thought to be organised into an eight-chain structure (Weissman, 1994). The TCR itself consists of two different polypeptide chains, termed α and β chain, bound to one another by a disulphide bond. These α:β heterodimers account for antigen recognition. Neither chain of the TCR heterodimer has a large cytoplasmic domain that might serve to signal the cell that the TCR has bound antigen. The signal transduction pathway starts

instead with a complex of proteins, known as the CD3-complex, which is stably associated with the TCR on the surface of T cells. These include the non-polymorphic CD3ε, CD3γ, CD3δ, and TCRζ chains, which are all essential for receptor assembly, cell-surface expression and signalling (Ashwell and Klusner, 1990).

The cytoplasmic domains of the signalling components contain tyrosine residues in conserved motifs termed immunoregulatory tyrosine activation motifs (ITAM). Many receptors that participate in immune responses feature multiple subunits and/or motifs that mediate signal transduction. Intriguingly, multiple copies of these motifs can be found in virtually all receptors that utilise ITAMs for signal transduction (Reth, 1989). The receptors contain no inherent kinase activity but, upon crosslinking by ligand binding, the tyrosines within the receptor ITAMs become phosphorylated by Src-family kinases, thus initiating signal cascades.

ITAMs consist of semi-conserved sequences of amino acids that contain two appropriately spaced tyrosines ($YXXL/IX_{6-8}YXXL/I$; where X denotes non-conserved residues) (Reth, 1989). These ITAMs appear as a single copy on the CD3γ, δ, and ε chains and as a triplicate repeat on the ζ chains (Cambier, 1995). This motif is crucial for TCR coupling to intracellular tyrosine kinases and hence absolutely required for all subsequent TCR signalling responses.

1.7.2 Anatomy of T cell signalling

The earliest recognisable event after TCR engagement by antigen is the induction of tyrosine phosphorylation by the Src kinases Lck and Fyn. How these Src kinases are activated exactly is unclear, but it involves additional recruitment to the cell membrane (Shaw et al., 1995).

1.7.2.1 Raft hypothesis

A clue as to how the TCR is brought into association with signal-initiating Src kinases came from the characterisation of lipid rafts. Cell membranes are comprised of a complex mixture of cholesterol, glycerophospholipids and sphingolipids. These constituents exhibit distinct biophysical properties; sphingolipids have longer and more saturated fatty acid chains and exhibit stronger lateral cohesion than glycerophospholipids, and cholesterol preferentially interacts with sphingolipids

rather than with unsaturated glycerophospholipids. Thus, these lipids behave in different ways when forming a bilayer, with sphingolipids showing a tendency to form distinct 'liquid-ordered' phases dispersed in the 'liquid-disordered' matrix formed by glycerophospholipids (Simons and Toomre, 2000).

Although lipid rafts, rich in cholesterol and glycosphingolipids, were identified initially because of their peculiar biophysical properties, they have been directly visualised in living cells (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998; Schutz et al., 2000). Rafts are now defined as dispersed liquid-ordered phase domains to which specific proteins are sequestered but from which others are excluded (Simons and Toomre, 2000). Thus rafts float in a sea of phospholipids providing a mechanism for the lateral sorting of membrane proteins.

Under resting condition, the TCR-CD3ζ complex seems to be excluded from or only weakly associated with the raft signalling platforms (Montixi et al., 1998), skewing the equilibrium of the receptor toward the non-raft regions of the membrane. Engagement of the TCR by its ligand induces TCR translocation into raft domains, shifting the equilibrium towards rafts, where the accumulation of supramolecular signalling complexes occurs, moved around by the actin cytoskeleton (Montixi et al., 1998; Lanzavecchia et al., 1999). These events, as well as T cell activation, can be suppressed by disrupting raft structure, indicating that membrane compartmentalisation is a pre-requisite for TCR signal transduction (Xavier et al., 1998).

Any factor that affects the process of receptor translocation into rafts could be predicted to have the potential to promote or dampen signalling. Indeed, access of receptors is altered during both T cell and B cell development, reflecting changes in the outcome of antigen engagement by the receptors. In DN T cells, the pre-TCR appears to localise into rafts without the need for ligation, providing a mechanism for receptor signalling for further development (Guo et al., 2000; Saint-Ruf et al., 2000). In contrast, in DP T cells, the crosslinked receptors fail to translocate into rafts, possibly providing a structural basis for different outcomes of signalling in mature and immature lymphocytes (Ebert et al., 2000).

The post-translational addition of lipids (by myristoylation, palmitoylation and prenylation) is an essential requirement for the targeting of many proteins to

membrane rafts, as well as for their correct functioning. Several molecules are associated with raft domains in T cells. Lck, the Src kinase responsible for initial tyrosine phosphorylation events following TCR ligation, is targeted to membrane rafts by the dual acylation of its N-terminus (Resh, 1994). Targeting to raft domains is crucial for Lck function, as shown by the fact that Lck mutants that are not acylated and do not localise to the plasma membrane are unable to signal properly (Kabouridis et al., 1997). Alternatively, it has been proposed that, in lymphocyte plasma membranes, Lck and Fyn kinases exhibit optimal activity in raft microdomains but encounter inhibitory conditions in surrounding membrane areas with a non-raft morphology (Ilangumaran et al., 1999).

In resting cells, rafts appear to be dynamic structures that are estimated to be small, but may fuse to form larger domains upon receptor ligand binding, to contribute to the immunological synapse (IS) (Janes et al., 1999).

1.7.2.2 The immunological synapse

Circulating T cells are rounded and non-polarised, with uniform radial distribution of membrane domains, receptors and microvilli on the cell surface (Sanchez-Madrid and del Pozo, 1999). Within minutes of contacting an APC, a T cell undergoes a dramatic polymerisation of actin filaments at the site of cell-cell contact. This specific molecular organisation is observed only at the interface between the DC and T cell. The interaction thus creates a specific physical site, termed the 'immunological synapse' (Grakoui et al., 1999), at which specific ligands and receptor molecules trigger and sustain the T cell activation process. Key to this mechanism is the DCs ability for high expression of MHC, accessory (CD80 and CD86) and adhesion (ICAM-1 and ICAM-3) molecules (Banchereau and Steinman, 1998).

The IS is currently viewed as a concentric structure, with a central supramolecular activation cluster (cSMAC) enriched in TCR, CD2 and CD28, that is surrounded by the peripheral SMAC (pSMAC), which is enriched in lymphocyte function-associated antigen-1 (LFA-1). Importantly, the large membrane molecules CD43 and CD45 are excluded from the IS (Johnson et al., 2000). Topological models of T cell signalling predict that those molecules with large extracellular domains would be excluded from sites of TCR – MHC/peptide interactions due to the small

distances between apposing membranes (~15 nm) (van der Merwe et al., 2000). This size-exclusion model is supported by the demonstration that elongated forms of CD2 can severely impair T cell activation (Wild et al., 1999). Close contacts may be achieved indirectly through cytoskeletal protrusions anchored to larger adhesion molecules like LFA-1 and ICAM-1, or more directly by smaller adhesion receptor pairs like CD2 and CD58 that would work immediately beside the TCR.

The formation of sSMACs may be driven by an initial TCR – MHC/peptide interaction and the subsequent recruitment of additional molecules, a process that is driven by T cell motility and promotes redistribution of molecules within the two membranes. Indeed, the formation of the mature IS is preceded by TCR signalling (Lee et al., 2002). Effector T cells polarise their microtubule organising centres (MTOC) toward APCs (Kupfer and Singer, 1989). Fluorescence studies show that redistribution of involved membrane structures occur within a few minutes and lead to a stable configuration for extended periods of time (Grakoui et al., 1999).

IS formation requires an active cytoskeletal rearrangement, which is closely associated with T cell activation (Wulfing and Davis, 1998). However, it can occur between T cells and DCs in the absence of exogenous antigen, a process associated with several T cell responses (Kondo et al., 2001; Revy et al., 2001). IS formation was found tightly associated with small calcium fluxes, increased tyrosine phosphorylation of TCRζ and Lck, recruitment of Zeta-chain Associated Protein of 70 kDa (ZAP-70) and prolonged T cell survival. Other receptor-ligand interactions besides the TCR – MHC/peptide ligation can affect actin organisation. For example, intergrin-dependent recruitment of actin crosslinking proteins such as talin to the contact site creates a dense network of filaments (Sedwick et al., 1999). Actin organisation is also important for the assembly of proteins involved in TCR signalling. Actin regulatory molecules like Wiskott Aldrich Syndrome protein (WASP), Fyn-binding protein (Fyb)/ Src homology (SH)2 domain-containing Leukocyte Protein of 76 kDa (SLP-76)-associated protein (SLAP) and Vav play important roles in actin remodelling at the synapse (Krause et al., 2000), together with the Rho family of guanosine triphosphatases (GTPases), including Cdc42, Rac and Rho. Thus, T cell signalling and actin remodelling are intricately interwoven for optimal T cell activation and differentiation.

Importantly, APCs regulate the lateral distribution of their surface MHC class II molecules also in micro-domains, even before contact with T cells and formation of the IS (Kropshofer et al., 2002). The local concentration of MHC molecules probably facilitates antigen presentation at low doses of antigen (Anderson et al., 2000). However, DCs contain only few MHC molecules in lipid rafts, the majority being found in tetraspan micro-domains, displaying specific peptides, co-localised with important ligands for the accessory molecules on T cells like B7.2, CD82 and CD9. This lateral association of MHC with adhesion and co-stimulatory molecules might facilitate synapse formation and T cell activation.

Complexes between peptide and MHC molecules are generally thought to have a low affinity for TCRs, with relatively fast off-rates, but they can nonetheless deliver sustained stimulation to the T cell (Davis et al., 1998). It is known that just a few MHC-peptide complexes on a DC can be sufficient to trigger T cells (Valitutti et al., 1995; Sykulev et al., 1996). A mechanism to increase the overall avidity of this interaction is the formation of TCR and accessory receptor arrays that interact with specific as well as non-specific MHC-peptide dimers. It was demonstrated that TCRs are enriched in the central region of the IS, viewed as a means to generate stable molecular complexes able to sustain the signal over time. In addition, T cell signalling is viewed as an ongoing process that is sustained by serial engagement and triggering of TCRs by MHC-peptide complexes (Valitutti et al., 1995).

The recent report by Lee et al. (2002), challenged the current ideas about the precise role of the IS in TCR signalling. They showed that the TCR resides at the periphery of the immature synapse upon first contact with an APC, whereafter it moves to the central position in the mature synapse. They subsequently showed that active Lck only co-localises with the external ring of TCR in the immature synapse, and that at the formation of the mature synapse, active Lck is undetectable. Also, active ZAP-70 was first found at the peripheral ring and only weakly at the centre of the mature synapse. They concluded that it appears that the initiation of signalling precedes the formation of the IS, and that synapse formation may not be directly involved in either the initiation or enhancement of TCR signalling. It does not exclude, however, that the IS provides an initiation point where engagement and activation of receptors other than the TCR takes place (van der Merwe and Davis, 2002).

1.7.3 Initiation of T cell signalling

It is not yet known how engaged TCRs are recruited into raft domains. It is possible that ligation of TCRs by MHC-peptide tetraspan micro-domains on the APC surface might decrease the lateral diffusion of the engaged receptors in the membrane and drive their recruitment into raft micro-domains. The limited space between the T cell and its participating APC may lead to exclusion of large receptors, including the tyrosine phosphatases CD45 and CD148, but would allow smaller receptors to remain in the contact zone. This receptor sorting is critical to the forming of the IS and topologically organisation of the SMAC. In addition, the TCR-CD3 complex undergoes a conformational change, leading to recruitment of actin-remodelling molecules (Gil et al., 2002).

Interestingly, upon first contact between T cell and APC, LFA-1 molecules are recruited to the centre of the contact area, whereas TCRs were found at the periphery of the immature synapse co-localised with activated Lck. This pattern is reversed shortly after, with the TCR and Lck in the centre, surrounded by LFA-1 in the mature synapse (Lee et al., 2002). Once TCRs are recruited into the immature synapse, the kinase-rich environment, created by the recruitment of Lck, allows subsequent phosphorylation of CD3ζ and the recruitment of ZAP-70, resulting in signal transduction. The underlying concept is that by keeping phosphatases apart from substrates of TCR-induced phosphorylation, the IS would protect the propagation of positive signals. Indeed, CD45, the most abundant membrane tyrosine phosphatase, is excluded from raft domains (Rodgers and Rose, 1996; Janes et al., 2000). The coalescence of raft microdomains induced by the formation of the IS will exclude CD45 from the signalling area and protect activated kinases and signalling motifs from dephosphorylation (Thomas, 1999).

A discreet pool of CD45 moves back into the central region of the mature synapse, adjacent to the area of TCR/MHC engagement (Johnson et al., 2000). The positive or negative role of this CD45 movement is unknown, but it may provide a mechanism to sustain Lck activity near engaged TCRs. However, these CD45 molecules are not found in the plasma membrane, but intracellularly, and thus may serve a quite different function. Many other phosphatases that may be involved in the regulation of TCR signalling are mostly expressed in T cells as cytosolic proteins and excluded from rafts (Gjorloff-Wingren et al., 2000). The importance of this was

demonstrated by deliberately targeting the SH2 domain containing tyrosine phosphatase (SHP-1) into the raft microdomains by using the N-terminal region of Lck, which profoundly inhibited CD3 induced tyrosine phosphorylation and Il-2 production (Su et al., 2001).

Exactly how Lck is activated during TCR engagement is unclear. Activation of Lck is negatively regulated through the carboxy-terminal (C-terminal) tyrosine phosphorylation site (Y505), which mediates intramolecular binding to its SH2 domain that places the kinase in a closed conformation leaving its kinase domain inaccessible (Sicheri et al., 1997). The removal of the C-terminal phosphate residue leads to Src kinase unfolding (Veillette et al., 1988). This conformational change frees up the SH2 domain that allows Lck to interact with new partners, including ZAP-70 (Di Bartolo et al., 1999). However, phosphopeptide-mapping experiments have shown that the majority of Lck in resting T cells is already dephosphorylated at Y505 (Ostergaard et al., 1989). Based on these data, it has been suggested that recruitment, and not activation of Lck may be the critical activation step (Shaw et al., 1995). Phosphorylation of inhibitory Y505 is controlled by the opposing actions of the protein tyrosine kinase (PTK) Csk and by the protein tyrosine phosphatase (PTP) CD45. CD45 is excluded from aggregated rafts, whereas the Csk docking protein Cbp is an acylated transmembrane protein that is concentrated in rafts (Kawabuchi et al., 2000). Under steady state conditions, Cbp is basally phosphorylated, allowing recruitment of constitutively active Csk to the rafts to keep present Lck quiescent. Upon triggering of the TCR however, Cbp is rapidly dephosphorylated leading to dissociation of Csk from lipid rafts (Torgersen et al., 2001).

The localisation of Lck in and around the IS is due to its unique N-terminal domain, which is acylated at its two cysteine residues (Resh, 1994), and binds to the cytoplasmic tails of the co-receptor molecules CD4 and CD8 (Barber et al., 1989). In the IS, the SH3 domain can engage with a proline-rich sequence in the cytoplasmic domain of CD28 (Holdorf et al., 1999), consequently stimulating its activity. Conversely, a second site of tyrosine phosphorylation (Y394) found in the activation loop of the kinase domain is autophosphorylated to activate the kinase for substrate phosphorylation. Interestingly, Y394 is also dephosphorylated by CD45, as well as by Proline-, Glutamic acid-, Serine-, and Threonine-rich [PEST]-domain Phosphatase (PEP) (D'Oro and Ashwell, 1999). Recruitment of Lck was shown to be delayed in CD4-deficient T cells compared with wild type, resulting in slow autophosphorylation

of Y394. In CD28-deficient T cells Y394 phosphorylation could be detected at comparable rate, however, it was quickly lost (Holdorf et al., 2002). This suggests that CD4 or CD8 initially recruit Lck and stimulate its autophosphorylation at the synapse, whereas CD28 sustains the phosphorylation state and activity.

Upon TCR crosslinking, the CD3 multichains enter rafts, supplying the ITAMs as substrates for kinase binding and phosphorylation. Full activation of the Src kinases also results, after several minutes, in the re-phosphorylation of Cbp, which recruits Csk back into the synapse, thus providing a mechanism by which signalling can be terminated (Brdicka et al., 2000; Torgersen et al., 2001). This suggests that all the machinery necessary for the initiation and termination of TCR signalling is concentrated in the IS.

1.7.4 Proximal T cell signalling

Following receptor engagement, phosphorylation of ITAM tyrosine residues by PTKs of the Src family of kinases is one of the earliest events in the signalling cascade (Weiss, 1993). Phosphorylation of both tyrosine residues within an ITAM is generally thought to be essential for signalling, and is required for efficient recruitment of the tandem SH2 domain-containing PTK ZAP-70 to the receptor complex. ITAM phosphorylation by a pool of active Src kinases is continuously opposed by PTPs (van der Merwe et al., 2000). This repressive effect is removed during the formation of the immunological synapse, allowing Src kinases to dominate the phosphorylation status of the ITAMs (Shaw and Dustin, 1997).

After its recruitment to the ITAM motifs, ZAP-70 is phosphorylated by Lck, resulting in its activation (Iwashima et al., 1994). Lck interacts directly with ZAP-70 through the binding of the Lck SH2 domain to the phosphorylated Y319 residue of ZAP-70 (Pelosi et al., 1999). This interaction is critical for sustained ITAM phosphorylation, leading to additional recruitment of ZAP-70 molecules and Lck substrates, such as the Tec kinase Itk (van der Merwe et al., 2000). Interestingly, not all the ITAMs appear to be phosphorylated on every occasion the TCR is triggered. Depending on the ITAM phosphorylation pattern, the TCRζ migrates during SDS-PAGE as either a 21- or 23-kD peptide (Kersh et al., 1998a; van Oers et al., 2000).

The molecules that bridge the activated TCR with its associated PTKs to the tyrosine kinase substrates play a critical role in TCR-mediated signalling. Propagation and amplification of the TCR signal is directed via adapter or linker proteins. The most prominent are SLP-76 and linker of activation of T cells (LAT). The latter, a 36/38 kDa transmembrane key signalling molecule localised in raft microdomains, is tyrosine phosphorylated by ZAP-70 and is, like Lck, associated with the co-receptor molecules CD4 and CD8 (Bosselut et al., 1999). LAT mutants lacking palmitoylation sites are excluded from rafts and do not become phosphorylated by ZAP-70, resulting in inefficient TCR-induced signal transduction (Zhang et al., 1998). LAT provides a scaffold by which multiple signalling molecules, such as phospholipase C (PLC)γ1 can be recruited. In addition, tyrosine-phosphorylated LAT also recruits multiple members of the growth factor receptor-bound protein (Grb2/Gads/Grap) family of adaptor proteins, which are able with their multiple protein-protein interaction domains to assemble macromolecular signalling complexes.

PLCγ1 tyrosine phosphorylation is one of the major determinants for calcium signalling, and requires the activity of three PTKs, Lck, ZAP-70 and Itk (Kurosaki and Tsukada, 2000). Consensus phosphotyrosine residues are responsible for the recruitment of PLCγ1 SH2 domains to LAT and consequently to bring it in close contact with the inner leaflet of the surface membrane. LAT also stabilises SLP-76 binding, which brings the Tec kinase Itk into this macromolecular complex, ultimately resulting in phosphorylation of PLCγ1 (Kurosaki and Tsukada, 2000). This catalytically activates PLCγ1, utilising phosphatidylinositol-4,5 biphosphate ($PI(4,5)P_2$), concentrated in plasma membrane rafts (Caroni, 2001), to generate inositol-1,4,5-triphophate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of calcium ions from intracellular stores, mainly the ER (Berridge et al., 1998). Once these stores are depleted, store-operated calcium channels in the surface membrane allow extracellular calcium influx. This results in a sustained elevation of intracellular calcium levels, which act as a trigger for transcriptional activation in the nucleus. Calcium binds to calcineurin, a calcium calmodulin-dependent serine phosphatase that dephosphorylates the nuclear factor of activated T cells (NFAT) (Baksh and Burakoff, 2000). This leads to the conversion of cytoplasmic NFAT to nuclear NFAT, capable of binding to sites in promoters.

DAG binds to a small 5 kDa zinc containing domain, called the protein kinase C homology-1, which has been identified in protein kinase C (PKC) and RasGRP

amongst others. T cells express multiple functionally distinct PKC isoforms. Calcium, DAG and other phospholipids regulate them. Among the many isoforms, only PKC-θ is recruited into the immunological synapse (Monks et al., 1997), where it is involved in the activation of the nuclear factor κB (NF-κB) pathway and possibly also the N-terminal c-Jun kinase (JNK) cascade (Khoshnan et al., 2000).

1.7.5 T cell signalling downstream of PTKs

In addition to PLCγ1 activation, PTKs affect phosphatidylinositol (PI) turnover through phosphoinositol-3 kinase (PI3K). The membrane phospholipid phosphatidylinositol is the precursor for a family of lipid second messengers, known collectively as phosphoinositides, which differ solely in the phosphorylation status of their inositol head group. The abundance of phosphoinositides makes it possible to target a large number of distinct proteins to a particular membrane without saturating the binding sites. In addition, the structurally distinct phosphoinositides, with rapid sequential interconversions between phosphorylated forms, can activate different downstream effectors.

LAT is also involved in regulation of the GTPase Ras and mitogen-activated protein kinases (MAPK) cascades. A central role, linking proximal signalling events to cascades of the MAPKs, is played by the guanine nucleotide binding protein p21Ras. The level of active Ras is determined by a balance of the rate of hydrolysis of bound GTP and the rate of exchange of bound GDP for cytosolic GTP. Receptor tyrosine kinases regulate Ras activity via interactions with guanine nucleotide exchange factors (GEF), such as Sos and RasGRP (Polakis and McCormick, 1993; Ebinu et al., 1998), and GTPase-activating proteins (GAP), such as p120-GAP (Downward et al., 1992). Activation of Ras triggers a cascade of the MAPK extracellular signal-regulated kinase (Erk), and is suggested to cross-talk to Rho GTPases, which in turn activate other MAPK families such as JNK and p38 (Genot et al., 1996).

There are three major groups of MAPKs in mammalian cells, Erks, p38 MAPK and JNK. Dual phosphorylation of a tyrosine and a threonine residue activates them. The sequence of the motif containing these two amino acids is different in each group of MAPKs. Each group is activated by a conserved protein cascade; Erks are activated by the MAP kinase kinases (MKK) MKK1 and MKK2; p38 MAPKs by

MKK3, MKK6 and possibly MKK4; while JNKs are activated by MKK4 and MKK7. In their turn these MKKs are activated by MAP kinase kinase kinases (MKKK), which are activated by different upstream signals with a prominent role for GTPases. Erks are the end of the cascade activated by the GTPase Ras via the MKKK Raf. In contrast, p38 MAPKs and JNKs are at the end of cascades initiated by Rac/Rho GTPases. All three kinases play an important role in the innate immune response, as well as in the adaptive immune response, where they are involved in the signalling cascades leading from TCRs to cytokine production and cellular responses to inflammatory cytokines (Dong et al., 2002).

Erk, downstream of Ras, is often involved in the regulation of cell growth and differentiation. There are two isoforms of Erk, Erk1 and Erk2, also known as p44/p42 MAPK. TCR engagement activates the Ras-Raf-MKK-Erk pathway, resulting in the expression of the activator protein-1 (AP-1) transcription factor Fos, as well as Myc (Dong et al., 2002). Fos is involved in transcriptional regulation of the AP-1 response elements in the Il-2 promoter (Jain et al., 1995).

The role of JNK in T cell activation and Il-2 production is somewhat more controversial. TCR ligation is required for the activation of JNK, but it is not clear what signals regulate the enhanced expression and activity of JNK proteins. Studies with JNK1 and JNK2 knockout mice suggest that both may have an important functional role in the establishment and maintenance of Th1 cells (Dong et al., 1998; Yang et al., 1998).

The p38 MAPK regulates IFNγ gene expression in Th1 cells but apparently does not affect Th2 cytokines (Dong et al., 2002). It regulates AICD in CD8 T cells but not in CD4 T cells, demonstrating that activation of the same signalling pathway can have different outcomes in the two T cell subsets (Merritt et al., 2000). Hence, activation of p38 MAPK results in increased production of IFNγ by CD4 Th1 cells and CD8 T cells, but also leads to a decrease in the number of effector CD8 T cells.

Activation of the Il-2 promoter by T cell signalling cascades also involves the NF-κB pathway (Harhaj and Sun, 1998). The exact mechanisms by which the NF-κB pathway is initiated by TCR ligation still needs to be clarified, but seems to involve the recruitment of PKCθ into the immunological synapse (Lin et al., 2000). NF-κB activation depends on a multisubunit cytosolic complex. It includes the catalytically

active IκB kinases IKKα and IKKβ, which form heterodimers with the non-catalytic subunit IKKγ (Regnier et al., 1997). The activated complex initiates the phosphorylation and subsequent degradation of the inhibitory IκBα and IκBβ proteins. This releases the NF-κB transcription factors, p65, c-Rel, RelA and p50, which were sequestered by IκBα and IκBβ, and allows them to enter the nucleus to initiate transcription of their target genes. One of these target genes is Il-2.

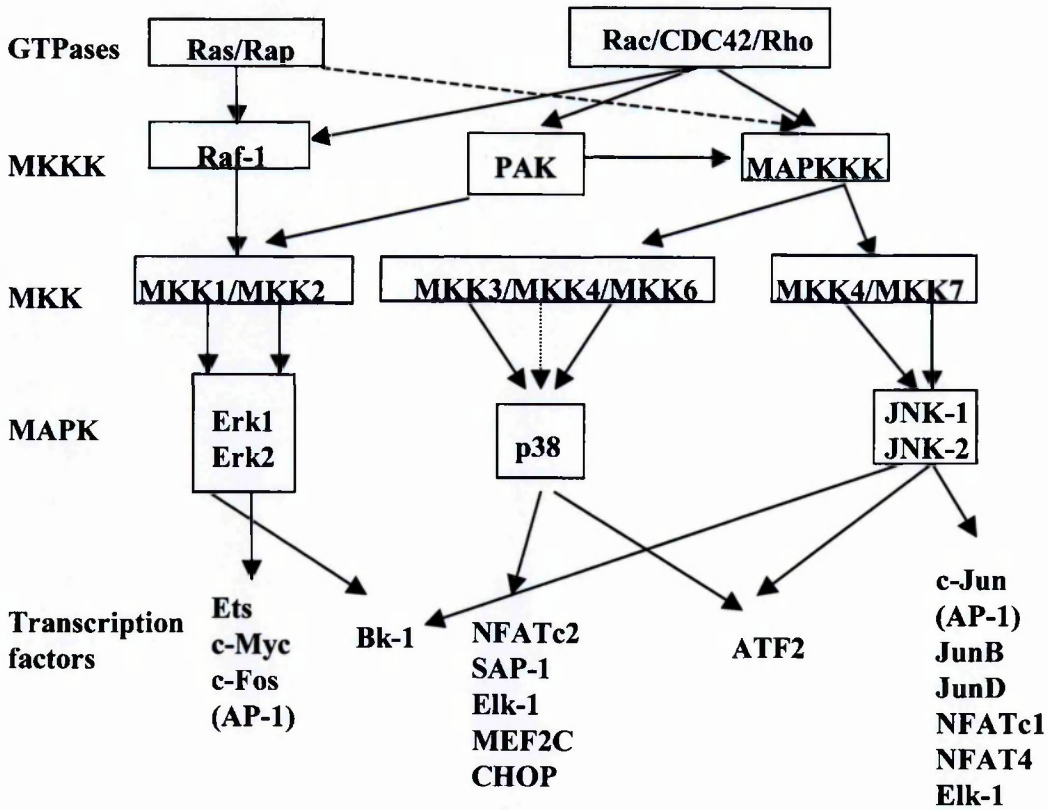


Figure 3. MAPK signalling pathways. Various stimuli lead to activation of the small GTPases like Ras and Rac, these initiate the various MAPK pathways, leading to Erk, p38 and JNK activation and resulting in activation of various transcription factors. The figure does not display all aspects of cross-talk between the three pathways, but illustrates the complexity involved at this level of signal transduction in T cells.

1.7.6 Accessory receptors

It is known that only a few receptor – MHC/peptide interactions are required to trigger and activate T cells (Valitutti et al., 1995). However, the affinity of the TCR for antigen is very low, unlikely to be sufficient to drive formation and stabilisation of the tight contact between T cell and APC. To maintain such a high degree of sensitivity and fidelity of T cell recognition, a number of co-stimulatory and accessory molecules are co-ordinately engaged in the immune recognition process. These include among others; CD2, CD4, CD8, CD28, LFA-1, CD9, CD44, CD48 and CD5.

The identification of co-stimulators led to the two-signal model for T cell activation, proposing that the first signal is provided by the TCR/CD3 complex, while a second signal is generated by engagement of T cell accessory receptors (Schwartz, 1990). Although numerous molecules have been implicated as accessory receptors, CD28 is the classical example. The interaction of CD28 on T cells with B7 family molecules on APCs is important for providing signal 2, since it up-regulates Il-2 production and T cell proliferation (Linsley and Ledbetter, 1993; June et al., 1994). However, the unification of the two-signal model with the immunological synapse model is currently challenged. An alternate view is that co-stimulators function to fine-tune the TCR signal by organising the T cell – APC contact site for optimal TCR engagement and subsequent signal transduction (Dustin and Shaw, 1999; Gett and Hodgkin, 2001).

1.7.6.1 The co-receptors CD4/CD8

The co-receptors CD4 and CD8 play an important role in TCR signalling, but the nature of their contribution is not clear. It is thought that CD4/CD8 increase the efficiency of formation of the IS, by enhancing contact between the TCR and MHC molecules. CD4/CD8 associates with the TCR following activation by recruitment of the TCR into rafts (Xavier et al., 1998). During activation, CD4 increases T cell sensitivity to antigen by 10- to 100-fold (Hampl et al., 1997). Part of this contribution stems from its ability to recruit the PTK Lck to the TCR/CD3 complex (Barber et al., 1989). But even without its cycloplasmic Lck-binding domain, CD4 still augments T cell reactivity by 3- to 9-fold (Janeway Jr, 1992).

Anti-TCR blockade prevents TCR/CD3 accumulation and abolishes accumulation of CD4. Thus CD4 accumulation seems dependent on TCR binding or signalling even though its ligand is present. This suggests that early TCR – MHC/peptide recognition serve to recruit CD4 to the interface and initiate signalling. Surprisingly, this is not a function of the cytoplasmic tail, although associated with the important signalling molecules Lck and LAT (Bosselut et al., 1999), since a deletion mutant behaved in the same way (Krummel et al., 2000).

In the mature synapse, CD4/CD8 is localised together with TCRζ at the centre of the synapse, but the co-receptor then progressively migrates out of the central zone (Krummel et al., 2000). These findings do not support a model in which CD4/CD8 act to stabilise the TCR – MHC/peptide complexes. Instead, they suggest that the function of these co-receptors may be to boost the early phase of activation. Thereafter, they are excluded from the cSMAC.

1.7.6.2 CD2 and LFA-1

Lymphocytes usually travel in a non-adhesive form through the blood and lymph but become adherent when they have to interact with other cells. The LFA-1 integrin and its cellular ligands ICAM-1 and -3 play a crucial role in this process because they are involved in adhesion and transmigration through endothelial cells of the blood vessels and stabilisation of cell-cell contacts with APCs. LFA-1 has a relative large extracellular domain (the LFA-1/ICAM-1 interaction spans ~30-40 nm), and by the formation of the IS is excluded from the cSMAC, but is found abundantly in the outer ring, stabilising the cell-cell contact site. Furthermore, LFA-1 has been demonstrated to increase its avidity for ICAM-1 after raft clustering, one potential mechanism by which TCR engagement increases LFA-1 adhesion (Krauss and Altevogt, 1999). LFA-1 does not deliver a co-stimulatory signal but facilitates the generation of the TCR signal, in particular at low antigen concentrations, by promoting adhesion of T cells to APCs, which can influence T cell activation and differentiation (Smits et al., 2002).

The membrane protein CD2, expressed on T cells and NK cells (and B cells in mice), is present in a different subregion of the pSMAC than LFA-1 (Grakoui et al., 1999), and promotes a tight homogeneous interaction between the membranes of the T cell and APC by specific interaction with its ligands CD48 and CD58 (Selvaraj et

al., 1987). This happens with similar physical dimensions as the TCR /MHC interactions (TCR/MHC, CD4/8/MHC and CD2/CD48 all span ~15nm). Its association with CD3 could serve to recruit the TCR to the contact surface. CD2/CD3 co-clustering increases TCR association with rafts and CD2 engagement facilitates TCR clustering, cytoskeletal reorganisation and receptor patterning at the TCR contact site (Dustin et al., 1998; Yashiro-Ohtani et al., 2000). These processes are mediated by the binding of CD2-Associated Protein (CD2AP), a protein containing three SH3 domains that binds to polyproline motifs in the cytoplasmic tail of CD2. Overexpressing a dominant-negative form of CD2AP blocked both CD2-triggered cytoskeleton polarisation and IS formation, illustrating the importance of adhesion molecules. However, T cells from mice lacking CD2 do not exhibit a significant phenotype; other molecules, topologically similar in size to CD2, like CD28 may compensate for some functions of it (Killeen et al., 1992).

Most studies on CD2-CD48 interactions have focussed on T cell CD2 binding to APC CD48, because human CD2 expression is limited to T cells. However, in murine immune systems CD2 is also expressed on APCs. Moreover, because CD48 is a glycosylphosphatidylinositol (GPI)-anchored molecule, expressed on T cells, binding to CD2 expressed on APCs may similarly facilitate adhesion and TCR contact. Indeed, CD48 can shorten the duration of TCR engagement required for commitment to T cell activation, by possibly inducing the migration and clustering of lipid rafts to the TCR contact cap, a process that requires the Lck SH3 domain (Patel et al., 2001). Besides these enhancer mechanisms, CD2-CD58 interactions have been implicated in tolerance induction due to the lack of additional co-stimulation (Punch et al., 1998; Wakkach et al., 2001).

So far, studies suggest that T cell engagement of the APC is first mediated by LFA-1 and CD2 in distinct domains of non-antigen-specific contact areas. These co-stimulators differ in how they associate with the plasma membrane and where they partition within the synapse. They facilitate T cell activation by formation of a signalling platform, which increases the number of receptors engaged.

