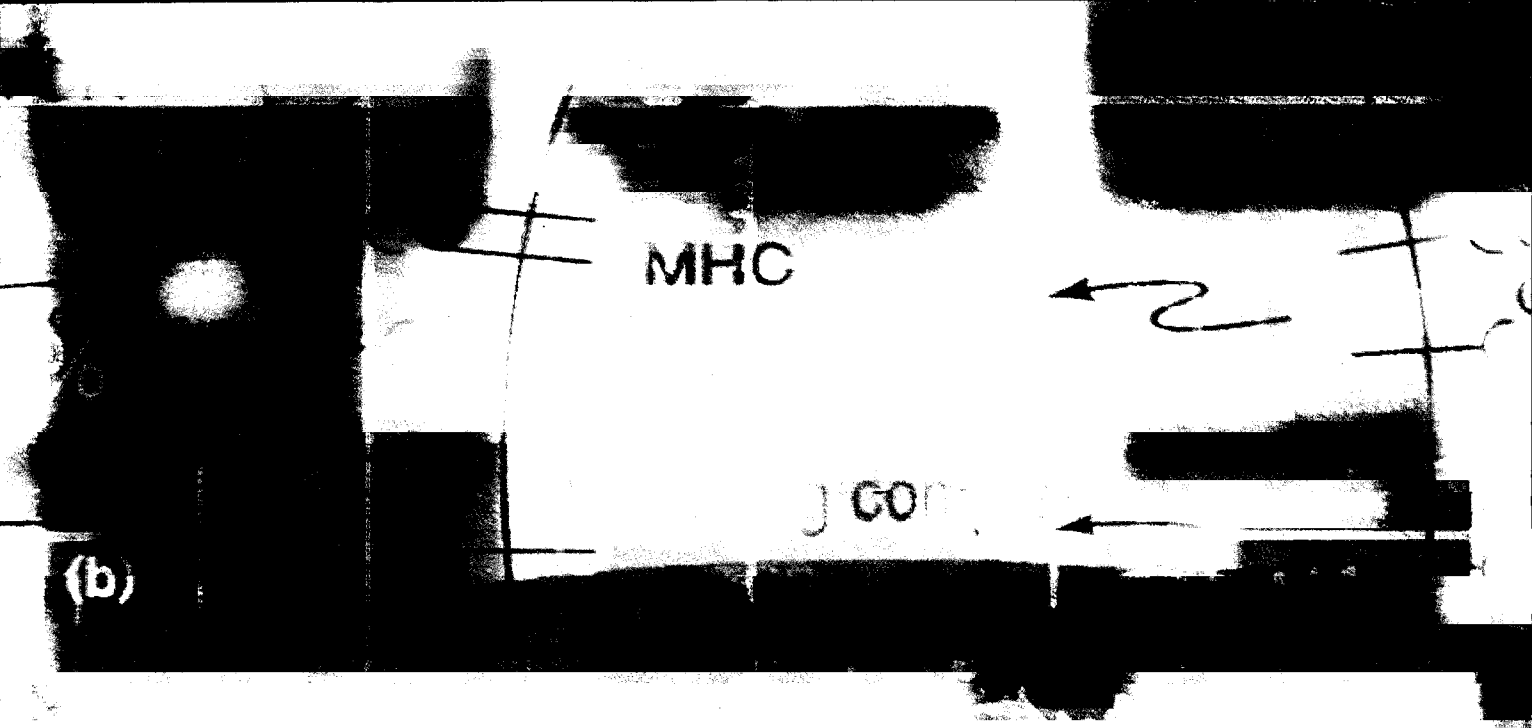
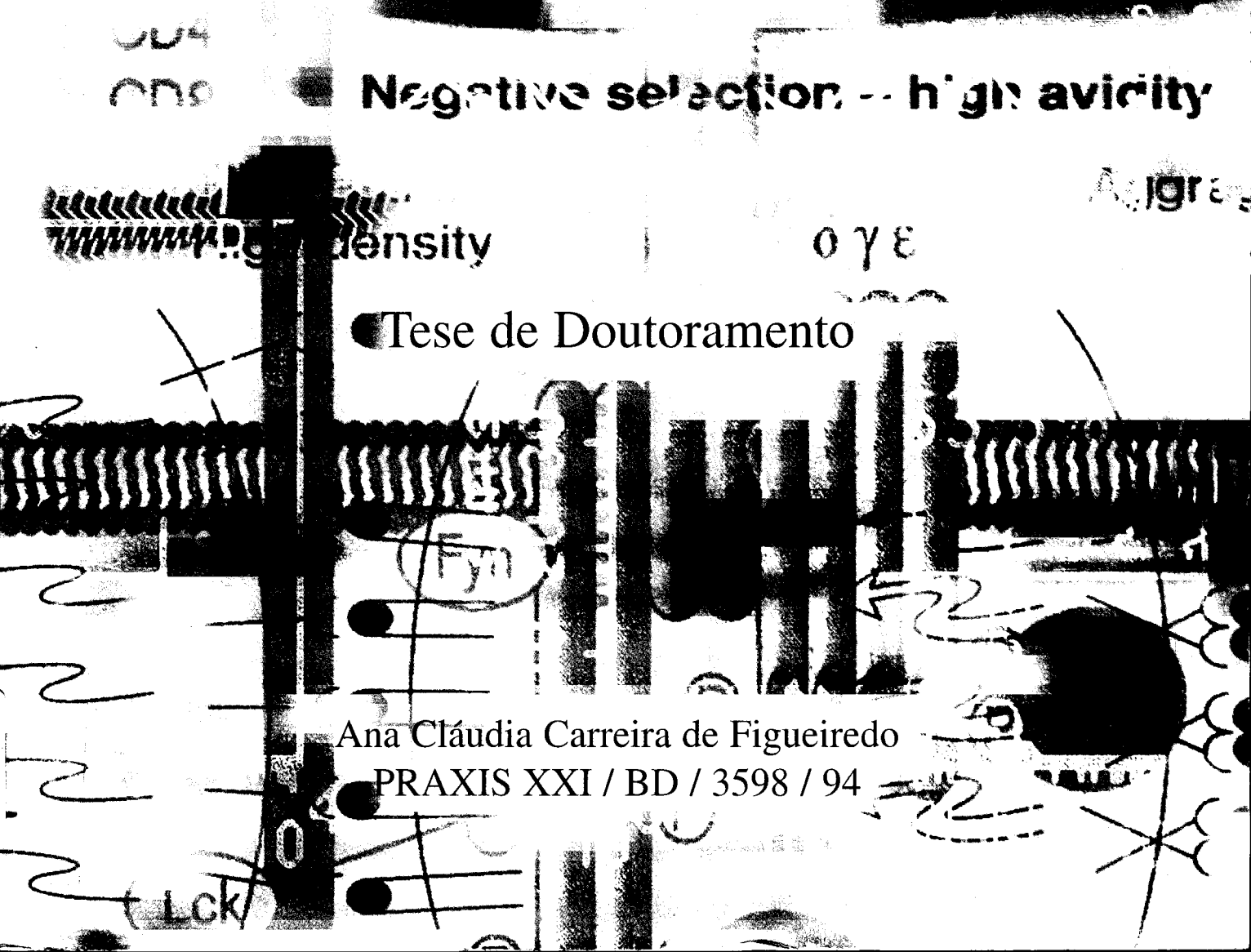


How the lack of the CD3γ chain affects T cell development and function



Tese de Doutoramento

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Aos meus pais, André e tia

CONTENTS

Abbreviations

APC	antigen presenting cell
β2-M	β2-microglobulin
BCR	B cell receptor
DDM	doublet differentiation mode
DN	double negative
DP	double positive
ER	endoplasmic reticulum
FTOC	fetal thymic organ culture
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factors
GPI	glycosyl phosphatidyl inositol
Grb2	growth-factor-receptor-bound protein-2
GTPase	guanosine triphosphatase
IL	interleukin
ISP	immature single positive
ITAM	immunoreceptor tyrosine based activation motifs
JNK	Jun amino-terminal kinase
LAT	linker for activation of T cells
LFA	leucocyte functional antigen
MAPK	mitogen-activated protein kinase
MEK	extracellular-signal regulated kinase kinase
MFI	mean fluorescence intensity

MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NK	natural killer
PI3-kinase	phosphatidylinositol 3'-hydroxyl kinase
PKC	protein kinase C
PLC- γ1	phospholipase C γ1
PMA	phorbol-12-myristate-13-acetate
pTα	pre-TCRα
PTK	protein tyrosine kinase
SH	Src-homology domain
Shc	SH2-containing protein
SLP-76	SH2-containing leukocyte protein 76
SOS	Son of Sevenless
SP	single positive
TAP	transporter associated with antigen presentation
TCR	T cell receptor

CHAPTER 1

INTRODUCTION TO THE THESIS

The immune system consists of a group of different cell types, which include macrophages, dendritic cells, natural killer cells, B and T lymphocytes. Its major function is generally considered to be the defense of the organism against the invasion of pathogenic agents. This function can be accomplished in a highly specific way by the lymphocytes. T and B lymphocytes can specifically recognize and respond to pathogenic agents (or antigens), due to the expression of highly variable and clonally distributed antigen receptors on their cell surface, i.e., the T cell receptors (TCRs) and the B cell receptors (BCRs), respectively (reviewed in Tonegawa, 1983; Kronenberg *et al.*, 1986 and Davis and Bjorkman, 1988). Based on the nature of their TCRs, T lymphocytes are divided in two major T cell lineages: the αβ and γδ T cells. The αβ T cells, which represent the major group of T lymphocytes, can be further subdivided into two major subsets. These are the $CD4^+$ and the $CD8^+$ T cells, which express CD4 and CD8 as co-receptor molecules, respectively. The $CD4^+$ T cells mostly recognize antigens in the context of major histocompatibility (MHC) class II molecules and are referred to as T helper cells. They regulate the responses of B lymphocytes and $CD8^+$ T lymphocytes. The $CD8^+$ T cells mostly recognize antigens in the context of MHC class I molecules and are referred to as cytotoxic T cells. They are involved in direct effector functions, which include the lysis of virus-infected cells (reviewed in Yewdell and Bennink, 1990).

The αβ TCR is a highly complex receptor. It is composed of at least six distinct polypeptides: the clonally distributed αβ heterodimer and the invariant chains TCRζ, CD3ε, -γ and -δ (reviewed in Clevers *et al.*, 1988a). TCR engagement mediates a broad range of functional and developmental responses. In mature T cells, engagement of the TCR activates T cells to proliferate, produce cytokines and mediate a variety of effector functions. In addition, it can trigger T cell unresponsiveness and T cell death. The αβ TCR and its immature counterpart, the pre-TCR, are also crucial regulators of αβ T cell development in the thymus (Figure 1.1).

In the αβ TCR complex, the αβ heterodimer constitutes the ligand binding unit of the receptor, while the associated CD3/TCR ζ chains are involved in receptor assembly and signal transduction (reviewed in Frank *et al.*, 1990 and Clevers *et al.*, 1988a). The complexity of the αβ TCR has raised the issue concerning the role of each one of the CD3/TCR ζ components. Are all of them absolutely required for TCR functioning or is there redundancy among the different subunits? Is each subunit mostly required for TCR assembly or, in addition, does it provide the TCR with unique signalling abilities? Are the different subunits equally essential for mature T cell functioning and T cell development? To address these questions, many studies have relied on the use of T cell lines. More recently, a large effort has been made to clarify the role of the different CD3/TCRζ TCR subunits *in vivo*, namely, their role on T cell development.

We have undertaken the investigation of the role of the CD3γ chain in T cell development and function. For that purpose, CD3γ null mice have been produced, by gene targeting in embryonic stem cells (Haks *et al.*, 1998). In these mice, T cell development was found to be

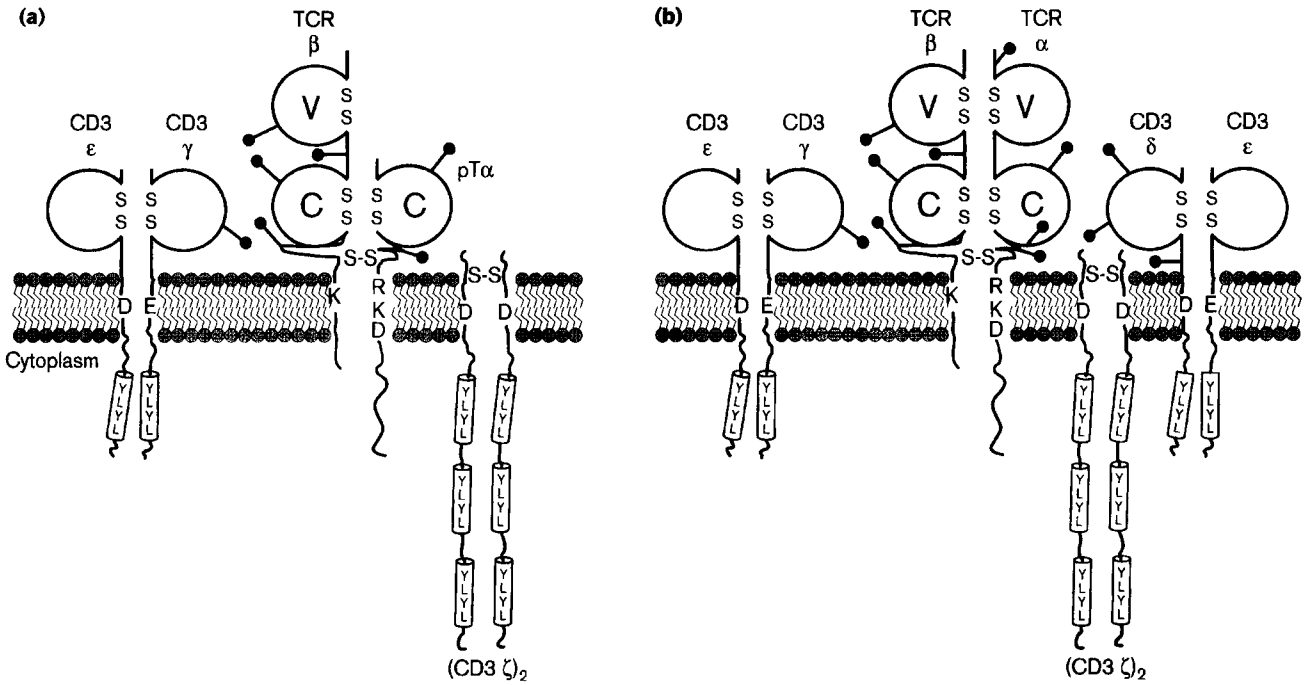

Figure 1.1: Putative subunit composition of the pre-TCR (a) and αβTCR (b).

severely blocked at an early T cell differentiation stage. Nevertheless, some T cells were found to be able to complete their maturational program and to colonize the peripheral lymphoid organs. How this absence of the CD3γ chain affects peripheral T cell functioning and late stages in thymic T cell development is the subject of this thesis.