1.7.6.3 CD28 and CTLA-4

CD28, the most prominently studied accessory receptor, is a disulphide-linked homodimeric glycoprotein, which is expressed on T cells and upregulated after T cell

activation. CD28 interacts with B7-1 and B7-2 on APCs (Linsley and Ledbetter, 1993; June et al., 1994). It contains a 41 amino-acid residue cytoplasmic domain that lacks any obvious catalytic capacity. However, like the TCR, CD28 becomes phosphorylated on tyrosine residues by Src family kinases and Tec family kinases (Itk) (Raab et al., 1995). In addition, the CD28 cytoplasmic tail contains a proline-rich motif, which can serve to recruit SH3 domain containing molecules, like Lck (Holdorf et al., 1999). Despite the potential of the phosphorylated CD28 tyrosine motif to regulate signalling via recruitment of SH2-containing molecules like PI3-K and Grb2 or Gads, it is not clear whether this is essential for CD28 co-stimulation *in vivo*.

High doses of antigen in the absence of co-stimulation do not manage to activate naive T cells, whereas in the presence of co-stimulation naive T cells respond to low doses of antigen with high efficiency (Viola and Lanzavecchia, 1996; Iezzi et al., 1998). It is known that co-stimulation via CD28, although to some extent dispensable as demonstrated in CD28-deficient mice (Shahinian et al., 1993), can dramatically enhance the response of naive T cells and prevent the induction of anergy. Double mutant mice lacking CD28 and CD2 are more impaired in T cell activation than are the corresponding single mutant mice (Green et al., 2000). This suggests a degree of redundancy among adhesion/co-stimulation molecules. The unique phenotype associated with CD28 deficiency is the inability to respond to a wide range of peptide antigens, in contrast to lacking CD2, CD48 or LFA-1, which alters the quantitative aspects of the T cell response to a given peptide-MHC complex.

The different attributes of the different accessory receptors correlate with the localisation of these molecules in the mature IS; CD28 is localised in the sSMAC, whereas LFA-1 is engaged in a different subregion of the pSMAC (Grakoui et al., 1999). Therefore, CD28 is perfectly positioned to directly manipulate TCR signals. TCR triggering is weak in the mature synapse, but sustained TCR signals are required for full T cell activation (Lee et al., 2002). As demonstrated recently, CD28 influences immediate and sustained TCR signalling by enhancing activation of Lck (Holdorf et al., 2002). CD4 or CD8 initially recruit Lck and stimulate its autophosphorylation, but CD28 sustains the phosphorylation state and activity. Thus, CD28 affects the earliest process associated with TCR signalling and formation of the IS, enhances engagement processes in the cSMAC and consequently sustains TCR signalling.

Co-stimulatory molecules also provide negative signals required to attenuate the immune response and to maintain peripheral T cell tolerance to protect against autoimmunity. The interaction of B7 molecules with the cytolytic T lymphocyte associated antigen 4 (CTLA-4) provides inhibitory signals required for down-regulation of the T cell response. CTLA-4 protein is not detectable in naive T cells, but it is upregulated upon T cell activation, where it is targeted to the endosomal compartment (Lindsten et al., 1993).

CTLA-4 is translocated by polarised exocytosis to the centre of the mature synapse, thereby directly able to inhibit TCR signalling in addition to blocking CD28 signals (Egen and Allison, 2002). It also binds to B7, but with much higher avidity than CD28 (van der Merwe et al., 1997). Upon binding, it delivers an opposing signal to the T cell compared with CD28, it reduces the production of Il-2 and restricts T cell expansion by reducing the production of cyclins and cyclin dependent kinases (Cdk) (Brunner et al., 1999). Interestingly, localisation of CTLA-4 to the synapse is favoured under conditions of strong TCR ligation, suggesting that it controls the amplitude of TCR signalling (Egen and Allison, 2002). Thus, the activation of a T cell is initiated by the TCR, sustained by CD28 located in the cSMAC and modulated by targeted exocytosis of CTLA-4 into the cSMAC.

1.8 Signalling in development, differentiation and anergy

The induction of a cellular response following engagement of signal transduction receptors is based on the co-ordinated activities of diverse intracellular signalling pathways. The spectrum of biological activity elicited by TCR-mediated T cell activation seems to correlate with the binding affinity of the TCR for its peptide-MHC ligand (Rabinowitz et al., 1996; Wulfing et al., 1997; Germain and Stefanova, 1999). However, the signals that drive T cells into activation or regulation are as yet poorly defined. The amount of ligand required to activate T cells varies according to the state of the T cells (naive or memory) and the number of adhesion and co-stimulatory molecules on the APC (Inaba and Steinman, 1984). T cells respond only when the number of triggered TCRs reaches an appropriate threshold, and this threshold can be adjusted by co-stimulatory molecules (Viola and Lanzavecchia, 1996). This suggests that TCR and co-stimulatory pathways synergise along signal transduction cascades (Tuosto and Acuto, 1998; Salojin et al., 1999; Sedwick et al., 1999; Tsuchida et al., 1999; Holdorf et al., 2002). APCs lacking adhesion and co-

stimulatory molecules may induce TCR triggering below a certain threshold, leading to T cell anergy (Ferber et al., 1994), while the inflamatory milieu may determine the combination and level of accessory molecules expressed on DCs, leading to differentiation of activated CD4 T cells into Th1, Th2 and T_{reg}.

Contributing to a model of T cell activation with hierarchical thresholds for different functional responses were studies analysing T cell responses demonstrating a significant heterogeneity within a clonal population during an immune response (Assenmacher et al., 1994; Bucy et al., 1994; Openshaw et al., 1995; Valitutti et al., 1995; Itoh and Germain, 1997). More recently, a correlation was found between the level of IFNγ secretion and Lck expression, suggesting a relationship between signalling strength and cell differentiation into effector cells with characteristic cytokine profiles (Slifka and Whitton, 2001).

The affinity of the TCR for the peptide-MHC complex determines the level of ITAM phosphorylation and thus seems an important determinant in the outcome of an immune response (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Love and Shores, 2000a). The TCRζ chain contains three ITAMs, including a total of six tyrosines that are phosphorylated in an ordered manner after TCR ligation (Kersh et al., 1998a). Recognition of a less potent ligand leads to partial phosphorylation of a subset of tyrosine residues, while a potent ligand, induces complete phosphorylation of the ITAMs. By viewing the phosphorylation process as a series of reversible sequential steps, it was proposed that weak ligands having a rapid dissociation, would only manage a limited amount of TCRζ phosphorylation, subsequently reducing the recruitment of ZAP-70, thus allowing the T cell to discriminate among antigens (Kersh et al., 1998b).

As a result of quantitative reduction in TCR signalling, possible qualitatively different signals determine the outcome of T cell activation. It is however still unclear whether these qualitative signals include novel signalling molecules or complexes. It is possible that monophosphorylated ITAMs recruit other molecules than ZAP-70, like negative regulators of TCR signalling (Love and Shores, 2000a). By using agonist and altered peptide ligands (APL) to stimulate T cells, several differences in signal transduction pathways have been reported. Stimulation of naive CD4 T cells with an agonist leads to sustained and high stoichiometry of phosphorylation of the TCRζ chain (p23), ZAP-70 recruitment and phosphorylation, phosphorylation of

LAT, sustained increase of intracellular calcium, activation of Erk and JNK and translocation of NFAT to the nucleus. In contrast, ligation with APLs can result in substoichiometric phosphorylation of TCRζ (p21), failure to recruit and activate ZAP-70, weak and transient calcium mobilisation and low levels of Erk and JNK activation (Sloan-Lancaster et al., 1994; Sloan-Lancaster et al., 1996a; Sloan-Lancaster and Allen, 1996b; Boutin et al., 1997; Leitenberg and Bottomly, 1999). Changes in TCR-mediated signalling or its downstream signalling cascades might drive the differentiation of the naive T cell into different response modes, varying from Th1/Th2 modes to T_{reg} cells and T cell unresponsiveness.

1.8.1 Signalling during T cell development

After structural studies in mature T cells showed the differential phosphorylation capacity of the TCRζ, it was speculated that weak and strong signals in the thymus also result in partial phosphorylation of ITAMs. They clearly amplify the TCR signal during thymic development, but data to date do not suggest that ITAMs qualitatively mediate positive or negative selection (Shores et al., 1997; van Oers et al., 1998; Ardouin et al., 1999; Love et al., 2000b). Interestingly, it was shown that the signalling properties of the TCR change during thymocyte maturation, differentially affecting responses to related peptide-MHC molecule complexes and contributing to this discrimination (Lucas et al., 1999).

More recent data suggest a duration model, where at very proximal points in the signalling pathway, positive selection signals are like negative selection signals, only shorter. High affinity interactions trigger a strong but transient signal whereas low affinity ligands trigger a weak but sustained one. This is also consistent with subsequent biochemical data on the role and duration of other key players in TCR signalling like PKC (Ohoka et al., 1997), Ras (Shao et al., 1997) and MAPKs (Sharp et al., 1997; Bommhardt et al., 1999; Mariathasan et al., 2001; Wilkinson and Kaye, 2001). When the signalling potential of the TCR was modified by substituting transgenic TCRζ chains containing either three, one, or no ITAMs for endogenous TCRζ, it was shown that negative selection could be converted to positive selection in the presence of TRCζ containing none or one ITAM (Love et al., 2000b). However, this process is more complex, since not only TCRζ but also the other CD3 chains, al containing one ITAM are actively involved in selection processes (Dave et al., 1997;

Delgado et al., 2000; Haks et al., 2002). The role of particular CD3 components in these events remains unclear. However, the multiplicity of CD3 components and their evolutionary conservation suggest that they may serve distinct functions.

Erk activation has been identified as a potential point where the signals of positive and negative selection qualitatively diverge (Alberola-Ila et al., 1996; Sharp et al., 1997; Sugawara et al., 1998). High affinity ligands stimulate a strong and transient activation of Erk, while low affinity ligands stimulate a much weaker activation but this is sustained over a longer time period (Werlen et al., 2000; Mariathasan et al., 2001). Importantly, similar results were obtained using mice deficient in one of the CD3 chains. CD3δ is critical for TCR signalling in positive selection, and in CD3δ-deficient mice Erk activation was severely impaired, while JNK and p38 activation were unaffected (Delgado et al., 2000). The underlying mechanism by which TCR signalling is linked to Erk activation in thymocytes points towards RasGRP (Dower et al., 2000; Priatel et al., 2002). TCR engagement leads to the activation of PLCγ, resulting in accumulation of DAG at the plasma membrane, subsequently recruiting RasGRP, which activates Ras. Interestingly, mice that have decreased levels of Grb2, capable of activating Ras by recruiting Sos, show impaired activation of JNK and p38, but normal activation of Erk (Gong et al., 2001). The sustained signal may be the result of the absence of TCR downregulation when low affinity ligands trigger the TCR. These data are especially important in light of data from other signalling systems where transient and sustained Erk activation led to different cell fates (Marshall, 1995; York et al., 1998).

Models based on signalling strength or duration have also been implicated in lineage commitment to CD4 or CD8. An important role here was suggested for the PTK Lck. A constitutively active form of Lck led cells expressing a MHC class I-restricted receptor to adopt the CD4 fate, while a catalytically inactive form of Lck forced cells expressing a MHC class II-restricted receptor to adopt the CD8 fate (Hernandez-Hoyos et al., 2000). Duration of the stimulus was shown to be important in *in vitro* studies, where high affinity ligands were presented by DCs. Short incubation times resulted in CD8+ thymocytes while long exposure to the ligand led to CD4+ thymocytes (Yasutomo et al., 2000). Again the Ras/Raf/Erk pathway has been implicated in this lineage decision (Bommhardt et al., 1999; Sharp and Hedrick, 1999).

Models applying for thymocyte selection in the thymus may apply to some extent in the periphery. Weak signals, or interactions with a short half-life, may lead to T cell survival (van Oers et al., 1994), intermediate signals to unresponsiveness, and only strong enough signals to fully activate T cells. Very strong signals would lead to clonal deletion, another way of peripheral tolerance (Webb et al., 1990).

1.8.2 Signalling in peripheral T cell differentiation

Differences in the organisation of TCR signalling pathways may also determine the differentiation of naive T cells into effector subsets with unique cytokine and immunoregulatory profiles. A change in TCR signalling potency can determine the type of cytokine that will be induced (Constant et al., 1995; Hosken et al., 1995; Boutin et al., 1997; Leitenberg and Bottomly, 1999). In the absence of a dominant cytokine, low potency TCR signals preferentially generate Il-4 production and Th2 differentiation. In contrast, priming naive CD4 cells with optimal doses of high affinity peptide favour Il-2 and IFNγ production resulting in Th1 differentiation.

Both subsets of Th cells require a spectrum of transcription factors to activate cytokine promoters. Th1 development is critically dependent upon transducer and activator of transcription (STAT) 4, class II transcription factor (CIITA), and T-bet, while Th2 development requires STAT6, GATA3, JunB, c-Maf and NFAT45 (Asnagli and Murphy, 2001). Most transcription factors are regulated by MAPKs, suggesting that differential activation of MAPKs affect the differentiation of CD4 T cells. Indeed, JNK2 has been reported to be an important factor for the polarisation of CD4 T cells towards a Th1 phenotype (Yang et al., 1998), while JNK1 is capable of blocking the development of a Th2 response (Dong et al., 1998). After differentiation, Th2 cells fail to induce JNK activation, while Th1 cells easily activate it. JNK1 acts by decreasing the nuclear NFATc abundance, required for the activation of the Il-4 promoter. JNK2, probably in synergy with other factors, positively regulates IFNγ production, and induces the expression of the β2 subunit of the Il-12 receptor (Murphy et al., 2000).

Thus, the ratio between transcription factors present in the nucleus determines the T cell differentiation fate. High levels of NFATc over NFATp and NFAT4 favour Il-4 expression and subsequent Th2 differentiation, whereas a reversal of this ratio interferes with Il-4 production and thus favours Th1 development. It is unclear

whether these differences in ratio of NFAT isoforms, as well as JNK activation, reflect TCR signalling potency. However, there are numerous links suggesting such a possibility. A sustained calcium flux promotes the nuclear relocation of NFAT proteins, as well as JNK1 activation, leading to NFATp and NFAT4 accumulation in the nucleus (Leitenberg and Bottomly, 1999). A potent agonist, promoting strong TCR signalling, sustained calcium release and high stoichiometry of ITAM phosphorylation could thus promote Th1 differentiation. In contrast, low potency ligands induce a short but sufficient burst of calcium flux to allow Erk activation and NFAT translocation to the nucleus (Sloan-Lancaster et al., 1996a; Leitenberg and Bottomly, 1999). In the absence of JNK activation, which possesses a higher threshold for activation than Erk (Gong et al., 2001), NFATc will dominate in the nucleus, favouring IL-4 production and Th2 differentiation.

In addition to the role of the TCR, a number of accessory molecules exert an effect on the polarity of T cell differentiation. These include contributions by CD4, CD45 and CD28 (Schweitzer and Sharpe, 1998). Their influence may reflect the stimulation of unique additional signalling pathways, but likely some synergies with the TCR cascades, thereby influencing thresholds for activation of downstream molecules.

1.8.3 T cell non-responsiveness/ anergy/ T_{reg}

Stimulating naive T cells in the absence of the CD28 signal can induce a state of unresponsiveness, *in vitro* often referred to as anergy. It is defined by the inability of individual T cells or T cell clones to produce Il-2 and to proliferate when restimulated with their appropriate antigen presented by APCs (Schwartz, 1992). This two-signal concept is also applicable *in vivo*, and can induce tolerance to organ grafts (Salomon and Bluestone, 2001). However, correlation between *in vitro* anergy and *in vivo* tolerance is not clear as yet, the more so since *in vivo* tolerance spans a variety of mechanisms from clonal deletion and ignorance to the induction of immunoregulatory T cell subsets.

Anergic T cells exhibit poor phosphorylation of TCRζ and CD3ε chains, are incapable of activating ZAP-70, Ras, JNK and Erk, and have impaired relocation of Grb2-Sos complexes to the membrane (Kang et al., 1992; Gajewski et al., 1994; Boussiotis et al., 1996; Fields et al., 1996; Mondino et al., 1996; Salojin et al., 1997).

As a result, transcription factors fail to bind to the Il-2 promoter thus disabling anergic cells from secreting this cytokine. However, they maintain their capacity to induce PLCγ1 phosphorylation and calcium flux, along with elevated levels of IP3, cyclic adenosine monophosphate (cAMP), and an increased Fyn kinase and Rap1A activity (Quill et al., 1992; Gajewski et al., 1995; Boussiotis et al., 1997). These findings suggest that anergy induction is dependent on the down-regulation of some pathways, while maintenance depends on others that are upregulated. Increased levels of cAMP lead to the induction of the cyclin-dependent kinase inhibitor 27 kD cyclin-dependent kinase inhibitor ($p27^{kip1}$). $p27^{kip1}$ blocks cell cycle progression and Il-2 production by associating with the c-Jun co-activator JAB1, thereby disrupting AP-1 binding sites in the Il-2 promoter (Boussiotis et al., 2000). In addition, cAMP activates the Exchange Protein directly Activated by cAMP (EPAC), a GEF that catalyses GTP exchange on Rap1.

Tolerance can also be induced in some peripheral T cell populations in the presence of CD28 (Wells et al., 2001). This kind of tolerance is dependent on the role of CTLA-4 but, interestingly, both forms of anergy show elevated levels of $p27^{kip1}$. When expressed on the cell surface, CTLA-4 is able to modulate several aspects of TCR signalling, including JNK and Erk phosphorylation, and the transcriptional activity of NF-κB, NFAT and AP-1 (Calvo et al., 1997; Alegre et al., 2001). The mechanisms behind CTLA-4 signalling are still poorly understood, but may include the recruitment of PTP to the TCR, inhibition of B7 binding to CD28 by competition, and disruption of signalling arrangements in the immunological synapse (Alegre et al., 2001).

Interestingly, the phenotypically distinct subset of CD4+/CD25+ T cells, which are suggested to play a role in maintenance of *in vivo* tolerance, are reported to exhibit constitutive CTLA-4 expression (Salomon et al., 2000). CTLA-4 also induces secretion of TGF-β, providing a possible explanation for some of the immunoregulatory capacities of these T cells (Chen et al., 1998). By removing the signal from engaged CTLA-4, suppressive activity could in some *in vitro* experiments be abrogated (Read et al., 2000; Takahashi et al., 2000).

The subpopulation of CD4+CD25+ T cells have been reported to be important in controlling autoreactive T cells *in vivo*; *in vitro* they have been shown to be hyporesponsive and suppressive (Papiernik, 2001). However, populations of T_{reg} are

described in a variety of experimental contexts; they have been identified using different cell surface markers and thus seem to be a heterogeneous population. Many questions regarding these cells remain as yet unresolved; these include their origin, their specificity and their mode of action. However, recent data indicate the existence of distinct pathways resulting in the generation of T_{reg}. Monospecific naive T cells can differentiate autonomously into antigen-specific, predominantly CD25-, T_{reg} cells in peripheral tissues. In addition, antigen expression in the thymus can lead to antigen-specific, predominantly CD25+, T_{reg} cells (Apostolou et al., 2002).

Insights into the mechanism of induction of T_{reg} cells are still very poor. However, Il-10 seems to play an important role, cells stimulated in its presence differentiate into a new subset of CD4 T cells, and these cells secrete high levels of Il-10 themselves. In addition, Il-10 pre-treated DCs are able to induce anergy (Steinbrink et al., 1997). These effects can only be observed in immature DCs; mature DCs are resistant to Il-10. The functional mechanism of Il-10 is associated with a reduction of antigen-presenting capacity, and stimulating T cells with anti-CD3 in the presence of Il-10 did not generate regulatory T cells (Wakkach et al., 2001). It down-regulates the expression of MHC molecules and essential accessory molecules like B7 and the adhesion molecule LFA-1, but not CD58 (Willems et al., 1994; Steinbrink et al., 1997; Wakkach et al., 2001). Since these are all important elements of the IS, this indicates that non-responsiveness is induced by interfering with proximal TCR signalling pathways. This is in parallel to *in vitro* protocols, where the absence of sufficient co-stimulatory signals results in anergy.

TGFβ has also been reported to enhance tolerance. Stimulation of naive CD4 T cells in its presence resulted in CD4+CD25+ T cells with potent suppresser activity on the development of CD8 T cells (Yamagiwa et al., 2001). The possibility was raised that CD4+CD25+ T cells produce TGFβ, whereupon they bind it to their cell surface, and deliver it directly to responder cells by a cell-cell contact-dependent delivery system (Nakamura et al., 2001). However, the mechanism behind TGFβ-induced tolerance is unknown, and many studies failed to find a role for it (Chen et al., 1994; Takahashi et al., 2000; Thornton and Shevach, 2000; Jonuleit et al., 2001; Read and Powrie, 2001). The only indication of a possible role came from Yamaguchi et al., (1997) who showed that TGFβ inhibited the maturation and differentiation of DCs, again suggesting that alteration of co-stimulatory molecules determines the differentiation of effector cells.

Interference with TCR signal transduction cascades in immortalised cell lines or genetically modified animal systems have provided valuable insights into complex system properties required to trigger a response. However, these techniques have limitations; molecules of interest can be expressed outside the targeted cell types, loss of crucial proteins can result in lethality or selection for cells that employed compensatory mechanisms, and immortalised cell lines have altered signalling properties and have lost their homing potential.

1.9 DNA- or protein-based modifications of cell functions

Many genes and their products have several roles in different tissues and at different stages of development. Phenotypes caused by genetic manipulations are often difficult to interpret since they might be due to differential expression in place or time. Conditional and cell-specific regulatory elements have been found to control transgene expression. Transcription of the gene of interest can now be kept under specific intracellular conditions when a specific promoter is activated. These regulatory signals are required to maintain specific activity. Furthermore, they should be reversible and specific for the target gene, and should not interfere with other cellular components or with cell metabolism (Lewandoski, 2001). During recent years, binary transgenic systems have been developed in which gene expression is controlled by the interaction of two compounds, such as Cre/loxP and the TetR-based transactivators. All these systems, however, rely on the laborious process of generating transgenic animals.

Cultured cells can be manipulated by the use of pharmacological agents and transfection of expression vectors via microinjection, electroporation, retroviral gene transfer, or association with cationic lipids and liposomes. While these approaches have been somewhat successful, problems such as the specificity of pharmacological inhibitors, low transfer efficiency, complex manipulation and cellular toxicity, precluded routine use of these methods, especially with fragile primary cells such as *ex vivo* T cells that are refractive to ordinary transfection methods. Additionally, reliance on the use of DNA results in very limited control over the exact amounts of protein expressed and present in the cytosol. Because timing of many events is a critical determinant of the outcome of biological processes, it would be useful to have

temporal control over the amount or activity of specific proteins, currently not feasible with genetic mutants.

1.9.1 Protein-transduction

A wide variety of methods have been proposed for the delivery of proteins into living cells. However, most of these methods were inefficient, caused high amounts of cell death, or resulted in protein trapping in intracellular vesicles without delivery into the cytoplasm. The explanation is that cells poorly take up most "information-rich" molecules, such as peptides and proteins, since they do not efficiently cross the lipid bilayer of the plasma membrane or of the endocytic vesicles (Lebleu, 1996). This is a major limitation for their *ex vivo* or *in vivo* use in fundamental studies or in clinical applications.

Until recently it had not been considered that some proteins might be internalised and directly targeted to the cell cytoplasm and nucleus without involving the classical endocytosis pathway. However, some transcription factors, involved in morphological processes during development, known as homeoproteins, can pass freely from cell to cell. They are secreted in an unconventional way and re-internalised by neighbouring cells where they can be found intact in the cytoplasm and nucleus (Joliot et al., 1991; Chatelin et al., 1996; Joliot et al., 1998). Green and Frankel first reported protein transduction, the internalisation of proteins into the cell from the external environment. They independently demonstrated that the Human Immunodeficiency Virus (HIV)-1 transactivator of transcription (Tat) protein could enter cells when added to the surrounding culture medium (Frankel and Pabo, 1988; Green and Loewenstein, 1988).

Since this initial observation, other proteins possessing this ability have been identified. These include the herpes simplex virus-1 DNA-binding protein VP22 (Elliott and O'Hare, 1997) and several homeobox-containing proteins of which Antennapedia (Antp) is the best known, and used to develop several penetrating peptides, the Penetratin family (Derossi et al., 1996; Han et al., 2000). The three protein-transduction domain (PTD) – containing proteins mentioned have the highest level of protein-transduction efficiency demonstrated so far and are internalised by all tested cell types and by approximately 100% of the cells present in culture (Derossi et al., 1996; Vives et al., 1997).

The apparent efficiency with which these PTD-containing proteins were shown to enter cells raised the possibility of utilising them as delivery vehicles for macromolecules. A method that allows the delivery of bioactive molecules into cells using membrane-permeable proteins has a great potential to modify cellular processes on a protein level. Indeed, PTD-containing peptides conjugated to proteins, DNA and RNA were shown to deliver their cargo intracellularly (Mann and Frankel, 1991; Fawell et al., 1994; Astriab-Fisher et al., 2000). The responsible transduction domain was identified as a short stretch of 11 to 16 amino acids, in Tat and Antp respectively (Schwarze et al., 2000). The Tat PTD, composed of six arginine and two lysine residues within a stretch of 11 amino acids, seems to be the most powerfull. It was shown to be capable of delivering biologically active fusion proteins, including the 120-kD β-galactosidase protein, into all mouse tissues after intra-peritoneal injection (Schwarze et al., 1999).

Taken together, the short sequence necessary for translocation, the power to translocate large full-length proteins that keep their biological activity after translocation, and the ubiquitous internalisation in any mammalian cell type, make PTDs a powerful tool for intracellular protein delivery. They have already successfully been used conjugated to several peptides, proteins, DNA and magnetic particles (Fawell et al., 1994; Bonifaci et al., 1995; Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999; Chellaiah et al., 2000; Lewin et al., 2000; Vocero-Akbani et al., 2001). The most impressive aspect of PTD-mediated delivery is its size independence. PTDs have been shown to deliver proteins in excess of 100 kD into cells in culture and animal systems. Interestingly, 40 nm super-paramagnetic iron nanoparticles conjugated to Tat PTD peptides were internalised into hematopoietic and neural progenitor cells and into peripheral T cells, without affecting cell viability, differentiation, proliferation or homing potential (Lewin et al., 2000; Dodd et al., 2001).

1.9.2 Mechanism of cellular internalisation

Despite the large number of potential applications of these PTD containing proteins, the mechanism by which translocation proceeds remains essentially unknown. Initial studies with homeoproteins, like Antp, suggested that interference with their helix structure was able to prevent cell entry, and thus that the helix structure has a key role in internalisation (Le Roux et al., 1993). However, chemical

synthesis of short sequences of this helix, one composed of the inverso D-isomer, one in which the order of the amino acids was reversed, and one in which the helix structure was broken by introducing proline residues, showed that all four peptides were internalised (Derossi et al., 1996). Similar results where obtained using the PTD domain of Tat, with the retro-inversion form of Tat having the highest translocation efficiency (Wender et al., 2000). This indicates that translocation of PTD domains across the plasma membrane does not require a helicoid conformation, and more importantly does not require a chiral receptor.

In agreement with the observation of apparent absence of a receptor, cellular internalisation of PTD-containing proteins occurs at 4°C, a temperature that abolishes active transport mechanisms involving endocytosis, as well as at 37°C.
This indicated that the transduction mechanism, besides being receptor-independent, is also energy-independent (Joliot et al., 1991; Derossi et al., 1996). Protein transduction seems therefore to be independent of the classical endocytosis pathways and is not specific for any cell type (Mann and Frankel, 1991; Derossi et al., 1996; Elliott and O'Hare, 1997; Vives et al., 1997). The latter is consistent with the notion that Tat is able to enter into a wide variety of human, rodent, and simian cell lines.

Sequence comparisons between Tat, Antp and VP22 PTDs show the presence of the basic positively charged amino acids arginine and lysine (Schwarze et al., 2000), corresponding to the sequence responsible for cellular uptake (Vives et al., 1997; Schwarze et al., 2000). This points out that charge may be an important factor for protein internalisation, whereby positively charged amino acids interact with negatively charged phospholipids. Indeed, by preparing a series of analogues of Tat-PTD alanine substitutions, it was shown that cationic residues play a critical role in cellular uptake (Wender et al., 2000).

The positively charged arginine residues in PTD domains are essential for translocation as deletion or substitution by alanine of a single arginine reduces internalisation (Vives et al., 1997; Wender et al., 2000). Their charge alone, however, is insufficient. Homopolymers of citrulline, an arginine isostere in which the nitrogen of the guanidine group is replaced with oxygen, showed no transduction activity (Mitchell et al., 2000). Indeed, a 9-mer of arginine residues is more efficient than Tat itself in cellular uptake, suggesting that the guanidinium groups play a greater role in facilitating internalisation than their charge alone. The number of arginine residues

within the Tat peptide appears to be the main determinant for maintaining a high translocation activity (Futaki et al., 2000; Wender et al., 2000). Moreover, the length of the poly-arginine peptide seems critical, as a maximal rate of internalisation was observed for a peptide 9 arginine residues in length. The D-isoform and the retro-inverso form of the poly-arginine peptide were found to internalise even more efficiently; the reason might be found in the higher stability of the D-isoform in serum-containing medium, as the rate of uptake was the same in serum-free medium (Wender et al., 2000).

A possible important clue about the mechanism of PTD-containing protein internalisation came from mixing these proteins with lipids extracted from rat brain. This provoked the formation of reverse micelles, not observed with chemically defined lipids or with non-internalised proteins (Berlose et al., 1996). Interestingly, the translocation of full-length Tat fusion proteins was shown to result in inactivation or denaturation of the conjugated protein, thus resulting in a linear protein without a specific structure (Bonifaci et al., 1995). Further studies revealed that the flexibility of the PTD backbone allows their adaptation to the concave surface of a micelle, and might be the explanation for the necessity of unfolding of conjugated proteins in order to get internalised. This was also illustrated by transduction of correctly folded Tat - green fluorescent protein (GFP) fusion protein into cells, which resulted in a high intracellular level of Tat-GFP, but with significantly altered kinetics and loss of GFP emission (Caron et al., 2001).

Tyagi et al. (2000) recently demonstrated that cell membrane heparan sulphate proteoglycans (HSPG), distributed ubiquitously on all mammalian cell types, might act as a receptor for extracellular Tat. Previously it had been shown that the addition of soluble heparin could modulate several of the biological properties of Tat (Rusnati et al., 1998). In addition it was now shown that specific lysases for HSPGs block Tat internalisation, and that cell lines with genetic defects in the metabolic pathways involved in the production of HSPGs fail to internalise Tat. A common motif for HSPG binding consists of a region rich in basic amino acids flanked by hydrophobic residues, like the PTD domain (Esko, 1991). Although other basic amino acid sequences are capable of binding HSPGs, these fail to be transduced over the membrane (Lappi et al., 1996), indicating that ionic interactions alone are not sufficient for internalisation. Only arginine-rich basic peptides seem to share this internalisation pathway (Futaki et al., 2000; Suzuki et al., 2002).

The current model of PTD internalisation proposes that protein is stabilised at the cell surface by electrostatic interactions between the positively charged arginine and lysine residues, and the negatively charged phospholipids and cell HSPGs. This loading may be sufficient to allow protein accumulation at the water-membrane interface thus destabilising the membrane organisation, allowing the formation of inverted micelles. It is also speculated that fusion of the Tat PTD peptide to the N-terminal region of proteins favours its steric accessibility to cellular structures involved in the translocation process. The micelles trap the protein in hydrophobic cavities, which might explain the importance of the hydrophobic residues arginine and lysine other than for their positive charge, and deliver it into the cytoplasm.

1.10 Aims of this study

The aim of this project is to study whether manipulation of the signalling pathways downstream of the TCR/CD28 receptor can result in distinct functional differentiation of monoclonal CD4+ T cell populations with known antigenic specificity. To this end I set out to develop a protein transfer mechanism capable of delivering proteins or peptides into naive *ex vivo* T cells that can compete with or inhibit endogenous proteins within the signalling pathways downstream of the TCR.

The initial focus was on the generation of a peptide delivery system and its ability for transferring full-length proteins and protein domains into naive *ex vivo* T cells. For this purpose fusion proteins with the Tat PTD domain were generated. Given the important position in the proximal signalling cascades downstream of the TCR, the aim was to focus on the PTKs Lck and ZAP-70 and to use domains of these fused to Tat-PTD that can interfere with their endogenous function. Using these, the aim was to analyse the consequences of modifying TCR-mediated signals on activation and differentiation of naive CD4 T cells.

2 Materials and Methods

2.1 Animals

The mice strains used were A18 TCR transgenic ($Rag1^{-/-}$ $C5^{-/-}$) (Zal et al., 1994), A1 TCR transgenic ($Rag1^{-/-}$ $C5^{-/-}$) (Zelenika et al., 1998), AND $Rag1^{-/-}$ (Kaye et al., 1989), F5 $Rag1^{-/-}$ (Mamalaki et al., 1993), BM3 $Rag^{-/-}$ (Sponaas et al., 1994), OT-1 $Rag1^{-/-}$ (Hogquist et al., 1994), and $TCR\alpha^{-/-}$ (Mombaerts et al., 1993). Polyclonal controls used were B10 ($H\text{-}2^{b}$) and A/J ($H\text{-}2^{a}$). All mice were kept in conventional, but pathogen-free, animal facilities at the National Institute for Medical Research (London, UK).

2.2 Cell lines, cultures and culture media

2.2.1 Cell lines and culture media

E6.1 Jurkat T cells were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 5% heat inactivated Fetal Calf Serum (FCS) (Gibco BRL), $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and $5x10^{-5}$ M β-mercaptoethanol (all Sigma).

CTLL-2 is an Il-2 dependent T cell line, ATCC cat. No. TIB 214.

The culture medium used for *ex vivo* cultures and cell lines was Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL) supplemented with 5% heat inactivated FCS (Gibco BRL), $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and $5x10^{-5}$ M β-mercaptoethanol (all Sigma). Medium for washing cells was Air-Buffered IMDM containing 25 mM HEPES and L-glutamine and supplemented with 0.21% NaCl, 100 U/ml penicillin, 100 μg/ml streptomycin and 12.5 mM NaOH (AB medium).

2.2.2 Determination of cell viability and numbers

Trypan blue (Sigma) at a final concentration of 0.08% in phosphate buffered saline (PBS) (10.1 g NaCl, 0.362 g KCl, 0.362 g KH_2PO_4, 1.449 g Na_2HPO_4 in 1 l H2O) was used to determine the viability of cells. Cells were counted in a 1:1 mixture of Trypan blue using a Neubauer counting chamber (BDH Ltd., UK) under the light microscope. Dead cells, stained blue, were excluded from counting.

2.2.3 Cholerae toxin B-subunit (CtxB) binding and toxicity tests

Tests were performed with different concentrations of commercial CtxB (Calbiochem-Novabiochem Corp.) and FITC-conjugated CtxB (Sigma). For toxicity assessments, CtxB was added to *ex vivo* splenic T cell cultures for up to three days. Anti CtxB mAb (Calbiochem-Novabiochem Corp.) was used to crosslink CtxB on the cell surface.

2.2.4 Bone Marrow Cultures

Bone marrow-derived dendritic cells were generated as described previously (Stockinger and Hausmann, 1994) with some modifications. Briefly, 5 10^6 bone marrow cells were cultured in 9 cm petri dishes (NUNC, Denmark) in 10 ml culture medium containing 10% supernatant of Ag8653 myeloma cells transfected with murine GM-CSF cDNA (about 25 U/ml). On day 4 of culture, non-adherent granulocytes were removed and GM-CSF medium was replaced. Loosely adherent cells were transferred to a fresh dish on day 6 of culture. From day 6 to day 8 these transferred loosely adherent cells were used as a source of DCs. DCs were incubated at 1×10^6/ml for 2–4 h at 37 °C with indicated concentrations of specific antigen and washed to generate antigen-pulsed APC.

2.2.5 *Ex vivo* cell cultures

Spleen and lymph nodes (Axillary, Branchial, Mesenteric, and Inguinal) were harvested from relevant mice strains and gently pressed through a mash using AB medium for resuspension of cells. Cells were washed once, and erythrocytes were removed by resuspending in 5 ml/mouse ACK lysis buffer (0.15 NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2-7.4) for 5 min at RT. Cells were washed, clumps were removed by passing cells through a 70 µm cell strainer (Falcon, Becton Dickinson Labware) and viable cells were counted.

2.2.6 Plate-bonnd anti-CD3 stimulation

Anti-CD3 antibodies (145-2C11) and 10 µg/ml anti-CD28 (37.51) antibodies were coated onto 24-well tissue-culture plates in PBS by over night (o/n) incubation at 4°C. Prior to use, cells were washed with AB medium, and kept at RT. Cells were put on the plates at 2 10^5/ml in 2 ml culture medium, followed by a short spin at 500 rpm for 1 minute. Cells were incubated for 4 hours at 37°C, resuspended, and replated on a non-coated 24-well plate for the rest of the experiment.

2.2.7 MACS selection

Cells were labelled with biotinylated antibody (Thy1.2 (CD90.2) (53-2.1), 1:500 (PharMingen)) at 10^7/ml total cells. Cells were washed by addition of 10x the volume of labelling buffer (PBS supplemented with 2 mM EDTA and 2% BSA). Thereafter, cells were resuspended in 90 µl labelling buffer per 10^7 total cells, and 10 µl of MACS streptavidin microbeads (MACS, Miltenyi Biotec, GmbH) per 10^7 total cells was added. Alternatively, single cell suspensions were directly labelled using mouse CD4 (L3T4) MACS microbeads (MACS, Miltenyi Biotec, GmbH). After 15 minutes at 6°C, cells were washed and resuspended in 500 µl of labelling buffer per 10^8 total cells. Cells were loaded into the AutoMACS (MACS, Miltenyi Biotec, GmbH) using the *POSSEL*

program for positive selection. After selection, recovered cells were washed, counted, and resuspended in 500 µl/10^6 cells 5% FCS IMDM culture medium.

2.3 Flow cytometry

2.3.1 Cell surface marker staining

Analytical flow cytometry was conducted using a Fluorescence Activated Cell Sorter (FACS) Calibur (Becton Dickinson), and the data were processed using CellQuest software (Becton Dickinson). Cells (not exceeding10^6/50 µl) were preincubated with unlabeled mAb to Fcγ III/II Receptor to minimize unspecific staining. Four-colour staining was performed with fluorescein isothiocyanate- (FITC), phycoerythrin- (PE), allophycocyanin (APC), and biotin- (Bio) conjugated mAbs followed by streptavidin PE Cy7 using forward and side scatter characteristics to exclude dead cells. All stainings were performed on ice and cells were washed with washing buffer (PBS, 2% FCS, 0.1% azide) in between staining. The Abs used for phenotypic analysis are summarised in Table 1.

Table 1. Specificity and name of anti-mouse Abs used for extra cellular staining.

Specificity	Conjugate	Clone name	Company
Fcγ III/II R	none	2.4G2	Home made
CD3	FITC	145-2C11	Home made
CD4	FITC	GK1.5	Pharmingen (San Diego, CA)
	PE	H129.19	Pharmingen (San Diego, CA)
	APC	RM4-5	Pharmingen (San Diego, CA)
CD5	FITC	53-7.3	Pharmingen (San Diego, CA)
CD8α	FITC	53-6.7	Pharmingen (San Diego, CA)
	PE	CT-CD8a	CalTag Laboratories (Burligame, CA)
	APC	53-6.7	Pharmingen (San Diego, CA)
CD25	FITC	7D4	Pharmingen (San Diego, CA)
	PE	PC61	Pharmingen (San Diego, CA)
	Bio	7D4	Pharmingen (San Diego, CA)
CD62L	Bio	MEL-14	Pharmingen (San Diego, CA)
CD69	FITC	H1.2F3	Pharmingen (San Diego, CA)
	PE	H1.2F3	Pharmingen (San Diego, CA)
	Bio	H1.2F3	Pharmingen (San Diego, CA)
CD44	FITC	IM7	Home made

	PE	IM7	Pharmingen (San Diego, CA)
	Bio	IM7	Pharmingen (San Diego, CA)
CD45RB	Bio	16A	Pharmingen (San Diego, CA)
CD95 (Fas)	Bio	Jo2	Pharmingen (San Diego, CA)
Streptavidin	PE Cy7		CalTag Laboratories (Burligame, CA)
TCRβ	FITC	H57-597	Pharmingen (San Diego, CA)
	PE	H57-597	Pharmingen (San Diego, CA)
	APC	H57-597	Pharmingen (San Diego, CA)

2.3.2 Apoptosis assay

For detection of early apoptosis, single cell suspensions were incubated with 20 μg/ml 7-Amino-actinomycin D (7-AAD; Sigma) for 20 min at 4°C as previously described (Lecoeur et al., 1997).

2.3.3 Intracellular staining

Cytokine production is induced by stimulating the cells with 500 ng/ml PdBu, 500 ng/ml Ionomycin and 10 μg/ml BrefeldinA for 4 hours at 37°C. Cells were collected, and the extracellular staining protocol followed to stain for surface markers. Thereafter, cells were resuspended in 100 μl 3% paraformaldehyde in PBS and incubated for 20 minutes on ice. Cells were washed, and resuspended in 0.1% NP40 PBS for 3 minutes, whereafter cells were washed and incubated with unlabeled mAb to Fcγ III/II Receptor, followed by specific cytokine Abs or isotype controls for 20 minutes each. Before acquisition, cells were given a final wash in PBS for 15 minutes to wash out non-bound Abs. The Abs used for cytokine profile analysis are summarised in Table 2.

Tabel 2. Specificity of anti-mouse Abs used for intra cellular staining.

Specificity	Conjugate	Company
Il-2	FITC	Pharmingen (San Diego, CA)
	PE	Pharmingen (San Diego, CA)
Il-4	PE	Pharmingen (San Diego, CA)
Il-5	PE	Pharmingen (San Diego, CA)
Il-10	APC	Pharmingen (San Diego, CA)
IFNγ	FITC	Pharmingen (San Diego, CA)
	PE	Pharmingen (San Diego, CA)
Isotype IgG1	FITC	Pharmingen (San Diego, CA)
Isotype IgG1	PE	Pharmingen (San Diego, CA)

2.3.4 Calcium flux

$3x10^6$ CD4+ T cells were resuspended in 500 µl IMDI with 1% FCS and a final concentration of Indo-1 AM (Sigma) of 2 µM. Cells were incubated for 30-40 minutes to load Indo-1 in the dark at 37°C. Thereafter, cells were washed and collected, resuspended in 500 µl 1% FCS IMDM and stained with appropriate antibodies for phenotypic staining and a suitable concentration of anti-CD3 (145-2C11) for 15 minutes in the dark at RT. Hereafter, cells are washed, collected and resuspended in 300 µl 1% FCS IMDM. Cells are kept shielded from light at RT before acquisition. Samples were allowed to achieve a baseline on the LSR Benchtop Flow Cytometer (Becton Dickinson) with time as a parameter on the x-axis and the FL5 (400/40nm)/FL4 (510/20nm) ratio on the y-axis for 1 minute. Hereafter, a calcium flux was induced using 20 µg/ml final concentration of goat anti-Armenian Hamster (Jackson Immuno Research laboratories Inc.) crosslinking antibody, whereafter data were collected for a further 5 minutes. Data analysis was performed using CellQuest (Becton Dickinson), FCS assistant (shareware) and Excel (Microsoft) software.

2.3.5 Tat internalisation

FITC-conjugated fusion proteins were added to the culture medium at desired concentrations for a limited period of time. Cells were collected, and washed twice with ice-cold PBS. Some fractions were treated with 5 μg/ml Trypsin for 15 minutes on ice. Subsequently, cells were washed into ice-cold PBS 0.1% Azide at pH 7.4 or pH 6.8. Cells were acquired on a FACS Calibur, whereby sheet fluid was replaced with PBS 0.1% Azide with appropriate pH.

2.4 Assays for T cell activation

2.4.1 Proliferation and Il-2 production

Splenocytes were cultured for the indicated time in round-bottom 96-well plates (2x105 /well) in the presence of the indicated amounts of specific antigen, or 24-well plates (1x105 /well) with the indicated amounts of plate-bound anti-CD3 (145-2C11) and 5 μg/ml anti-CD28 (37.51). Transfer of 50 μl aliquots of supernatant to fresh flat-bottom 96-well plates together with 5000/well Il-2-dependent CTLL cells assessed Il-2 production.

Incorporation of [^{3}H] thymidine by CTLL was measured 24 hours after transfer of supernatant. Alternatively, the AlamarBlue™ (Biosource) assay was used to measure quantitatively the proliferation of cells based on detection of metabolic activity. The system incorporates an oxidation-reduction indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. Data were collected by monitoring fluorescence at 590nm using a Luminescence Spectrometer LS50B (Perkin Elmer) analyzing data with FL WinLab software (Perkin Elmer).

2.4.2 Cytokine ELISA

IFNγ- and Il-10 production was measured in a sandwich Enzyme Linked Immunosorbent Assay (ELISA) using a pair of antibodies. 50 µl supernatants were transferred to 96-well flat bottom plates (Dynatech) coated o/n with 10 µg/ml AN18 (rat) anti-mouse IFNγ or 5 µg/ml JES52CA5 (Rat) anti-mouse Il-10, and blocked with PBS supplemented with 5% FCS and 5% horse serum and incubated for two hours at RT. Following washes with PBS/0.05% Tween 20 (Sigma) biotinylated R4-6A2 (rat γ1) anti-mouse IFNγ or anti-Il-10 (pharmingen) (1:2000) was applied for 1 hour RT. Wells were extensively washed and bound IFNγ was detected using Streptavidin-Horseradish Peroxidase (HRPO) (1:2000) (Southern Biotechnology) for 1 hour RT, whereafter detection of the bound conjugate Ab was performed using 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma) substrate, activated by the addition of 1 µl/ml 30% H_2O_2. Absorbances were read at 414 nm on a Multiskan EX (Labsystems) analyzing data with Genesis software (Labsystems).

2.5 Engineering of Tat expression vectors

2.5.1 Digestions and ligations

All restriction enzyme (Roche) digestions were performed in the appropriate buffers at 37°C for at least 2 hrs, unless otherwise mentioned. Restriction enzymes were removed prior to further manipulation with use of the Bio 101 Geneclean Spin kit (Anachem Ltd) or Qiagen DNA purification column (Qiagen Ltd), according to the suppliers manual. Before ligation with T4 DNA ligase (Roche), DNA fragments were excised from agarose gels and purified with use of the Geneclean Spin kit. After linearising vectors, the terminal phosphates were removed using Shrimp Alkaline Phosphatase (Roche) by incubating 30 minutes at 37°C. Ligations were performed as described by Sambrook et al. (1989).

Oligonucleotides were annealed following resuspension of both complementary primers at the same molar concentration in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA). The suspension was thereafter brought to 95°C in a heatblock for 2 minutes, whereafter the block was allowed to cool to room temperature on the workbench (45-60 minutes). The resulting mix was stored short term at 4°C until use, or long term at -70°C.

2.5.2 Polymerase Chain Reactions

PCRs were performed with use of 2 units *Taq* polymerase (Perkin Elmer) for checking of oligonucleotides and colony screening, 1 unit *Pfu* Turbo DNA polymerase (Stratagene Ltd) for general cloning purposes, or 2 Units Pfu DNA polymerase (Perkin Elmer) for sequencing, in the appropriate buffer supplemented with 40 mM dNTPs (Roche), 4 µl of each desired primer at optical density (OD) 1, and approximately 20 ng of template DNA or in case of screening colonies, a few bacteria. Generally the following PCR program was followed; 50 seconds 95°C denaturation, 50 seconds 60°C annealing, and depending on the size of the desired product an elongation time at 72°C, repeated for 30 cycles.

2.5.3 Competent cells and transformations

DH5α (dcm$^-$) E. coli bacteria for general cloning purposes were made competent using the TSB method, from 200 ml fresh bacteria grown in Luria Bertani (LB) broth at 37°C to OD_{600} 0.4-0.6. Thereafter, cells were cooled on ice for 10 minutes, collected and resuspended in 20 ml ice-cold TSB (LB supplemented with 10% PEG 3350 (BDH chemicals Ltd), 5% DMSO (Sigma), 20 mM $MgCl_2$, pH 6.5, filter-sterilised), distributed into 500 µl aliquots, and stored at -70°C after snap freezing in liquid nitrogen. Competent DH5α were incubated 20-30 minutes on ice mixed together with DNA, followed by a heat shock of 42°C for 40 seconds; hereafter they were allowed to express the Ampicilin resistance gene for 1 hour at 37°C whereafter they were plated on nutrient agar plates containing 100 µg/ml Ampicilin (Sigma) or 50 µg/ml Carbicillin (Novagen). Alternatively, the Top10 one-shot kit (Invitrogen) was used for low efficiency cloning, according to the suppliers' manual.

Expression of proteins was achieved using BL21 (Novagen) as host, transformed according to the suppliers' manual.

2.5.4 Sequencing

All generated vectors were sequenced using the ABI Prism 373 Genetic Analyzer (Perkin Elmer) according to the manufacturer's directions. Approximately 500 ng of plasmid DNA were used for sequence analysis.

2.5.5 Generation of Tat protein expression vectors

pET-TAT was constructed by inserting two double-stranded oligomeric nucleotides (P1 and P2) encoding the 11-amino acid TAT PTD (YGRKKRRQRRR) into the pET-15b vector (Novagen) under the control of a strong bacteriophage T7 promoter. Subsequent vectors were constructed by inserting a PCR fragment encompassing the desired cDNA flanked by engineered restriction sites and a HA-tag in frame into pET-TAT. In addition a complete pTAT-HA vector was generated for future use, inserting the 9-amino acid HA epitope tag (YPYDVPDYA), P3 and P4 into pET-TAT.

pET-TAT Lck-wt was generated using oligonucleotides P5 and P6, spanning the entire cDNA of Lck, amino acid 1 to 509. pET-Tat Lck NSH2 was generated using oligonucleotides P5 and P7, spanning wild type Lck from the first amino acid to the valine at position 240. pET-Tat Lck SH2 was generated using primers P8 and P7, spanning the Lck wt sequence from amino acid 102 to 240.

Alternatively, two double-stranded oligomeric nucleotides encoding the 11-amino acid Tat-PTD and the C-terminal His-tag, P11, P12, P13 and P14, were inserted into the pET-15b vector. Thereafter, a PCR fragment encompassing the desired region and at the 5' site the HA-tag flanked by engineered restriction sites was inserted, thereby replacing its original N-terminal His-tag. This enabled the expression of desired proteins with a free N-terminus.

Table 3. Oligonucloutides used in this study.

P1	**Tat F**	GGAATTCCATATGTATGGCAGGAAGAAGCGGAGACAGCGACG AAGAGGGGATCCATA
P2	**Tat R**	TATGGATCCCCTCTTCGTCGCTGTCTCCGCTTCTTCCTGCCAT ACATATGGAATTCC
P3	**HA F**	CGCGGATCCTGGTTACCCATACGACGTCCCAGACTACGCTGGA TGA*GAATTC*TGATCACGC
P4	**HA R**	GCGTGATCA*GAATTC*TCATCCAGCGTAGTCTGGGACGTCGTATG GGTAACCAGGATCCGCG
P5	**Lck 1-24 F**	ATTTGATCATATGGGCTGTGTCTGCAGCTCAAAC
P6	**Lck 1527-1506 R + HA**	ATTTGATCAAGCGTAGTCTGGGACGTCGTATGGGTAAGGCTGG GGCTGGTACTGGCCC
P7	**Lck 720-698 R + HA**	ATTTGATCAAGCGTAGTCTGGGACGTCGTATGGGTAAACTTCC CATTCGTCCTCCCACC
P8	**Lck 306-327 F**	ATTTGATCACCTGACGACTGGCCAAGAAGGC
P9	**ZAP70 1-21 F**	CGCGGATCCTATGCCAGACCCCGCGGCGCAC
P10	**ZAP70 792-769 R + HA**	CGCGGATCCAGCGTAGTCTGGGACGTCGTATGGGTACCTGAGG CGTTGCTGGCACTG
P11	**Tat F**	GGAATTCCATATGGGATATGGCAGGAAGAAGCGGAGACAGCG ACGAAGAGGAGGTCTCGAGCAAG
P12	**Tat R**	CTTGCTCGAGACCTCCTCTTCGTCGCTGTCTCCGCTTCTTCCTG CCATATCCCATATGGAATTCC
P13	**Tb His F**	CTTGCTCGAGGGCCTGGTGCCGCGCGGCAGCAGCGGACATCATC ATCATCATCACAGCAGCGGCTAGGATCCCGA
P14	**Tb His R**	TCGGGATCCTAGCCGCTGCTGTGATGATGATGATGATGTCCGCT GCTGCCGCGCGGCACCAGGCCCTCGAGCAAG

2.6 Biochemistry

2.6.1 Peptide synthesis

Tat-PTD (CYGRKKRRQRRRGGYPYDVPDYA) and Tat-PTD-Z70I (MAYGRKKRRQRRRGGKLILFLLLLAKKK) were prepared with an automated peptide synthesizer (ABI431A) by using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry with HBTU as the peptide-coupling reagent. Cleavage from the resin was achieved by using 90% trifluoroacetic acid (TFA) / 5% thioanisole / 2.5% H_2O / 2.5% ethane-dithiol for 1.5 hrs. The reaction mixture was paper-filtered whereafter the peptide was precipitated and washed with methyl-tertiary-butyl-ether. The ether was removed by rotary evaporation, and the resulting solid was solved in H_2O, freeze-dried o/n, and purified by RP-HPLC (H_2O/CH_3CN in 0.1% TFA). The products were recovered by lyophilisation and characterised by electrospray mass spectrometry.

2.6.2 Tat fusion protein expression and localisation

A single colony from an agar plate was used to inoculate 10 ml LB medium containing appropriate antibiotic and grown o/n. 10 µl of culture was added to 10 ml fresh LB medium. Expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) (0.5 – 1.0 mM final concentration) when the optical density (OD_{600}) reached approximately 0.5. Samples of 1.5 ml were taken after 2, 5, 8 hours and o/n (15-18 hours) incubation. The pellet was taken up in 200 µl lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 1 mM EDTA) and cells were disrupted by sonication using an Ultrasonic sonicator at 60% duty cycle for 2-3 times 20 sec. Lysates were cleared from cell debris, and supernatants were analyzed as the cytosolic fraction; the pellet suspension was used as the insoluble fraction and resuspended in an equal volume of 8M Urea buffer (8M Urea, 50 mM Tris pH 7.5; 300 mM NaCl, 1 mM EDTA).

2.6.3 Tat fusion protein expression and purification

3 l Terrific Broth (TB) containing 50μg/ml Carbenicillin (Novagen) was inoculated with a suspension of high expressing BL21 (Novagen) cells picked up from three overnight-incubated thinly colonised agar plates containing 50 μg/ml Carbenicillin. Tat fusion proteins were purified by sonication of 1 g of wet weight bacterial pellet per 10 ml 5.8 M Guanidine HCl lysis buffer (5.8 M Guanidine HCl (Fluka Biochemica), 50 mM Tris pH 8.0 (BDH laboratory supplies), 200 mM NaCl (BDH laboratory supplies), 2 mM $MgCl_2$ (BDH laboratory supplies), 0.1% Triton X-100 (Sigma), 5 mM β-mercaptoethanol (Sigma), 5 mM Imidazole (Fluka Biochemica), 200 mM PMSF (Sigma)). The resulting suspension was cleared by centifugation at 10,000 g for 20 minutes. The supernatant was applied on a Nickel-NitriloTriacetic Acid (Ni-NTA) column (Qiagen), and allowed to flow through by gravity. The column was washed once with 2 column volumes of lysis buffer, thereafter with 2 column volumes of washing buffer 1 (5M Guanidine HCl, 50 mM Tris pH 8.0, 500 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 15 mM Imidazole) and 2 column volumes of washing buffer 2 (5M Guanidine HCl, 50 mM Tris pH 7.5, 500 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 30 mM Imidazole). Protein bound to the Ni-NTA column was eluted using with 2x1 column volume of Elution buffer (4M Guanidine HCl, 50 mM Tris pH 7.5, 500 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 500 mM Imidazole, 100 mM PMSF). All fractions collected were routinely checked for their protein content by Sodium Dodisyl Sulphate- Protein acrylamide Gel Electrophoresus (SDS-PAGE).

2.6.4 Tat fusion protein slow desalting

Collected protein eluate from the Ni-NTA column was brought to a concentration of 2 mg/ml by the addition of elution buffer. Oxidation of the protein was allowed by incubating the eluate for three days at room temperature under slow stirring in an open bottle. The solution was concentrated using a stirred-cell concentrator (Amicon) with a 10 kD cut-off PES membrane (Millipore) to a volume of 25-40 ml. Thereafter it was slowly dripped over 18 hours into 1 l vigorously stirring Solubilisation buffer 1 (2 M

Urea (Sigma), 1 M NaCl, 50 mM Glycine (BDH laboratory supplies, UK), 20 mM HEPES pH 7.5 (Sigma), 5 mM EDTA (Sigma), 20 % Glycerol (BDH laboratory supplies), 100 mM PMSF, 2 mg/ml PepstatinA (Sigma) and 2 mg/ml Leupeptin (Sigma)) at 4°C. The resulting solution was incubated slowly stirring for three days at 4°C. More protein inhibitors were added (100 mM PMSF, 2 mg/ml PepstatinA and 2 mg/ml Leupeptin), the solution was filtered over a 45 µm PES membrane bottle-top filter (Nalgene) and concentrated to 50-60 ml using a stirred-cell concentrator with a 10 kD cut-off PES membrane. This was slowly dripped over 18 hours into 1 l vigorously stirring Solubilisation buffer 2 (1 M NaCl, 50 mM Glycine, 20 mM HEPES pH 7.5, 5 mM EDTA, 20 % Glycerol, 100 mM PMSF, 2 mg/ml PepstatinA and 2 mg/ml Leupeptin) at 4°C and subsequently incubated for three days under slow stirring. More protein inhibitors were added (100 mM PMSF, 2 mg/ml PepstatinA and 2 mg/ml Leupeptin), the solution was filtered over a 45 µm PES membrane bottle-top filter and concentrated to 40 ml using a stirred-cell concentrator with a 10 kD cut-off PES membrane. The resulting solution was dialysed over two days against PBS at 4°C using Slide-A-lyser cassettes (Pierce), whereafter the protein content was determined using the Lowry method, and subsequently the protein solution was brought to the desired concentration using a stirred-cell concentrator with a 10 kD cut-off PES membrane. Protein content was checked by SDS-PAGE and Coomassie blue staining after filter sterilisation using 0.2 µm syringe filters (Sartorius AG, Germany). Obtained solutions were thereafter stored at 4°C.

2.6.5 Tat fusion protein rapid desalting

Tat fusion proteins were purified by sonication of 1 g of wet weight bacteria pellet per 10 ml 8 M Urea lysis buffer (8 M Urea, 50 mM Hepes pH8.0, 100 mM NaCl, 10% Glycerol, 5 mM Imidazole). The resulting suspension was cleared by centrifugation at 10.000 g for 20 minutes. The supernatant was applied on a Ni-NTA column (Qiagen), and allowed to flow through by gravity. The column was washed once with 2 column volumes of lysis buffer, thereafter with 2 column volumes of washing buffer (8M Urea, 50 mM Hepes pH 8.0, 100 mM NaCl, 10% Glycerol, 25 mM Imidazole). Protein bound to the Ni-NTA column was eluted using with 2x 1 column volume of Elution buffer (8M Urea, 50 mM

Hepes pH 8.0, 100 mM NaCl, 10% Glycerol, 500 mM Imidazole). 15 ml of the eluted fraction was mixed with 5 ml of Source 30Q or Source 30S (Amersham Biosciences) in a total volume of 15 ml Dilution buffer (20 mM Hepes, 10% Glycerol, pH and NaCl concentration is protein dependent, see Figure 15C), and incubated for 30-60 min on a rotator. The Source material was collected by centrifugation, washed with Buffer A (20 mM Hepes, 10% Glycerol, pH and NaCl concentration is protein dependent, see Figure 15C), and protein was eluted with 10-15 ml of Buffer B (20 mM Hepes, 10% Glycerol, 1 tablet of protease inhibitor (Roche), pH and NaCl concentration is tailor made for each fusion protein, see Figure 15C). The eluted fraction was subsequently slowly diluted in PBS + 10% Glycerol, dialysed against PBS + 10% Glycerol and filter sterilised. All fractions collected were routinely checked for their protein content by SDS-PAGE.

2.6.6 Western Blot Analysis

For checking the expression of desired fusion proteins, a fresh colony was taken from an o/n grown plate and cultured in 10 ml LB medium to OD_{600}=0.5; expression was induced by 0.5 mM IPTG for 3-6 hours at 30°C. Thereafter the equivalent of 1 ml OD_{600}=1 was lysed in 100 µl lysis buffer (8 M Urea, 50 mM Hepes pH 8.0, 100 mM NaCl) and 10-20 µl sample were analysed by SDS-PAGE and transferred to nitrocellulose (Immubilon-P, Millipore) or stained with Coomassie-blue.

Blots were blocked with 5% Albumin in PBS-Tween, and stained with Abs in 0.5% Albumin in PBS-Tween. The anti-bodies used for Western blots were: 12CA5 (1:2000) anti HA mAb (Roche), anti His_6 mAb (1:1000) (Santa Cruz), ant-P-Tyr (4G10) (1:12,500), ProteinA-HRPO (Amersham Life Sciences) (1:5000).

Blots were developed using Enhanced Chemo Luminescence (ECL) (PerkinElmer Life Sciences) and exposed for the desired time to X-ray film (Kodak).

Blots were stripped of bound Abs by washing with an 100 mM Glycine pH3.0 solution for 30 min, where after blots were tested for residue Ab by ECL.

2.6.7 Biochemical Tat binding assay

To measure binding and endocytosis of Tat, $5x10^6$ cells were incubated in 2 ml RPMI with indicated Tat concentrations for varying lengths of time. Cells were washed three times with ice-cold PBS and trypsinised for 15 min on ice where indicated. After treatment, the cells were washed in cold PBS and harvested in lysis buffer at 10^6 cells/ 40 µl (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, and10 mM sodium pyrophosphate).

2.6.8 FITC conjugation

Fusion proteins were dialysed into 0.25 M carbonate buffer at alkaline pH of 9.3. FITC isomer (Molecular Probes), dissolved at 10 mg/ml in DMSO was added to give 100 µg FITC/mg protein, followed by an o/n incubation, rotating at 4°C. Subsequently, conjugated protein was dialysed against multiple changes of PBS. Conjugated protein was aliquoted and stored at 4°C in 0.01% Azide.

2.6.9 Protein A antibody coupling

200 ml packed protein A beads (Pharmacia Biotech) in a 1.5 ml Eppendorf tube were washed in PBS-A to wash out the ethanol. Subsequently, beads were equilibrated to 0.2 M Boric Acid pH 9.0. Antibodies and protein A beads were mixed (5-10 µg Antibody per 10 µl packed beads) in an Eppendorf tube for 1 hour at RT, extra Boric Acid was added to make the volume 1 ml. Beads were washed 4 times with 10 volumes 3M NaCl, 50 mM Boric Acid pH 9.0 by centrifugation and aspiration. Beads were resuspended in 1.5 ml of 3 M NaCl, 50 mM Boric Acid pH 9.0 for IgG1 or 0.2 M Boric Acid pH 9.0 for IgG2 plus 20 mM Dimethylpimelimidate (Sigma). Beads and antibodies were mixed on a rotator for a further 30 min, thereafter the reaction was stopped by washing the beads once in 0.2 M ethanolamine pH 8.0 at RT for 2 h while gently mixing. Beads were washed once with PBS-A, followed by a 100 mM Glycine pH 3.0 wash to remove all non-covalently bound

antibodies. This was followed by two washing steps in PBS-A and a final wash into PBS 0.01% Azide.

2.6.10 Immune precipitations

10^8 Jurkat T cells were used for each fraction. Cells were grown for the desired time in medium containing the desired concentration of Tat-PTD fusion protein. Thereafter cells were collected and resuspended in 1 ml medium. 180 µl 100 mM sodium ortho-pervanadate (Sigma) were activated 15 minutes prior to use with 20 µl 1 M H_2O_2, giving a 100-times stock solution. 10 µl were added to the 1 ml Jurkat cells for 15 min at 37°C. Subsequently, cells were collected and lysed in 1 ml 1% Triton lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 20 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium ortho-pervanadate and per 50 ml a tablet of protease inhibitors (Roche)). Cells were lysed during 1 h rotating at 4°C. DNA and cell debris were spun out by 15 min centrifugation 14,000 rpm at 4°C. Supernatant was transferred and pre-cleared with protein A beads for 30 min at 4°C, thereafter beads were removed, and protein A beads coupled to antibody (anti-HA, 12CA5 (Roche); or 33.1 anti-ZAP-70 mAb (kindly provided by J. Tite, Glaxo Smith Kline) were added for an o/n incubation rotating at 4°C. Subsequently, supernatants were removed, and protein A beads were washed 5 times with 1 % Triton X-100 lysis buffer. After the last wash, 100 µl of loading buffer were added, and samples were stored at -20°C until boiling for 3 min at 95°C and loading of 50 µl on a SDS-PAGE maxi gel.

"The most beautiful experience we can have is the mysterious" (Albert Einstein)

3 Results

The major limitation for introducing proteins and peptides into live cells is their poor uptake by cells due to the low efficiency with which they cross the lipid bilayer of the plasma membrane or of endocytic vesicles. The delivery of these compounds therefore depends on physical disruption of the membranes in order to allow entry to the cytosol, as achieved during microinjection and electroporation. Alternatively, delivery might be accomplished using a vehicle that facilitates the crossing of lipid membranes without causing major disruption, such as cationic lipids and liposomes. At the start of this study, the potential of one such vehicle, the B subunit of *E.coli* enterotoxin (Etx) was considered.

In order to introduce potential signal attenuators into naive *ex vivo* T cells, the most appropriate and effective delivery vehicle had to be found. Any effect of the delivery vehicle itself can potentially disturb the balance of the signalling pathways in resting T cells, and may influence the eventual differentiation and the effector functions once the naive T cells are activated in its presence. Therefore, initial studies started with testing EtxB and its influence on naive T cells.

3.1 The use of EtxB as a delivery vehicle

A number of proteins from plant and bacteria are highly toxic to mammalian cells because of their ability to enter the cytosol and attack essential constituents. The majority of these toxins are referred to as AB-toxins. The A moiety generally has enzymatic activity and modifies a cellular target upon entry into the cytosol. The B moiety, consisting of one or more subunits, serves as a vehicle to bind the toxin to the cell-surface via a specific receptor and thereafter translocates the A moiety to the cytosol, in most cases by receptor-mediated endocytosis.

The B subunit of *E.coli* enterotoxin (Etx) and cholera toxin (Ctx) are internalised via the cell surface ganglioside G_{M-1} that is ubiquitously expressed on all cells (Lencer et al., 1992). Both toxins are structurally and functionally almost

identical. Indeed, the two toxins share 80% amino acid identity. As members of the AB toxins, and more precisely the AB5 toxins, the A-subunit is responsible for the toxic effect while the B-subunit is regarded as a non-toxic carrier needed for delivery of the A-subunit into target cells. In case of Etx and Ctx, the B-subunit carrier is a doughnut-shaped pentameric ring formed by five B-subunits. In the holotoxin, the A-subunit is located on one side of the plane of the B pentamer opposite the side that binds the receptor (Figure 4). Previous studies have established that the B-subunit pentamer can be used to deliver peptides attached by either chemical or genetic conjugation into the cytosol and nucleus of eukaryotic cells (Loregian et al., 1999). As such this protein has the unique capacity to deliver substances into resting primary cells that are otherwise very resistant to transfection.

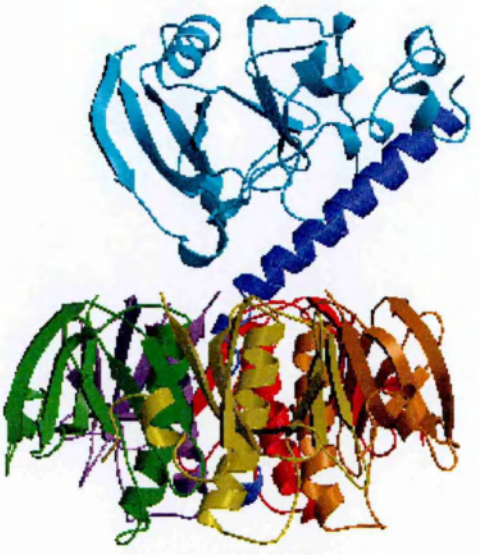


Figure 4. Ribbon crystal structure of *Escherichia coli* enterotoxin I. The top part of the toxin consists of the bioactive A subunit, shown in the shades of blue. The A subunit is raised above a platform made up by five B subunits, and is generally considered as the non-toxic carrier. It delivers the A subunit to the cells by binding G_{M-1} and allowing its subsequent internalisation and delivery into the cell cytoplasm (Sixma et al., 1993).