CHAPTER 2

THE T CELL RECEPTOR
A REVIEW ON ITS COMPOSITION AND FUNCTION

Contents

2.1 Introduction

The αβ T cell receptor (TCR) is a multisubunit complex comprising a clonotypic TCRα-TCRβ heterodimer, non-covalently associated with invariant polypeptides. In this complex, the polymorphic TCRαβ constitutes the ligand binding unit of the TCR complex, while the monomorphic polypeptides are required for TCR assembly and cell surface expression, as well as for signal transduction. We will review here the composition and biogenesis of this receptor complex and that of its immature counterpart, the pre-TCR. In addition, we will review the role of these receptors in T cell activation and T cell development.

2.2 The αβ TCR components

TCRα and TCRβ chains are type I transmembrane proteins and members of the Immunoglobulin superfamily. Both polypeptides can be divided into five domains: two extracellular immunoglobulin-like domains, a membrane proximal hinge region, a transmembrane segment and a short cytoplasmic tail of five amino acid residues. The membrane distal immunoglobulin-like domain of the TCRα and TCRβ constitute the variable fraction of the TCR; the hinge region contains the cysteine residues responsible for the linkage of the two chains; and the transmembrane segments of TCRα and TCRβ contain one and two positively charged amino acid residues, respectively. Both chains are glycoproteins with a molecular weight of 40-50 kDa and 28-32 kDa in the glycosylated or non-glycosylated form, respectively (reviewed in Kronenberg *et al.*, 1986).

TCRα and TCRβ chains are encoded by variability (V), diversity (D), joining (J) and constant (C) gene segments that undergo rearrangement during T cell differentiation to yield functional genes (reviewed in Gellert, 1992). The murine TCRβ locus contains approximately 20 V segments (Vβ), 2 D segments (Dβ), 12 J segments (Jβ) and 2 C gene segments (Cβ). The Dβ, Jβ and Cβ gene segments are organized into two clusters each containing 1 D, 6 J and 1 C segments (Lai *et al.*, 1987). The murine TCRα locus comprises about 100 V segments (Vα), 50 J segments (Jα) and a single C gene segment (Cα) (Koop *et al.*, 1992). Assembly of TCRβ and TCRα gene segments is sequential. For TCRβ, one of the Dβ segments is first juxtaposed to one of the Dβ segments; subsequently, a Vβ segment is juxtaposed to the Dβ-Jβ fusion; the rearranged stretch is joined to a Cβ gene segment. Assembly of the TCRα locus involves the joining of a Vα to a Jα segment which is followed by the fusion of the joined Vα-Jα to the Cα gene segment. The TCRβ and TCRα membrane distal immunoglobulin-like domains are encoded by the joined Vβ-Dβ-Jβ and Vα-Jα segments, respectively. Their variability is therefore accounted for by the aleatory juxtaposition of the different genetic segments and by

the addition and/or deletion of nucleotides at the rearranged junctions. The remainder of the TCRβ and TCRα chains are encoded by the Cβ and Cα genetic segments, respectively. The Cβ gene segments consist of four exons; these encode the membrane proximal immunoglobulin-like domain, the hinge-like domain, the transmembrane region and the cytoplasmic tail, respectively. TCRβ and TCRα murine locci are located on chromosomes 6 and 14, respectively (reviewed in Marrack and Kappler, 1986).

The monomorphic subunits of the TCRαβ can be divided into two groups: the CD3 complex and the ζ-family proteins. The CD3 complex consists of three proteins referred to as γ, δ and ε. The CD3γ and δ are both glycoproteins of 21 kDa and 25 kDa, respectively; the CD3ε is not glycosylated and has a molecular weight of 20kDa (reviewed in Clevers *et al.*, 1988b). The three chains are members of the immunoglobulin superfamily and are highly homologous to each other (Gold *et al.*, 1987a). They all consist of one extracellular immunoglobulin-like domain (79-99 amino acid residues), a transmembrane domain – which comprises 26-27 amino acid residues including one negatively charged –, and a cytoplasmic tail (45-65 amino acid residues). The genes coding for all three CD3 chains are closely linked within 50-300 kb on human chromosome 11 and mouse chromosome 9 (van den Elsen *et al.*, 1985; van den Elsen *et al.*, 1986; Saito *et al.*, 1987; Gold *et al.*, 1987b); CD3γ and δ coding genes lie within 1.5kb of each other, in a head-to-head orientation (Saito *et al.*, 1987). The CD3γ and δ genes are organized into seven and five exons, respectively (van den Elsen *et al.*, 1986; Saito *et al.*, 1987). Comparison of CD3γ and δ gene exon/introns indicate that the two genes are highly homologous. The CD3γ gene differs from the CD3δ gene by the presence of a 24bp exon (exon 2), which does not have a counterpart in CD3δ, a 9bp insertion on exon 3 (which is equivalent to CD3δ exon 2) and by a separated exon encoding a 3' untranslated region (van den Elsen *et al.*, 1986; Saito *et al.*, 1987). The CD3ε gene is organized into eight exons (Clevers *et al.*, 1988b): the first exon (64-bp) is untranslated, the second and third (102-bp and 18-bp, respectively) encode the leader peptide, exon 4 (15-bp) encodes the N terminus of the mature protein, exon 5 (216-bp) encodes the extracellular domain, exon 6 (168-bp) encodes the transmembrane segment and the intracellular domain is encoded by exons 7 and 8 (47-bp and 844-bp, respectively) (Clevers *et al.*, 1988b).

The ζ family members constitute a group of structurally and functionally related proteins which include ζ, η (an alternatively spliced form of ζ), and FcεRIγ (Kuster *et al.*, 1990 and reviewed in Klausner *et al.*, 1990). Approximately 90% of the αβTCR complexes comprise a ζ-ζ homodimer. While, in the remaining 10% of TCR complexes, the ζ chain is disulfide-linked to the η chain. The ζ and η chains are non-glycosylated proteins with no sequence or structural homology to the CD3 chains or the TCRβ and TCRα chains. The molecular weight of the ζ and η chains is 16kDa and 22kDa, respectively. Their coding gene is located on mouse chromosome 1 (Baniyash *et al.*, 1988; reviewed in Klausner *et al.*, 1989).

Although all members of the TCR complex have been identified, their stoichiometry has not been completely determined. Several stoichiometries have been proposed, namely: (i) one TCRαβ heterodimer: one CD3δε dimer: one CD3γε dimer: one TCRζζ homodimer (Koning *et al.*, 1990; de la Hera *et al.*, 1991; Hou *et al.*, 1994; Punt *et al.*, 1994a), (ii) two TCRαβ heterodimers: two CD3δε/CD3γε dimers: one TCRζζ homodimer (Exley *et al.*, 1995) and (iii) two TCRαβ heterodimers: three CD3εδ/CD3γδ dimers: one TCRζ homodimer (Thibault and Bardos, 1995). At present, the generally accepted TCR stoichiometry is that of a protein complex comprising one TCRαβ heterodimer, one CD3γε dimer, one CD3δε dimer and one TCRζζ homodimer (Figure 1.1). In murine T cells expressing a human CD3ε, in addition to the murine CD3ε chain, it has been observed that the murine CD3ε can be co-precipitated and co-modulated with its human counterpart, and vice versa. This has been taken as an indication for the presence of at least two CD3ε chains per TCR complex (Blumberg *et al.*, 1990; de la Hera *et al.*, 1991). In murine T cells, transgenic for two different TCRβ and two different TCRα chains, there is no co-modulation nor co-precipitation of two different TCRβ or TCRα chains, which suggests that there is only one TCRβ and one TCRα chain per TCR complex (Hou *et al.*, 1994; Punt *et al.*, 1994a). In addition, the quantification of the relative expression levels of CD3ε and TCRβ, by immunofluorescence, indicates that the two chains are present in a 2:1 ratio on the T cell surface (Punt *et al.*, 1994a). A major drawback in this model is that, according to this stoichiometry, the TCR complex is expected to display a net -3 charge, at the transmembrane level. A transmembrane neutral charge would be expected if the TCR complex comprises two TCRαβ heterodimers, two CD3γε/δε dimers and one TCRζζ homodimer. Moreover, the size of the TCR complex, as estimated by centrifugation on density gradients, has been shown to correspond closer to a complex with two TCRαβ heterodimers, rather than to a TCR complex with one TCRαβ heterodimer (Exley *et al.*, 1995).

2.3 The αβ TCR biogenesis

Assembly of the TCRαβ complex occurs in the endoplasmic reticulum (ER) and proceeds in a highly ordered manner, which involves: (i) formation of noncovalently associated pairs of δε and γε proteins, (ii) assembly of α, β proteins with δε and γε pairs to form αδε and βγε intermediate complexes, (iii) joining of αδε and βγε chains sub-complexes, followed by disulfide bonding of CD3-associated α, β proteins to yield incomplete αβδεγε complexes, and finally (iv) addition of disulfide-linked ζζ homodimers to form the complete αβδεγεζζ TCR complex (Minami *et al.*, 1987a; Alarcon *et al.*, 1988; Klausner *et al.*, 1990; Williams *et al.*, 1991; Kearse *et al.*, 1995a).

The efficiency of TCR assembly in the ER determines the receptor density at the T cell surface. Single subunits that fail to join the complex are retained in the ER and subsequently degraded, whereas partial complexes are targeted to lysosomal compartments for destruction. Both incomplete and complete TCR complexes egress from the ER to the Golgi complex, but only complete TCR complexes are efficiently transported to the cell surface (Sussman *et al.*, 1988; Chen *et al.*, 1988; Klausner *et al.*, 1990; Williams *et al.*, 1991; Kearse *et al.*, 1995a). The molecular determinants underlying the subunit-specific interactions and the degradation of single TCR-CD3 subunits in the ER have been the subject of intensive research, but are still not fully known. Experimental evidence supports an assembly model based on salt bridges between charged residues within the transmembrane domains of the individual TCR subunits (Cosson *et al.*, 1991). In addition, it has been shown that the extracellular domains of the TCR subunits are essential for the assembly of the TCR complex and to rescue single subunits from degradation in the ER (Wileman *et al.*, 1993; Dietrich *et al.*, 1996). In particular, it has been shown that specific sites on the CD3γ chain extracellular domain are crucial for TCR assembly. These crucial sites are also conserved in the primary structure of the CD3δ chain, which has led the authors to suggest that the CD3γ and CD3δ chains share a common binding motif in CD3ε (Dietrich *et al.*, 1996). In agreement with this notion, the CD3γ and CD3δ chains have been found to compete for binding to the CD3ε subunit (Geisler, 1992; Huppa and Ploegh, 1997).

The molecular chaperone calnexin (IP90, p88) is also involved in the assembly of the TCR, and has been found in association with partially assembled TCR complexes, devoid of ζ subunits (Hochstenbach *et al.*, 1992; David *et al.*, 1993). Since calnexin associates with all TCR subunits except TCR-ζ (Hochstenbach *et al.*, 1992; David *et al.*, 1993; Kearse *et al.*, 1994a; Rajagopalan *et al.*, 1994), it has been suggested that it promotes the assembly of $\alpha\beta\gamma\delta\varepsilon_2$ complexes. In particular, calnexin has been shown to significantly prolongue the half-life of newly synthesized TCRα proteins (Kearse *et al.*, 1994a). Another putative chaperon in TCR assembly is the calnexin related molecule, calreticulin. Indeed, calreticulin was found to specifically associate with nascent TCRα and TCRβ proteins (van Leeuwen and Kearse, 19996). Calreticulin is an ubiquitous endoplasmic reticulum chaperone (reviewed in Michalak *et al.*, 1998) and has also recently been shown to be expressed on cell surface of activated T cells in association with MHC class I molecules (Arosa *et al.*, 1999).