3.1.1 Binding of CtxB to naive T cells

The first criterion for the delivery vehicle is its capacity to bind to naive murine T cells. The B subunits of EtxB and CtxB are internalised via the cell surface ganglioside G_{M-1} that is reported to be ubiquitously expressed on all cell types (Lencer et al., 1992). To confirm this, FITC-conjugated CtxB, 80% homologous to EtxB, was used to determine the capacity of the B-subunit pentamer to bind to *ex vivo* murine T cells (Figure 5).

Figure 5.

A single cell suspension of A/J strain splenocytes was incubated for 20 minutes with 1 μg/ml FITC-conjugated or non-conjugated CtxB, and stained with PE-conjugated anti-CD4 or anti-CD8 followed by FACS analysis. Splenocytes incubated with antiCD4 and **(A)** non-conjugated CtxB, or **(B)** FITC-conjugated CtxB. Splenocytes incubated with anti-CD8 and **(C)** non-conjugated CtxB, or **(D)** FITC-conjugated CtxB.

Alternatively, a single cell suspension of A/J strain splenocytes was incubated for 20 minutes with indicated amounts of FITC-conjugated CtxB, and stained with PE-conjugated anti-CD4 (●) or anti-CD8 (□) **(E)**.

Binding of CtxB to CD4+ and CD8+ polyclonal T cells.

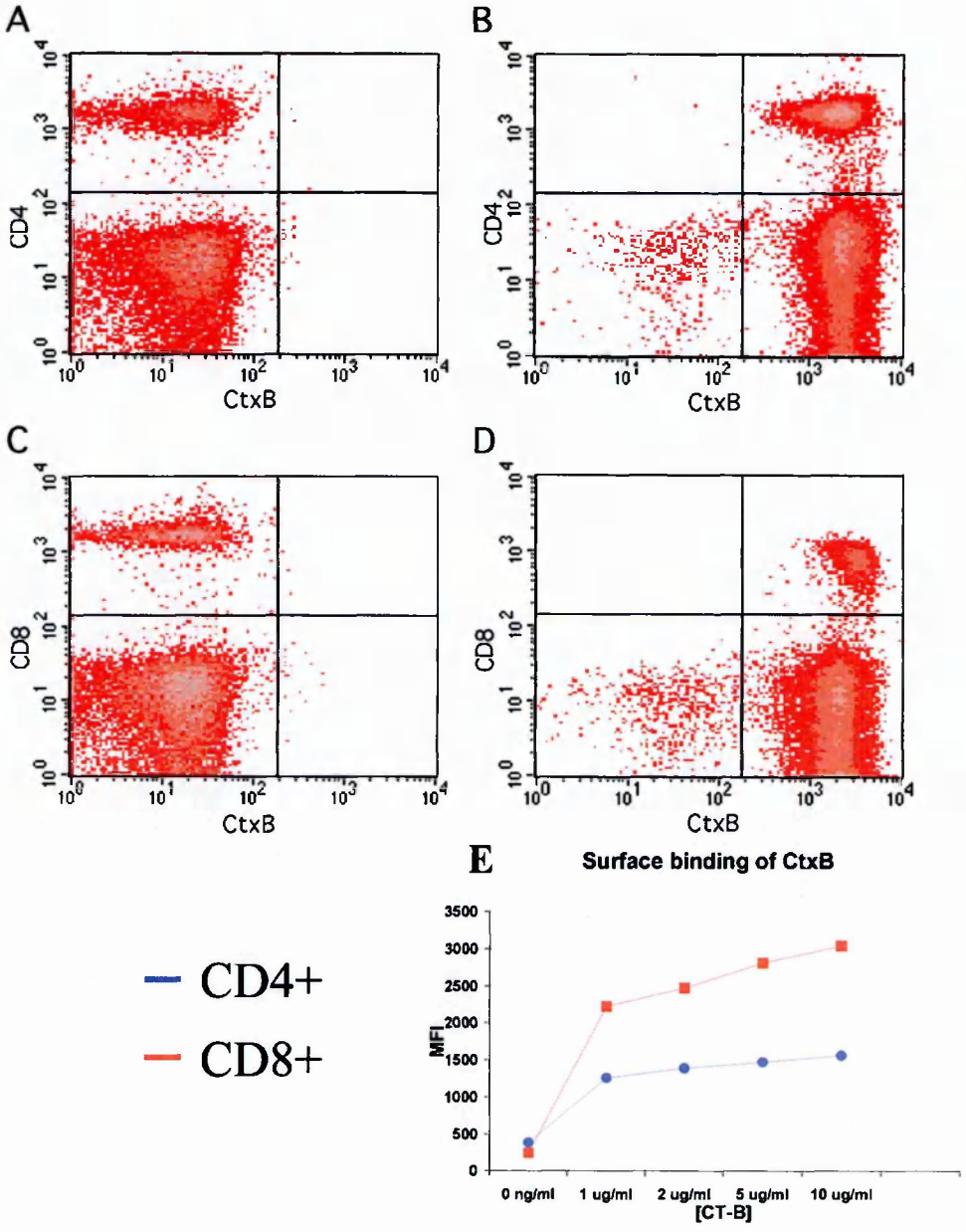


Figure 5 shows the ability of virtually all cells, and 100% of both subsets of murine T cells, CD4+ as well as CD8+, to bind CtxB molecules on the surface. In addition, comparing Figure 5B and 5D, CD8+ T cells seemed capable of binding more CtxB than CD4+ T cells. This difference in binding capacity was studied in more detail, and Figure 5E shows that murine CD8+ T cells have the capacity of binding twice as much CtxB than murine CD4+ T cells. The capacity to bind naive T cells of both subsets meets the first criterion to use EtxB as a potential delivery vehicle for TCR signalling attenuators

3.1.2 Effect of EtxB on naive T cells

A subsequent criterion is that CtxB/EtxB should not have an effect on cell physiology by itself, so that the effect of the delivered molecules alone or in conjunction with a T cell receptor-mediated signal can be assessed. The enterotoxin B subunit has been shown to signal calcium release and Il-2 production in Jurkat T cells, but this was dependent on crosslinking surface-bound EtxB with anti-B-subunit antibodies and has not been reported in the absence of crosslinking (Gouy et al., 1994). The effect of enterotoxin B subunit on naive polyclonal and transgenic T cells had not been studied in detail yet and this was the focus of the next experiments.

Different concentrations of CtxB varying from 25 ng/ml to 25 μg/ml were added to *ex vivo* splenic T cells in culture for up to three days. The influence of CtxB was studied at the level of proliferation, by tritiated thymidine incorporation, apoptosis (7AAD), cytokine production (IL-2 and IFNγ) and the expression of the surface markers CD4, CD8, CD69, CD62L, CD25, CD44, CD45RB, CD95 and the TCR, by FACS analysis.

Figure 6.

Splenic single cell cultures of the polyclonal B10 strain were incubated with indicated amounts of CtxB for up to 3 days, and stained with PE-conjugated anti-CD4 **(A, C, E)** or anti-CD8. **(B, D, F)**. Shown are the expression levels of CD69 **(A, B)**, CD25 **(C, D)**, and CD44 **(E, F)** as determined by FACS analysis on live T cells. For comparison, cells were stimulated with 10 µg/ml anti-CD3 (mAb 145-2C11) as positive control. The figure shows mean percentages ± SD of positive cells from two individual experiments.

Influence of CtxB on surface marker expression of polyclonal murine CD4+ and CD8+ T cells.

CD4+ CD8+

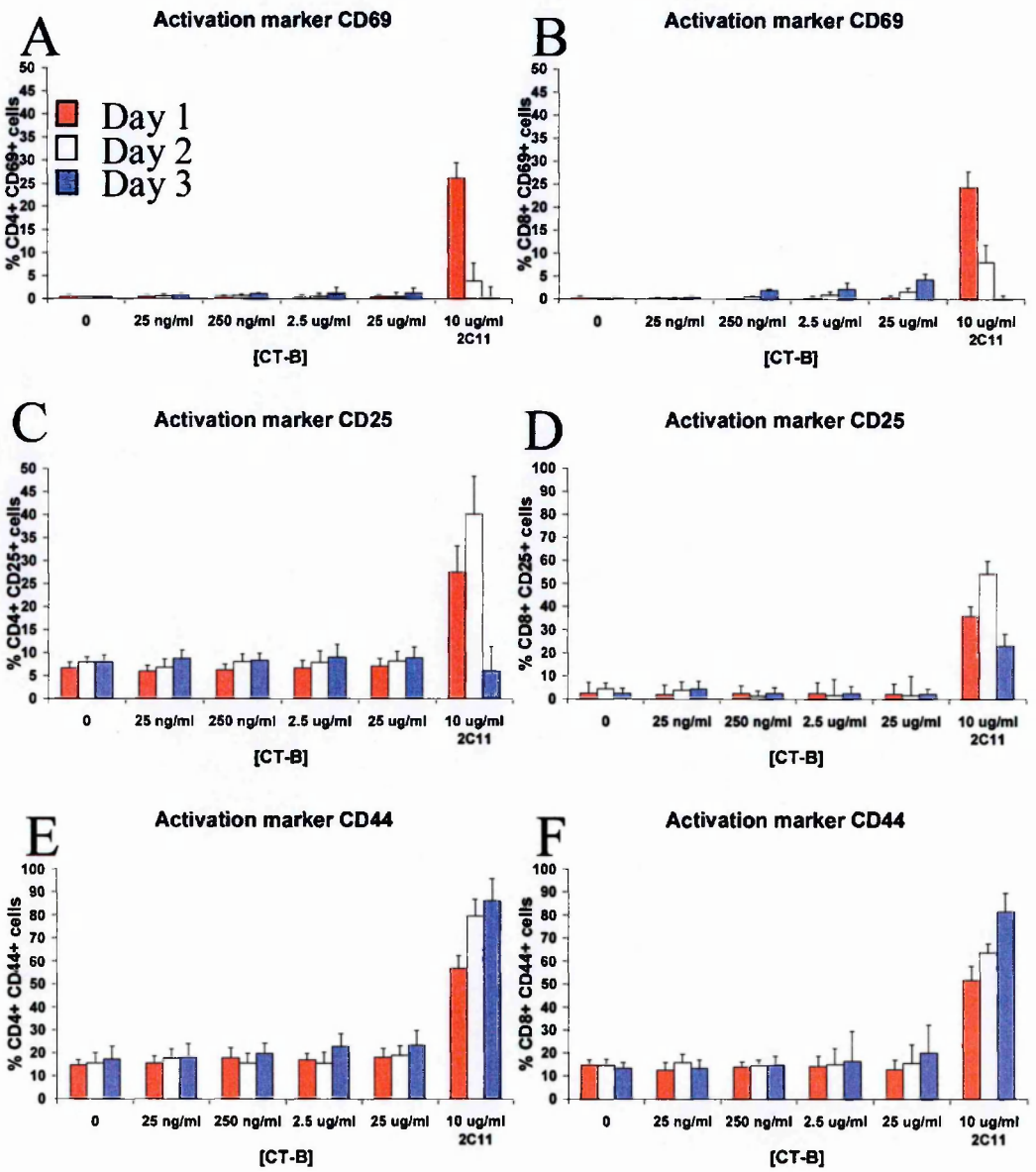


Figure 6 shows results obtained using the polyclonal mouse strain B10, and shows three important activation markers, the early marker for T cell activation CD69, the intermediate marker CD25 and the late activation marker CD44. Similar results were obtained using the polyclonal mouse strain A/J, the MHC class II-restricted, Rag-negative TCR-transgenic strains A18 (Zal et al., 1994), A1 (Zelenika et al., 1998), and AND (Kaye et al., 1989), and the MHC class I-restricted, Rag-negative TCR-transgenic mice strains F5 (Mamalaki et al., 1993), BM3 (Sponaas et al., 1994) and OT-1 (Hogquist et al., 1994). No significant differences in cell surface marker expression could be observed, as shown in Figure 6, for CD25 and CD44. In addition no significant differences were found for the tested markers CD4, CD8, CD45RB and the TCR.

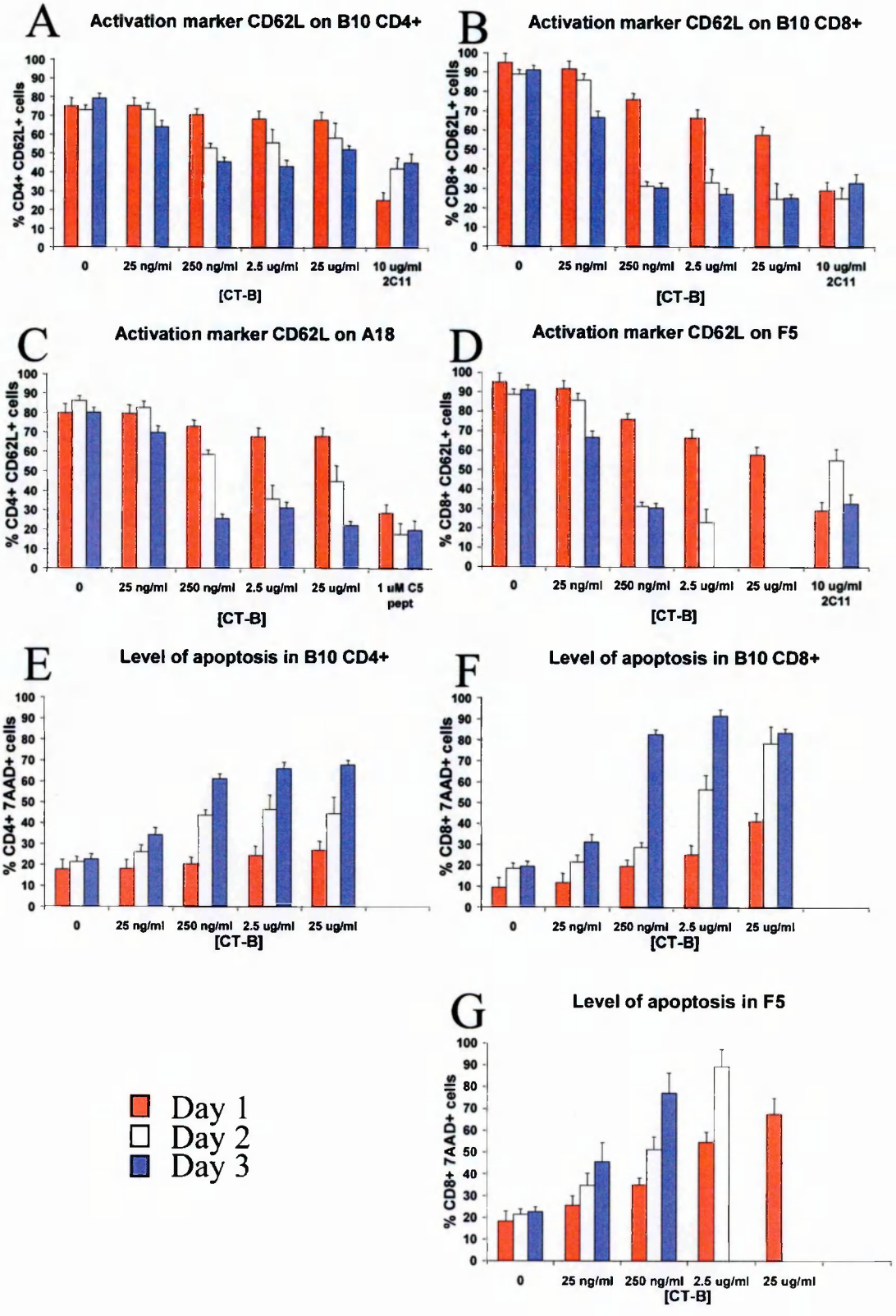
In contrast, CD69 levels do seem to increase slightly in a dose-dependent way over time in the CD8+ T cell subset (Figure 6B). However, the results obtained on the time points after day 1 are greatly biased in this subset due to the low cell numbers left for analysis. In addition to changes in CD69, the expression level of the L-selectin, CD62L, declined in a dose-dependent manner over time during culture with CtxB (Figure 7A-D). This was observed in both T cell subtypes, but more prominently in the CD8+ subset. The differences observed between both T cell subsets probably reflect the difference in binding capacity of CtxB (Figure 5). When comparing polyclonal T cell populations with monoclonal TCR-transgenic T cells, it was further noticed that the effects of CtxB were more evident on the latter (Figure 7).

The most significant influence of CtxB on *ex vivo* T cells in this study was its effect on their survival (Figure 7E-G). The CD8+ subset of T cells and, to a somewhat lesser extent the CD4+ subset, show high susceptibility to apoptosis in the presence of CtxB. Culture with 2.5 μg/ml CtxB results in almost 100% cell death within 2 days for the TCR-transgenic mouse strains F5 (Figure 7H) and OT-1, and within 3 days for the CD8+ subset in the polyclonal mouse strains B10 (Figure 7F) and A/J.

Figure 7.

Splenic single cell cultures of the polyclonal B10 strain **(A, B, E, F)** the MHC class II-restricted Rag-negative TCR-transgenic strain A18 **(C)**, or the MHC class I-restricted, Rag-negative TCR-transgenic strain F5 **(D, G)** were incubated with indicated amounts of CtxB for up to 3 days, and stained with PE-conjugated anti-CD4 **(A, C, E)** or anti-CD8. **(B, D, F, G)**. Shown are the expression levels of CD62L **(A, B, C, D)** and uptake of 7AAD **(E, F, G)** as determined by FACS analysis. For comparison, cells were stimulated with 10 μg/ml anti-CD3 (mAb 145-2C11) as positive control. The figure shows mean percentages ± SD of positive cells from two individual experiments. Panels **D** and **G** have data missing due to high levels of cell death.

Influence of CtxB on CD62L expression and apoptosis



In this study, addition of a crosslinking antibody against surface-bound CtxB on *ex vivo* T cells, resulted in an enhancement of the observed effects on cell death, shedding of CD62L and upregulation of CD69; however, the high amount of T cell death prevented accurate analysis of these results.

The initial aim of this study was to generate a peptide delivery system. This delivery system needs to fulfil two important criteria; it should be capable of binding to naive *ex-vivo* T cells and subsequently facilitate the entry of fused proteins in sufficient quantity, and the vehicle itself should be inert to allow analysis of the transferred signalling attenuators. This study shows that EtxB/CtxB is capable of binding *ex vivo* T cells; however, the results show that it is not an inert delivery vehicle, and therefore not suitable for the intended aims of this study.

3.2 Determining the influence of HIV-1 Tat-PTD on *ex vivo* T cells

Several other proteins capable of intracellular delivery have been described and offer an alternative to receptor-mediated endocytosis. The most powerful seems to be the HIV-1 Tat transactivator protein, which is efficiently taken up by cells. The domain responsible for this translocation has been ascribed to an 11 amino acid region and has already successfully been used to transfect cells with wild-type full-length peptides that stayed biologically active after translocation across the membrane.

The biological effects of the 11 amino acid Tat-PTD peptide, YGRKKRRQRRR, on activation markers, cytokine profile, survival and proliferation of transgenic and polyclonal T cells was, as in the case of EtxB, the focus of initial experiments. Several reports showed the inhibitory effect of the full-length Tat protein on T lymphocyte proliferation and gene transcription (Ensoli et al., 1993; Helland et al., 1991; Holloway et al., 2000; Viscidi et al., 1989), describing its possible role in enhancing viral infection and replication. However, these effects require several domains of the full-length protein, and the PTD itself has not been reported to have any of these effects on its own.

To test this on murine T lymphocytes, a peptide was synthetically generated containing the Tat-PTD, a glycine bridge and an HA-tag, CYGRKKRRQRRR-GGYPYDVPDYA. Primary T cells from monoclonal TCR-transgenic mouse strains; from both the CD4+ (A18, AND and A1) and the CD8+ (F5, BM3 and OT-1) subsets as well as polyclonal T cells (A/J, B10), were cultured in the presence of this peptide for up to three days with concentrations varying from 0.5 μM to 10 μM.

Figure 8.

Splenic single cell cultures of the Rag-negative TCR-transgenic strains AND **(A, C, E)** and F5 **(B, D, F)** were incubated with indicated amounts of Tat-PTD peptide for up to 3 days, and stained with PE-conjugated anti-CD4 **(A, C, E)** or anti-CD8. **(B, D, F)**. Shown are the expression levels of CD69 **(A, B)**, CD25 **(C, D)**, and CD44 **(E, F)** as determined by FACS analysis on live T cells. For comparison, AND and F5 T cells were stimulated with their respective antigen, 1 μM MCC or 10 nM NP68, as positive controls. The figure shows mean percentages ± SD of positive cells from three individual experiments.

Influence of Tat-PTD on surface marker expression of TCR-transgenic murine CD4+ and CD8+ T cells.

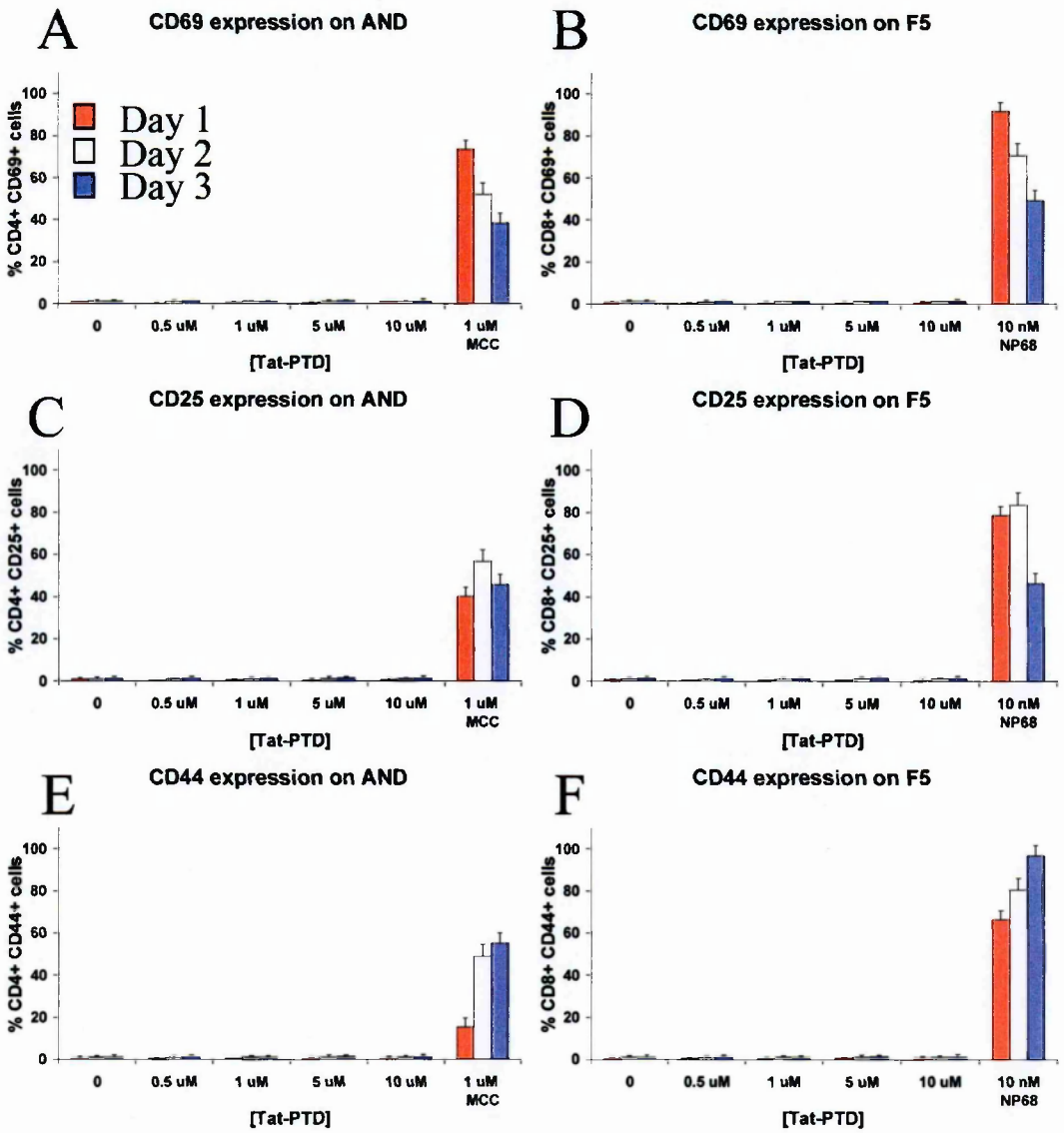


Figure 8 shows a summary of several experiments in which the influence of the Tat-PTD peptide was assessed on monoclonal primary T cell populations. AND and F5 strains are shown as representatives of the CD4+ and CD8+ subpopulations respectively. Depicted in Figure 8 are the early marker for T cell activation CD69, the intermediate marker CD25 and the late marker CD44. These and other surface markers tested, including CD4, CD8, TCR, CD45RB, CD62L and CD95, did not show any alteration in their expression pattern compared with the medium control.

The results show that the Tat-PTD peptide has no effect on cell surface marker expression in *ex vivo* naive T cells when compared with medium controls. In addition, levels of apoptosis were studied in greater detail. *Ex vivo* naive T cells were cultured in the presence of the Tat-PTD peptide, in concentrations up to 10 μM and followed over a period of 3 days. These tests were conducted on the polyclonal strain B10, and the TCR-transgenic strains AND and F5. Figure 9 shows that no difference could be observed between medium controls and Tat-PTD-treated cells with regard to apoptosis levels.

The aim of this study is to use the Tat-PTD peptide as a delivery vehicle for TCR signalling attenuators, to ascertain if manipulation of the signalling pathways downstream of the TCR/CD28 receptor can result in distinct functional differentiation of T cell populations after a stimulus. In order to assess the effect of the delivered molecules on their own, the Tat-PTD peptide should not interfere with a TCR-mediated signal. The next experiments therefore focussed on priming naive T cells in the presence of the Tat-PTD peptide in order to study this.

Influence of Tat-PTD on apoptosis levels in murine CD4+ and CD8+ T cells

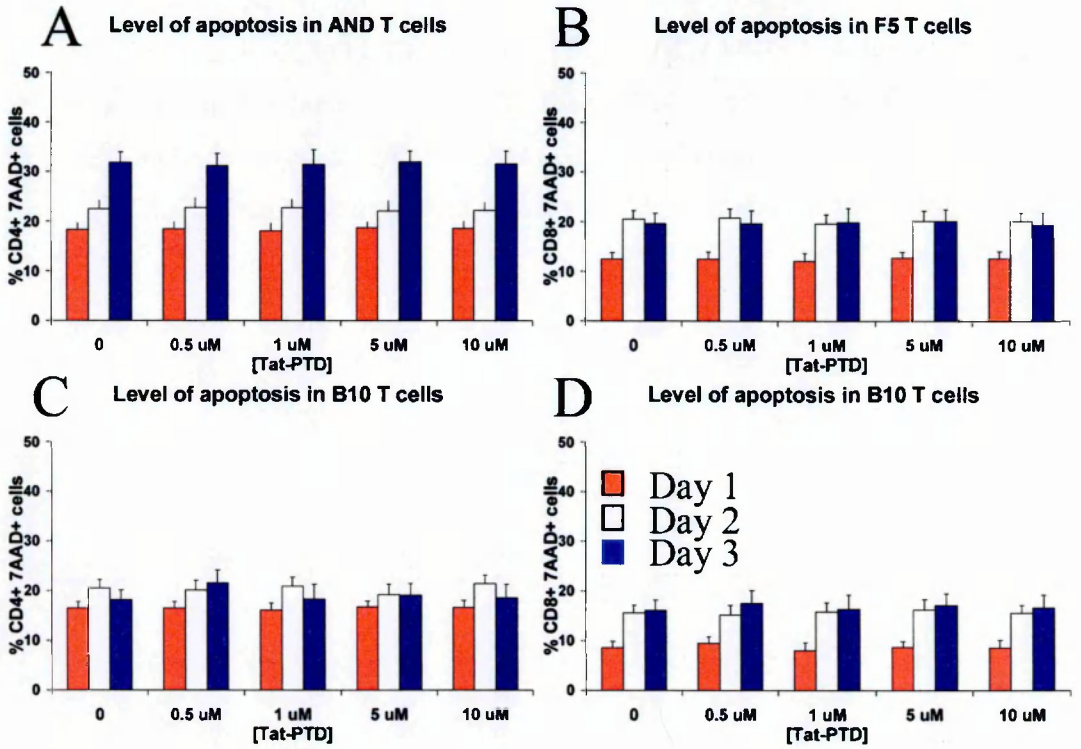


Figure 9. Splenic single cell cultures of the Rag-negative TCR-transgenic strains AND **(A)** and F5 **(B)** and the polyclonal strain B10 **(C, D)** were incubated with indicated concentrations Tat-PTD peptide for up to 3 days, and stained with PE-conjugated anti-CD4 **(A, C)** or anti-CD8. **(B, D)**. Shown is the uptake of 7AAD, as determined by FACS analysis. The figure shows mean percentages ± SD of positive cells from three individual experiments.

Figure 10.

Splenic single cell cultures of the Ragneg TCR-transgenic strains AND **(A, C, E)** and F5 **(B, D, F)** were incubated with 10 μM Tat-PTD peptide for up to 3 days, and stained with PE-conjugated anti-CD4 **(A, C, E)** or anti-CD8. **(B, D, F)**. Shown are the expression levels of CD69 **(A, B)**, CD25 **(C, D)**, and CD44 **(E, F)** as determined by FACS analysis on live T cells. AND and F5 T cells were stimulated on day 0 with indicated concentrations of their respective antigenic peptide, MCC or NP68. The figure shows mean percentages ± SD of positive cells from three individual experiments.

Influence of the presence of Tat-PTD on surface marker expression of TCR-transgenic murine CD4+ and CD8+ T cells during priming.

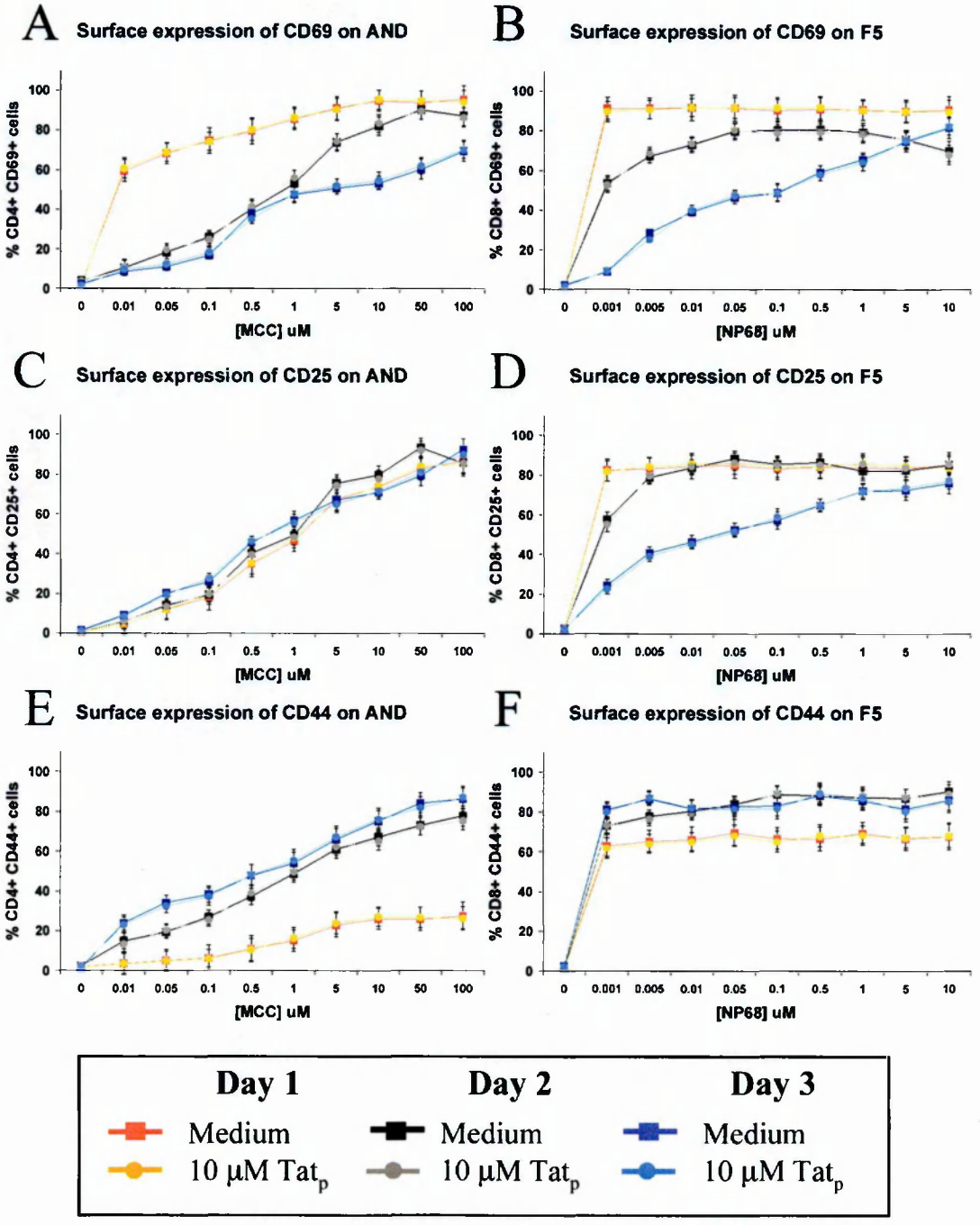


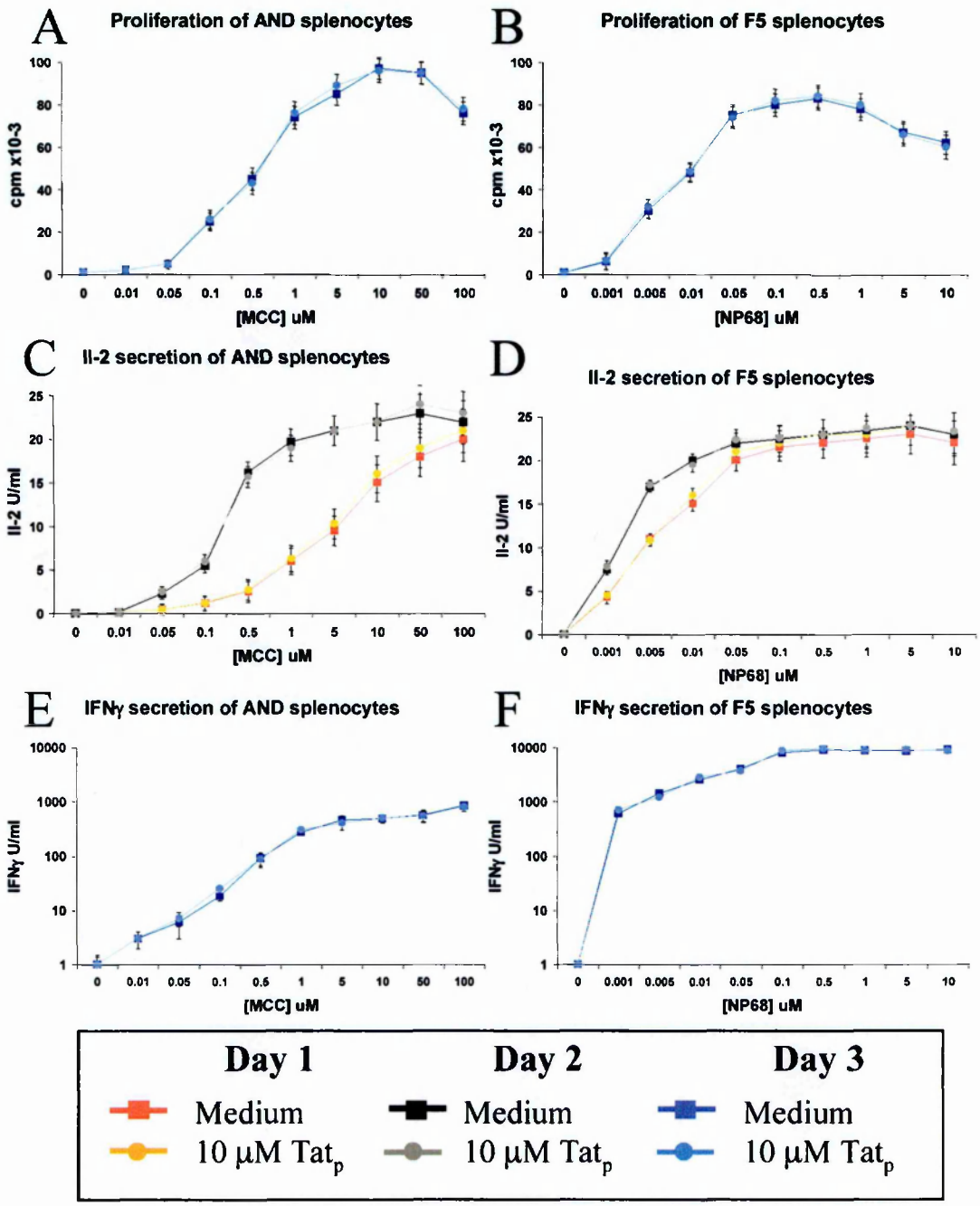
Figure 10 represents a summary of several experiments in which the influence of the Tat-PTD peptide on T cell activation was assessed on monoclonal primary T cell populations. The AND and the F5 strains are shown as representatives of the CD4+ and CD8+ subpopulations respectively. Similar to Figure 8, depicted are the early marker for T cell activation CD69, the intermediate marker CD25 and the late marker CD44. These and the other surface markers tested, including CD4, CD8, TCR, CD45RB, CD62L and CD95, did not show any alteration in their normal expression pattern when cells were stimulated with their specific antigenic peptide in the presence of up to 10 μM Tat-PTD, compared with medium controls. This applies to the proportion of cells changing their expression level, the total level of surface molecules and the pattern of expression over time.

In addition to activation markers, the influence of the Tat-PTD peptide on proliferation, and the production of the cytokines Il-2 and IFNγ was also assessed. Figure 11 shows a summary of these results. No effect of the Tat-PTD peptide could be observed on the level of Il-2 and IFNγ production and on proliferation, when compared with medium controls in these cultures over a period of 3 days. Not only did the overall level of Il-2 production not change, but in the presence of Tat-PTD the kinetic profile over time was also identical.

Figure 11.

Splenic single cell cultures of the Rag-negative TCR-transgenic strains AND **(A, C, E)** and F5 **(B, D, F)** were incubated with 10 μM Tat-PTD peptide for up to 3 days. Shown are the incorporation of [^{3}H] thymidine at day 3 after stimulation with the respective specific antigenic peptide, MCC or NP68 **(A, B)**, Il-2 secretion in the medium as measured in a CTLL-assay at day 1 and 2 after stimulation with the respective specific antigenic peptide **(C, D)**, and IFNγ secretion in the medium as measured by ELISA at day 3 after stimulation with the respective specific antigenic peptide **(E, F)**. The figure shows mean percentages ± SD of positive cells from two individual experiments.

Influence of Tat-PTD on proliferation, and cytokine production of TCR-transgenic murine T cells



3.3 Production of Tat-PTD fusion proteins

The previous results indicated that the Tat-PTD peptide itself does not affect naive T cells, and therefore may serve as a suitable carrier for the intended aims of this study. In order to transfer signalling attenuators into naive T cells, fusion proteins with the Tat-PTD peptide were generated.

3.3.1 Targets in the TCR signalling cascade

Activation of the PTK Lck following TCR engagement is responsible for phosphorylation of ITAM tyrosine residues, one of the earliest events in the TCR signalling cascade (Weiss, 1993). The two critical tyrosines in each ITAM are independently phosphorylated, resulting in the generation of differently phosphorylated TCRζ isoforms, p21 and p23, predominantly found in resting peripheral spleen and lymph node T cells and in activated T cells, respectively. The 21-kD isoform is generated by the phosphorylation of the four tyrosines located in ITAMs 2 and 3, whereas p23 is fully phosphorylated on all three ITAMs (Figure 12) (van Oers et al., 2000). Analysis of phosphorylated TCRζ isoforms using site-specific antibodies have shown that p21 predominantly contains singly phosphorylated ITAMs, while p23 predominantly contains dually phosphorylated ITAMs (Kersh et al., 1998a). This sequential phosphorylation of the six tyrosine residues of the TCRζ was found to be highly ordered, and completion of phosphorylation steps is dependent on the nature of the ligand; full phosphorylation was found to depend on the strength of TCR triggering. This indicates that each phosphorylation step may establish a threshold for T cell activation.

Stimulation of T cell clones with altered peptide ligands (APLs), predominantly results in the p21 TCRζ isoform, that either antagonises or anergises the T cell response. Thus, there is a correlation between the induction of particular isoforms of TCRζ and the functional responsiveness of T cells (Kersh et al., 1999; Sloan-Lancaster and Allen, 1996b).

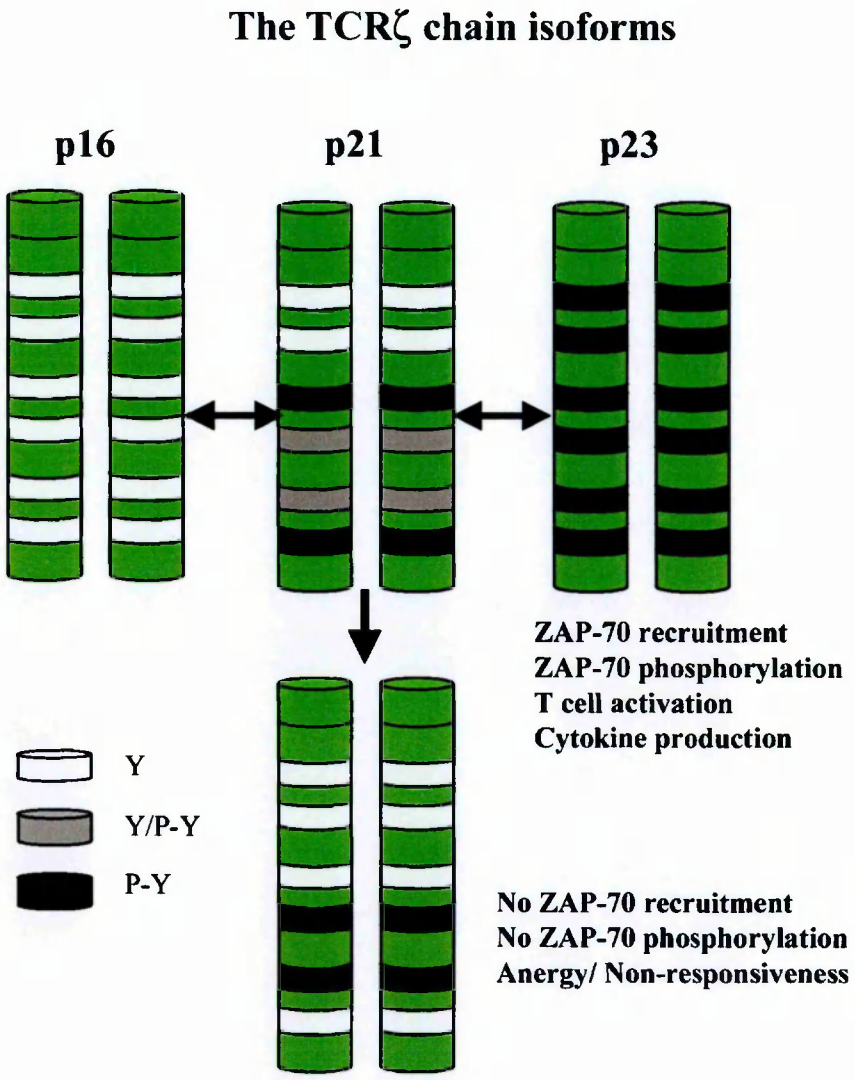


Figure 12. The TCRζ chain has multiple isoforms, based on its phosphorylation state. The non-phosphorylated form, p16, is the predominant form found in cell lines, hybridomas and in *ex vivo* cell cultures after 24 hours. The p21 semi-phosphorylated isoform is found in spleen and lymph node T lymphocytes, and is capable of binding ZAP-70. Upon TCR triggering, all ITAMs are phosphorylated, resulting in the p23 isoform that recruits ZAP-70 and allows subsequent activation of the T cells. T cells activated with specific APLs can express a different isoform of p21, which does not allow ZAP-70 binding, and results in T cell anergy or non-responsiveness.

Only dually-phosphorylated ITAMs recruit the crucial PTK molecule ZAP-70 via its tandem SH2 domain (Iwashima et al., 1994). Hence, p21 can be found associated with a pool of ZAP-70 molecules; these, however, are not phosphorylated. Since there are three ITAMs in each TCRζ, this allows up to three ZAP-70 molecules to bind each chain. Indeed, recombinant ZAP-70 binds the phophorylated TCRζ in a 3:1 molar ratio (Weissenhorn et al., 1996), providing a mechanism for signal amplification. Although all doubly-phosphorylated ITAMs will compete with each other, there is a hierarchy in affinity of ITAM1>ITAM2>ITAM3 (Isakov et al., 1995).

Binding of ZAP-70 to the ITAMs results in the phosphorylation of multiple residues of ZAP-70, regulating its activity (Watts et al., 1994; Di Bartolo et al., 1999). Crucial for its kinase activity is phosphotyrosine residue 319, with which it recruits the SH2 domain of Lck (Di Bartolo et al., 1999; Pelosi et al., 1999), allowing phosphorylation of Y493 by Lck and subsequent autophosphorylation of Y292 and Y492 (Chan et al., 1995).

The central question that this study tries to address is whether manipulation of signalling pathways can drive T cells into different response modes. Both Lck and ZAP-70 are likely candidates to set such thresholds for T cell activation and both have been reported to play a role in induction or maintenance of T cell anergy. The relative importance of these kinases for lymphocyte activation has been evaluated by gene disruption in the mouse. However, the possibility of compensatory function of other proteins complicates the interpretation of these studies. In addition, the use of immortalised cell lines does not reflect the signalling mechanism in naive T cells. Therefore, these molecules, involved in the most proximal TCR signalling events, became the target of this study in which specific peptide-based inhibitors of individual proteins were generated, to help clarify the role that these enzymes play in signalling pathways and differentiation of T cells.

The Tat-PTD should allow the entry of these specific inhibitors into naive *ex vivo* T cells, which are otherwise difficult to transfect. The specific inhibitors were designed to be capable of competing with important protein-protein interaction sites of the endogenous Lck and ZAP-70 molecules. The function of ZAP-70 in TCR

signalling depends on its recruitment to the plasma membrane by dually-phosphorylated TCRζ chains, its subsequent phosphorylation and interaction with Lck. The interaction between ZAP-70 and the TCRζ depends entirely on its tandem SH2 domains. Therefore, when these are introduced in sufficiently high concentrations, one might expect them to compete with endogenous ZAP-70, thereby reducing the amount recruited to the plasma membrane and decreasing downstream signals. Therefore, a genetic fusion was generated between the Tat-PTD and the truncated form of ZAP-70, consisting only of its two SH2 domains (Tat-Zap70$(SH2)_2$) (Figure 13).

The PTK Lck is responsible for the phosphorylation of the TCRζ chains and a tyrosine residue on ZAP-70. It binds with its unique N-terminal part to the co-receptors CD4 and CD8, allowing it to come into close contact with the TCR signalling machinery. In addition, Lck has two other motifs for protein-protein interactions, a SH2-domain and a SH3 domain. The SH2 domain enables its recruitment to phosphotyrosine residues like Y392 in ZAP-70, which permits subsequent phosphorylation of Y493 determining ZAP-70 kinase activity (Chan et al., 1995), and the SH3 domain allows interaction with CD28 to sustain Lck activity (Holdorf et al., 1999). In order to interfere with possible recruitment and function of Lck, as in the case of ZAP-70, a kinase-truncated molecule was genetically fused to the Tat-PTD. With the intention of allowing acylation, myristoylation and recruitment of Lck to the plasma membrane, to compete with endogenous Lck, a fusion protein was generated that left the N-terminus of Lck free of interfering tags as well as the Tat PTD (Lck^{NSH2}-Tat), and a second one that would not allow this recruitment because of the His-tag and Tat-PTD at the N-terminus (Tat-Lck^{NSH2}) (Kabouridis et al., 1997) (Figure 13). In addition, a smaller version was generated, expressing its specific SH2 domain only (Tat-Lck^{SH2}). Finally, wild type Lck was fused to the Tat-PTD at the N- or C-terminus, allowing to test Lck functionality in the Jurkat mutant cell line JCaM1, containing a defect in the expression of functional lck (Straus and Weiss, 1992).

Overview of the generated Tat-PTD fusion proteins

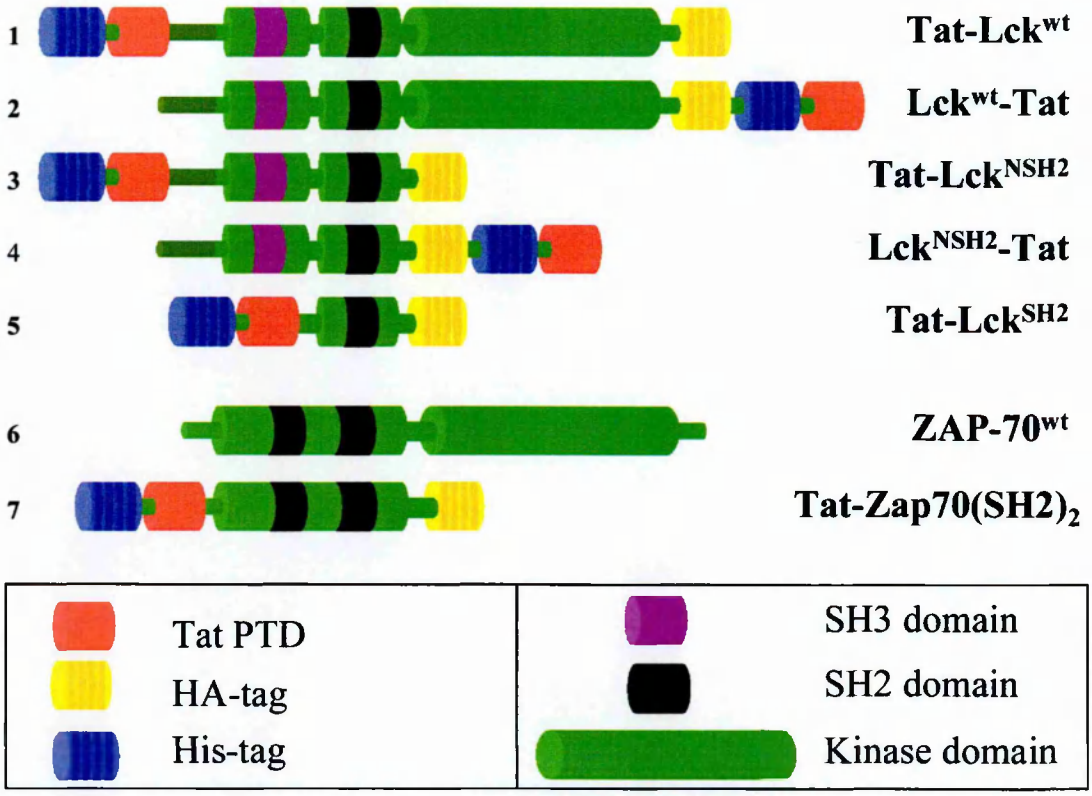


Figure 13. Contructs 1 and 2 represent wild-type Lck (Lck^{wt}) cloned in between a His-tag for purification purposes and the Tat-PTD at the N-terminus, and a HA-tag for immuno-detection purposes at the C-terminus (Tat-Lck^{wt}), or with a free N-terminal end and all modifications at the C-terminus (Lck^{wt}-Tat), respectively. Constructs 3 and 4 represent truncated forms of Lck, lacking its kinase domain, but maintaining its unique N-terminus and SH3 and SH2 domain, in between tags (Tat-Lck^{NSH2}), or with a free N-terminus (Lck^{NSH2}-Tat), respectively. Construct 5 represents the SH2 domain of Lck only (Tat-Lck^{SH2}), cloned in between His and HA tags. Construct 6 is wild-type ZAP-70 (ZAP-70^{wt}), and construct 7 represents the kinase-truncated form of ZAP-70 (Tat-Zap70$(SH2)_2$), leaving only its characteristic tandem SH2 domain, cloned in between both tags.

The functionality of the fusion partners depends greatly on their refolding capacity after translocation across the cell membrane, since cellular uptake by Tat-PTD is preceded by an unfolding step. The refolding properties are different for every protein, and therefore, full-length Lck was also included as a fusion partner. This would allow testing the functionality of Lck in this system by reconstitution of the functional Lck-negative mutant Jurkat T cells, JCaM1 (Goldsmith and Weiss, 1987).

3.3.2 Expression of Tat-PTD fusion proteins

cDNA fragments of ZAP-70 and Lck were cloned into the pET-Tat-HA vector, Figure 14A, derived from the pET15b vector, and sequenced to check for irregularities. After transduction into Bl-21 bacteria, expression of the desired fusion products was tested in small-scale cultures. Two single colonies were grown up, expression was induced with IPTG for four hours and total cell lysates were tested for the presence of full-length fusion proteins by separation by SDS-PAGE and Western blotting. Since two tags were engineered into the proteins, a His-tag at the N-terminus, and an HA-tag at the C-terminus (Figure 13), immuno-blotting for these tags would reveal if the desired protein is expressed at full-length. Figure 14 shows the expression of the following Tat-PTD fusion proteins; Tat-Lck^{wt}, Tat-Lck^{NSH2}, Tat-Lck^{SH2} and Tat-Zap70(SH2)$_2$. Panel B shows staining for the His-tag, located at the N-terminus, and panel C shows the same blot stained for the HA-tag, located at the C-terminus. Since panel B and C show identical patterns of staining, at the expected molecular weights of the intact full-length fusion proteins, it was concluded that the engineered vectors expressed the complete fusion proteins.

Figure 14.

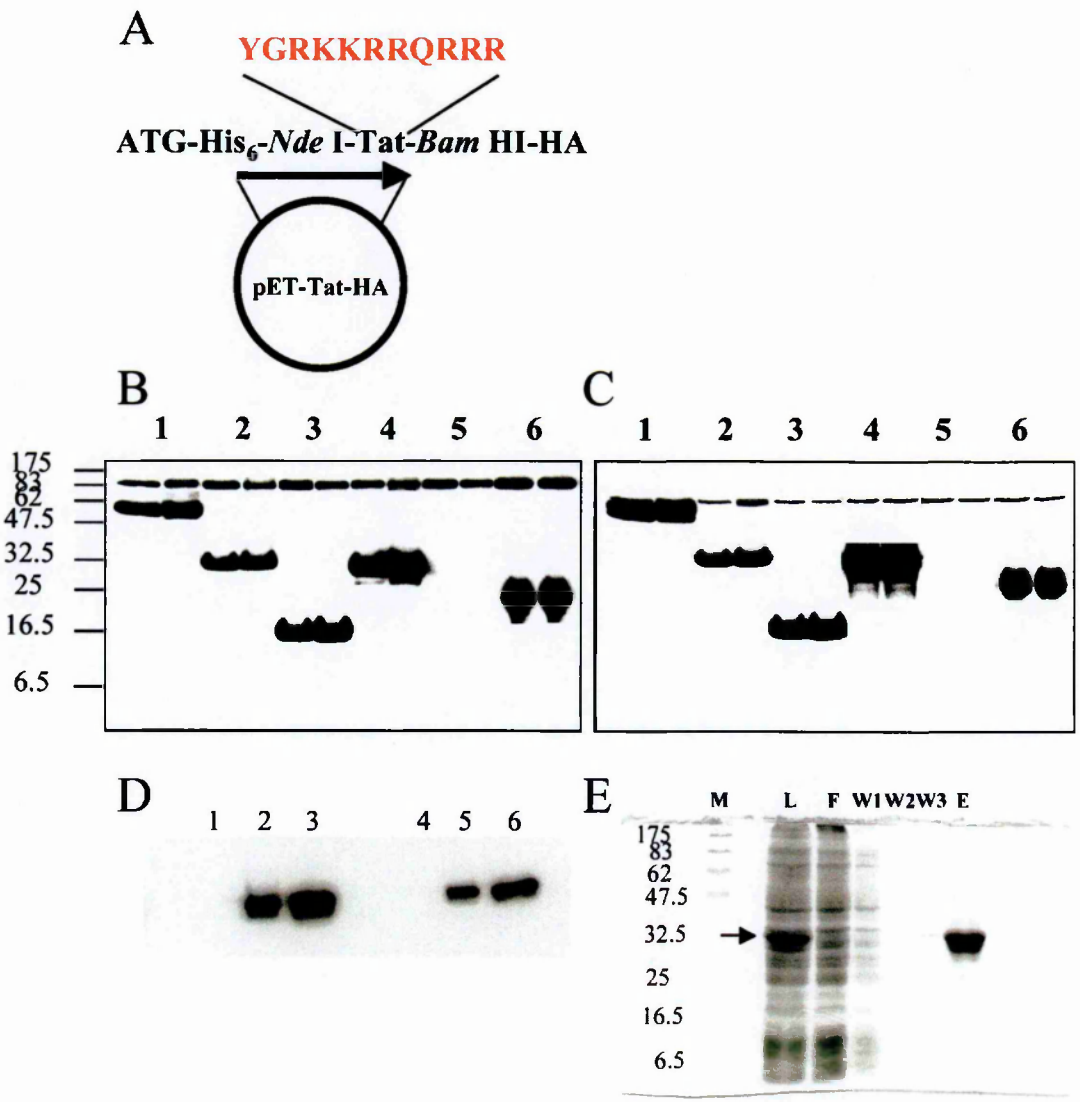
Fragments of cDNA were cloned into the pET-Tat-HA vector **(A)**, containing a His-tag, the Tat-PTD and a HA-tag.

Expression of the genetically engineered Tat-PTD fusion proteins was tested by SDS-PAGE and Western blotting. Total cell lysates were separated on a 12% SDS-PAGE gel, blotted onto nitrocellulose, and stained with anti-His mAbs **(B)**; subsequently the blot was stripped and reprobed with anti-HA mAbs (12CA5) **(C)**. Shown are lysates of two individual colonies of; **(1)** Tat-Lck^{wt}, **(2)** Tat-Lck^{NSH2}, **(3)** Tat-Lck^{SH2}, **(4)** Tat-Zap70$(SH2)_2$, **(5)** empty vector and **(6)** control protein containing both tags.

The expression levels over time, and the solubility of the expressed fusion proteins was tested by separating the soluble and insoluble fractions of cell pellets taken at time intervals of 5 and 8 hours after IPTG induction **(D)**. Depicted here are the soluble fraction **(lanes 1, 4)**, the insoluble fraction **(lanes 2, 5)** and the total cell lysate **(lanes 3, 6)** of Tat-ZAP-70$(SH2)_2$ fusion protein expressed by Bl-21, 5 hours **(lanes 1, 2, 3)** and 8 hours **(lanes 4, 5, 6)** post-IPTG induction.

Purification of expressed fusion proteins was done with the help of the engineered His-tag and Ni-NTA columns **(E)**. Shown here is a Coomassie Blue staining of the total cell lysate before applying it to the Ni-NTA column **(L)**, the flow through **(F)**, the first **(W1)**, second **(W2)** and third **(W3)** washing steps and finally the eluted fraction **(E)** of a Tat-Zap70$(SH2)_2$, (indicated by the arrow), purification, separated on a 12% SDS-PAGE gel.

Expression and isolation of Tat-PTD fusion proteins



To obtain maximum amounts of protein, the expression kinetics of each fusion protein was tested. Inoculated cultures were grown to OD_{600}=0.5, whereafter expression of the fusion proteins was induced with 0.6 mM IPTG. Samples were taken at 2, 5 and 8 hours post-induction and checked for total amount of fusion protein by SDS-PAGE. This expression system performed optimally when cultures were grown at 30°C with 0.6 mM IPTG for 4 to 6 hours.

Important for the purification purposes was to know if the target proteins were expressed in a soluble form, or in inclusion bodies. Therefore, bacteria expressing each Tat-PTD fusion protein were collected 5 or 8 hours post-IPTG induction, and divided into a soluble and insoluble fraction. Figure 14D depicts the study for Tat-ZAP-70$(SH2)_2$, and shows that the shorter time interval of 5 hours results in higher expression of this fusion protein per weight of bacterial pellet. More importantly, it shows that the majority of this protein is expressed in inclusion bodies, which was the case for all fusion proteins tested in this study.

3.3.3 Purification of Tat-PTD fusion proteins

The expression of the fusion proteins in the insoluble fraction required purification of these proteins under denaturing instead of native conditions. Most proteins in inclusion bodies are solubilised with detergents or denaturants such as 8 M Urea or 6 M Guanidine HCl. Urea solutions must be used promptly and not stored for long periods of time because of the formation of the Urea isomer ammonium cyanate; the cyanate ions can covalently modify primary amines on the target protein (Stark et al., 1960). Both denaturants were initially used for these studies, but the more stable denaturant Guanidine HCl was chosen in the final protocol.

The chosen method of purification, immobilised-metal affinity chromatography using the engineered 6x His-tag, does not depend on tertiary structure, which is lost once proteins are denatured. The advantage is that the 6x His-tag will be fully exposed so that binding to the chelating ligand will improve, and the efficiency of the purification will be maximised by reducing the potential for non-specific binding. Utilising the Tat-PTD system for intracellular delivery, efficient translocation is dependent on an unfolding step to enable the membrane translocation,

and this is followed by a refolding step whereby several proteins are reported to regain their biological function (Bonifaci et al., 1995).

A general purification protocol for all Tat-PTD fusion proteins was established using small-scale cultures and optimal conditions were determined empirically. Bacterial pellets were dissolved in a 5.8 M guanidine HCl lysis buffer supplemented with 5 mM imidazole, and with help of additional sonication. The cell debris was spun out, and the resulting fraction was applied to a Nickel-Nitrilotriacetic acid (Ni-NTA) column and allowed to flow by gravity. The column was washed with different concentrations of imidazole to decrease non-specific binding, and finally the bound proteins were eluted in a high concentration imidazole buffer. Figure 14E shows a purification of Tat- Zap70$(SH2)_2$, and shows that the majority of the target protein has bound to the Ni-NTA column, comparing the lysates before (**L**) and after (**F**) application to the Ni-NTA column. Washing resulted in the clearing of non-specifically bound proteins, where the last wash sometimes elutes a minor amount of the target protein (**W3**), and subsequently the highly purified fusion proteins were successfully eluted (**E**).

3.3.4 Slow desalting of denatured Tat-PTD fusion proteins

The ultimate use of the Tat fusion proteins requires them to be added to the medium in which *ex vivo* T cells are cultured. This means that the high concentrations of denaturants used during purification need to be removed from the final protein solution. When proteins are purified under denaturing conditions, some renaturation may be necessary to allow the proteins to be soluble in physiological buffers, for which the conditions must be determined empirically for each target protein. Table 4 shows an overview of different strategies, and recommendations from the literature that have been tried alone and in combination throughout this study. During a long period of trial and error, conditions were determined to establish a protocol allowing solubilisation of Tat fusion proteins in a physiological buffer in sufficient quantity to allow multiple experiments from one round of purification.

Table 4: Overview of different strategies tried during this study to allow solubilisation of Tat fusion proteins.

	Reagents	Effect
Dilution		Reducing denaturants
Dialysis	Slide-a-lyser	Removing denaturants
Desalting	PD-10 columns/Mono Q/S	Removing denaturants
Protein concentration	10-50 µg/ml	
Cosolvents	Glycerol 5-20%	Stabilising hydrophobic interactions
	Glucose 10%	Decrease unfolding rate
	Sucrose 10%	Decrease unfolding rate
	Ethanol 5-20%	Stabilising hydrophobic interactions
	Anions : phosphate and sulphate	Decrease unfolding rate
	Cations : HEPES	Decrease unfolding rate
Oxidising/ Thiol reagents	Glutathione/oxidised Glutathione Oxidation by air Cu^{2+}	Allowing/ inhibiting formation of cysteine bridges
Salts	100 mM KCl 150-500 mM NaCl 2 mM $MgCl_2$	Prevent ionic interactions
pH	Neutral / pI	Enhance solubility
Protease inhibitors	0.5 mM PMSF 0.05-2 mg/ml aprotinin 2 µg/ml pepstatin 2-5 µg/ml leupetin	Suppress proteolysis
Immobilised refolding	Denaturant gradient in high salt and 20% glycerol	Prevent intermolecular interactions
Amino acids	L-arginine; Glycine	Enhance solubility
Other additives	$MgCl_2$; $CaCl_2$	Essential ions

A general method of Tat fusion protein production was published in 2000 (Schwarze et al., 2000); this protocol formed the basis for the protein production in this study, but failed to generate high yields of Tat-PTD fusion proteins. In short, recombinant proteins are isolated from the bacterial pellet by sonication in 6 M Guanidine HCl and purified over a Ni-NTA affinity column. After purification, the denaturant will have to be removed. This can be done rapidly using a PD-10 desalting column, Mono Q/S columns, or dialysis against a physiological buffer like PBS. Initial attempts to remove the denaturant in this way all failed due to extensive aggregation of the target protein. The addition of various cosolvents like salts and glycerol did not lead to any improvement.

The solubility of the Tat fusion proteins will depend on their complexity and the structure that is energetically the most favourable. The fusion proteins used in this study all have a SH2 domain in common. Additional domains are: the unique N-terminus of Lck that is not known to favour a particular structure, the SH3 domain which forms an additional complexity, and the complex kinase domain in the Lck^{wt}, making Lck^{wt} likely the most complex and thus most difficult protein to solubilise. None of the proteins in the initial stages, however, allowed rapid removal of the denaturant. Slow dilution and step-wise dialysis against decreasing concentrations of the denaturant offered an alternative for denaturant removal. Dilution was found to be the most desirable, since this led to higher amounts of soluble protein. The protein is diluted many fold (>10) directly but slowly into refolding buffer to achieve low concentration conditions that promote a folding state whereby the protein is soluble and disfavours aggregation. The critical point of removing the denaturant was around the 2M concentration, and a 2M Urea solution served as the first dilution buffer. Thereafter, a Tris- or HEPES-based buffer served as the second dilution buffer. After dilution steps, the volumes were kept at reasonable levels using a stirred-cell concentrator. The final step in the solubilisation protocol was a dialysis step, whereby the protein was dialysed against 10% glycerol in PBS, allowing removal of residual chemicals and a cell-compatible end product.

The presence of a high number of aromatic and aliphatic residues is thought to decrease the solubility of proteins and contribute to aggregation (Burgess, 1996). These hydrophobic interactions can be minimised by including mild detergents and

glycerol in the solubilisation buffers. The use of glycerol in this study did enhance protein yield considerably at concentrations between 10 and 20%.
Another frequently used approach is to dilute the protein in order to decrease intermolecular aggregation, already applied in this study for denaturant removal. The net charge of the target protein can also be used as a guide for the determination of buffer conditions that may enhance solubility. The high amount of positively charged residues, arginines and lysines, in the Tat-PTD peptide, suggested that pH adjustments might result in greater solubility and recovery of soluble protein. Adjustments of pH in this study did not result in significant improvements, however.

The presence of cysteine residues and disulfide bonds in a fusion protein helps to indicate what refolding reagents to use. All fusion proteins in this study contain between 4 and 10 cysteine residues, but these are not used to create disulfide bonds *in vivo*. Thiol redox reagents, such as oxidised/reduced glutathione, can be added to catalyse disulfide bond formation and to inhibit the formation of non-productive disulfide intermediates. As reported previously, although Lck does not form disulfide bonds, the redox state affects its solubility (Yasukawa et al., 1995). Alternatively, the target protein can be fully oxidised by the use of $CuSO_4$ or $NaSeO_3$ and exposure to air under slow stirring. The latter has proved to be the most powerful method tested during this study.

After many trials and adjustments it proved difficult to obtain high enough quantities of the Tat-PTD-Lck^{wt} fusion protein. Since the only aim of making this protein was testing its refolding and functional capacity after transduction, as a model for the other Lck domains-containing fusion proteins, it was decided to abandon the production of this particular fusion protein. The other proteins in Figure 13, could all be produced by stepwise dilution of the denaturant; first in a high salt HEPES buffer supplemented with 2M Urea, 20 % glycerol and 50 mM Glycine, secondly in a high salt HEPES buffer supplemented with 20% glycerol and 50 mM Glycine, thereafter a final dialysis step followed against 10% glycerol in PBS.

3.3.5 Rapid desalting of denatured Tat-PTD fusion proteins

Although removal of denaturants by slow dilution resulted in about 10 mg of fusion protein per isolation, it seemed possible that alternative stratagies might give higher yields of protein. Rapid removal from 8M Urea by PD-10 desalting-columns and with ion exchange chromatography was successfully reported for several proteins before. This method has been reported to give the highest yield and quality of Tat-PTD fusion proteins (Vocero-Akbani et al., 2001).

The general method of Tat fusion protein production, published by Schwarze et al (2000), proposes the use of ionic exchange chromatography and FPLC equipment. However, initial small-scale experiments using Mono Q or S resin resulted in precipitation of eluted protein or irreversible binding to the resin, and the technique was therefore abandoned. At a later stage, attempts were made using single step elutions, thereby avoiding the use of automated FPLC or HPLC equipment. The purification of proteins by ionic exchange chromatography is largely dependent on their isoelectric point (pI), however, due to the use of high molar Urea solutions and the addition of a highly positively charged Tat-PTD amino-sequence, some fusion proteins used in this study were found to bind to Mono Q or S resin independently of their theoretical pI (Figure 15C).