2.4 Signalling through the αβ TCR in mature T cells

Engagement of the TCR by antigen, superantigen or anti-TCR antibodies induces a complex cascade of events which eventually leads to T cell differentiation and clonal expansion. One of the earliest measurable biochemical events upon TCR engagement is the activation of

protein tyrosine kinases (PTKs) and concomitant phosphorylation of several cellular proteins on their tyrosine residues. The TCR subunits themselves have no intrinsic tyrosine kinase activity. Instead, the cytoplasmic tails of CD3 and TCRζ subunits possess amino acid sequences, termed the immunoreceptor tyrosine based activation motifs (ITAMs), which are absolutely required for TCR signalling (reviewed in Wange and Samelson, 1996). The ITAM sequences are present in one copy in the CD3 subunits and three copies in the TCRζ subunit. They consist of two tyrosine residues spaced 9 to 11 amino acid residues apart with isoleucine or leucine residues positioned three residues carboxy-terminal to each tyrosine (YXXL X_{6-8} YXXL) (Reth, 1989). Upon TCR engagement, the ITAM sequences are phosphorylated on their tyrosine residues and become docking sites for different SH2 (Src homology 2) domain containing proteins (Figure 2.1).

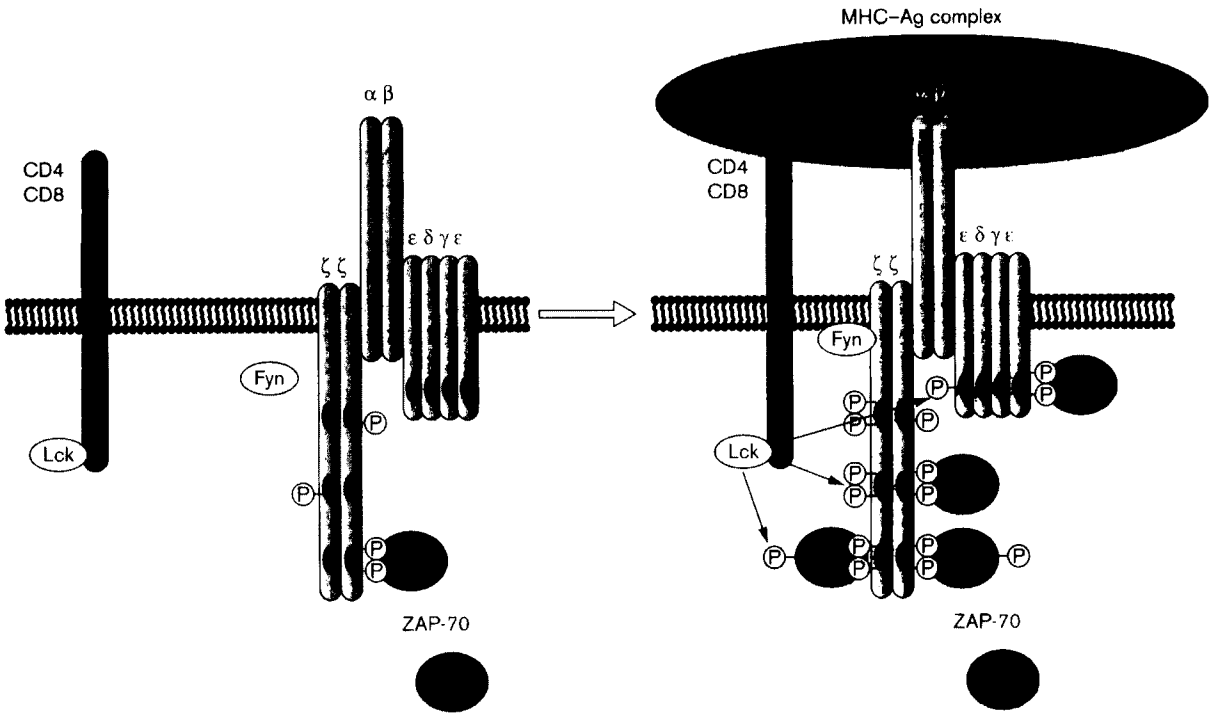

Figure 2.1: TCR proximal signalling events upon TCR engagement.

Two families of protein tyrosine kinases (PTKs) are sequentially involved in early TCR signalling, the Src and Syk families. The Src family comprises, at least, eight known mammalian members (Src, Fyn, Yes, Lyn, Hck, Fgr, Lck and Blk) (reviewed in Bolen and Brugge, 1997). The Src PTKs have a constitutive capacity to associate with the plasma membrane and share a common domain organization. These include the catalytic domain (Src homology 1, SH1), a phosphotyrosine binding domain (Src homology 2, SH2), a proline-rich sequence binding domain (Src homology 3, SH3) and a plasma membrane targetting domain (Src homology 4, SH4).

A central role for the Lck Src PTKs family member in TCR signalling has been demonstrated. Lck is expressed in T cells and NK cells (Biondi *et al.*, 1991; reviewed in Ravichandran *et al.*, 1996 and Binstadt *et al.*, 1997). In T cells it associates with the CD4 and CD8 TCR co-receptors, as well as, with other T cell surface molecules (Veillette *et al.*, 1988; Levin *et al.*, 1993a). The Lck enzymatic activity is activated upon TCR triggering. T cell activation is severely compromised in Lck-deficient Jurkat T cells (Straus and Weiss, 1992), as well as in peripheral T cells of Lck-deficient mice (Molina *et al.*, 1993; Wen *et al.*, 1995). Fyn is another member of the Src family of PTKs which appears to play a role in TCR signalling. Although, it is widely expressed, a unique form of Fyn is present in hematopoiteic cells as a consequence of alternative splicing of the seventh exon that encodes sequences in the Fyn SH1 domain (Cooke and Perlmutter, 1989). Fyn is constitutively associated with the TCR chains, as well as with GPI-linked molecules such as Thy-1 (Samelson *et al.*, 1990; Thomas and Samelson, 1992). TCR signalling is enhanced in T cell hybridomas transfected with a constitutively activated form of Fyn (Davidson *et al.*, 1992) as well as in T cells from Fyn transgenic mice (Cooke *et al.*, 1991). Nevertheless, Fyn-deficient mice show no clear defect in T cell activation upon TCR triggering (Appleby *et al.*, 1992; Stein *et al.*, 1992).

The mechanism underlying Src PTK activation, upon TCR triggering, is not well understood. Autophosphorylation within their own SH1 domain is thought to represent a critical event in the activation process (Johnson *et al.*, 1996; reviewed in Bolen and Brugge, 1997). In addition, the enzymatic activity of the Src PTKs has been shown to be negatively regulated by the phosphorylation of a conserved tyrosine residue near their carboxy-terminus (Cooper and Howell, 1993). The Csk PTK and the CD45 phosphatase have been implicated in the phosphorylation and dephosphorylation, respectively, of this tyrosine residue (Brickell, 1992; Mustelin and Burn, 1993; Chow *et al.*, 1993; Hata *et al.*, 1994). Both enzymes appear to play a major role in the regulation of Src PTKs enzymatic activity and TCR signalling. Csk-deficient cell lines display hyperactivity of different members of the Src PTKs family (Imamoto and Soriano, 1993; Nada *et al.*, 1993). Overexpression of Csk in mouse T cells suppresses T cell activation upon TCR triggering (Chow *et al.*, 1993). T cells deficient in CD45 demonstrate hyperphosphorylation of the C-terminal negative regulatory tyrosine residue of Lck and exhibit marked deficiencies in TCR-mediated signalling events, including ZAP-70 phosphorylation and activation (Pingel and Thomas, 1989; Koretzky *et al.*, 1990; Iwashima *et al.*, 1994).

Besides the Src kinases, also the Syk protein kinases play a key role in TCR signalling. The Syk family of PTKs comprises two members: ζ associated protein-70 (ZAP-70) and Syk. These molecules are structurally similar. They consist of two phosphotyrosine binding SH2 domains and one catalytic SH1 domain (Weiss and Littman, 1994; Bolen, 1995; DeFranco, 1995). ZAP-70 is expressed exclusively in T cells and NK cells (Chan *et al.*, 1994a). Upon TCR engagement, ZAP-70 is phosphorylated on tyrosine residues, activated and becomes associated

with the TCR chains (reviewed in Weiss and Littman, 1994). The fundamental role of ZAP-70 in TCR signalling is well illustrated by the fact that ZAP-70 deficiency severely hampers T cell activation in both Jurkat T cells and mouse peripheral T cells (Arpaia *et al.*, 1994; Chan *et al.*, 1994b; Elder *et al.*, 1994; Negishi *et al.*, 1995; Gelfand *et al.*, 1995; Taylor *et al.*, 1996). Syk is expressed in most hemopoietic cells, although the level of expression varies in different cell types. For instance, thymocytes express 3-4 fold higher levels of Syk than peripheral T cells (Chan *et al.*, 1994a; Law *et al.*, 1994). As for ZAP-70, TCR engagement leads to the activation of Syk and its association with the TCR chains (Chan *et al.*, 1994a). Nevertheless, this PTK does not appear to be essential for αβ T cell development. While, Syk-deficient mice die shortly after birth, in irradiated mice, reconstituted with Syk-deficient fetal liver cells, αβ T cell development proceeds normally (Cheng *et al.*, 1995; Turner *et al.*, 1995). Moreover, ZAP-70 and Syk appear to differ in their process of activation. While ZAP-70 activation involves its association to the TCR chains and its phosphorylation by Src PTKs, binding of Syk to the TCR chains is sufficient for its activation, through an autophosphorylation-dependent mechanism (Shiue *et al.*, 1995; Rowley *et al.*, 1995; Kimura *et al.*, 1996).

Src and Syk PTKs catalyse the phosphorylation of several cellular proteins, including the TCR subunits themselves (reviewed in Samelson and Klausner, 1992), the enzyme PLC-γ1 (phospholipase C-γ1) (Secrist *et al.*, 1991 and Weiss *et al.*, 1991), the nucleotide exchange factor Vav (Bustelo *et al.*, 1992 and Margolis *et al.*, 1992) and the adaptor proteins c-cbl (Donovan *et al.*, 1994 and Reedquist *et al.*, 1994), Shc (Ravichandran *et al.*, 1993), SLP-76 (Reif *et al.*, 1994; Buday *et al.*, 1994 and Jackman *et al.*, 1995) and LAT (Zhang *et al.*, 1998). Adapter molecules are molecules which lack enzymatic and transcriptional domains, but have multiple motifs and domains for intermolecular association. As mentioned before, once phosphorylated, the CD3/TCRζ ITAM sequences become docking sites for SH2 domain containing proteins. Subsequently, numerous proteins become associated with the TCR, either directly or indirectly. These include the ZAP-70 and Syk kinases (Chan *et al.*, 1991; Chan *et al.*, 1994a and reviewed in van Oers and Weiss, 1995), PLC-γ1 (Dasgupta *et al.*, 1992), the guanine nucleotide exchange factors SOS (Reif *et al.*, 1994) and Vav (Wu *et al.*, 1996 and Marengere *et al.*, 1997), the small GTPases of the $p21^{ras}$ superfamily and a number of adapter molecules such as c-cbl, Grb2, Shc, SLP-76 and LAT (for a recent review see van Leeuwen and Samelson, 1999).

PLC-γ1 has been shown to be recruited to the TCR through its SH2 domain-mediated association with the tyrosine phosphorylated LAT (Sieh *et al.*, 1994; Finco *et al.*, 1998). In addition, it has been shown that PLC-γ1 can interact with phosphorylated Syk (Law *et al.*, 1996). Once recruited to the plasma membrane, PLC-γ1 is phosphorylated on serine and tyrosine residues (Park *et al.*, 1991; Weiss *et al.*, 1991) and becomes activated. PLC-γ1 catalyses the hydrolysis of plasma membrane inositol phospholipids into inositol polyphosphates and

diacylglycerols, which trigger an increase in cytoplasmic free calcium and the activation of the protein kinases C (PKC), respectively. These are crucial molecular events in the process of T cell activation (reviewed in Weiss and Imboden, 1987). Indeed, T cell treatment with calcium ionophores and phorbol esters, which lead to a rise in intracellular free calcium and the activation of PKC, mimic TCR-induced T cell activation (Weiss *et al.*, 1984 and reviewed in Berry and Nishizuka, 1990).

Another TCR induced signalling pathway involving inositol lipid metabolism and activation of PKC is triggered by the enzyme phosphatidylinositol 3'-hydroxyl kinase (PI3-kinases). PI3-kinase comprises a regulatory 85 kDa subunit, which contains two SH2 domains and one SH3 domain, and a 110kDa catalytic subunit. Upon TCR engagement, PI3-kinase is recruited to the TCR and its catalytic subunit is phophorylated on serine residues (reviewed in Reif *et al.*, 1993). Experimental evidence indicates that PI3-kinase can associate with other TCR signalling molecules either throught the SH2 domains of its 85kDa subunit or via interaction of its two proline-rich sequences and Src SH3 domains (Prasad *et al.*, 1993a; Prasad *et al.*, 1993b; Amrein *et al.*, 1993; Vogel and Fujita, 1993; Ramos-Morales *et al.*, 1994; Fukazawa *et al.*, 1995a; Fukazawa *et al.*, 1995b). Once activated, the PI3-kinase catalyses the phosphorylation of phosphoinositides at the D-3 hydroxyl of the myo inositol ring to generate polyphosphoinositides PtdIns(3)P, PtdIns(3,4)P2 and PtdIns(3.4.5)P3. The direct downstream targets of the phosphorylated phosphoinositides is not well known, but it has been shown that, they can modify the catalytic activity of PKC *in vitro* (reviewed in Rudd *et al.*, 1994; Liscovitch and Cantley, 1994; Cantrell, 1996).