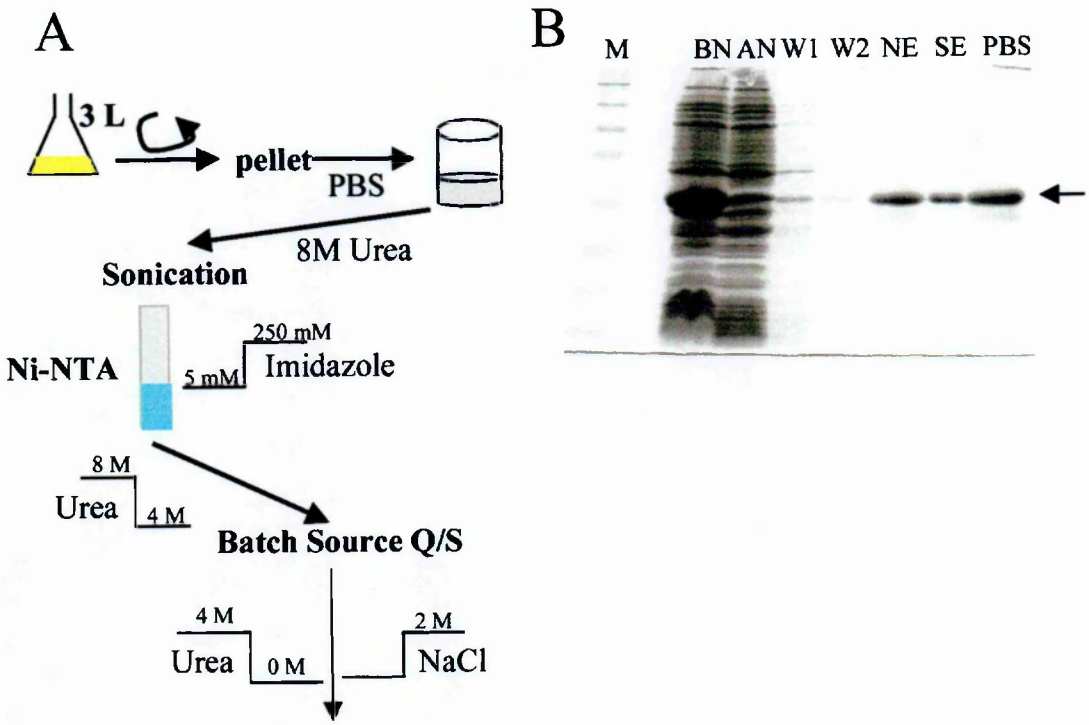
Because 6 M Guanidine HCl is a more stringent denaturant than 8 M Urea, this is in some cases favoured above Urea, because it allows better exposure of the His-tag purification leader. However, due to the ionic nature of Guanidine HCl it can not be used in conjunction with ion exchange chromatography, and when used for purification, needs to be replaced with 8 M Urea prior to ion exchange chromatography. Figure 15B shows that denaturation and isolation under 8 M Urea conditions works equally well for the proteins used during this study, and since the denaturant is removed very quickly using this method, 8 M Urea was chosen as denaturant. Once the fusion proteins are eluted from the Ni-NTA column, they were diluted in a dilution buffer, which has to be tailored to each individual fusion protein, with specific sodium chloride concentration and pH (Figure 15C) resulting in a 4 M Urea solution. Since Tat-proteins bind to Mono Q or S more or less strongly

depending on their pI, optimal conditions for each protein need to be determined empirically.

As a general method, newly purified proteins were added to 5 ml Source Q in batch form, equilibrated with 20 mM Hepes, pH 8.0, or Source S, equilibrated with 20 mM Hepes, pH 6.5. If no or weak protein binding is observed with one resin, regardless of predicted pI, the use of other Source resin is indicated. If strong binding is observed, but poor yield in the eluate, several modifications can reduce the effective avidity of the protein to the resin. The goal is to decrease the avidity of the protein for the resin in Urea to the point of obtaining a reversible binding. The sample and resin can be equilibrated with increasing amounts of NaCl, until recovery yield is high. Alternatively or in combination, the pH of the buffer can be decreased (Source Q) or increased (Source S) in steps until improvements in yield in the eluate.

After collection of the eluate, generally containing 1-2 M NaCl, some proteins may be desalted to physiological salt concentrations using a PD-10 desalting column. Proteins used in this study, however, still showed a tendency to precipitate, and were therefore diluted in PBS 10% glycerol in three steps, thereby increasing the volume, and thus reducing the protein concentration allowing the protein to stay soluble. Precipitated protein was filtered-out using a 45µm syringe filter prior to overnight dialysis against PBS 10% glycerol. On average, 50-80 mg of protein was obtained from a 3 l starting culture. Figure 15A shows a schematic purification protocol.

Rapid desalting of denatured Tat-PTD fusion proteins



C

Tat fusion partner	Theoretical pI	Source	pH for binding	[NaCl] for binding	pH for elution	[NaCl] for elution
$Zap70(SH2)_2$	8.7	S	8.0	200 mM	10	2 M
Lck^{NSH2}	6.1	S	6.5	50 mM	8	2 M
Lck^{SH2}	9.2	Q	8.0	50 mM	8	2 M
Lck^{wt}	5.8					

Figure 15. (A) Rapid purification protocol. **(B)** Purification of Tat-Lck^{NSH2} over Ni-NTA resin and Source S resin. Crude lysate (BN) from 3 L culture was applied to the Ni-NTA resin, allowed to flow through (AN) by gravity, washed twice (W1, W2), eluted (NE), applied to Source S, washed, eluted (SE), diluted and dialysed against PBS (PBS). 10 µl of each fraction were resolved by SDS-PAGE and stained with Coomassie blue. **(C)** Overview of rapid desalting conditions used for the Tat-PTD fusion proteins generated during this study.

3.4 The use of a small Zap70 inhibitor peptide

Since numerous technical problems were encountered with the purification of large fusion proteins, interest was aroused by two recent reports showing the use of chemically synthesised protein transduction domains fused to a short peptide sequence (Nishikawa et al., 2000; Bonny et al., 2001). Nishikawa et al. (2000) screened a tyrosine-oriented peptide library, substituting the tyrosine residue by a phenylalanine residue. They identified a specific and competitive peptide inhibitor of ZAP-70 (IC_{50}~10 μM in kinase assay). By delivering this peptide at high concentrations to the interior of Jurkat T cells, making use of the penetratin system, they showed diminished phosphorylation levels of proteins known to be ZAP-70 substrates, as well as inhibition in transcriptional activation of the Il-2 gene upon CD3 crosslinking.

This approach circumvents the problems encountered with protein solubility, and the described peptide-sequence (KLILFLLLL) can be synthesised covalently attached to the Tat-PTD. It offers an alternative approach of the original aims of this study. Instead of utilising large protein domains, short peptide-sequences designed to bind to and to interfere with protein-protein binding domains can be transferred into naiveT cells in order to compete with endogenous proteins with the aim of modifying T cell differentiation.

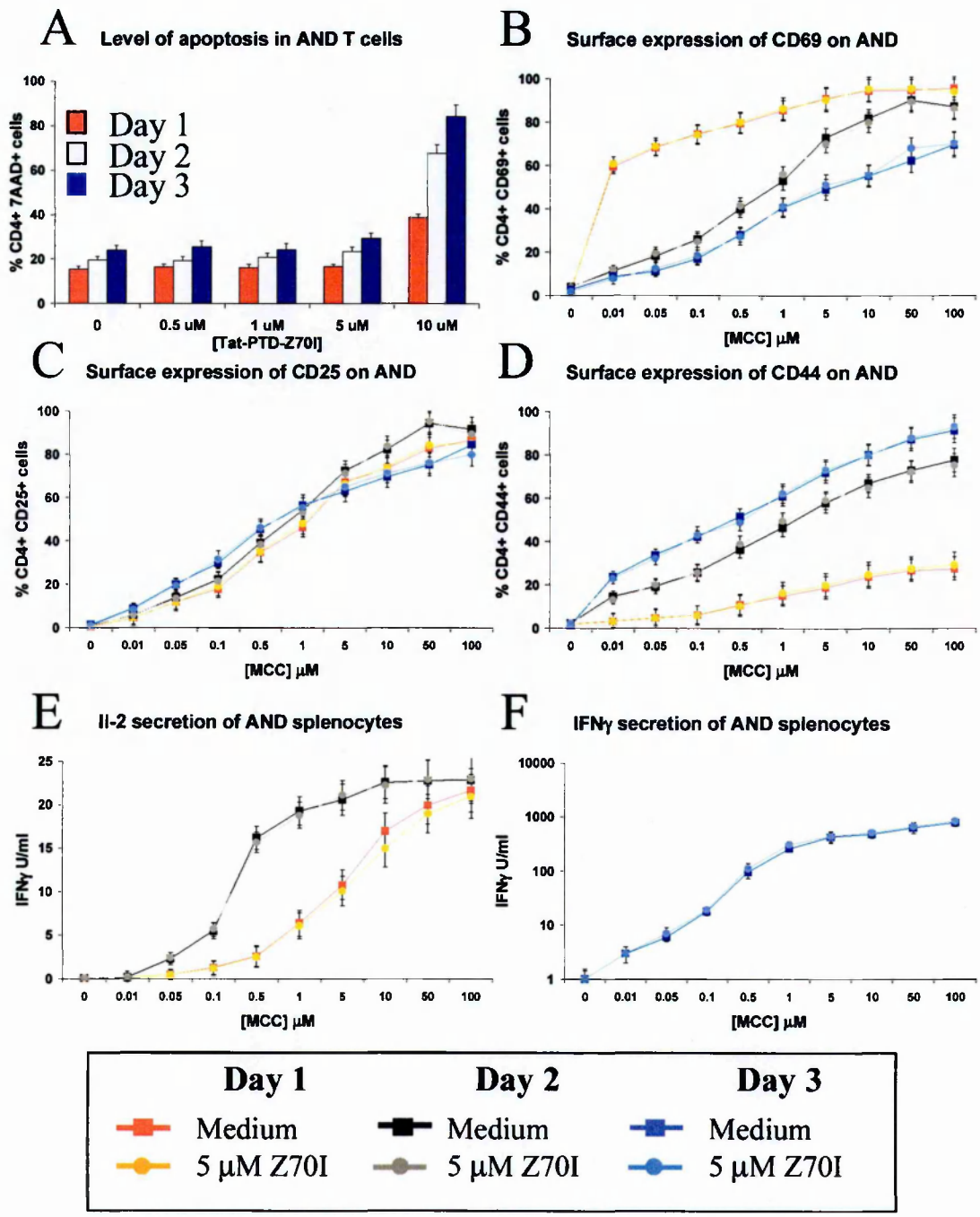
Concentrations varying from 0.1 μM to 50 μM of the peptide, named Tat-PTD-Z70I, were tested on monoclonal and polyclonal T cell populations, in the presence or absence of antigen-specific stimulation or CD3 crosslinking. T cell populations from different TCR-transgenic and polyclonal mice (A1; BM3; OT-1, A-strain and B10) were tested for activation markers and cytokine production as described before, but none of them showed diminished activation in the presence of Tat-PTD-Z70I. Figure 16 shows the results of one of the tested transgenic strains, AND, and the absence of any influence of the reported Zap-70 inhibitor peptide at the tested concentrations on the activation markers CD69, CD25 and CD44 as well as the cytokines Il-2 and IFNγ. As shown in panel A, the peptide appears to be toxic for *ex vivo* T cells at concentrations above 5 μM. Concentrations reported to inhibit Jurkat T cells, ranging from 10-100 μM, could therefore not be tested and the Tat-PTD-Z70I peptide was found not suitable for the intended aims of this study.

Figure 16.

Splenic single cell cultures of the Rag-negative TCR-transgenic strain AND were incubated with indicated concentrations of Tat-PTD-Z70I peptide for up to 3 days, and stained with PE-conjugated anti-CD4. Shown is the uptake of 7AAD, as determined by FACS analysis **(A)**.

Splenic single cell cultures of the Rag-negative TCR-transgenic strain AND were incubated with 5 µM Tat-PTD-Z70I peptide for up to 3 days, stimulated on day 0 with indicated concentrations of the specific antigenic peptide MCC, and stained with PE-conjugated anti-CD4. Shown are the expression levels of CD69 **(B)**, CD25 **(C)**, and CD44 **(D)** as determined by FACS analysis. Il-2 secretion in the medium as measured in a CTLL-assay at day 1 and 2 after stimulation with the specific antigenic peptide **(E)**, and IFNγ secretion in the medium as measured by ELISA at day 3 after stimulation with the specific antigenic peptide **(F)**. The figure shows mean percentages ± SD of positive cells from two individual experiments.

Influence of the small peptide, KLILFLLLL, a reported ZAP-70 inhibitor, on activation of AND T cells



3.5 Transduction of Tat-PTD fusion proteins

Following production of Tat-PTD fusion proteins in soluble form and in a physiological buffer the first question to be addressed was if Tat-PTD is indeed capable of intracellular delivery of the fused proteins. The first protein that to be produced in larger quantities, Tat-Zap70$(SH2)_2$, was used to test if Tat-PTD could deliver large proteins into T cells.

3.5.1 Cell entry of slowly desalted Tat-PTD fusion proteins

The first experiments were set up with Jurkat T cells. Cells were incubated with slowly desalted Tat-PTD fusion protein for various times, thereafter cells were washed, lysed and loaded on a SDS-PAGE gel. In order to discriminate between membrane-bound fusion protein and cytoplasmic protein, treated cell fractions were split in two, and one of the fractions was left untreated, while the other was treated with trypsin, removing extracellular proteins.

Figure 17A shows that after 10 minutes fusion proteins were pulled down with the cells, indicating binding. However, upon trypsin treatment, this fraction disappeared, indicating that it had not entered the cells. Upon prolonged exposure to the fusion protein, for 1 and 4 hours, it was still detectable after trypsin treatment, showing that it had entered the cells, since intracellular proteins are protected from trypsin by the plasma membrane. This indicates that a fraction of the fusion protein had entered the cells.

The most important point for this study was to test if Tat-PTD fusion proteins are able to cross the membrane of naive *ex vivo* T cells. In order to test this, fusion proteins were labelled with the fluorochrome FITC, which has the characteristic of being quenched at acidic pH levels. Since cells keep their interior milieu at physiological levels with the help of ion channels, intacellular FITC will be able to emit, while extracellular FITC is quenched by low pH. This principle is shown in Figure 17B, where T cells are stained with FITC-conjugated anti-TCR antibodies.

Acquisition at neutral pH leads to a high FITC signal, while an acidic pH of 6.8 quenches the extracellular bound antibodies.

Co-cultures of naive *ex vivo* T cells with FITC-conjugated Tat-Zap70$(SH2)_2$ could now be assessed for the uptake of fusion protein. Figure 17C shows that after 90 minutes of co-culture, and washing, acquired cells at pH 7.4 gave a strong positive signal. Upon acquisition at an acidic pH, part of this signal was lost, but it was still above background levels. In comparison, co-cultured cells were treated with trypsin, and subsequently acquired at pH 7.4, showing identical signal intensity. This suggests that Tat-PTD is indeed capable of delivering large proteins into naive T cells. In addition, Figure 17C shows that the majority of T cells are transduced with the fusion protein.

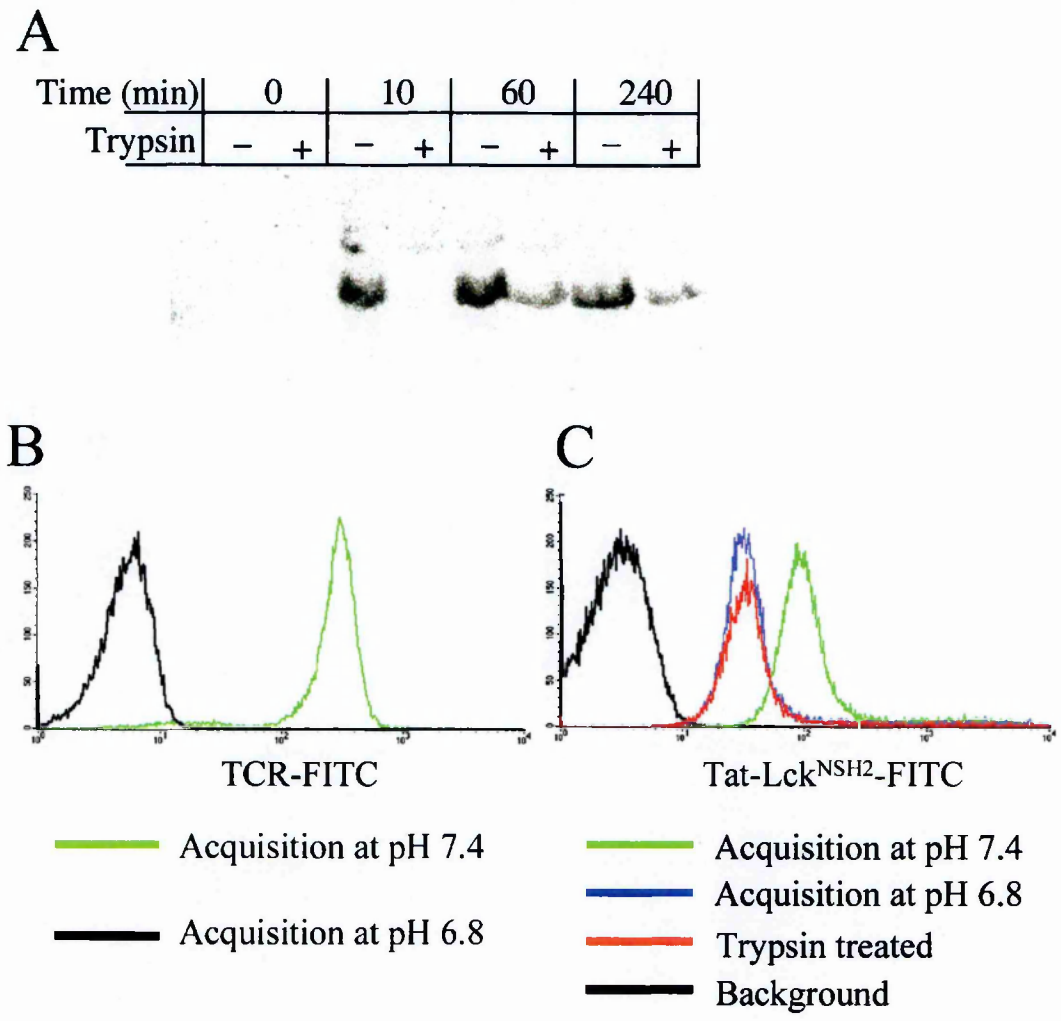
Figure 17.

(A) $5x10^6$ Jurkat T cells were incubated for indicated minutes with slowly desalted 200 nM Tat-Zap70$(SH2)_2$ at 37°C. They were then collected, washed twice, split into two fractions of which one fraction was treated with 10 µg/ml trypsin for 15 min on ice. Cells were subsequently washed with cold PBS, lysed, and loaded onto a 12% SDS gel. After transfer, blots were stained with anti-His-tag or anti-HA (12CA5) antibodies. Shown here is the result of anti-HA staining; anti-His staining gave identical results.

(B) MACS-purified B10 T cells were stained with FITC-conjugated anti-TCR antibody, and were subsequently acquired by FACS at pH 7.4 or pH 6.8.

(C) MACS-purified B10 T cells were incubated for 90 minutes with or without 2 µM FITC-conjugated Tat-Zap70(SH2)2, thereafter cells were left untreated or treated with 5 µg/ml trypsin for 15 min on ice, followed by acquisition on a FACScalibur at pH 7.4 or pH 6.8.

Translocation of slowly desalted Tat-Zap70(SH2)$_2$ across the plasma membrane.



3.5.2 Kinetics of cell entry of slowly desalted Tat-Zap70(SH2)$_2$ fusion protein

This study is based on the hypothesis that transduced proteins can interfere with TCR signalling events during T cell priming. This implies that the fusion protein should be present at high enough concentrations just before and during the stimulus. Timing is probably an essential parameter in these experiments, and therefore it is important to know the kinetics of transduction of naive T cells. It is important to know how long it takes before the fusion proteins are taken up, and when they are at their highest concentration inside the cell.

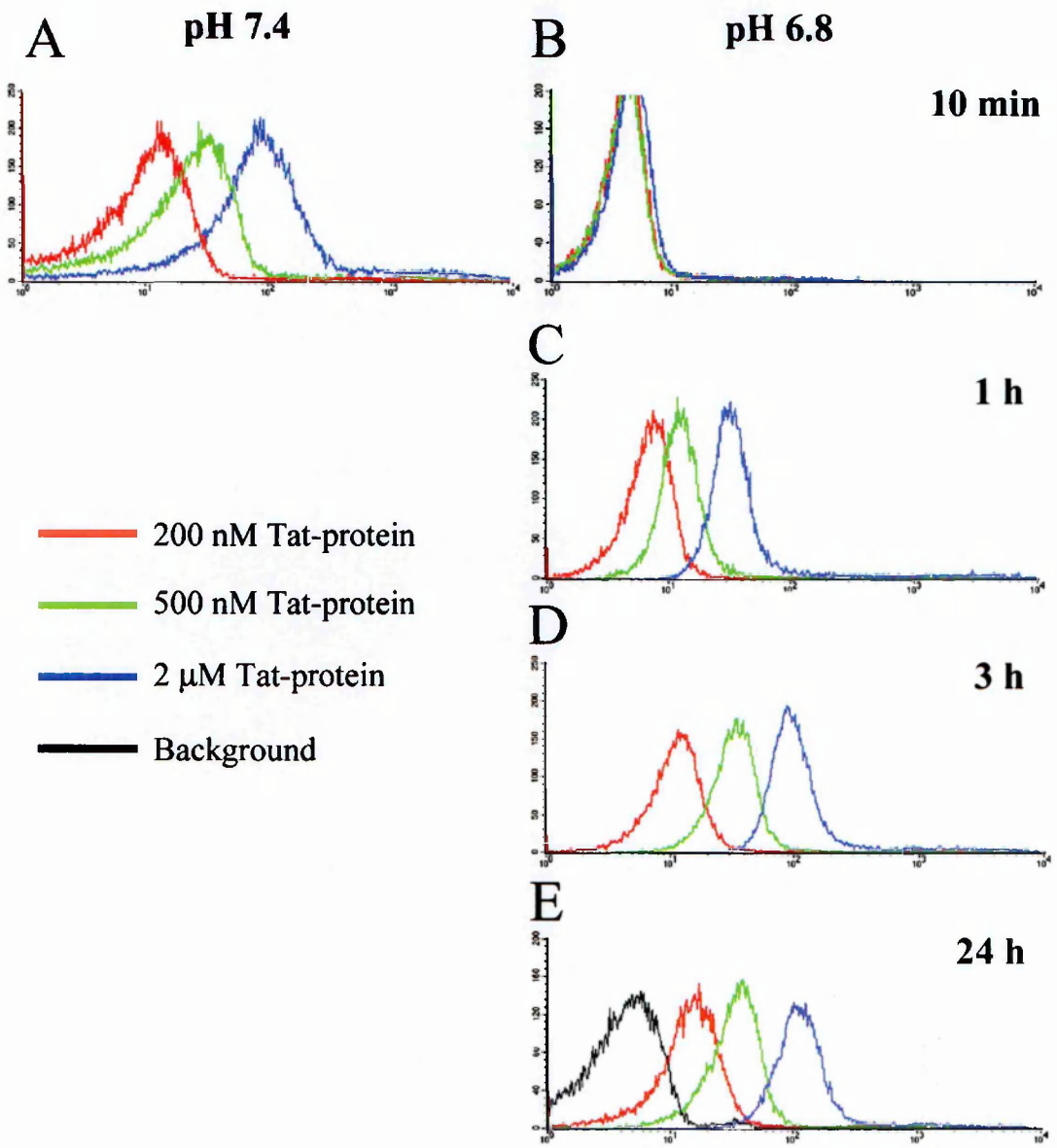
In order to test this, different concentrations of slowly desalted Tat-Zap70(SH2)$_2$-FITC were added to the medium, and primary Magnetic Activated Cell Sorter (MACS)-selected $CD4^+$ T cells were cultured in this medium for different periods of time. Figure 18 shows that after an incubation period of 10 minutes, cells are loaded on the outside, in a concentration-dependent way, with the Tat fusion proteins (Figure 18A), but no fluorescence can yet be detected inside the cells (Figure 18B). After one hour (Figure 18C), FITC-conjugated protein has entered the cell, and it can be observed that a higher extracellular fusion protein concentration correlates with a higher intracellular concentration of protein. The maximum intracellular levels are reached within 3 hours of coculture (Figure 18D), as compared with the 24 hours incubation period (Figure 18E). Identical results were obtained using FITC-labelled Tat-Lck^{NSH2} and Lck^{NSH2}-Tat.

At the tested concentrations, protein uptake does not seem to be dependent on a limited amount of binding sites, and intracellular levels reflect the extracellular levels. The uptake of the protein does take some time, however; an incubation period of three hours results in maximum levels of intracellular protein concentrations under these culture conditions.

Figure 18.

MACS-purified $CD4^+$ B10 T cells were co-cultured with indicated concentrations of FITC-conjugated slowly desalted Tat-Zap70$(SH2)_2$, thereafter cells were acquired by FACS at indicated times of 10 minutes **(A, B)**, 1 h **(C)**, 3 h **(D)**, and 24 h **(E)** at pH of 7.4 **(A)** or pH 6.8 **(B, C, D, E)**.

Dose- and time-dependent uptake of slowly desalted Tat-Zap70(SH2)$_2$



3.5.3 Functionality of slowly desalted Tat-Zap70(SH2)$_2$

Although it could be verified that Tat-PTD fusion proteins were efficiently internalised, this does not provide any information about their status. Since it is known that in order to be transported across the cell membrane the Tat-fused protein is first unfolded (Bonifaci et al., 1995), it is important to test if the fused protein is properly refolded and performs its intended function. Tat-Zap70(SH2)$_2$ is designed to compete with endogenous ZAP-70 by binding to the same phosphorylated tyrosine residues in ITAMs with its tandem SH2 domains. In order to do this, both SH2 domains should be properly refolded (Isakov et al., 1995). Properly refolded Tat-Zap70(SH2)$_2$ should then be capable of pulling down TCRζ chain from activated T cells. As shown in Figure 13, all constructs were engineered to contain an HA-tag, thus allowing specific immune-precipitation of Tat-Zap70(SH2)$_2$. Association of CD3 chains with Tat-Zap70(SH2)$_2$ versus endogenous Zap70 can then be compared.

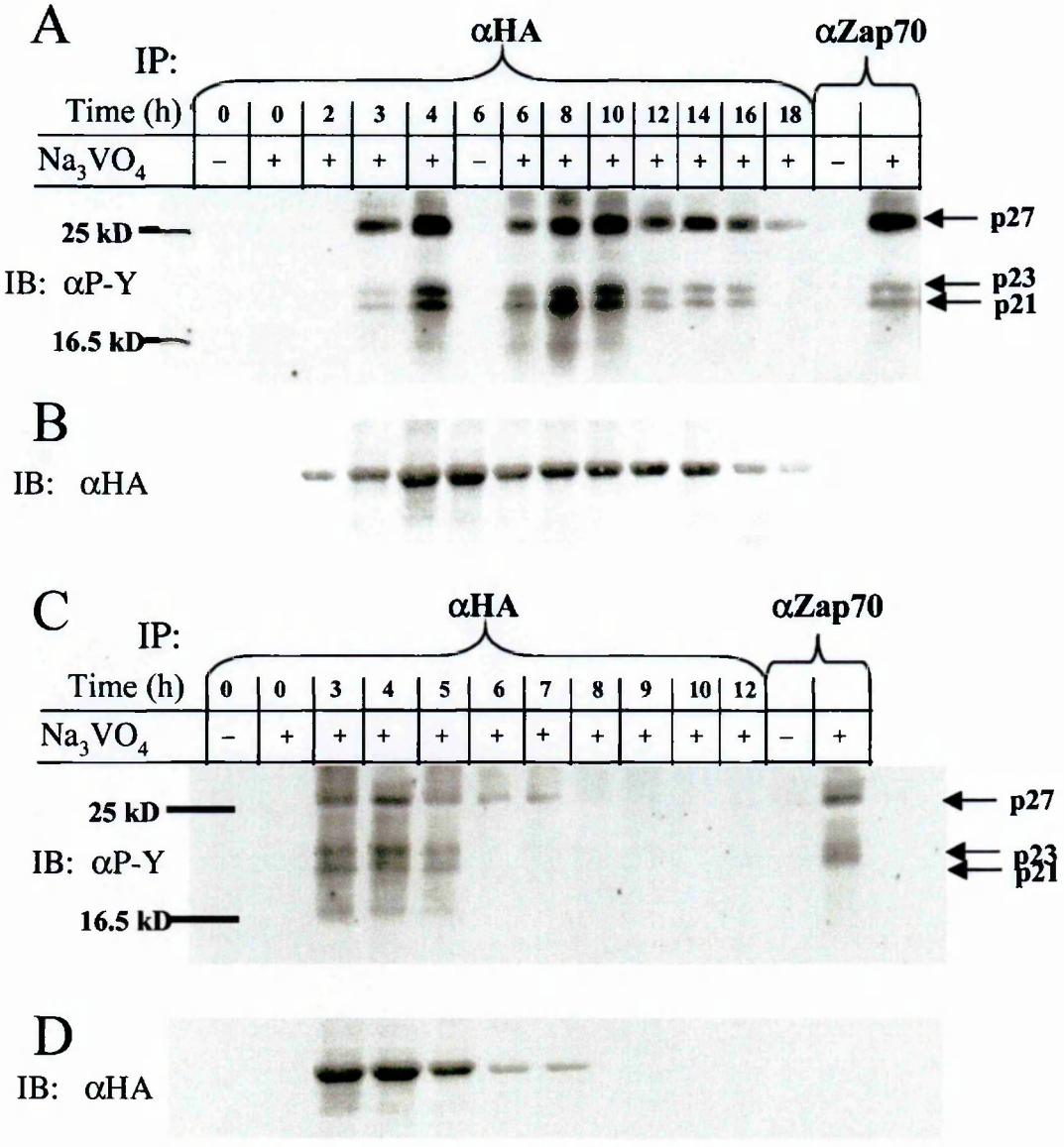
The immune-precipitations of ZAP-70 and Tat-Zap70(SH2)$_2$ in Figure 19, show identical bands at the expected sizes of p21 and p23, the two TCRζ isoforms, and p27, CD3ε. These are first detected 3 hours after coculture. Although a fraction of the fusion protein would already have been internalised by 1 hour after coculture (Figure 18), this appears to be not yet capable of binding phosphorylated ITAMs. Since the immune-precipitated protein was not equivalent for the different time points, evident from panel B, it is not possible to draw quantitative conclusions for the different time points. However, Tat-Zap70(SH2)$_2$ is clearly capable of binding phosphorylated ITAM motifs, indicating the presence of a refolded and functionally active fraction of this protein in the cells.

Figure 19.

(A) Jurkat T cells (10^8) were grown in the continuous presence (or absence) of 4 μM slowly desalted Tat-Zap70(SH2)$_2$. Cells were collected at indicated time points (in h) after the initial start of the culture, and phosphorylation was induced by 1 mM sodium ortho-vanadate (Na_3VO_4) where indicated (+). Subsequently, cells were lysed and Tat-Zap70(SH2)$_2$ and ZAP-70 and associated proteins were analysed by immune-precipitations (IPs) with anti-HA antibody (12CA5) or with anti-ZAP-70 antibody (33.1). Tyrosine-phosphorylated proteins were detected (IB) by using an anti-phosphotyrosine antibody, 4G10 (-P-Tyr), and are indicated by the arrows, and pulled-down Tat-Zap70(SH2)$_2$ was detected by using anti-His antibody **(B)**. The migration of molecular mass markers is indicated on the left. Data are representative of three experiments.

(C) Jurkat T cells (10^8) were grown in the presence of 3 μM slowly desalted Tat-Zap70(SH2)$_2$; at time point 4 hours, cells were washed and cultured in fresh medium without Tat-Zap70(SH2)$_2$. Cells were collected at indicated time points (in h) after the initial start of the culture, and phosphorylation was induced by 1 mM sodium ortho-vanadate where indicated (+). Subsequently, cells were lysed, and Tat-Zap70(SH2)$_2$ and ZAP-70 and associated proteins were analysed by IPs with anti-HA antibody (12CA5) or with anti-ZAP-70 antibody (33.1). Tyrosine-phosphorylated proteins were detected using an anti-phosphotyrosine antibody, 4G10 (-P-Tyr), and are indicated by the arrows, and pulled-down Tat-Zap70(SH2)$_2$ was detected by using anti-HA antibody **(D)**. Data are from a single experiment only.

Refolding and functionality of slowly desalted Tat-Zap70(SH2)$_2$



In the continuous presence of Tat-Zap70$(SH2)_2$ in the culture medium, coprecipitation of TCRζ can be observed up to 16 hours after addition of the fusion protein. In contrast, when the cells are washed after the initial loading period of 4 hours, the fusion protein is only capable of pulling down TCRζ for up to one hour after the wash step (Figure 19C). This suggests that the fusion protein easily leaves the cell again, or is rapidly turned over in the cytosol. This implies that future experiments require the constant presence of Tat fusion protein in the medium before and during manipulation.

3.5.4 Cell entry of rapidly desalted Tat-PTD fusion proteins

It has been reported for some Tat-PTD fusion proteins, that the kinetics of translocation across the cell membrane between fully and partially denatured proteins can be different (Nagahara et al., 1998; Caron et al., 2001). Since slowly desalting may induce the Tat-PTD fusion proteins to adopt a partially folded structure, this might have slowed down the translocation across the plasma membrane. In order to test this, newly generated rapidly desalted Tat-Lck^{NSH2} and Lck^{NSH2}-Tat were FITC-labelled and added to MACS-separated $CD4^+$ T cells. This allowed comparison of dose- and time-dependent uptake between slowly and rapidly desalted Tat-PTD fusion proteins. Figure 20 shows that after exposure of T cells to rapidly desalted FITC-labelled Tat-PTD fusion proteins, the proteins translocate across the membrane more rapidly compared with slowly desalted fusion proteins (Figure 18). The amount of protein transferred across the cytoplasmic membrane is still concentration dependent, as shown in Figure 20A and C, for Tat-Lck^{NSH2} and Lck^{NSH2}-Tat respectively. Optimal intracellular concentrations were reached after only 30 minutes of co-culture with both the N-terminally and the C-terminally placed Tat-PTD, shown in Figure 20B and D.