The GTPase $p21^{ras}$ is the TCR proximal component of conserved signal transduction pathways known as the mitogen-activated protein kinase (MAPK) cascades (reviewed in Su and Karin, 1996 and Henning and Cantrell, 1998). The activation state of $p21^{ras}$, like that of all GTPases, is controlled by two main classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) promote the transition from the inactive GDP-bound to the active GTP-bound form and GTPase-activating proteins (GAPs) stimulate GTPase inactivation. The activation mechanism involves a guanine-nucleotide-dependent conformational change in two regions of the Ras protein referred to as the Switch I and Switch II domains (reviewed in Macara *et al.*, 1996). Three MAPK cascades have been identified: the extracellular signal regulated kinase (ERK) 1/2, the Jun N-terminal kinases (JNKs) and the p38 MAPK. Raf-1 is the Ras downstream component of the ERK 1/2 MAPK signalling pathway.

TCR induced Ras activation requires the translocation of the nucleotide exchange factor mSos to the plasma membrane. This has been shown to occur via the adapter proteins Grb2 and LAT (Buday *et al.*, 1994; Holsinger *et al.*, 1995). Upon TCR engagement, mSOS and Grb2, which are constitutively associated, translocate to the plasma membrane through binding of the Grb2 SH2 domain to the tyrosine phosphorylated LAT. This adapter protein is an 36 kDa integral

membrane protein and a substract for the Syk PTKs (Zhang *et al.*, 1998). Once at the plasma membrane, SOS has been shown to activate Ras, by catalysing its conversion from a GDP to a GTP-bound form. GTP-Ras has a high affinity to Raf-1 and recruits this serine/threonine kinase to the plasma membrane (Koide *et al.*, 1993; Hallberg *et al.*, 1994; Marais *et al.*, 1998; reviewed in Finney and Herrera, 1995). Raf-1, which is normally located in the cytosol in an inactive state, becomes activated once translocated to the plasma membrane (reviewed in Morrison and Cutler, 1997). The mechanism of this activation is not well understood. Mutations in Raf-1 or Ras, which prevent their association, block Raf-1 activation (Fabian *et al.*, 1994 and Luo *et al.*, 1997). Nevertheless, Raf-1-Ras association is not sufficient for Raf-1 activation (Traverse *et al.*, 1993; Stokoe and McCormick, 1997; Tamada *et al.*, 1997) and a plasma membrane targeted Raf-1 form becomes independent of Ras for its activation (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Phosphorylation of both serine/threonine and tyrosine residues appear to play a role in Raf-1 activation, as Raf-1 activity can be abolished by treatment with serine/threonine or tyrosine specific protein phosphatases (Kovacina *et al.*, 1990; Turner *et al.*, 1993; Jelinek *et al.*, 1996 and reviewed in Morrison and Cutler, 1997). The crucial role of Ras and Raf-1 in T cell activation has been clearly demonstrated. Transient transfection of T cells with constitutively active forms of Ras or Raf-1 either induce hallmarks of T cell activation or promote TCR-induced activation. On the other side, dominant negative forms of Ras or Raf-1 have the ability to block T cell activation upon TCR engagement (Rayter *et al.*, 1992; Woodrow *et al.*, 1993; Owaki *et al.*, 1993; Wotton *et al.*, 1993; Izquierdo *et al.*, 1993; Izquierdo *et al.*, 1994a; D'Ambrosio *et al.*, 1994; Taylor-Fishwick and Siegel, 1995).

2.5 The role of TCR in thymic T cell development

Two forms of the TCR control αβ T cell development in the thymus. These are the mature αβ TCR described above, and an immature TCR form, referred to as the pre-TCR. This pre-TCR is minimally composed of a TCRβ chain covalently associated with an invariant polypeptide, the pre-TCRα (pTα), and non-covalently associated with the same components of the CD3-TCRζ complex, which are also part of the mature TCR. The pre-TCR and the mature αβ TCR control two different checkpoints in αβ T cell development, which are referred to as β-selection and positive/negative selection, respectively. β-selection, the first TCR-mediated checkpoint, ensures that only thymocytes that have undergone a productive TCRβ rearrangement can progress in their maturation. Positive/negative selection is responsible for modulating a mature αβ TCR repertoire able to recognize foreign antigens in the context of self-MHC molecules, but devoid of harmful autoreactivity. The two TCR-mediated checkpoints in αβ T cell development, as well as the nature of the pre-TCR, are considered in detail, below.

2.5.1 αβ T cell development in the thymus

αβ T cell development in the thymus proceeds in accordance to a strictly regulated developmental program. Based on the differential expression of a number of cell markers, the process of T cell differentiation can be subdivided into distinct stages. The most broadly used markers have been the CD4 and CD8 TCR co-receptors: The most immature thymocytes are $CD4^-CD8^-$ (double negative, DN); these mature into $CD4^+CD8^+$ (double positive, DP) and finally into either $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive, SP). The immature $CD4^-CD8^-$ population is also referred to as triple negative (TN), since thymocytes do not express detectable levels of TCR on their cell surface, in contrast to a minor mature $CD4^-CD8^-$ population. The TN thymocytes comprise only 1-3% of all thymocytes in the adult thymus. This population can be further subdivided into four subpopulations based on the differential expression of c-kit, CD44 and CD25. The most immature TN thymocytes are $c\text{-}kit^+CD44^+CD25^-$; at this stage the TCRα and β loci are still in germ line configuration, as well as the genes for the γδ T cell receptor. These TN thymocytes are not yet fully committed to the T cell lineage, as cells within this population can serve as progenitors to natural killer (NK) cells, B cells, T cells and thymic dendritic cells (Wu *et al.*, 1991; Ardavin *et al.*, 1993; Godfrey *et al.*, 1993; Carlyle *et al.*, 1997). Upon acquisition of CD25 cell surface expression, the TN thymocytes become $c\text{-}kit^+CD44^+CD25^+$; these are the so-called pro-T cells, in which some steps towards T cell commitment have occurred. The TCRβ loccus, as well as TCRγ and TCRδ loci, undergoes rearrangement at the next developmental stage, the $c\text{-}kit^-CD44^-CD25^+$ (early pre-T cells). Finally, these cells downregulate CD25 expression and reach the end stage as a TN thymocyte, becoming $c\text{-}kit^-CD44^-CD25^-$ (late pre-T cells). At the late pre-T cell stage commitment to the T cell lineage is irreversible (Godfrey *et al.*, 1993; Godfrey *et al.*, 1994; Wilson *et al.*, 1994; reviewed in Shortman and Wu, 1996). The TN $c\text{-}kit^-CD44^-CD25^-$ progress to the $CD4^+CD8^+$ stage via CD4 or CD8 immature single positive (ISP) intermediates. At the DP stage, thymocytes undergo rearrangement of the TCRα locus and low expression of TCRαβ complexes becomes detectable on the thymocytes cell surface. TCR^{lo} DP thymocytes upregulate their TCRαβ cell surface expression and finally downregulate the expression of either CD8 or CD4 co-receptors to become $CD4^+CD8^-$ or $CD4^-CD8^+$ single positives, respectively (Figure 2.2) (reviewed in Robey and Fowlkes, 1994; von Boehmer, 1995; Malissen and Malissen, 1996; Fehling and von Boehmer, 1997).

In the developmental program, summarized above, thymocyte maturation from the $c\text{-}kit^-CD44^-CD25^+$ into the $c\text{-}kit^-CD44^-CD25^-$ TN thymocyte subpopulation is controlled by the pre-TCR. Maturation of the DP into SP thymocytes is dependent on the specificity of the αβ TCR expressed by DP thymocytes. The ability of the DP TCRs to interact with thymic self-peptide-self-MH dictates the fate of the DP thymocytes into further maturation (positive

selection), induced cell death (negative selection) or cell death by neglect. The nature of the pre-TCR will be reviewed, as well as the two TCR-mediated checkpoints, in αβ T cell development.

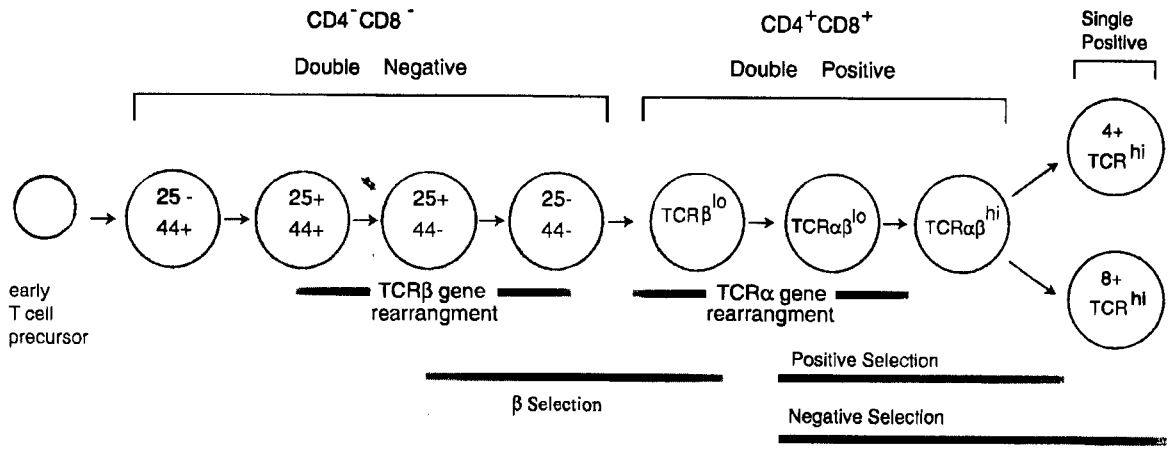

Figure 2.2: Schematic representation of T cell development in the thymus.

2.5.2.1 The pre-TCR: its components

It has become evident that the DN to DP thymocyte transition is under the control of a TCR form. T cell development is completely arrested at the DN stage in *scid* mice and mice lacking RAG-1 or RAG-2 expression, which are unable to rearrange their TCR loci (Bosma *et al.*, 1983; Habu *et al.*, 1987; Bosma and Carroll, 1991, Shinkai *et al.*, 1992; Mombaerts *et al.*, 1992a). Subsequent experiments suggested that the TCRβ is a component of this TCR form: expression of a productively rearranged TCRβ gene in *scid* mice and RAG-deficient mice was shown to be sufficient to allow T cell maturation into the DP stage (Shinkai *et al.*, 1993; Shores *et al.*, 1993). It was also evident that, at this early stage in development, TCRβ chain was not associated with TCRα: at the DN stage, the TCRα gene is still in its germ line configuration; moreover, T cell development beyond the DN stage is severely blocked in mice deficient for TCRβ, but not in mice lacking TCRα (Mombaerts *et al.*, 1992b).

In contrast to pre-T cells, *scid* mice derived immature T cell lines, transfected with a productively rearranged TCRβ chain gene express high levels of TCRβ at their cell surface (Groettrup *et al.*, 1993). Biochemical analysis revealed that these cell surface expressed TCRβ chains are covalently associated with a 33kDa glycoprotein, the pre-TCRα (pTα). A TCRβ-pTα heterodimer was also detected on the cell surface of thymocytes from TCRα-deficient mice and it has become the hallmark of the so-called pre-TCR (Groettrup *et al.*, 1993). Several cDNA clones encoding murine pTα chain have been isolated and sequenced (Saint-Ruf *et al.*, 1994). The pTα is

a type I transmembrane protein, which belongs to the immunoglobulin superfamily. It has a single extracellular Ig-like domain, which exhibits little homology with any other antigen-receptor molecule. Its transmembrane domain contains two positively charged amino acid residues, as the counterpart domain of TCRα. Its cytoplasmic tail is approximately 31 amino acid residues long. The murine pTα is encoded by a non-rearranging gene located on chromosome 6, in the vicinity of the MHC locus (Fehling *et al.*, 1995a). pTα mRNA is detected in all TN thymocyte subpopulations, reaching its peak in the $CD44^{-}CD25^{+}$ TN stage. pTα transcripts are still detected in DP, but not in SP thymocytes (Saint-Ruf *et al.*, 1994; Bruno *et al.*, 1995).

The exact identity of CD3 chains associated with the TCRβ-pTα heterodimer has been difficult to establish. In TCRβ transfected *scid* mice derived immature T cell lines, the cell surface expressed TCRβ chain is strongly associated with CD3ε and CD3δ, but weakly or not associated with CD3γ and TCRζ (Groettrup *et al.*, 1992; Groettrup *et al.*, 1993). On thymocytes derived from TCRα-deficient mice, the TCRβ has been reported to be strongly associated with CD3ε and CD3γ, but not with CD3δ and TCRζ (Jacobs *et al.*, 1994). Another study indicates that, virtually all TCRβ-pTα heterodimers expressed on the surface of TCRα-deficent thymocytes include the CD3δ, γ and ε; while approximately half of the complexes include TCRζ (Berger *et al.*, 1997). These conflicting data illustrate that biochemical analysis of the pre-TCR composition is hampered by its extremely low cell surface expression. Gene targeting experiments have therefore contributed significantly to determination of the identity of the crucial CD3 components for pre-TCR function.

Mice lacking TCRζ or TCRζ and TCRη show a 2-30-fold reduced thymic cellularity (Malissen *et al.*, 1993; Ohno *et al.*, 1993; Liu *et al.*, 1993). Although the $CD44^{-}CD25^{-}$ TN thymocyte subpopulation is nearly absent in these mice (Crompton *et al.*, 1994), some DN thymocytes mature to the DP stage. This is in agreement with the biochemical evidence for a weak association of TCRζ with the pre-TCR. Considering the hypothesis that the FcεRIγ chain might partially substitute TCRζ, mice lacking all ζ family members have been produced (Shores *et al.*, 1998). T cell differentiation in these mice is indistinguishable from TCRζ/η-/- mice, which supports the notion that ζ family members are not essential components of the pre-TCR. A crucial role for CD3 components in pre-TCR function, was revealed by the absolute T cell developmental arrest at the DN stage in mice with a targeted CD3ε gene (Malissen *et al.*, 1995). Nevertheless, this study did not allow to distinguish the contribution of the individual CD3 components, because these CD3ε-/- mice also displayed a reduction in the amount of CD3γ and had no detectable CD3δ mRNA (Malissen *et al.*, 1995). Although the CD3δ chain has been identified as a structural component of the pre-TCR (Berger *et al.*, 1997), DN to DP thymocyte maturation appears unaffected in CD3δ-deficient mice (Dave *et al.*, 1997); which suggests that CD3δ is dispensable for pre-TCR function. CD3γ-deficient mice provide evidence for an essential role of the CD3γ, as a component of the pre-TCR. In these mice, T cell development is severely blocked at the $CD44^{-}$

$CD25^+$ TN stage (Haks *et al.*, 1998). Nevertheless, the T cell developmental arrest is not as absolute as the one observed in mice lacking all CD3 components. In the CD3γ-deficient mice, some DN thymocytes do mature into the DP stage. To address the possibility that the CD3δ might partially substitute CD3γ in its function, mice lacking both CD3δ and CD3γ were generated. In such CD3δ and CD3γ double deficient mice, T cell development is absolutely arrested at the $CD44^-CD25^+$ TN stage (Wang *et al.*, 1998). Most recently, mice lacking the CD3ε but not CD3γ and CD3δ, have been generated. In these mice, T cell development is absolutely arrested at the $CD44^-CD25^+$ TN stage (DeJarnette *et al.*, 1998). Taken together, the information derived from gene targeted mice suggests that the essential CD3 components of the pre-TCR are the CD3ε and CD3γ chains; and that CD3δ can partially or inefficiently substitute the CD3γ chain.

Besides TCRβ-pTα heterodimer and CD3 subunits, it is possible that additional, yet to discover, pre-TCR components exist. Two additional components have been found in association with the pre-TCR: the chaperon molecule, calnexin (Takase *et al.*, 1997), and a homodimer of an alternatively spliced product of the CD3γ gene (Takase *et al.*, 1998). This alternatively spliced CD3γ product lacks the CD3γ transmembrane segment and its expression on the cell surface is restricted to immature thymocytes. The function of this protein remains to be identified.

2.5.2.2 Pre-TCR function

A complex set of events is associated with thymocyte progression through the pre-TCR mediated checkpoint. These include cellular differentiation, extensive cellular proliferation and cessation of further rearrangement (allelic exclusion) at the TCRβ locus. All together, these events result in enrichment for thymocytes that have undergone a productive TCRβ rearrangement (Pearse *et al.*, 1989; Dudley *et al.*, 1994; reviewed in Malissen *et al.*, 1992). While it is clear that the pre-TCR is required for T cell development beyond the β-selection checkpoint, it is not clear which of the events associated with this checkpoint are a direct consequence of pre-TCR signalling.

Since the pre-TCR is expressed at extremely low levels on the cell surface (reviewed in Borst *et al.*, 1996), two critical issues in understanding pre-TCR function are whether it requires cell surface expression, and whether it involves ligand binding. Indeed, there are precedents for signalling by intracellular forms of surface receptors, such as the platelet-derived growth factor receptor (Huang and Huang, 1988). To address the question of the requirement for pre-TCR cell surface expression, transgenic mice expressing a rearranged TCRβ gene linked to an endoplasmic reticulum (ER) retrieval signal have been generated (O'Shea *et al.*, 1997). This TCRβ transgene was shown to be unable to signal the cessation of endogenous TCRβ

rearrangements, cellular proliferation or differentiation of DN thymocytes. This indicates that exit of the pre-TCR from the ER/cis-Golgi is necessary for pre-TCR function. However, these experiments do not distinguish a requirement for the pre-TCR to be exposed on the cell surface for extracellular ligand recognition or for assembling intracellular signalling components. At present a putative ligand for the pre-TCR has not been identified. In addition, information derived from transgenic mice strongly suggests that pre-TCR function does not involve extracellular ligand binding. An important study in this regard showed that a TCRβ transgene lacking its variable ligand binding domain is capable of promoting DP thymocyte differentiation and inhibiting the further rearrangement of endogenous TCRβ loci (Krimpenfort *et al.*, 1989). Most recently, the hypothesis of extracellular ligand binding by the constant domains of TCRβ and/or pTα appears to have been excluded. TCRβ and pTα transgenes lacking all the extracellular Ig-like domains were shown to be fully capable of promoting DN into DP thymocyte differentiation (Irving *et al.*, 1998).

2.5.2.3 Signalling through the pre-TCR

Although it is not known which events trigger pre-TCR signalling, it is clear that pre-TCR derived signals are crucial for the further differentiation of DN thymocytes. The role of the different CD3 subunits in pre-TCR signalling, as well as the identity of effectors of a downstream pre-TCR signalling pathway have been addressed in a number of studies. Yet, as for the pre-TCR composition, also these studies are hampered by the low pre-TCR cell surface expression level.

Crosslinking of the pre-TCR expressed on the surface of TCRβ transfected *scid* mice derived T cell lines induces tyrosine phosphorylation of CD3ε and TCRζ, recruitment and phosphorylation of ZAP-70 and Syk and a rapid rise in intracellular calcium levels (Punt *et al.*, 1991; Groettrup *et al.*, 1992; van Oers *et al.*, 1995; Groettrup *et al.*, 1993). There is abundant evidence for a linkage between CD3 signalling and DN thymocytes differentiation. Treatment of RAG-deficient or TCRβ-deficient fetal thymic organ cultures (FTOC), or injection of RAG-deficient mice with anti-CD3ε monoclonal antibody, induces DN into DP thymocyte differentiation (Levelt *et al.*, 1993; Jacobs *et al.*, 1994; Shinkai and Alt, 1994; Levelt *et al.*, 1995). In addition to the CD3 components, it has been hypothesized that the pTα cytoplasmic tail contributes to the signal transduction capacity of the pre-TCR. The observation that the murine pTα cytoplasmic tail contains two potential serine and threonine phosphorylation sites, as well as proline-rich sequences that might constitute a binding motif for SH3 protein domains (Saint-Ruf *et al.*, 1994) is consistent with that hypothesis. Nevertheless, the finding that the human pTα cytoplasmic tail shows little homology with the corresponding murine sequence

suggested that the pTα cytoplasmic tail is not crucial for pre-TCR signalling (Del Porto *et al.*, 1995). This notion was further strengthened by the observation that a transgene encoding a tailless pTα is nearly as capable, as a full-length pTα chain transgene, in restoring DN to DP thymocyte differentiation in pTα-deficient mice (Fehling *et al.*, 1997).

As for the mature TCR, signalling by the pre-TCR is thought to be initiated by tyrosine phosphorylation of the CD3 ITAM motifs with subsequent recruitment of SH2-domain containing proteins. Since the different ITAMs vary in their amino acid sequences, the question has been raised whether ITAMs of different CD3 chains associated with the pre-TCR perform identical or unique functions. At present, experimental evidence strongly indicates a redundancy of the different CD3 ITAMs on pre-TCR function. It has been shown that reconstitution of TCRζ-deficient mice with a full length TCRζ transgene is as capable in promoting DN to DP thymocyte differentiation, as reconstitution with a TCRζ transgene lacking one, two or all three ITAMs (Shores *et al.*, 1994). Most recently, mice with a CD3γ chain lacking its ITAM motif have been produced (unpublished results). In these mice T cell development is indistinguishable from wild type mice. Therefore, the CD3γ chain appears to be an essential structural component of the pre-TCR, but its signalling function is not unique. An experiment using chimeric transgenes has given further support to the notion that the different ITAM motifs are redundant in pre-TCR function. RAG-deficient mice were reconstituted with chimeric transgenes consisting of the human IL-2 receptor α chain extracellular domain coupled to either CD3ε or TCRζ cytoplasmic domain. Crosslinking of these chimeric transgenes has demonstrated that both CD3ε and TCRζ cytoplasmic tails are capable of promoting DN to DP thymocyte differentiation (Shinkai *et al.*, 1995).

Early signalling by the mature TCR involves two scr family protein kinases, Fyn and Lck, and the Syk kinases ZAP-70 and Syk (reviewed in Weiss and Littman, 1994). Therefore, the hypothesis has been raised that these molecules might also be involved in pre-TCR signalling. Indeed, an essential role for the src kinases has been demonstrated, in pre-TCR signalling. In mice lacking Lck, T cell development is severely arrested at the DN thymocyte stage (Molina *et al.*, 1993). Nevertheless, this T cell developmental arrest is not absolute and it was suggested that Fyn might partially substitute Lck in its function. Indeed, while T cell development appears undisturbed in Fyn-deficient mice (Appleby *et al.*, 1992), in mice double deficient for Lck and Fyn T cell development is absolutely arrested at the DN thymocyte stage (van Oers *et al.*, 1996; Groves *et al.*, 1996). Further evidence for the crucial role of Lck in pre-TCR signalling has been obtained. Thymocytes from mice expressing a dominant negative form of Lck show a profound developmental arrest at the DN stage (Levin *et al.*, 1993b); while, a constitutively activated form of Lck is capable of promoting DN to DP thymocyte differentiation in RAG-deficient mice (Mombaerts *et al.*, 1994). Mice lacking the CD45 phosphatase also exhibit a partial blockage at the DN to DP thymocyte transition (Byth *et al.*,

1996). Since the CD45 phosphatase activates Src-family kinases, by dephosphorylating their carboxy terminal negative regulatory tyrosines (reviewed in Neel, 1997), T cell development blockage in CD45-deficient mice further implies a role for src-kinases in pre-T cell development. Indeed, an increase in hyperphosphorylated and inactive Lck and Fyn protein tyrosine kinases can be detected in CD45-deficient thymocytes (Stone *et al.*, 1997). In addition, an inactive form of the Csk protein kinase, a negative regulator of the Src-family kinases, has been shown to promote thymocyte differentiation beyond the DN stage (Schmedt *et al.*, 1998). This further strengthens the notion that activation of src kinases is a crucial signalling event for DN to DP thymocyte transition.

In contrast to Lck, neither ZAP-70 nor Syk, the two family members of the Syk PTKs, appeared to play a crucial role in pre-T cell development. DN to DP thymocyte differentiation is not affected in Syk-deficient mice (Turner *et al.*, 1995; Cheng *et al.*, 1995). In mice deficient for ZAP-70, either by a point mutation in the kinase domain of the molecule or by deletion of the entire ZAP-70 gene, pre-T cell development also appears undisturbed (Negishi *et al.*, 1995; Wiest *et al.*, 1997). Nevertheless, in mice double deficient for Syk and ZAP-70, T cell development is completely arrested at the pre-TCR mediated checkpoint (Cheng *et al.*, 1997). This indicates that the protein tyrosine kinases of the Syk family are crucial for pre-TCR signalling. Moreover, it suggests that ZAP-70 and Syk are highly interchangeable in their pre-TCR signalling function.

Engagement of a mature TCR form results in the rapid activation of the GTPase Ras (Downward *et al.*, 1990 and reviewed in Izquierdo *et al.*, 1995), which is a point of convergence for many receptor/tyrosine kinase-induced pathways (reviewed in Medema and Bos, 1993; Marshall, 1993; Bos, 1995 and Izquierdo *et al.*, 1995). Experimental evidence suggests that Ras is also involved in pre-TCR signalling. While a dominant negative form of Ras is not capable of inhibiting murine DN to DP thymocyte differentiation (Swan *et al.*, 1995), a constitutively active form Ras promotes differentiation and expansion of DP thymocytes in RAG-deficient mice (Swat *et al.*, 1996). The adaptor protein SLP-76, which is tyrosine phosphorylated upon TCR engagement, has also been shown to be crucial for pre-TCR signalling. In mice deficient for SPL-76, T cell development is partially blocked at the DN stage (Clements *et al.*, 1998; Pivniouk *et al.*, 1998). Vav, the nucleotide exchange factor for the Rho family of GTPases (reviewed in Boguski and McCormick, 1993), is another TCR signalling protein which has been shown to be crucial for pre-TCR signalling. Vav-deficient mice show a reduced thymic cellularity with defective DN to DP thymocyte differentiation (Tarakhovsky *et al.*, 1995; Fischer *et al.*, 1995; Zhang *et al.*, 1995).