Dose- and time-dependent uptake of rapidly desalted Lck^{NSH2} Tat-PTD fusion proteins

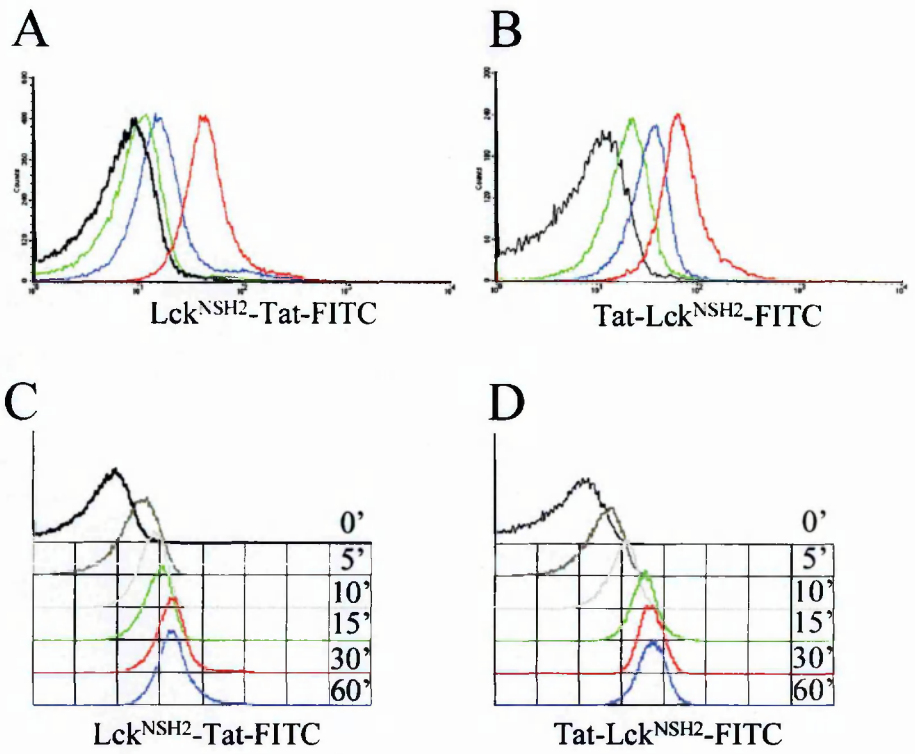


Figure 20. MACS-purified CD4$^+$ B10 T cells were co-cultured with FITC-conjugated rapidly desalted Lck^{NSH2}-Tat **(A, C)** or Tat-Lck^{NSH2} **(B, D)**, thereafter cells were washed and acquired by FACS at pH 6.8. **(A)** Cells were co-cultured with 0 nM (▬), 100 nM (▬), 250 nM (▬) and 1 µM (▬)Lck^{NSH2}-Tat, or **(B)** with 250 nM (▬), 500 nM (▬) and 1 mM (▬) Tat-Lck^{NSH2} for 1 h. **(C, D)** Cells were incubated with indicated amount of time (min) with 1 µM Lck^{NSH2}-Tat **(C)** or Tat-Lck^{NSH2} **(D)** respectively.

3.6 Effect of Tat-PTD fusion proteins on T cell differentiation

The first part of this project was completed with the generation and testing of the Tat-PTD delivery system. Tat-PTD was shown to be an inert delivery vehicle, capable of tranducing large proteins into naive T cells. In addition, Figures 18 and 19 have provided an estimate for the time restrictions in which Tat-Zap70$(SH2)_2$ protein is internalised and functionally active. It takes over an hour to be internalised, but biological activity can only be observed after 3 hours of coculture. When cells are washed after the initial loading step, the effect of Tat-Zap70$(SH2)_2$ is lost within two hours; however, if it is continuously present in the medium, biological activity can be observed up to at least 16 hours after the start of the culture in constantly dividing Jurkat T cells.

In order to evaluate the effects of the generated Tat-PTD fusion partners on signal transduction and cell response modes, we chose to start with a look at a very early event after TCR triggering; the calcium flux.

3.6.1 Calcium fluxes

The calcium-dependent pathway is turned on after TCR ligation and is essential for cytokine gene expression. Furthermore, it plays an important role in activation of differentially expressed transcription factors (Dolmetsch et al., 1997). Amongst others, calcium signalling results in nuclear translocation of the transcription factor NFAT after it has been dephosphorylated by calcineurin, a calcium-dependent phosphatase. The transcription factor NFAT is known to play a role in both the expression of the Il-4 and IFNγ genes (Kubo et al., 1994; Campbell et al., 1996).

Calcium is a ubiquitous intracellular signal responsible for controlling numerous cellular processes. At one level its action is simple; cells at rest have a low concentration of intracellular free calcium, but upon activation it rises rapidly. Calcium signals are, however, capable of regulating many processes due to their versatility in terms of speed, amplitude and spatio-temporal patterning. More

variations are achieved through the interactions of calcium with other signalling pathways.

Calcium signals are directly downstream of ZAP-70, which upon recruitment to the TCR and phosphorylation by Lck, phosphorylates LAT. LAT provides the scaffold through which multiple signalling molecules, such as PLCγ1, can be recruited. Upon tyrosine phosphorylation PLCγ1 becomes a major determinant for calcium signalling, requiring the activity of all three PTKs, Lck, ZAP-70 and Itk (Kurosaki and Tsukada, 2000). Since Tat-PTD fusion proteins were made with the purpose of interfering with the endogenous PTKs: Lck and ZAP-70, initial tests were focused on its influence on calcium fluxes in naive T cells.

Figure 21A shows the dose-response curve of CD3-stimulated $CD4^+$ B10 lymphocytes. It reveals that anti-CD3 stimulus with doses above 1 μg/ml results in a maximum response in Il-2 production. In order to reveal a possible difference in calcium signalling in the presence of Tat-PTD fusion proteins, sub-optimal concentrations of the anti-CD3 stimulus were used to maximise the chance of observing an effect induced by the fusion proteins. Three concentrations were chosen: an optimum stimulus of 5 μg/ml, as a positive control, and the two sub-optimum concentrations of 1 μg/ml and 0.3 μg/ml.

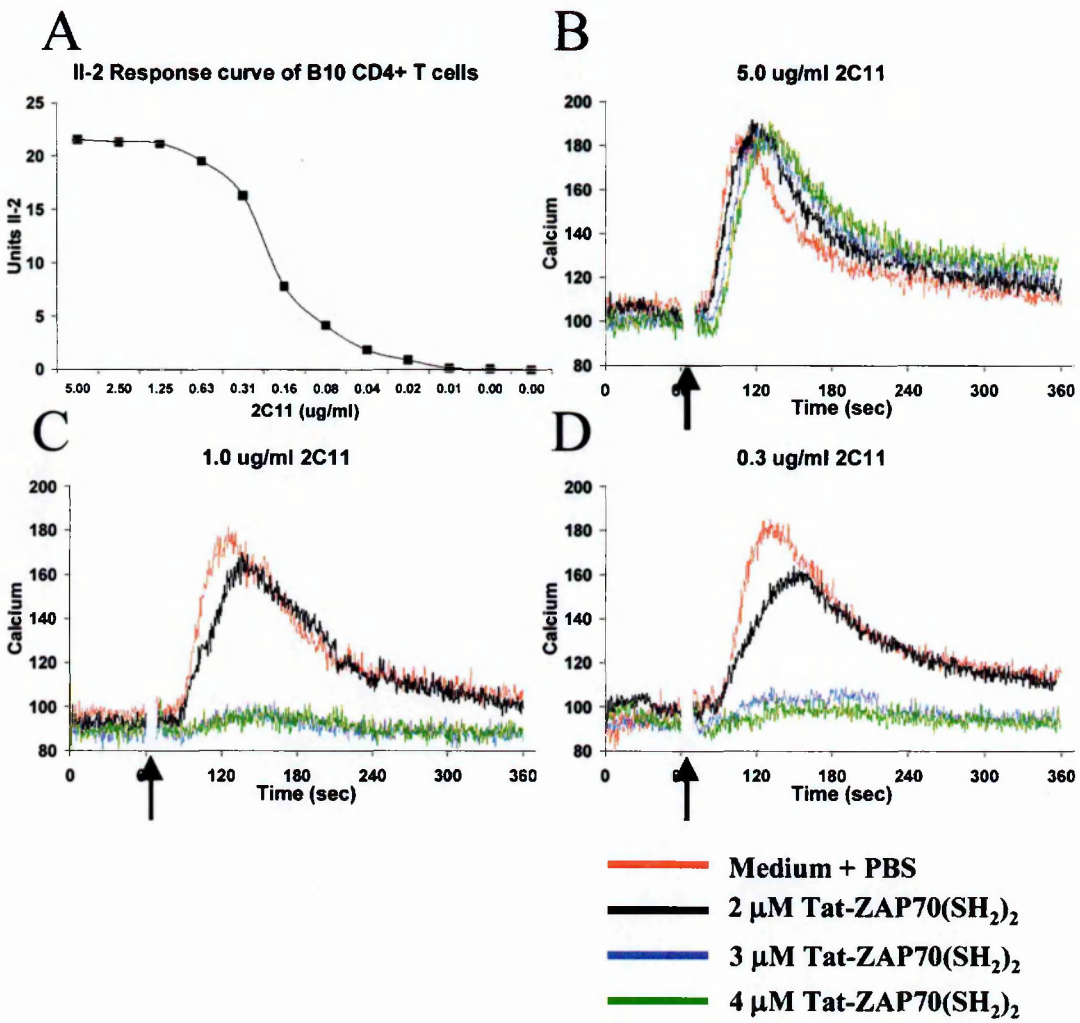
Stimuli with 5 μg/ml anti-CD3 resulted in an amplitude of calcium flux equal in all T cell cultures regardless of the presence of Tat-Zap70$(SH2)_2$ fusion protein (Figure 21B). However, in the presence of increasing concentrations of Tat-Zap70$(SH2)_2$, a slight concentration-dependent shift in timing could reproducibly be observed. Lowering the stimulus to 1 μg/ml anti-CD3 (Figure 21C), showed a profound effect of the fusion protein. At the 2 μM concentration of Tat-Zap70$(SH2)_2$ there was a small delay in reaching the maximum flux, and the maximum amplitude reached was lower. This effect was even more dramatic upon lowering the TCR stimulus to 0.3 μg/ml (Figure 21D). At Tat-Zap70$(SH2)_2$ concentrations of 3 μM and higher the calcium flux was completely lost when a sub-optimal stimulus was delivered (Figure 21C, D).

Figure 21.

(A) $CD4^+$ MACS-selected B10 T cells were incubated for 4 hours on plates coated with indicated amounts of anti-CD3 antibody (2C11), and 10 μg/ml anti-CD28 (37.51). Recovered cells were cultured overnight, and Il-2 concentrations in the medium were determined by a CTLL assay. Results are representative of two independent tests.

(B, C, D) $CD4^+$ MACS-selected B10 T cells were incubated for 4 hours with indicated amounts of slowly desalted Tat-Zap70$(SH2)_2$, and stained with PE-conjugated anti-CD4, labelled with non-conjugated anti-CD3 (2C11) in concentrations of 5 μg/ml **(B)**, 1 μg/ml **(C)** and 0.3 μg/ml **(D)**, and loaded with indo-1. Baselines for intracellular calcium levels were established for 1 minute after which a calcium flux was induced with anti-Armenian Hamster crosslinking antibody, indicated by an arrow, and subsequently recorded for a further 5 minutes. Results are representative of three independent tests.

Influence of slowly desalted Tat-Zap70(SH2)$_2$ on calcium signalling



After the observations of an altered calcium flux in the presence of the Tat-PTD fusion protein Tat-Zap70$(SH2)_2$, the kinase-truncated version of Lck was tested in the same way. As depicted in Figure 13, two constructs were generated; Tat-Lck^{NSH2}, containing Tat-PTD and the His-tag at the N-terminus, thereby inhibiting the recruitment of this protein to the plasma membrane, and Lck^{NSH2}-Tat, containing all tags and the Tat-PTD at the C-terminus, allowing the free unique N-terminus to direct this protein to the plasma membrane. Tat-Lck^{NSH2} could therefore serve as a diabled protein control for Lck^{NSH2}-Tat.

As shown in Figure 22, Tat-Lck^{NSH2} had no effect on calcium flux when cocultured with MACS-selected $CD4^+$ B10 T cells at concentrations of 1 and 2 μM. No effect was noticed when cells were stained with decreasing concentrations of anti-CD3, from 5 μg/ml (Figure 22A), 1 μg/ml (Figure 22B) and 0.3 μg/ml (Figure 22C). Lck^{NSH2}-Tat, however, produced a dramatic decrease in calcium flux at a concentration of 1 μM, and even more prominent at 2 μM. This effect seems independent of the concentration of anti-CD3 used, identical profiles could be observed using 5 μg/ml (Figure 22A), 1 μg/ml (Figure 22B) and 0.3 μg/ml (Figure 22C) anti-CD3. Anti-CD3 is incapable of triggering a full calcium flux in the presence of Lck^{NSH2}-Tat but as shown in Figure 22D, the lipophilic calcium ionophore ionomycin, capable of circumventing the proximal signalling molecules by directly transporting calcium out of the ER, can overcome this inhibition.

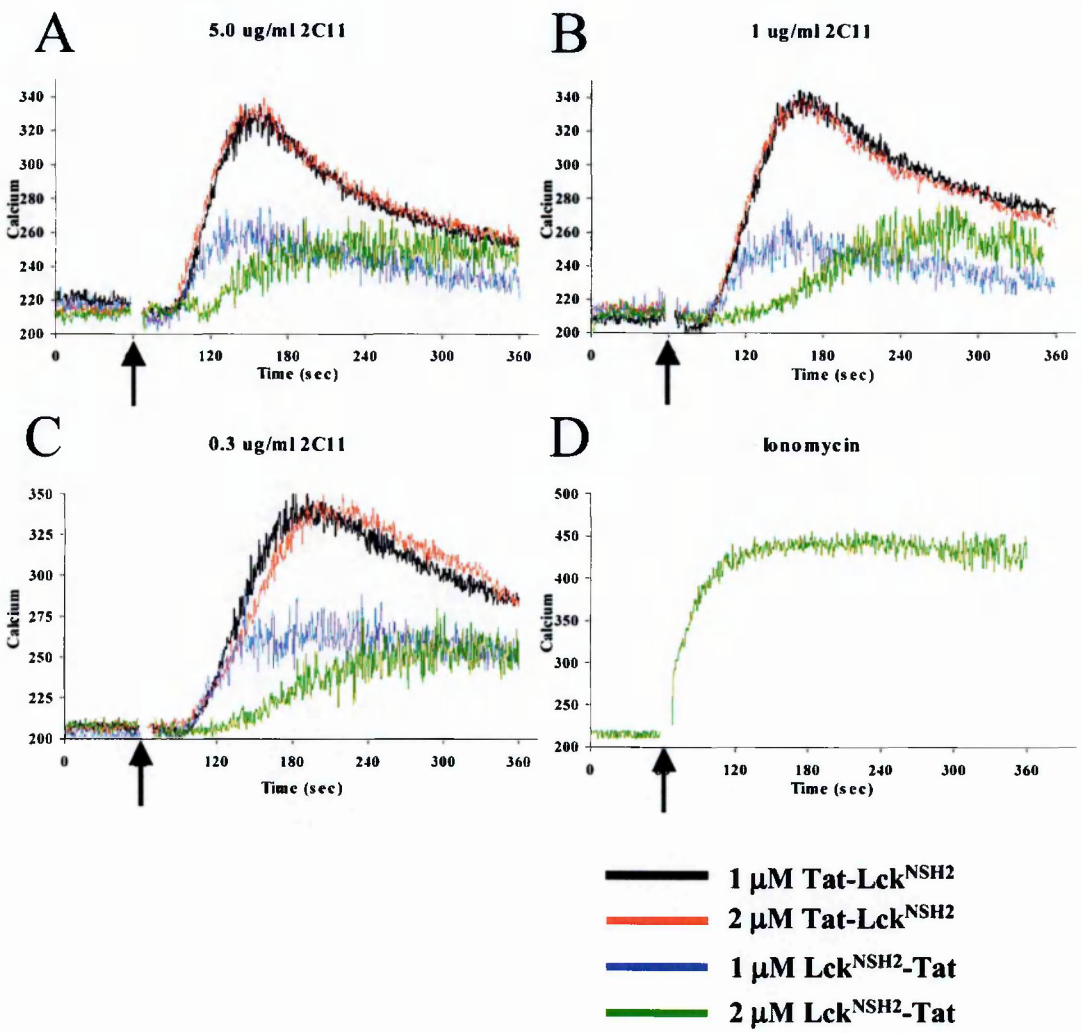
3.6.2 T cell differentiation

Since Lck^{NSH2}-Tat showed a profound effect on intracellular free calcium levels after anti-CD3 stimulation, and Tat-Lck^{NSH2} could serve as a disabled control fusion protein, these fusion proteins were tested for their ability to influence T cell differentiation. MACS-selected $CD4^+$ B10 T cells were incubated with 1 or 2 μM fusion protein for 4 hours, whereafter cells were stimulated with plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (10 μg/ml) for 4 hours. Subsequently, collection of the cells and culturing them on new plates separated the cells from the stimulus. Proliferation was tested by the Alamar-blue-method. Figure 23A shows that

Figure 22.

(A, B, C, D) $CD4^+$ MACS-selected B10 T cells were incubated for 4 hours with indicated amounts of slowly desalted Tat-Lck^{NSH2} (—, —), or Lck^{NSH2}-Tat (—, —), and stained with PE-conjugated anti-CD4, labelled with non-conjugated anti-CD3 (2C11) in concentrations of 5 μg/ml **(A)**, 1 μg/ml **(B)** and 0.3 μg/ml **(C)**, and loaded with indo-1. Baselines for intracellular calcium levels were established for 1 minute after which a calcium flux was induced with a crosslinking antibody, indicated by an arrow, which was subsequently recorded for a further 5 minutes. **(D)** As a control for their calcium signalling capacity, cells pretreated with 2 μM Lck^{NSH2}-Tat were exposed to 50 nM ionomycin.

Influence of slowly desalted Lck^{NSH2} Tat fusion proteins on calcium signalling



proliferation was impaired and at background level in cells pre-treated with 2 μM Lck^{NSH2}-Tat, but not different from medium controls in the presence of the control protein Tat-Lck^{NSH2}.

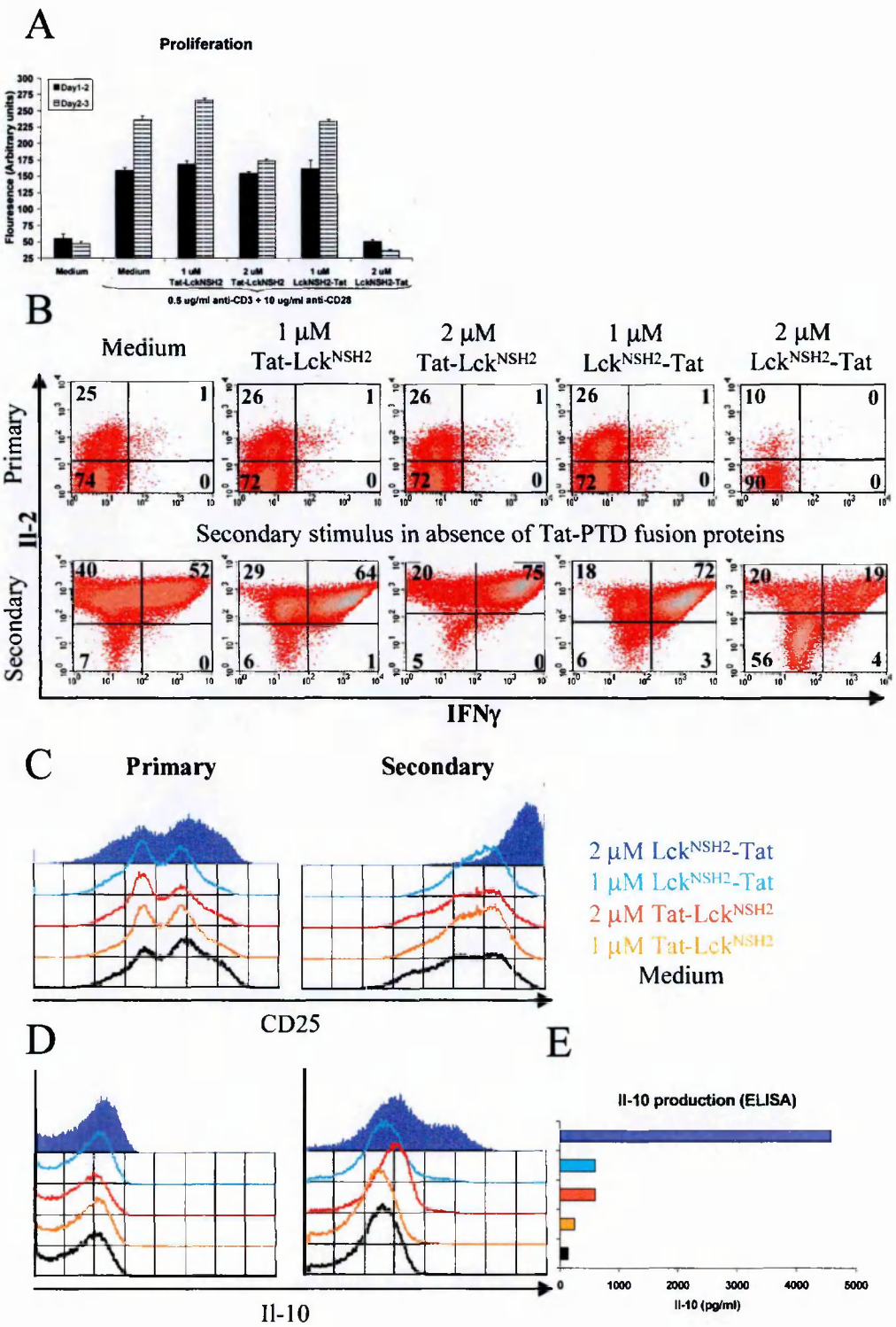
Levels of the surface markers CD69, CD44, CD25, CD4 and TCR were assessed on three days after the primary stimulus. In cells that had been exposed to 2 μM Lck^{NSH2}-Tat a delayed response was apparent. The early activation marker CD69 increased more slowly compared with the other conditions, and did not reach maximum levels. Upregulation of the late activation marker CD44 was also delayed (data not shown). The left panel of Figure 23C shows that no differences were observed in CD25 expression after the primary stimulus. However, a difference in the proportion of Il-2 producing cells was noticed (Figure 23B top row) by intracellular FACS-analysis at day 6 after primary stimulus. Cultures that had received their CD3 stimulus in the presence of 2 μM Lck^{NSH2}-Tat developed about 60% fewer Il-2 producing cells. Little IFNγ (Figure 23B top row) and no Il-4 (data not shown) and Il-10 (Figure 23D left panel) producing cells were observed after the primary stimulation.

Cells were collected at day 7 after primary stimulus, and rechallenged with plate-bound anti-CD3 (1.0 μg/ml) and anti-CD28 (10 μg/ml) for 8 hours in the absence of any fusion protein. Thereafter, cytokine profiles and cell surface marker expression were tested 5 days after the secondary stimulus. Figure 23B (bottom row) shows that the majority of cells that were originally stimulated in the presence of 2 μM Lck^{NSH2}-Tat failed to produce Il-2 and IFNγ upon restimulation, while the majority of cells in all other conditions produced Il-2 and in addition 50 to 70% produced IFNγ. Figure 23C and E (right panels) show that those cells that were primed in the presence of 2 μM Lck^{NSH2}-Tat showed increased levels of CD25, and a considerable fraction (23%) produced Il-10 upon rechallenging in the absence of any Tat-PTD fusion proteins. The production of Il-10 was confirmed by an Il-10 ELISA using the supernatants at day 5 after rechallenging of cultured cells (Figure 23E).

Figure 23.

$CD4^+$ MACS-selected B10 T cells were incubated for 4 hours prior to the primary stimulus only with indicated amounts of slowly desalted Tat-Lck^{NSH2}, or Lck^{NSH2}-Tat where indicated, wherafter cells were stimulated (primary stimulation) with plate-bound 0.5 µg/ml anti-CD3 (2C11) and 10 µg/ml anti-CD28 (37.51). At day 7 after primary stimulation, cells were collected and restimulated (secondary stimulus) with plate-bound 1.0 µg/ml anti-CD3 (2C11) and 10 µg/ml anti-CD28 (37.51). **(A)** After the primary stimulation, proliferative responses were measured by the Alamar-blue method, o/n after Day 1 and Day 2. The panel shows mean values ±SD. **(B)** Cells were restimulated with 500 ng/ml Ionomycin, 500 ng/ml PdBu and 10 µg/ml BrefeldinA for 4 hours, and their cytokine profile was determined by intracellular FACS analysis at day 6 after primary stimulus (upper panels) and day 5 after secondary stimulus (lower panels). Plotted is Il-2 against IFNγ, whereby the percentage of cells is given in each quadrant. **(C)** Cells were stained for extracellular markers; shown here is their CD25 profile 6 days after the primary and 5 days after the secondary stimulus. **(D)** Cell profiles for Il-10 at day 6 after primary stimulation, and day 5 after secondary stimulation. **(E)** At day 5 after secondary stimulus, supernatants were tested for their Il-10 concentration by ELISA.

Influence of Lck^{NSH2} Tat-PTD fusion proteins on T cell differentiation



"For those who believe no explanation is necessary. For those who do not none will suffice." (Dunninger)

4. Discussion

Signalling events have been well studied in T cell hybridomas and T cell lines, however, events in immortalised cells may not reflect the *in vivo* situation (Astoul et al., 2001), and their use greatly limits functional assessment of T cell modes of action. It is as yet poorly understood how changes in TCR-mediated signalling and its downstream signalling cascades drive the differentiation of the naive T cell into different response modes. The aim of this project, therefore, was to target *ex vivo* T cell populations with signalling attenuators and subsequently study the effect on T cell differentiation *in vitro*.

The use of *ex vivo* T cells, however, greatly limited the manipulation strategy. Naive T cells are known to have a very short half-life *in vitro* due to high levels of programmed cell death (Perandones et al., 1993), which limits the use of a genetic delivery strategy. The ability to express proteins that can alter cellular phenotypes has been largely limited to recombinant genetic approaches in the past. The circumvention of genetic approaches by the delivery of full-length proteins directly into cells is problematic due to the restrictions imposed by the cell membrane. Proteins can, nevertheless, cross this barrier either after transient membrane permeabilisation, or by endocytosis involving surface interactions, receptors and vesicular compartments. Primary T cells, however, restrict the use of membrane permeabilisation methods due to their fragility.

Certain naturally occurring macromolecules enter cells through an active transport mechanism, termed receptor-based endocytosis. One such group of proteins, known as AB-toxins, are highly toxic to mammalian cells because of their ability to enter the cytosol and attack essential constituents. The delivery of compounds imported through receptor-based endocytosis is selective, but limited to cells expressing a particular receptor. Furthermore, the receptor determines the rate of compound uptake, and often targets delivery to an organelle.

An alternative to receptor-based endocytosis is a poorly understood mechanism, independent of receptors, transporters and endocytosis, termed adsorptive endocytosis or non-chiral receptor mechanism. Although this technology was originally described in 1988, little was reported in the subsequent 10 years. In the last few years, significant steps have been taken to advance this technology into a broadly applicable method that allows rapid introduction of proteins into primary cells.

The potential of two delivery vehicles was investigated during this study; the B subunit of *E.coli* enterotoxin (EtxB) that enters cells via receptor-based endocytosis and the protein transduction domain of the HIV-1 Tat protein (Tat-PTD) that gains entry via adsorptive endocytosis.

Can EtxB serve as an inert delivery vehicle to target ex vivo T cells?

The mucosal adjuvanticity of cholera- and enterotoxin and the potential of their B subunits to serve as an oral vaccine carrier have prompted interest in coupling of immunogenic peptides to these proteins (Dertzbaugh and Elson, 1993; O'Dowd et al., 1999). In addition, it was shown that the B subunit is capable of successful delivery of short peptides into eukaryotic cells (Loregian et al., 1996). This initiated our attempt to explore the possibility of using the B subunit of *E. coli* heat-labile enterotoxin as a recombinant carrier for receptor-mediated delivery of larger protein domains into *ex vivo* T cells.

After confirming binding of the EtxB homologue CtxB to naive T cells, experiments focussed on the effect of CtxB on T cell physiology. From the start, a most striking effect was found on T cell numbers upon addition of CtxB to *in vitro* T cell cultures. A closer look at T cell survival rates and apoptosis revealed a dramatic increase in levels of apoptosis when T cells were cultured in the presence of CtxB. This induction of high levels of apoptosis after a short exposure to CtxB excluded the use of CtxB as an inert delivery vehicle, making it unsuitable for the intended aims of this study. The induction of apoptosis may, however, offer an explanation for the

reported inhibitory effect of CtxB/EtxB on T cell activation (Francis et al., 1990; Woogen et al., 1993), and the depletion of CD8+ T cells in *in vitro* cultures (Nashar et al., 1997).

Ctx and Etx are recognised as two of the most potent mucosal adjuvants identified, able to greatly enhance antibody responses to co-administered antigens. However, their toxicity precluded usage in human vaccines. Although earlier studies suggested that adjuvant activities were completely dependent on the ability of the catalytic A subunit to raise intracellular cAMP levels, it is now well established that this is not the case. G_{M1} binding was found essential and responsible for both immunogenicity and adjuvant activity in the absence of the A subunit (Guidry et al., 1997; de Haan et al., 1998). The adjuvant potency of CtxB/EtxB, measured by the humoral response to co-administered antigens, can be solely attributed to CtxB/EtxB binding to APCs. It has been shown that CtxB activates DCs and B cells via crosslinking of G_{M1}, thereby increasing the expression of costimulatory molecules and the production of antigens (Li and Fox, 1996; Nashar et al., 1997). This raised the possibility of using the B subunit pentamer as a strong adjuvant, but data reported here and by others suggest that also the B subunit alone still possesses strong lymphotoxic properties. This might greatly limit the use of CtxB/EtxB as an immunoadjuvant for inclusion in human vaccines.

What causes the dramatic effect of CtxB on T cell apoptosis?

Cholera toxin was known to have immuno-modulating effects, but this was initially solely attributed to the A subunit's enzymatic activity (Imboden et al., 1986; Gray et al., 1988) and to depend on cell type and experimental system used. Rat, but not mouse, thymocytes were shown to be stimulated upon binding of CtxB, possibly via crosslinking of G_{M1} by the multivalent B subunit (Spiegel et al., 1985). In a later study, the effect of CtxB was shown to include acute changes in the concentration of cytoplasmic free calcium. Since this effect was absent in calcium-free medium, this

suggested that CtxB does not induce a release of calcium from intracellular stores, consistent with the observation that inositol trisphosphate was not produced upon CtxB binding, and that PKC and DAG levels were unaffected. Earlier criticism of A subunit contamination, resulting in the observed effects, was ruled out by the absence of increased cAMP levels. Similar results were obtained using human B cells (Dugas et al., 1991).

In the human Jurkat T cell line, however, an increase in intracellular free calcium concentrations could only be observed upon crosslinking surface-bound CtxB with anti-CtxB antibodies (Gouy et al., 1994). This calcium response was shown to involve both extra- and intracellular calcium, and to partially deplete the CD3-dependent and inositol trisphosphate-sensitive intracellular calcium pool and to result in Il-2 production. Tyrosine phosphorylation of PLCγ-1 could only be observed in Jurkat cells that express high levels of G_{M1}.

The reason for the dramatic effect on apoptosis levels in *ex vivo* murine T cells reported in this study might be related to the mechanism by which CtxB gains entry to the cytosol. The pentamer recognises, binds to and subsequently clusters the pentasaccharide chain of ganglioside G_{M1}, present in high amounts on the T cell membrane. The physiological function of G_{M1} is not well understood, but it has been implicated in various signal transduction pathways depending on cell type and experimental system used. CtxB has previously been shown to contribute to activation of TCR-mediated signalling via raft aggregation (Janes et al., 1999; Janes et al., 2000), suggesting that the pro-apoptotic effect seen here may be due to inappropriate TCR signalling in primary T cells. These various signals, amongst others, include calcium release and phosphorylation events (Dixon et al., 1987; Duchemin et al., 2002; Fang et al., 2002). Recent data have shown that crosslinking of G_{M1} directly activates a pre-existing calcium channel (Fang et al., 2002). The observed increase of the early T cell activation marker CD69, most prominently seen after additional crosslinking of G_{M1} with anti CtxB antibodies in this study, may well reflect this increase of cytoplasmic free calcium (D'Ambrosio et al., 1994).

Upon receptor binding of the toxin, entry to the cytosol is allowed. This involves endocytosis of the holotoxin-receptor complex, stimulated by the binding of the B subunit pentamer to G_{M1} (Tsai et al., 2001). G_{M1} is found in the raft microdomains on T cells, and CtxB is routinely used to make these visible. Recent insights into Cholera toxin entry have demonstrated that binding to G_{M1} anchors the toxin to rafts, whereby cholesterol and sphingolipids play an important role in trafficking the toxin into the Golgi system (Wolf et al., 2002).

Membrane sphingolipids constitute a unique signalling pathway that links surface receptors to the nucleus, with ceramide and other sphingomyelin products serving as second messengers. Models for glycosphingolipid-induced apoptosis generally favour an endocytotic vesicle pathway with ceramide release from vesicle compartments (Gulbins et al., 1995; Hannun and Obeid, 1995). Breakdown products, like ceramide and sphingosine act as anti-proliferative agents and regulators of apoptosis. Thus, the apoptosis induced by CtxB may be the result of the entry mechanism of CtxB, triggered by binding to G_{M1}, and the direct result of products generated by the lipid metabolism involved (Janes et al., 1999). In addition, second messengers derived from ceramide and sphingomyelin, in conjunction with a rise in intracellular calcium levels, may stimulate the PKC/Ras pathway and subsequent expression of CD69.