Downstream Ras, the possible involvement of the mitogen-activated protein (MAP) kinase cascade, ras-->raf-->MEK-->ERK, in pre-TCR signalling, has been investigated. While it has been demonstrated that a catalitically inactive form of MEK-1 does not affect murine pre-

T cell differentiation *in vivo* (Swan *et al.*, 1995; Alberola-Ila *et al.*, 1995); a different experimental approach has provided evidence for a role of MEK-1 in pre-TCR signalling (Crompton *et al.*, 1996). Using retrovirus mediated gene transfer, introduction of a dominant-negative MEK-1 into TCRα-deficient fetal thymic organ cultures has been shown to inhibit DN into DP thymocyte differentiation. Furthermore, the dominant-negative MEK-1 also abolished the development of DP thymocytes in anti-CD3ε-treated RAG-/- fetal thymic lobes (Crompton *et al.*, 1996).

In mice deficient for TCRβ or pTα, i.e., mice unable to assemble a TCRβ-pTα heterodimer, a small number of DP thymocytes is generated (Mombaerts *et al.*, 1992b; Fehling *et al.*, 1995b). The origin of the DP thymocytes in TCRβ-deficient mice has been obscure and the possibility that they belong to the γδ T cell lineage has been considered (Mombaerts *et al.*, 1992b). In pTα-deficient mice, however, some DP thymocytes clearly belong to the αβ T cell lineage, as they express TCRαβ on their cell surface. This indicates that, although the TCRβ-pTα is crucial for the generation of large numbers of DP thymocytes from DN precursors, it is not the only TCR able to mediate DN to DP transition. Indeed there is experimental evidence for a role of both TCRαβ and TCRγδ in mediating DN into DP thymocyte differentiation (Buer *et al.*, 1997; Kang *et al.*, 1998).

2.5.3 Positive/negative selection

Mature T cells display two fundamental functional characteristics. They respond to peptides presented in the context of self-MHC molecules and are devoid, at least largely, of harmful reactivity towards self antigens. These phenomena are referred to as MHC restriction (Zinkernagel and Doherty, 1974) and self-tolerance, respectively. A large body of experimental evidence indicates that MHC-restriction and self-tolerance are the result of a stringent selection process occurring in the thymus, at the transition from DP to SP thymocytes (reviewed in Jameson *et al.*, 1995; Kisielow and von Boehmer, 1995) Indeed, only a small fraction of DP thymocytes further mature to the SP stage (Surh and Sprent, 1994).

DP thymocytes express a highly diverse TCRαβ repertoire, derived from the randomly rearranged TCRβ and TCRα genes. The ability of the TCR to bind self-peptide-self-MHC complexes on thymic stromal cells determines the developmental fate of each cell (reviewed in Jameson *et al.*, 1995 and Kisielow and von Boehmer, 1995). Most DP thymocytes bear TCRs that cannot recognise thymic MHC-peptide complexes, and these cells die within 3-4 days through a process termed death by neglect (Huesmann *et al.*, 1991; Shortman *et al.*, 1991). Recognition of self-MHC-self-peptide can trigger either apoptosis (negative selection) or survival and DP differentiation to CD4 or CD8 SP thymocytes (positive selection). The

mechanisms governing positive versus negative selection and DP differentiation to CD4 versus CD8 SP thymocytes have been the subject of intensive investigation.

Direct evidence for the involvement of self-MHC molecules in positive selection has been obtained through the analysis of TCR transgenic mice, as well as through the analysis of mice deficient for MHC class I or class II molecules (Sha *et al.*, 1988; Kisielow *et al.*, 1988b; Teh *et al.*, 1988; Scott *et al.*, 1989; Kaye *et al.*, 1989; Koller *et al.*, 1990; Zijlstra *et al.*, 1990; Grusby *et al.*, 1991; Viville *et al.*, 1993). In addition, the presence of self-MHC in combination with the cognate antigen was shown to induce negative selection of DP thymocytes via clonal deletion (Kisielow *et al.*, 1988a; Murphy *et al.*, 1990; Swat *et al.*, 1991; Iwabuchi *et al.*, 1992; Zal *et al.*, 1994). DP deletion was also observed in response to superantigens (reviewed in Kappler *et al.*, 1988 and MacDonald *F.*, 1988). A role for self-peptides in positive selection was suggested by the analysis of T cell development in mice with mutated MHC molecules. It was shown that the ability of the MHC molecules to induce positive selection correlates with their ability to present peptides (Nikolic-Zugic and Bevan, 1990; Sha *et al.*, 1990; Jacobs *et al.*, 1990). Further evidence for a role of self-peptides in positive selection was provided by the analysis of T cell development in fetal thymic organ cultures from MHC class I deficient mice, either due to a lack of TAP-1 or β2-M. These studies demonstrated that addition of single peptides or peptide mixtures could restore the otherwise deficient CD8 T cell differentiation in these organ cultures (Ashton-Rickardt *et al.*, 1993; Hogquist *et al.*, 1993 and reviewed in Bevan *et al.*, 1994).

Both positive and negative selection appear to operate on thymocytes at the same stage of development. Also, both selection processes result from the interaction of the TCR with thymic MHC+peptide complexes. A widely accepted model for the mechanisms leading to positive/negative selection is the differential avidity model (reviewed in Ashton-Rickardt and Tonegawa, 1994). This model postulates that the outcome of the TCR interaction with a ligand depends on the avidity of the T cell-thymic stromal cell interaction, it depends on the affinity of the TCR for that particular ligand and on the cell surface densities of the TCR and the ligand. According to the differential avidity model, a low affinity TCR interaction results in positive selection, while a high affinity interaction leads to negative selection (Figure 2.3). This model is supported by much experimental evidence. Analysis of the development of TCR transgenic thymocytes in fetal thymic organ cultures from MHC deficient mice has demonstrated that positive or negative selection depends on the nature of the peptides added to the organ cultures (Hogquist *et al.*, 1994; Ashton-Rickardt *et al.*, 1994; Sebzda *et al.*, 1994). Direct measurements of the affinities between the TCR and peptide-MHC complexes demonstrated that, in one particular system, the TCR had an higher affinity for negatively selecting peptide-MHC complexes than for positively selecting ones (Alam *et al.*, 1996). Furthermore, it has been shown that low concentrations of one single peptide can induce positive selection, whereas a high

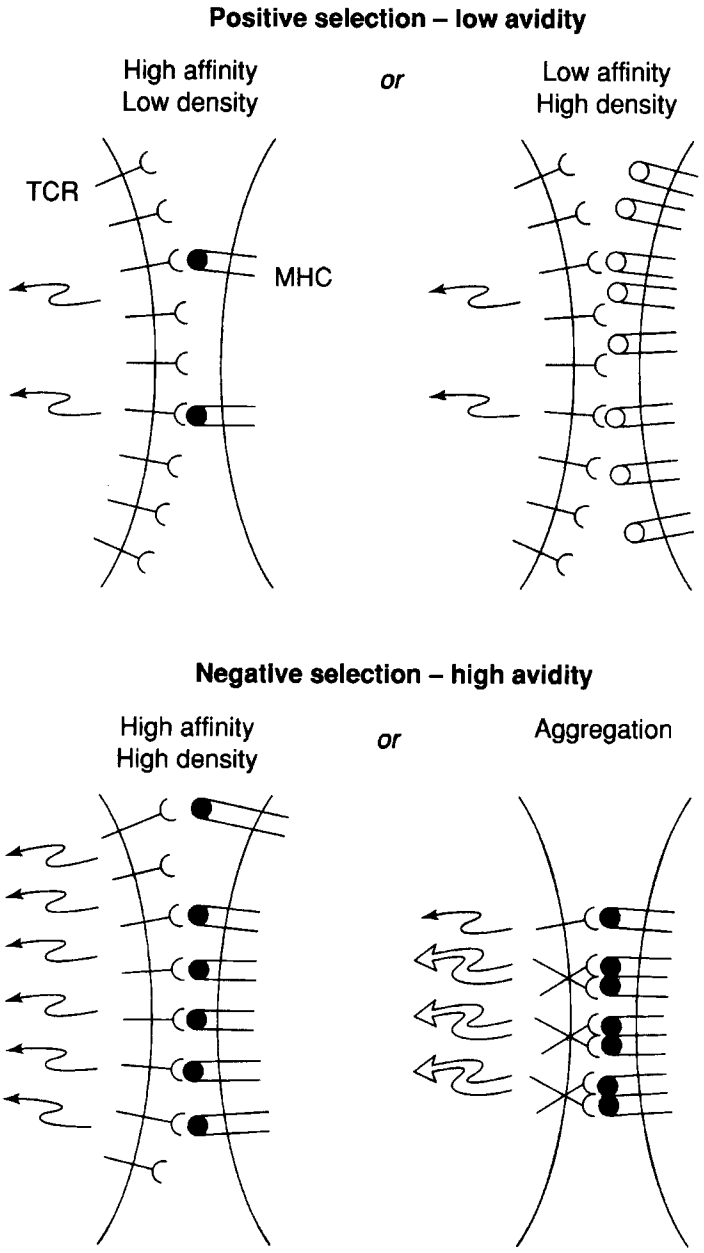

Figure 2.3: Differential avidity model for positive/negative selection. The differential avidity model postulates that low avidity TCR/MHC-peptide interactions lead to negative selection (a), while high avidity TCR/MHC-peptide interaction lead to negative selection.

concentration of the same peptide causes negative selection of the reactive thymocyte population (Ashton-Rickardt *et al.*, 1994; Sebzda *et al.*, 1994). In addition, experimental evidence indicates that the avidity of the TCR-peptide/MHC interaction is also dependent on the density of other T cell/APC molecules such as the CD4 and CD8 co-receptors, LFA and CD28 (re-viewed in Amsen and Kruisbeek, 1998).

Nevertheless, some experimental observations are difficult to reconcile with the differential avidity model. Some peptides are only able of inducing either positive selection or negative selection, over a wide range of concentration (Hogquist *et al.*, 1994; Hogquist *et al.*, 1995; Sebzda *et al.*, 1996). Also, peptides that are able to inhibit either positive or negative selection induced by another peptide have been described (Mamalaki *et al.*, 1992; Mamalaki *et al.*, 1993; Spain *et al.*, 1994). To explain these observations, another model for positive/ /negative selection has been proposed. This is the agonist-antagonist balance model (reviewed in Williams *et al.*, 1997). This model is based on the assumption that a single DP thymocyte can interact with numerous different peptide ligands, and on the notion of peptide analogues. The notion of a single DP thymocyte interacting with different peptide ligands is supported by the

finding that multiple peptides have the ability to select a single TCR (Hogquist *et al.*, 1997; Hu *et al.*, 1997), and multiple different TCRs can be selected in mice in which a single peptide/MHC complex predominates (Ignatowicz *et al.*, 1996; Fukui *et al.*, 1997; Ignatowicz *et al.*, 1997; Tourne *et al.*, 1997; Grubin *et al.*, 1997; Surh *et al.*, 1997; Sant'Angelo *et al.*, 1997). Peptide analogues are peptides that differ from the antigenic peptide (or agonist peptide) in minor amino acid residues. Peptide analogues include both antagonist peptides (peptides able to inhibit the T cell response to the agonist peptide (De Magistris *et al.*, 1992) and partial agonist peptides (peptides which selectively stimulate certain T cell responses (Evavold and Allen, 1991; Evavold *et al.*, 1993; Sloan-Lancaster *et al.*, 1993; Windhagen *et al.*, 1995). The agonist-antagonist model postulates that the fate of a single DP thymocyte is the result of the sum of signals derived from the interaction of their TCRs with different peptide ligands, which may include agonist, partial agonist and/or antagonist peptides.

Regardless of whether positive versus negative selection involves TCR ligation to one single ligand or to numerous ligands, both models imply that signals generated by the TCR upon engagement of different ligands must be quantitatively or qualitatively different. At the TCR proximal level, positive and negative selection inducing signals appear to be qualitatively indistinguishable. Both positive and negative selection of DP thymocytes proceed unimpaired in Fyn-deficient mice (Appleby *et al.*, 1992; Stein *et al.*, 1992), while Lck appears to be required for both selection processes. In mice double transgenic for the anti-HY TCR and for a dominant-negative Lck transgene, positive selection in female mice is strongly inhibited. An effect on negative selection, in male mice, is not observed, but deletion of endogenous superantigen reactive thymocytes is reduced (Hashimoto *et al.*, 1996). Further support for a role of src PTKs in positive and negative selection is obtained from the analysis of T cell development in CD45-deficient mice. In these mice, T cell development is severely blocked at the DP to SP thymocyte transition. Moreover, in fetal thymic organ cultures from these mice, TCR triggering with anti-CD3 antibodies fails to induce DP apoptosis (Kishihara *et al.*, 1993; Byth *et al.*, 1996; Wallace *et al.*, 1997). ZAP-70 has also been shown to be essential for both positive and negative selection. ZAP-70-deficient mice have an increased number of DP thymocytes and no CD4 or CD8 SP thymocytes (Negishi *et al.*, 1995; Wiest *et al.*, 1997).