$CD8^+$ T cells were more susceptible to apoptosis following treatment with CtxB than $CD4^+$ T cells. This might reflect the capacity of $CD8^+$ T cells to bind twice the amount of CtxB, compared with CD4+ T cells, as was observed here and previously reported (Elson et al., 1995). The reason why CD8+ T cells contain almost twice as much G_{M1}, and are more sensitive to apoptosis, is poorly understood. It might reflect a possible difference in their raft composition. It is known that activated T cells always show higher levels of G_{M1} expression than resting T cells, due to *de novo* synthesis induced by T cell activation (Tuosto et al., 2001). The synthesis of raft components allows effector cells to be more responsive than naive T cells. $CD4^+$ and $CD8^+$ T cells are also known to possess different proliferative responses (Foulds et

al., 2002). $CD8^+$ T cells become committed to differentiating fully into effector cells rapidly upon stimulation and respond with substantial proliferation (van Stipdonk et al., 2001), while $CD4^+$ T cells differentiate into distinct sets of polarised cells and show a reduced proliferative capacity compared with $CD8^+$ T cells. The differences in proliferative patterns and clonal expansion of $CD4^+$ and $CD8^+$ T cells may result from the composition of their respective rafts, indicated by different expression of G_{M1}.

Can Tat-PTD serve as an inert delivery vehicle to target ex vivo T cells?

Since EtxB could not be used as a delivery vehicle, attention was turned to protein transfer domains. Of the three best-characterised PTDs, Tat-PTD was found most suitable for this project. The protein transfer technique was extensively studied with help of Antp; its domain responsible for protein transduction was identified, and amino acid substitution resulted in a number of PTD peptides together termed the penetratin system (Derossi et al., 1998). However, these peptides have only shown the capacity to deliver short peptide sequences up to 100 amino acids long (Derossi et al., 1998), while this study aimed at the delivery of large protein domains and full-length proteins. Tat-PTD was shown to be capable of transducing large proteins, including β-galactosidase, caspase-3, Rho-A, and superoxide dismutase, (Vocero-Akbani et al., 1999; Barka et al., 2000; Chellaiah et al., 2000; Kwon et al., 2000). Furthermore, the technique, using Tat-PTD, was reported to be capable of delivering biologically active proteins into the mouse by IP-injections, to all tissues, including the brain (Schwarze et al., 1999).

The main advantage of this internalisation procedure is the low toxicity and high uptake by all tested cell types. The cellular internalisation occurs at 4°C and 37°C and cannot be saturated, strongly suggesting a transduction mechanism that is energy- and receptor-independent (Joliot et al., 1991; Derossi et al., 1996; Derossi et al., 1998). However, it is not known whether there is an upper size limit, and if internalisation rate and bioactivity are correlated with the complexity of the cargo. In

addition, the highly positively charged residues may result in lack of solubility of the fusion proteins.

As a transcripton factor, Tat is also able to trans-activate other cellular genes, including those encoding cytokines like TNFα, Il-2 and Il-6 (Buonaguro et al., 1992; Scala et al., 1994; Westendorp et al., 1994), and cell survival-related proteins like p53 and Bcl-2 (Li et al., 1995; Zauli et al., 1995). Several reports also showed an inhibitory effect of the full-length Tat protein on T lymphocyte activation, proliferation and gene transcription (Viscidi et al., 1989; Helland et al., 1991; Ensoli et al., 1993; Holloway et al., 2000). This, however, did not exclude the possible use of the Tat-PTD as an inert delivery vehicle for proteins into T cells. The cysteine-rich region of Tat mediates the formation of metal-linked dimers, and is essential for its function (Garcia et al., 1988; Ruben et al., 1989). The delivery vehicle Tat-PTD only contains the 11 amino acids basic region, and lacks the essential regions necessary for dimerisation and transcription activity. At the start of this project, the effect of Tat-PTD on T cells was largely unknown, and was thus the focus of initial experiments.

The short peptide, comprising of the 11 amino acids of Tat-PTD and a 9 amino acid HA-tag, did not influence the basic expression levels of activation markers, CD4, CD8 and TCR, nor was any effect seen on basal levels of apoptosis in these *in vitro* T cell cultures. The presence of 10 μM Tat-PTD in the culture medium during priming with specific antigen or anti-CD3 did not result in any alteration of the expression levels of cell surface markers nor the kinetics of their expression compared with medium controls. In addition, no influence of Tat-PTD on T cell proliferation, and on kinetics and levels of secretion of the cytokines Il-2 and IFNγ could be observed.

These results were not unexpected, since Tat-PTD consists only of the basic region of full-length Tat, which requires other domains for its suppressive activity on T cell activation and its influence on gene transcription. The absence of any influence

of Tat-PTD on the parameters tested meant that it fulfilled the first criterion to serve as an inert vehicle for protein delivery into naive T cells.

Can Tat-PTD deliver large proteins across the naive T cell membrane?

The second criterion important for the potential usefulness of Tat-PTD was the capacity to deliver large protein fragments across the cell membrane of naive *ex vivo* T cells. Full-length Lck and protein domains from the signalling molecules Lck and ZAP70, involved in TCR-mediated proximal signalling processes, were genetically fused to Tat-PTD and expressed in Bl-21 *E.coli.* Sonication of bacterial pellets in 8M urea or 6M guanidine HCl allowed the disaggregation of all insoluble protein into a soluble form. The use of denaturant produced the highest yield of protein for purification, especially since all tested fusion proteins were expressed in inclusion bodies. An additional effect of using a denaturant was that purified proteins were unfolded, which enhanced their ability to transduce across the cell membrane for most previously reported Tat-fusion proteins (Nagahara et al., 1998). Only minor optimisation was required to purify high yields of fusion proteins that were pure by Coomassie Blue gel analysis from the denatured fraction.

The major pitfall experienced when preparing Tat-fusion proteins was the high occurrence of precipitation of the unfolded proteins after placing them into aqueous buffers. Since these fusion proteins were intended for treatment of *ex vivo* naive T cells, a very fragile cell type, any denaturant present had to be removed, and the proteins had to be solubilised into a physiological buffer. The original protocol achieves this by rapid removal of the denaturant, yielding Tat-fusion proteins with higher transduction efficiency than those prepared under native conditions (Nagahara et al., 1998).

Rapid removal of 8M urea by PD-10 desalting columns was successfully reported before, although it lead to inferior yields compared with ion-exchange

chromatography (Vocero-Akbani et al., 2001). Trials with the fusion proteins generated in this study, however, failed to yield soluble protein during PD-10 desalting. Removal of protein precipitation upon aggregation, by centrifugation or filtering, thereby reducing the precipitation cascade, did not improve the protein yield. Since some proteins may precipitate at high concentrations after elution, several dilution steps were introduced, which only marginally improved protein yields. The same was observed with attempts to reduce hydrophobic interactions by supplementing the buffers with glycerol, sucrose or glucose, by preventing ionic interactions with high salt concentrations and by enhancing protein solubility by the addition of the amino acids; arginine and glycine. The yield of soluble protein obtained with this method of desalting greatly depended on the properties of the protein of interest.

Initially, comparable results were obtained for all generated fusion proteins with rapid desalting strategies. Structurally the proteins share some homology, they are derived from highly conserved PTKs and share a highly conserved SH2 domain. None of the previously reported Tat-fusion proteins contained such a domain, and solubility conditions needed to be established empirically. Using a slow desalting method, it was found that the longer and the more complex the fusion partner, the more difficult it was to keep the proteins soluble, and the smaller the yield of soluble protein obtained. During the course of this project it proved to be impossible to obtain sufficient quantities of soluble Tat-Lck^{wt}, containing a SH1, SH2, and SH3 domain, and allowing further experiments, while the smallest construct, Tat-Lck^{SH2} consisting of the SH2 domain only, proved to be the most successful in initial trials using methods of slow desalting.

Since rapid desalting did not result in sufficient quantities of soluble fusion proteins, a slow desalting strategy was followed. A similar strategy was also applied to the purification of Tat-Ras, where 8M urea was removed via dialysis and dilution-steps (Hall et al., 2001; Myou et al., 2002). Sufficient amounts of soluble fusion proteins, for several experiments, could be obtained. However, a concern about using

a slow desalting method was that proteins might re-adopt their native or partly native structure, which could compromise their transduction across the cell membrane.

The internalisation of partly denatured Tat-eGFP in several cell types was reported to be decreased in efficiency and speed compared with completely denatured fusion proteins (Caron et al., 2001). Rapid removal of the denaturant creates denatured Tat-PTD fusion proteins that may have a higher potential of functional activity than those prepared under slow folding techniques. The absence of a biological response with some native Tat-fusion proteins may reflect this inefficiency of native protein to achieve required intracellular concentrations to show a response. It was reported that, prior to internalisation, Tat-fusion proteins undergo an unfolding step (Bonifaci et al., 1995), which implies that proteins with higher order minimal structure and with high entropy levels are more efficiently transduced than low entropy correctly folded ones. This was also illustrated with the use of eGFP; not only was the internalisation process slower, the protein lost its fluorescence during the translocation process.

In this study, slowly desalted Tat-PTD fusion proteins containing $Zap70(SH2)_2$ or Lck^{NSH2} were conjugated to the fluorochrome FITC. Transduction across the plasma membrane could be shown by FACS analysis whereby extracellular fusion protein was quenched with a low pH or by digestion with trypsin. As reported for other Tat-PTD fusion proteins, close to 100% of cells were transduced with FITC-conjugated fusion protein in this study. In addition, as indicated by the narrow width of the FACS histogram peak, all cells received similar amounts of fusion protein.

Subsequently, fusion protein uptake by *ex vivo* T cells was followed over several hours. The optimum intracellular concentration of FITC-labelled fusion protein was established around three hours of coculture, much slower than previously reported with other Tat-PTD fusion partners, where maximum levels of intracellular protein concentrations were reached between 5 and 60 minutes (Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999; Barka et al., 2000; Chellaiah et al.,

2000; Kwon et al., 2000; Hall et al., 2001; Myou et al., 2002). The results are in accordance with reports making use of a similar approach, whereby extracellular bound protein is quenched (Hall et al., 2001). Reports claiming more rapid transduction, however, did not exclude extracellular bound protein (Nagahara et al., 1998; Vocero-Akbani et al., 1999). The observed delay in transduction corresponded with the onset of functionality of Tat-Zap70$(SH2)_2$, which was only capable of co-precipitating TCRζ and CD3ε after an incubation time of at least 3 hours. For most previously described Tat-PTD fusion partners, functionality was observed correlating with the moment a maximum level of intracellular protein concentration was reached, except for eGFP, which reaches this only after 14 hours.

The delay in fusion protein crossing the cell membrane found in this study, compared with previous reports for other proteins might have reflected a different folding state, caused by the slow desalting method. The slow dilution method might prompt proteins to adopt a partly native structure or at least a more complex one than in the fully denatured state. Comparisons between denatured and native protein in their ability to induce biological responses have shown that denatured protein readily induced cellular responses at low concentrations, while native protein failed to do so even at the highest concentrations tested (Nagahara et al., 1998).

The protein transduction process in this study as reported by others was found to be concentration dependent (Nagahara et al., 1998; Schwarze et al., 1999; Vocero-Akbani et al., 1999; Barka et al., 2000; Chellaiah et al., 2000; Kwon et al., 2000; Hall et al., 2001; Myou et al., 2002). When the extracellular protein concentration is changed, Tat-PTD fusion proteins are reported to shift the equilibrium and subsequently transduce back out of the cells, as was also the case with fusion proteins tested in this study. This happens despite the fact that once inside the cell, denatured proteins can be correctly refolded by chaperones, and thus could shift the equilibrium due to a more complex structural state, thereby reducing the reverse transduction out of the cells. It is unknown what proportion of transduced, intracellular fusion protein adopts a correctly folded state. However, testing the functionality of Tat-Zap70$(SH2)_2$

in Jurkat cells by assessing their capacity to co-precipitate with TCRζ showed it was rapidly lost upon washing, suggesting that not all transduced protein may have been correctly refolded.

Attempts to find a method allowing rapid desalting of the generated Tat-PTD fusion proteins continued, since the transduction rate across the plasma membrane of slowly desalted fusion proteins was found slow, as compared with previous reported proteins, and acquired protein yields were still low. Initial rapid desalting trials, the use of PD-10 desalting columns and fast dialysis, had failed due to high occurrence of protein precipitation, and small scale tests with ion-exchange columns also resulted in protein precipitation or, in the case of Tat-Zap70$(SH2)_2$, to irreversible binding to the column. This problem of irreversible binding to ion-exchange chromatography columns was reported before for some Tat-fusion proteins (Becker-Hapak et al., 2001), and might be due to a combination of the highly positive charged Tat peptide and the structure of the fusion partner. However, the method of rapidly desalting by ion-exchange was favoured above other techniques by other groups, reporting superior results (Nagahara et al., 1998). I therefore resorted to using Resource resin in batch form, whereby the use of FPLC or HPLC equipment could be avoided. The introduction of Q and S resin in batch format, and single elution steps, allowed successful solubilisation of high amounts of denatured Tat-PTD fusion proteins into aqueous buffer. This method was successful for all fusion proteins generated during this study. However, each protein still required empirical testing of the optimal solubilisation conditions, regarding choice of column material, ionic strength of the buffers and pH.

A potential concern of using rapidly desalted protein was that, although transduction may be more efficient, refolding and thus functionality may be compromised. Many proteins have been transduced under denaturing conditions after rapid desalting. However, for some it has been reported that they need to be purified from a soluble fraction to become efficiently refolded *in vivo* (Schwarze et al., 2000). This suggests that complete denatuation of some proteins leaves the Tat-PTD fusion

protein in a state that has a high transduction efficiency but a poor rate of refolding. Rapidly desalted fusion proteins tested during this study indeed showed enhanced uptake efficiency. Maximum intracellular levels were reached, in accordance with previous reports, in about 30 minutes. However, data about the function of these proteins are currently outstanding, and will be the focus of further study. It is likely that an incubation period of several hours prior to manipulation of the transduced cells is still required. Others have shown that refolding of some proteins, even with high efficiency transduction rates, can take several hours whereafter functionality is observed (Nagahara et al., 1998; Caron et al., 2001). This presumably depends on the complexity of the fusion partner of interest.

Based on these data, Tat-PTD fulfilled its function as an inert delivery vehicle for large protein domains, which it was shown to be able to transduce across the T cell plasma membrane, in all cells with equal distribution. Furthermore, it was shown that Tat-Zap70(SH2)2 was functional 3 hours after coculture, indicating that fusion partners containing SH2 domains can be properly refolded. Although a rapid desalting method was established to solubilise denatured Tat-PTD fusion protein into aqueous buffer, and it was shown that these proteins transduce more efficiently across the plasma membrane compared with slowly desalted fusion proteins, data about refolding and function still need to be obtained. Since these parameters were established for slowly desalted fusion proteins, these were used in subsequent experiments.

The interest in transduction domains in recent years has led to the development of even more powerful peptides that might be able to boost the usage of this technique (Futaki et al., 2000; Wender et al., 2000), especially for large protein domains and full-length proteins. Although mutants in animals can provide useful data and insights into the importance of proteins, the resultant phenotype is a consequence of a complex developmental program and adaptive mechanisms so that the precise function of the affected molecules might be obscured. Transduction has already been effectively used at determining the regulatory role of small GTPases

(Chellaiah et al., 2000). Constitutively active and dominant-negative forms can be generated and added to cell cultures, allowing experiments to be performed on primary cells that are otherwise difficult to manipulate, and avoiding labour intensive work of generating transgenic animals. It is, however, impractical to expect Tat-mediated delivery to work for every protein, and to the right location.

Can TCR-mediated signalling and T cell differentiation be influenced by the introduction of competitor proteins?

After differentiation and emigration from the thymus to the peripheral immune organs, T cells are functionally immature and have the capacity, upon encountering antigen, to develop into different functional subsets of effector T cells, each with their own cytokine profile. It is still poorly understood, however, what drives these cells into different response modes.

Activation and differentiation in the periphery is determined by at least three factors. The first part of the signal for T cell activation is delivered by the TCR/CD3, after interaction with peptide/MHC on APCs, and is influenced by the concentration of available peptide. The second part of the signal is provided by a number of costimulatory or accessory molecules, whose expression levels determine the extent of signal amplification. Both signals are influenced by the duration of the interaction between T cells and APCs, determining the amount of signal that can be accumulated. In recent years, evidence has accumulated that hierarchical thresholds for proliferation and differentiation determine the generation of various effector T cell modes (Iezzi et al., 1998; Langenkamp et al., 2000; Langenkamp et al., 2002). The polarisation of Th1 versus Th2 $CD4^+$ T cells is clearly influenced by the nature of these two signals (Seder and Paul, 1994), and T_{reg}/non-responsive or anergic cells are classically generated in the complete absence of the second costimulatory signal (Schwartz, 1990). The mechanisms that account for the generation of these various effector T cells remain, however, unclear.

The signalling cascades determining the final outcome of a T cell response are based on a number of biochemical events, with the interactions between two or more proteins. This interaction is an absolute requirement for transferring the extracellular signal to the nucleus where all signals are integrated, resulting in an appropriate response. This study tried to explore the possibility to influence the outcome of a T cell response by introducing signalling domains that can compete with endogenous signalling molecules. To this end, the Tat-PTD protein transduction system was successfully tested as an inert method to deliver large proteins into naive T cells.

The PTKs Lck and ZAP-70 are tyrosine phosphorylated as one of the earliest events after TCR stimulation. They are involved in recruiting signalling molecules to the TCR-signalling complex with their high affinity interaction domains. These high affinity domains, SH2 and SH3, are well defined and have highly specific substrates, allowing the generation of high affinity competitor proteins that are capable of blocking specific protein-protein interactions. This, together with the correlation of abrogated Lck and ZAP-70 tyrosine kinase activities in anergic T cells (Faith et al., 1997), made Lck and ZAP-70 good candidates for interfering and altering TCR-mediated signalling in an attempt to drive T cells into different response modes.

Interfering with the function of ZAP-70 was attempted with two Tat-PTD fusion proteins, one containing a previously reported short peptide sequence inhibitor (Nishikawa et al., 2000), and one containing a ZAP-70 truncation mutant lacking the entire kinase domain, previously shown to function as a dominant negative mutant (Northrop et al., 1996; Qian et al., 1996).

The short peptide sequence inhibitor of ZAP-70 was identified using an affinity-based library screen, and although designed as an inhibitor of SH2 domain interactions, was found to act as an inhibitor at the catalytic pocket (Nishikawa et al., 2000). A membrane-permeable version was made by fusing it to an Antp-based penetratin peptide and its ability to block T cell responses was investigated in Jurkat T cells. Upon CD3 cross-linking the peptide specifically diminished phosphorylation

levels of proteins known to be ZAP-70 kinase substrates; PLCγ and LAT. In addition, it was shown to inhibit 70% of Il-2 gene expression at a concentration of 10 μM. In this study, the identical peptide sequence was fused to the Tat-PTD sequence and tested on *ex vivo* naive T cells.

In contrast to the penetratin construct in Jurkat T cells, the peptide inhibitor fused to Tat-PTD did not show any influence on T cell proliferation, expression of surface markers and the secretion of the cytokines Il-2 and IFNγ. This is unlikely to be due to the use of Tat-PTD. Although the mechanism of transduction across a lipid bilayer is currently unknown, both PTDs have been successfully used to transduce peptide sequences and share the presence of basic amino acids shown to be responsible for penetration of the membrane. It was established that the full-length Tat protein is internalised via the ubiquitous heparan sulphate proteoglycans (Tyagi et al., 2000), though more recently it was suggested that the short Tat-PTD is taken up by a route that does not involve heparan sulphates (Silhol et al., 2002), indicating that different mechanisms for cellular internalisation may exist.

The absence of an inhibitory effect of the small ZAP-70 inhibitor is more likely due to the use of *ex vivo* T cells and peptide inhibitor concentrations used. The reported 70% inhibition in an *in vitro* kinase assay was found around 10 μM peptide concentration, and not much inhibition may be expected in *ex vivo* cells at that concentration extracellularly since only a part of the added peptide will actually enter the cells. The concentration used to obtain 70% inhibition of transcriptional activation of a reporter construct for the Il-2 gene reported by Nishikawa et al. in Jurkat T cells was also 10 μM, by stimulating the cells with plate bound anti-CD3ε and 10 ng/ml PMA. An additional problem with the use of *ex vivo* T cells was that doses above 5 μM proved to be toxic and only peptide inhibitor concentrations up to 5 μM could be tested. The possibility remains that the inhibition in Jurkat cells was a feature of altered signal transduction pathways in a transformed cell line.

Recruitment of ZAP-70 to the TCR is crucially dependent on its tandem SH2 domain (Iwashima et al., 1994). It was previously shown that interfering with the recruitment of ZAP-70, via a PTPase-resistant ITAM peptide analogue (Wange et al., 1995), or a kinase-truncated mutant (Qian et al., 1996), can specifically suppress TCR-mediated phosphorylation of endogenous ZAP-70 activation of NFAT, Vav and Erk phosphorylation and recruitment of Grb2. When using a CD8-ζ chimeric receptor, it was shown that the kinase-truncated mutant was constitutively bound to the hyperphosphorylated CD8-ζ chimera, suggesting that it competes for the same binding places as wild type ZAP-70. However, these data were all obtained using Jurkat T cells, which prohibited the study of T cell differentiation and response modes after altering TCR-mediated signals. Fusing the kinase-truncated ZAP-70 mutant to Tat-PTD, generating Tat-Zap70$(SH2)_2$, allowed the assessment of the influence of this dominant-negative mutant on TCR-mediated signalling and T cell differentiation in *ex vivo* naive T cells.

For the use of large fusion partners to Tat-PTD, slowly desalted, kinetics of transduction time and functionality were established during this study. The time between the addition of fusion protein to the cells and reaching maximum intracellular protein levels and protein functionality, meant that experiments testing the influence of the presence of the Tat-PTD fusion partners on T cell priming and function were preceded by a 4 hour incubation period prior to stimulation.

In order to evaluate the effects of Tat-PTD fusion proteins on early signal transduction, calcium fluxes were studied as a first indication of altered TCR signalling processes. It had been reported that ZAP-70-negative mutants of the Jurkat T cell line fail to flux calcium in response to anti-CD3 monoclonal antibody (Williams et al., 1998). However, calcium mobilisation has been measured in T cell clones and naive CD4+ peripheral T cells under partial-agonist conditions that do not activate ZAP-70 (Sloan-Lancaster et al., 1994; Madrenas et al., 1995; Boutin et al., 1997).

In this report, Tat-Zap70$(SH2)_2$ was shown to influence calcium fluxes in a concentration-dependent and anti-CD3 antibody dose-dependent manner. This indicates that Tat-Zap70$(SH2)_2$ is capable of competing with endogenous ZAP-70, and altering the proximal signalling processes. Furthermore, this suggests that ZAP-70 function is regulated by the strength of TCR triggering, since high doses of anti-CD3 showed a full calcium flux in amplitude and speed, while lowering the anti-CD3 concentration resulted in a delayed response with lower amplitude when using 2 μM Tat-Zap70$(SH2)_2$, and abolished the calcium flux at 3 μM Tat-Zap70$(SH2)_2$ concentration. This might be the result of multiple binding sites for ZAP-70 at the TCR. The total of 10 ITAM motifs allow, in theory, the recruitment of more or less ZAP-70 depending on the strength of signal. Tat-Zap70$(SH2)_2$ may be able to block some of these sites, but not all, since a supra-optimal dose of anti-CD3 allowed a full calcium flux.

In order to further assess the influence of Tat-Zap70$(SH2)_2$, a control protein will need to be generated. All SH2 domains have an arginine residue crucial for SH2 domain function since it is responsible for making direct contact with the phosphotyrosine residue substrate (Eck et al., 1993). Substitution of this arginine residue by lysine will abolish SH2 domain function. In the case of ZAP-70, this residue is found at positions 37 and 191 (Hatada et al., 1995). Although the C-terminal SH2 domain shows the highest affinity, mutation of either single SH2 domain decreases the Kd by > 100-fold (Bu et al., 1995), and will be sufficient to act as a disabled control protein.

The effect observed on calcium flux in this study demonstrates a potentially interesting phenomenon, since it seems to be influenced by the strength of TCR signalling. Full activation of ZAP-70 results in supplying the IS with LAT, PLCγ, PKCθ and Grb2 (Blanchard et al., 2002). The latter is involved in activating Ras, Erk, JNK and p38 via the Grb2/Sos pathway leading to antigen-driven growth and differentiation. Interestingly, RasGRP1 was recently implicated in transducing low-grade TCR signals possibly necessary for T cell survival and differentiation (Priatel et

al., 2002). The observation by Gong et al. (2001) led to the hypothesis that T cell selection is coupled to the utilisation of the RasGRP1 and Grb2/Sos pathways and, hence, the activation of MAPKs (Yun and Bevan, 2001). This might be applicable to peripheral T cell differentiation; insufficiently strong signals will thereby activate Ras and Erk via RasGRP1 leading to T cell anergy or altered effector functions, while strong signals will activate Ras, Erk, JNK and p38 via the Grb2/Sos pathway leading to effector T helper cells. In addition, ZAP-70 was most recently implicated to directly target the dual-specificity PTPase VHR that can inactivate the MAPKs Erk2 and JNK (Alonso et al., 2003). VHR phosphorylation by ZAP-70 may give the TCR control over the duration of the Erk2 and JNK responses. The circumstances and kinetics of this are currently unknown, but since ZAP-70 plays a differential role in T cell anergy (Sloan-Lancaster et al., 1994), this may explain the differences in relative activation profiles of Erk and JNK in these cells.

The protein-protein interactions regulating Lck's activity and proximity to effectors, made it a likely candidate for spatial regulation during T cell activation. Two truncated Lck proteins were fused to Tat-PTD, allowing the transduction of T cells, whereafter these fusion proteins can potentially compete for identical protein-protein interactions as endogenous Lck. Since the SH2 domain was described essential for the initiation of signalling events, it was fused to Tat-PTD (Tat-Lck^{SH2}). However, the recruitment of Lck is known to be dependent on its N-terminal region (Kabouridis et al., 1997), and a kinase-truncated version, maintaining the N-terminus, SH3 and SH2 domains was fused to Tat-PTD. The dependency on its recruitment for activation allowed the generation of a disabled protein that contains the Tat-PTD and His-tag at the N-terminus, thereby prohibiting this protein from coming into proximity with its substrates.

Only the latter two, Tat-Lck^{NSH2} and Lck^{NSH2}-Tat, were tested for their influence on T cell activation and differentiation. When B10 CD4$^+$ T cells were pre-treated with these fusion proteins, a distinct fusion protein dose-dependent alteration in calcium flux could be observed after anti-CD3 crosslinking. This pattern could only

be observed when using Lck^{NSH2}-Tat, which has a free N-terminus, while the pattern observed with Tat-Lck^{NSH2} was identical to medium controls. In contrast to Tat-$Zap70(SH2)_2$, this alteration was not influenced by the concentrations of anti-CD3, suggesting that the effect is independent of TCR signalling strength. This is in accordance with Ehrlich et al. (2002), who failed to observe a difference in temporal and spatial patterns of Lck recruitment between strong and weak agonists.

Treatment with 1 μM Lck^{NSH2}-Tat still resulted in a minor flux with similar timing as that of medium controls, but the amplitude was much reduced. Treatment with 2 μM Lck^{NSH2}-Tat completely eliminated the initial spike in the calcium flux, although a gradual increase in intracellular free calcium levels could be observed at later time points. This may indicate that the initial signal from the TCR to intracellular channels (IP_3-receptors) for rapid release of intracellular calcium stores is inhibited, while the slower plasma membrane calcium ATPases are allowed to raise intracellular free calcium levels. This might eventually result in a delayed, but possibly altered, stimulation of the treated T cells.

The alteration in calcium flux was indicative of altered TCR signalling, and encouraged the assessment of the effect of Lck^{NSH2}-Tat on T cell differentiation and function. Since Tat-Lck^{NSH2} was unable to alter the calcium response upon TCR stimulation, this protein served as a disabled protein control in the next experiment. The loss of functionality observed with Tat-$Zap70(SH2)_2$ after 1 hour when cells were washed after pre-treatment with the fusion protein, made it necessary, after the initial loading phase, to keep T cells in medium containing the Tat-PTD fusion proteins during T cell priming. Some fusion partners might need to be present for some time after the primary stimulus, depending on their mode of action. The tested proteins in this study were therefore only washed out after the first overnight incubation.

Upon stimulation with plate-bound anti-CD3 and anti-CD28 for 4 hours, MACS-selected $CD4^+$ B10 T cells were assessed during the first three days for their

proliferative response and their expression of surface markers. The Tat-Lck^{NSH2} conditions showed identical results to the medium control, as expected. Treatment with 1 μM Tat-Lck^{NSH2}, despite showing an altered calcium flux, did not show an alteration in the proliferative response, or in the expression of surface markers. Treatment with 2 μM Lck^{NSH2}-Tat, on the other hand, showed a distinct effect on T cell proliferation, which was as low as that seen in unstimulated cells. Some alterations were observed in the expression of CD69, CD25 and CD44, and their up-regulation as compared with anti-CD3- and anti-CD28-stimulated controls was delayed. CD69 expression also never reached maximum levels, and the cells were initially smaller, not reaching the full blast stage. However, at 6 days after the initial stimulus, cells treated with 2 μM Lck^{NSH2}-Tat were increased in size, and showed upregulation of CD69, CD25 and CD44. If one relates this to the calcium flux data, these results indicate a delayed and altered T cell activation. This was also reflected in the cytokine profile at this stage. CD4$^+$ T cells pretreated with 2 μM Lck^{NSH2}-Tat showed a 60% decrease in the number of Il-2 producing cells, suggesting a functional difference between these and control conditions.

Challenging the primed cells with plate-bound anti-CD3 and anti-CD28 for a second time in the absence of any fusion proteins should highlight the polarisation of effector T cells. The cells pretreated with 2 μM Lck^{NSH2}-Tat during their primary stimulus showed increased expression levels of CD44 and CD25 compared with 2 μM Tat-Lck^{NSH2} and medium controls. This suggests that the signal transduction machinery was changed during the primary stimulus resulting in an altered response upon subsequent stimulation. The cytokine profile showed that the majority of these cells (56%) failed to produce Il-2 and IFNγ, in contrast to the control conditions, in which over 90% of the cells produced Il-2 and over 50% IFNγ, indicating that the response mode of the cells pretreated with 2 μM Lck^{NSH2}-Tat during their priming was significantly altered. The control cells showed a typical Th1 profile that was expected after anti-CD3/CD28 stimulation. Cell populations producing the Th2 type cytokines Il-4 and Il-10 were absent in these conditions. Interestingly, cells in the 2

μM Lck^{NSH2}-Tat condition failed to produced Il-4 but a population of 23% produced Il-10.

The absence of a proliferative response upon stimulation, and the failure to produce Il-2 and IFNγ, shows strong similarities with anergic T cells that arise from incomplete T cell activation (Schwartz, 1996). While anergy results from partial stimulation, it is considered to be an active signalling process requiring triggering of the TCR and the mobilisation of calcium (Macian et al., 2002). By using Lck^{NSH2}-Tat, recruitment of endogenous Lck and thus Lck function is partially blocked. This results in incomplete T cell stimulation, which fails to show the initial spike in calcium mobilisation, but allows a more gradual increase in intracellular free calcium levels. Functional uncoupling of the TCR and Lck activation was previously observed in human anergic CD4+ T cells (Fujimaki et al., 2001). Measurements of calcium transients in single cells have shown that weak agonist peptides elict lower levels of calcium mobilisation than strong agonosts (Rabinowitz et al., 1996; Sloan-Lancaster et al., 1996a). Experiments in the presence of the extracellular calcium chelator EGTA may block the induction of the phenotype found in this study, and give an indication of the importance of extracellular calcium mobilisation in the induction of the observed phenotype.

In addition to T cell anergy, the prolonged higher levels of the surface marker CD25 after primary stimulation and increased levels of CD25 after restimulation observed during this study using Lck^{NSH2}-Tat, indicates similarities with *in vivo* T_{reg} cells (Read and Powrie, 2001). Several groups have shown that $CD4^+CD25^+$ T cells are hyporesponsive and fail to produce Il-2. Accumulating data suggest that these anergic or T_{reg} cells, instead of being functionally inert, may develop a phenotype with functional importance in negative regulation of naive T cells. This regulation could be passive, by competing with normal naive T cells for survival factors, or local cytokines or ligand binding. On the other hand, regulation could be the outcome of an active mechanism. Some of these cells have been reported to produce Il-10 upon restimulation (Groux, 2001; Shevach, 2002), known to inhibit T cell functions (Akdis

and Blaser, 2001). It is currently unclear if the cells produced in the presence of Lck^{NSH2}-Tat in this study resemble these regulatory cells, and if they possess any *in vitro* or *in vivo* suppressive activity. This will, however, be the focus of continued research.

Further experiments in the near future will be aimed at defining the molecular mechanism behind the induction of the observed phenotype by Lck^{NSH2}-Tat. The role of endogenous Lck, which may be essential for a low or altered signal, may be determined by using the inducible Lck knock-in system (Legname et al., 2000). In these mice it is possible to stop the expression of Lck at a desired moment in time. This enables comparison between T cells still harbouring Lck and those that have lost its expression. Treatment of these cells with Lck^{NSH2}-Tat will allow assessment of whether endogenous Lck activity is required to induce the phenotype observed in treated $CD4^+$ B10 T cells during this study and if Lck is involved in the altered pathways maintaining this state.

This study has established that the N-terminal region of Lck is an absolute requirement for altering TCR-mediated signalling by using Tat-Lck^{NSH2}, which has a blocked N-terminal region, and was unable to alter TCR signalling. In addition to directing Lck^{NSH2}-Tat to the IS, the N-terminal region may compete with endogenous Lck for binding sites at the CD4 coreceptor, thereby reducing the amount of recruited wild type Lck upon TCR triggering. The SH2 domain in Lck^{NSH2}-Tat may also be important for competing for protein-protein interactions with endogenous Lck, since it is essential for the formation of the trimolecular complex containing Lck, CD4 and CD3 (Rudd et al., 1991), and for interaction with ZAP-70, leading to its subsequent phosphorylation and activation. The role of the SH2 domain in Lck^{NSH2}-Tat can be demonstrated by using the R154K mutant, which has a non-functional SH2-domain (Straus et al., 1996). Alternatively, the SH3 domain, which is responsible for interactions with the coreceptor CD28 (Holdorf et al., 1999), might play a role. T cell anergy can be induced when T cells are stimulated in the absence of CD28 stimulation. The SH3 domain has been shown to be a link between TCR-mediated and

CD28-derived signals, and to determine Lck activity in the IS (Holdorf et al., 2002). The influence of this domain can be demonstrated by removing it from the fusion protein construct.

More distal events determining the fate of Lck^{NSH2}-Tat treated T cells and their involvement in altered signalling pathways may reflect findings in previous reports studying T cell anergy. Biochemical studies with Lck^{NSH2}-Tat-treated, stimulated and rechallenged cells will assess levels of TCR, Lck, Fyn and ZAP-70 phosphorylation, and the activation levels of the three MAPKs with help of phospho-specific antibodies giving insight into which pathways are altered as a result of Tat-Lck^{NSH2} treatment.

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