At a level more distal to the TCR, some distinctions have been found between signals leading to either positive or negative selection. Experimental evidence for a role of calcium flux in negative selection has been obtained from *in vitro* studies. Intracellular Ca^{2+} chelators have been shown to reduce the ability of DP thymocytes to undergo apoptosis in response to their specific TCR ligand (Vasquez *et al.*, 1994; Kane and Hedrick, 1996). While, inhibitors of the calcium-activated phosphatase calcineurin appear to have little effect on DP thymocyte negative selection by antigen or superantigen (Wang *et al.*, 1995). In contrast, administration of calcineurin inhibitors, *in vivo* or in FTOC has been shown to inhibit the differentiation of SP thymocytes (Gao

et al., 1988; Urdahl *et al.*, 1994; Wang *et al.*, 1995; Anderson *et al.*, 1995). Further analysis of the effect of calcium chelators and calcineurin inhibitors on positive and negative selection led to the notion that calcium and/or calcineurin activation may play a role in setting the threshold for both selection processes. Indeed, calcineurin inhibitors can block negative selection by weakly deleting ligands (Wang *et al.*, 1995; Kane and Hedrick, 1996). Also blocking of extracellular calcium flux can abrogate negative selection in response to weak but not strong affinity TCR ligands (Kane and Hedrick, 1996). Finally, the magnitude of calcium influx in response to different peptide ligands was found to correlate with their ability to induce positive versus negative selection of TCR transgenic thymocytes. Peptides that induce a higher level of calcium flux were shown to be more efficient in triggering negative selection, whereas peptides that induced a lower level of calcium influx were more prone to trigger positive selection (Mariathasan *et al.*, 1998).

A clear distinction between positive and negative selection signalling has been found upon the analysis of the possible involvement of the Ras-Raf-MEK-Erk pathway in these processes. Indeed, this signalling pathway appears to be involved in positive, but not in negative selection. Mice transgenic for a dominant-negative form of Ras, Raf or MEK1 display a deficient T cell development beyond the DP stage (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995; O'Shea *et al.*, 1996), while, expression of a constitutively active Raf form has been shown to promote the differentiation of DP to SP thymocytes (O'Shea *et al.*, 1996). In contrast to their deleterious effect on positive selection, dominant-negative forms of Ras or MEK 1 have no apparent effect on the ability of DP thymocytes to undergo negative selection by superantigens or peptide antigens (Alberola-Ila *et al.*, 1995; Swan *et al.*, 1995). Considering that, in single transgenic mice, the block in positive selection is not absolute, and that the observed effect on positive versus negative selection might involve an altered signalling threshold, mice double transgenic for dominant-negative forms of Ras and MEK 1 were produced. In these mice, DP thymocyte positive selection is almost completely blocked, while negative selection proceeds unimpaired (Alberola-Ila *et al.*, 1996). Vav, a nucleotide exchange factor for the Rho family of GTPases (reviewed in Collins *et al.*, 1997 and Cantrell, 1998), is another signalling protein which has been shown to be involved in positive, but not negative selection. Vav-deficient thymocytes display a profound defect in positive selection, while negative selection is only marginally affected (Fischer *et al.*, 1995; Tarakhovsky *et al.*, 1995; Zhang *et al.*, 1995; Turner *et al.*, 1997).

2.5.4 CD4 versus CD8 T cell lineage commitment

Positively selected DP thymocytes differentiate to either CD4 or CD8 SP thymocytes. As they further mature, most DP thymocytes bearing class II MHC-specific

TCRs retain the expression of CD4, whereas those bearing class I MHC-specific TCRs retain expression of the CD8 co-receptor (reviewed in Fowlkes and Schweighoffer, 1995; Kisielow and von Boehmer, 1995). Although the DP TCR specificity clearly dictates the choice between the CD4 versus CD8 T cell lineage, the mechanism involved in this process remains controversial (reviewed in von Boehmer, 1996). Two major models for T cell lineage commitment have been proposed. These are the instructional and the stochastic/selective model, which will be discussed below.

The stochastic/selective model (Chan *et al.*, 1993a) postulates that, upon TCR engagement, T cell lineage commitment occurs randomly, some cells retain the appropriate co-receptor, while other cells retain a co-receptor that is mismatched with the TCR specificity. Subsequently, upon re-engagement of the TCR and CD4 or CD8 by the same peptide-MHC ligand, only thymocytes bearing a matched co-receptor and TCR specificity are allowed to further mature. The stochastic/selective model is mostly based on the study of so-called transitional cells, i.e., $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocytes. These have been considered to represent CD4 and CD8 committed T cells, on their way to downregulate CD8 or CD4, respectively. Analysis of T cell development in MHC-deficient mice has demonstrated the existence of $CD4^+CD8^{lo}$ and $CD4^{lo}CD8^+$ thymocytes in the absence of MHC class II and MHC class I, respectively (van Meerwijk and Germain, 1993; Davis *et al.*, 1993; Chan *et al.*, 1993a). Moreover, it has been shown that further maturation of transitional cells, with a mismatched TCR and co-receptor, can be partially rescued by the expression of an appropriate co-receptor transgene (Davis *et al.*, 1993; Corbella *et al.*, 1994; Robey *et al.*, 1994).

Nevertheless, it is not certain that the CD4/CD8 phenotype of the so-called transitional cells reflects their T cell lineage commitment. $CD4^{lo}CD8^+$ cells, at least, have been shown to give rise to both CD8 and CD4 SP thymocytes (Lundberg *et al.*, 1995; Lucas *et al.*, 1995; Suzuki *et al.*, 1995; Benveniste *et al.*, 1996; Lucas and Germain, 1996). Furthermore, even in mice constitutively expressing CD4 or CD8 transgenes, the generation of T cells with a mismatched TCR and co-receptor is invariably inefficient (Davis *et al.*, 1993; Robey *et al.*, 1994; Itano *et al.*, 1994; Paterson *et al.*, 1994; Chan *et al.*, 1994c; Baron *et al.*, 1994; Corbella *et al.*, 1994). These observations support the instructional model for T cell lineage commitment. According to this model, T cell lineage commitment is determined by co-engagement of TCR/CD4 or TCR/CD8 on DP thymocytes. It postulates that the CD4 and CD8 co-receptors deliver distinct signals to the DP thymocytes, which trigger the downregulation of CD8 and CD4, respectively (reviewed in Robey and Fowlkes, 1994).

In agreement with the notion of co-receptor differential signalling, it has been shown that a hybrid protein, consisting of the CD8 extracellular and transmembrane domains and the cytoplasmic domain of CD4, triggers the development of large numbers of CD4 T cells with MHC class I-restriction (Itano *et al.*, 1996). A similar finding was obtained with an hybrid

protein comprising the CD8 extracellular domain and the CD4 transmembrane and cytoplasmic domains (Seong *et al.*, 1992). Up to now, it is not known what distinguishes CD4 versus CD8 derived signals. One signalling protein that has been considered is Lck. Indeed, Lck binds non-covalently to a common CXCP motif present in the cytoplasmic domains of both CD4 and CD8, but it associates more weakly with CD8 (Shaw *et al.*, 1990; Turner *et al.*, 1990). Consequently, crosslinking of CD4 has been shown to activate Lck more strongly than crosslinking of CD8 (Veillette *et al.*, 1988; Wiest *et al.*, 1993; Ravichandran and Burakoff, 1994). Nevertheless, it has been found that thymocytes expressing CD4 or CD8 mutant proteins, unable to associate with Lck, can still differentiate into the CD4 or CD8 T cell lineage, respectively (Chan *et al.*, 1993b; Killeen and Littman, 1993).

A third major model for T cell lineage commitment is the instructional/default model. It postulates that CD8 T cell differentiation requires an instructional signal, while differentiation towards the CD4 T cell lineage occurs by default (Suzuki *et al.*, 1995; Benveniste *et al.*, 1996). In support of this model, stimulation of DP thymocytes *in vivo* or in organ cultures with anti-CD3 antibodies results in the differentiation of CD4, but not CD8 SP cells (Groves *et al.*, 1995; Kearse *et al.*, 1995b; Cibotti *et al.*, 1997). Likewise, *in vitro* treatment of DP thymocytes with the phorbol ester 12-myristate 13-acetate (PMA) and the calcium ionophore ionomycin was shown to induce DP thymocyte differentiation into the CD4, but not CD8 T cell lineage (Ohoka *et al.*, 1996). Furthermore, a deficient form of ZAP-70 in humans has been found to result in a complete loss of CD8 T cells, but only in a partial defect in the maturation of CD4 T cells, suggesting that there may be more stringent signalling requirements for CD8 lineage T cells (Arpaia *et al.*, 1994; Chan *et al.*, 1994b; Elder *et al.*, 1994).

2.6 Concluding remarks

This review illustrates the key role of the pre-TCR and αβ TCR in T cell development and function. We have described how the absence of CD3γ chain affects pre-TCR function. In the following chapters, the consequences of a CD3γ-deficient αβ TCR, in T cell functioning, positive/negative selection and T cell lineage commitment, will be presented.

CHAPTER 3

MATERIALS AND METHODS

Contents

3.1 Animals

CD3γ-deficient mice were produced on a mixed FVB/129 Ola genetic background (Haks *et al.*, 1998). CD3γ-deficient mice used in the present study were produced by breeding of mice homozygous for the CD3γ deletion. As control mice, we have used either both of the parental strains (FVB and 129 Ola) or (FVB x 129 Ola) F1 mice. SJL/J mice were used for MLR. All mice were bred at the experimental animal department of the Netherlands Cancer Institute (Amsterdam, The Netherlands) and kept under specific pathogen-free conditions. Unless otherwise specified, mice were used at 6-9 weeks of age.

3.2 Antibodies used in flow cytometry analysis

The following antibodies were used for flow cytometry analysis: FITC labeled anti-TCRβ (H57-597), anti-CD69 (H1-2F3), anti-CD4, anti CD8β.2 (53-7.3) and anti-CD5 (53-7.3); phycoerythrin labeled anti-CD4, anti-CD44 (IM7) and anti-HSA (heat stable antigen); biotin labeled anti-CD8β.2 (53-5.8), anti-CD4, anti-CD25 (7D4), anti-CD69 and anti-L-selectin (CD62L); and APC labeled anti-CD8α (53-6.7). Phycoerythrin labeled anti-CD4 was purchased from Caltag, Burlingame, CA; the remaining antibodies are from PharMingen, San Diego, CA.

3.3 Flow cytometry analysis

Lymphoid organs were processed into single cell suspensions in PBS containing 0.5% BSA (Boehringer Mannheim GmbH, Germany) and 0.02% sodium azide (Merck, Darmstadt, Germany). Maximally $0.5x10^6$ cells were stained per sample. Antibody was added in 20μl per sample, the cells were incubated on ice for 30 minutes and subsequently washed twice. Incubation with biotin-labeled antibodies was followed by incubation with streptavidin-tri-color or streptavidin-phycoerythrin conjugate (Caltag, Burlingame, CA) and two washing steps. Between $15x10^3$ and $30x10^3$ cells were analysed per sample, using either a Becton Dickinson FACScan or a Becton Dickinson FACSCalibur for data acquisition, and CellQuest software for data analysis.

3.4 Cell cycle analysis

$1\text{-}2x10^6$ cells per sample were double stained with the FITC labeled antibodies anti-CD4 and anti-CD8β.2, as described above. Subsequently, the surface labeled cells were ressuspended in 1ml of ice cold 70% ethanol in PBS and incubated on ice for 1-2 hours. After this incubation, the cells were washed twice in PBS supplemented with 0.5% BSA and 0.02% sodium azide. The

cells were resuspended in PBS containing 0.1% sodium citrate, 0.1% Triton X-100, 50µg/ml propidium iodine (Sigma) and 20µg/ml RNase A (Boehringer Mannheim GmbH, Germany), and incubated for 1 hour, at 4 ^{0}C, in the dark. 20x10^3 cells were analysed per sample using a Becton Dickinson FACScan and the CellQuest software. Data acquisition was performed at low flow and with the DDM on.

3.5 Peripheral T cell purification

Peripheral T cells were purified from pools of mesenteric, popliteal and axillary lymph nodes. The lymph nodes were brought into single cell suspensions in Iscove's modified Dulbecco's medium (Gibco/BRL, Paisley, UK), supplemented with 10% FCS (Biowhittaker, Verviers, Belgium), 2x10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Boehringer Mannheim GmbH, Germany). The cells were incubated with anti-MHC class II antibody (M5/114; American Type Culture Collection, Rockville, MD), 30 minutes on ice. Subsequently, the lymph node cells were depleted of antibody binding cells through magnetic bead depletion using a mixture of goat anti-mouse Ig beads (Advanced Magnetics, Cambridge, MA) and sheep anti-Rat Ig beads (Dynal, Oslo, Norway). In some experiments, lymph node cells were enriched for T cells by passage through nylon wool columns (Polysciences, Inc, via Brunschwig chemie, Amsterdam, The Netherlands).

3.6 DP thymocytes purification

Thymocytes were brought into single cell suspension in PBS supplemented with 0.5% BSA. 5x10^6 thymocytes per sample were double stained for CD4 and CD8β.2 (phycoerythrin and biotin labeled, respectively). Subsequently double positive thymocytes were sorted using a MoFlo cytomation, Inc or FACStar Plus (Becton Dickinson).

3.7 Peripheral T cell activation assay

Purified T cells or lymph node cells enriched for T lymphocytes were resuspended in supplemented Iscove's modified Dulbecco's medium, as described above. 1x10^6 cells/ml were incubated in flat bottom 96 well plates (Costar, Corning, NY), pre-coated with anti-CD3ε (145.2C11) or anti-TCRβ (H57-597) and anti-CD28 (37.51) mAbs, at 37 ^{0}C in humidified incubator containing 5% CO_2. After 16-20 hours incubation, the cells were harvested and analysed for the expression of the CD69, by flow cytometry.

3.8 Peripheral T cell apoptosis assay

Purified lymph node T cells were resuspended in supplemented Iscove's modified Dulbecco's medium and incubated in 6 well plates (Costar, Corning, NY) in the presence of PMA (2ng/ml) and Ionomycin (0.1µg/ml) (Sigma). After 2 days incubation, at 37 ^{0}C in humidified incubator containing 5% CO_2, the cells were harvested and washed. Subsequently, the cells were incubated in the presence of 50 U/ml IL-2 (PharMingen, San Diego, CA.), in 6 well plates, for two additional days. The cells were harvested and viable cells were separated on a lymphoprep (Nycomed Pharma As, Norway) density gradient. Viable cells were incubated in flat bottom 96 well plates either pre-coated with anti-CD3ε mAb or in the presence of PMA (20ng/ml) and Ionomycin (500ng/ml). After 12 hours incubation at 37 ^{0}C, 5% CO_2, the cells were harvested, stained with propidium iodide and analysed by flow cytometry.

3.9 Western blot

Purified lymph node T cells were resuspended in supplemented Iscove's modified Dulbecco's medium and incubated in 24 well plates (Costar, Corning, NY), at 3-4 x10^6 cells per well. After overnight incubation at 37 ^{0}C, 5% CO_2, the cells were harvested and incubated with anti-CD3ε mAb (10µg/ml), 30 minutes on ice. Subsequently, the cells were washed twice with cold medium and goat anti-hamster antibody (Pierce, Rockford, IL) was added at 10µg/ml in warm (37 ^{0}C) medium. After a 2.5 minutes incubation at 37 ^{0}C, the reaction was stopped by addition of ice-cold PBS. The cells were subsequently lysed for 30 minutes on ice. The lysis buffer consisted of 20mM Tris at pH 7.6 (Boehringer Mannheim GmbH, Germany), 150mM NaCl, 2mM EDTA (Merck, Darmstadt, Germany), 1% Triton X-100 (Sigma) and freshly added protease/phosphatase inhibitors 2 µg/ml Leupeptin (Boehringer Mannheim GmbH, Germany), 1mM PMSF, 10mM NaF and 1mM Na_3VO_4 (Sigma). Lysates were cleared by centrifugation at 14000 g for 10 minutes at 4 ^{0}C. Protein concentration was determined using the bicinchininic acid protein assay (Pierce, Rockford, IL). Proteins from total cell lysates (10µg per lane) were resolved on 10% SDS-PAGE (9.9% acrylamide, 0.3% bisacrylamide, 0.4M Tris at pH 8.8, 0.1% SDS, 0.05% APS and 0.1% TEMED (Bio-Rad Laboratories, Hercules, CA) and transferred onto nitrocellulose membranes (Schleider & Schuell, Dassel, Germany), using a Trans-blot semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA) and a transfer buffer consisting of 24.8mM Tris at pH 8.3 (Boehringer Mannheim GmbH, Germany), 0.2M Glycine and 20% methanol (Merck, Darmstadt, Germany). Membranes were blocked overnight at 4 ^{0}C, with PBS 1% BSA, 0.02% Tween (Merck, Schuchardt, Germany). Subsequently, the membrane was incubated with the anti-phosphotyrosine antibody (4G10) (Upstate Biotechnology Inc., Lake Placid, NY) in PBS 1% BSA, 0.02% Tween, 4 hours room temperature.

The membrane was washed 3-4 times and incubated with a rabbit anti-mouse peroxidase-conjugate (Dako), two hours at room temperature. The membrane was washed and the blots were developed using enhanced chemiluminescence (Amersham Intl, Buckinghamshire, UK).

3.10 TCR downregulation assay

Purified lymph node T cells were resuspended in supplemented Iscove's modified Dulbecco's medium, at $1x10^6$ cells/ml concentration, and incubated in flat-bottom 96 well plates either pre-coated with different concentrations of anti-CD3ε or in the presence of different concentrations of PMA. After 16-20 hours incubation at 37 ^{0}C, 5% CO_2, the cells were harvested, stained with anti-TCR and their level of αβTCR cell surface expression was analysed by flow cytometry.

3.11 Mixed lymphocyte reaction assay

Purified lymph node T cells were cultured in the presence of irradiated SJL/J or autologous spleen cells, in 96 round-bottom well plates, 200 $x10^3$ T cells and $100x10^3$ spleen cells per well. After 3 days of culture (at 37 ^{0}C, 5% CO_2), the cells were pulsed with 1 μCi of [^{3}H] thymidine and incubated for 6 additional hours. Subsequently, the cells were harvested onto an UNIFilter GF/C plate, using a Filtermate 196 (Packard). 25 μg/ml of a scintillation liquid (Microscint-O, Packard) was added to the plates and finally the [^{3}H] thymidine uptake was measured using a microplate scintillation counter (Packard).

3.12 *In vitro* positive selection assay

Thymocytes were brought into single cell suspension in supplemented Iscove's modified Dulbecco's medium. The cells were incubated at $2x10^6$ cells/ml concentration in flat-bottom 96 well plates pre-coated with anti-TCRβ and anti-CD28. After 16-20 hours of incubation at 37 ^{0}C in 5% CO_2, the cells were harvested and triple stained for CD4, CD8β.2 and CD69, or CD5. Subsequently, the cells were analysed by flow cytometry.

3.13 *In vitro* negative selection assay

Thymocytes were brought into single cell suspension in supplemented Iscove's modified Dulbecco's medium. The cells were incubated at $2x10^6$ cell concentration in round-bottom 96

well plates (Costar, Corning, NY). Induction of thymocyte deletion by TCR triggering was performed by incubating the thymocytes with 0.5 x10^6/ml B7.1 transfected P815 mastocytoma cells (DNAX, Palo Alto, CA) and different concentrations of soluble anti-TCR. Thymocyte deletion by glucocorticoids was performed by incubating thymocytes with different concentrations of dexamethasone (Sigma). After 16-20 hours incubation at 37 ^{0}C, 5% CO_2, the cells were harvested, double stained for CD4 and CD8β.2 and analysed by flow cytometry.

3.14 Co-receptor re-expression assay

TCRβ^{hi} HSA$^+$ thymocytes were purified as follows: Thymocytes were brought into single cell suspension in PBS supplemented with 0.5% BSA. The cells were double stained for TCRβ and HSA and TCRβ^{hi} HSA$^+$ thymocytes were sorted using a MoFlo cytomation, Inc or FACStar Plus (Becton Dickinson). Purified TCRβ^{hi} HSA$^+$ thymocytes were incubated with 0.02% EDTA with or without trypsin (10μg/ml), 30 minutes at 37 ^{0}C, 5% CO_2. Subsequently, the cells were washed, resuspended in supplement IDDM and further incubated at 37 ^{0}C, 5% CO_2. After overnight incubation, the cells were harvested, double stained for CD4 and CD8β.2 and analysed by flow cytometry.

CHAPTER 4

RESULTS AND DISCUSSION ON THE ROLE OF CD3γ CHAIN IN PERIPHERAL T CELL FUNCTIONING

Contents

4.1 Introduction

CD3γ deficiency causes a severe block in T cell differentiation at the developmental checkpoint under the control of the pre-TCR (Haks *et al.*, 1998), as detailed earlier in chapter 2. Nevertheless, some thymocytes do complete their maturational program and do colonize the peripheral lymphoid organs. Small numbers of both $CD4^+$ and $CD8^+$ T cells can be found in the lymph nodes and spleens of these mice. As in wild type mice, the majority of peripheral T cells in the CD3γ-deficient mice belong to the αβ T cell lineage. Indeed, the absence of the CD3γ chain appears to affect the differentiation of both αβ and γδ T cells (Haks *et al.*, 1998). Interestingly. this is in contrast with the phenotype of CD3δ-deficient mice. Mice lacking the CD3δ chain show a developmental arrest of T cells belonging to the αβ T cell lineage, while differentiation of γδ T cells appears to proceed undisturbed (Dave *et al.*, 1997). Besides the low T cell numbers, as compared to the numbers present in wild type mice, two notable features characterize the peripheral T cells in mice lacking the CD3γ chain. One of these is the ratio of CD4 to CD8 T cells, the other is the cell surface expression level of the αβ TCR. As compared to wild type mouse T cells, CD3γ-deficient mouse T cells display a decreased CD4:CD8 T cell ratio and an approximately 10-fold reduced level of cell surface TCR expression (Figure 4.1).

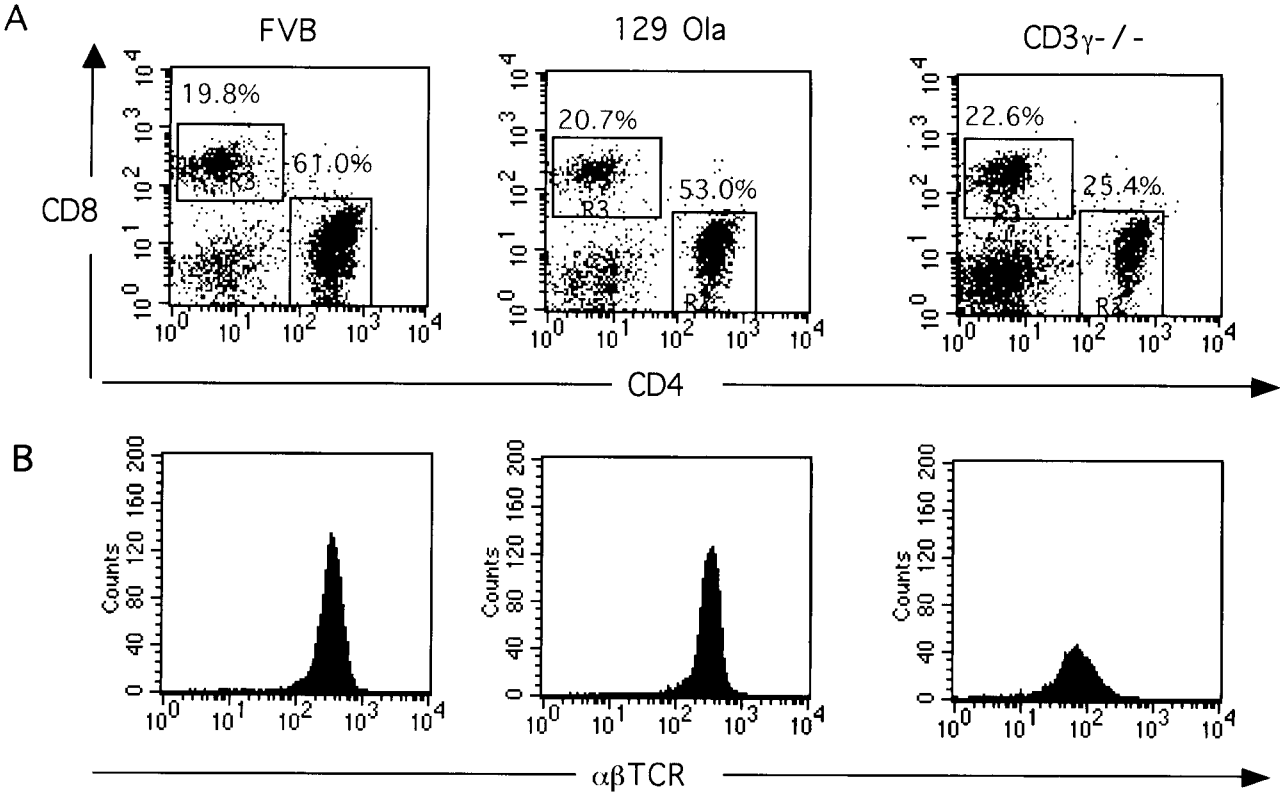

Figure 4.1: Lymph node T cell phenotype. Flow cytometry analysis of the percentage of $CD4^+$ and $CD8^+$ T cell subsets among all lymph node cells (A) and αβTCR expression level among lymph node peripheral T cells, i.e., $CD4^+/CD8^+$ cells (B).

The exact composition of the αβ TCR expressed on T cells from the CD3γ-deficient mice is not known yet. Nevertheless, it is clear that these TCR complexes do not integrate the CD3γ chain. Northern blot analysis has revealed that CD3γ-/- thymocytes lack any detectable expression of CD3γ mRNA (Haks *et al.*, 1998). How the relatively low levels of cell surface expression of TCR complexes devoid of the CD3γ chain affects T cell function is the subject of this chapter.

4.2 A relatively high fraction of CD3γ-deficient peripheral Tcells display an activation phenotype

We have initiated our study on the functionality of peripheral CD3γ-deficient T cells by analysing *ex-vivo* lymph node T cells for their resting/activation or naive/memory phenotype. There are currently no phenotypic markers that reliably distinguish effector from memory T cells. Nevertheless, it is possible to phenotypically distinguish naive T cells from effector or memory T cells. Naive and effector/memory mouse T cells differ in their expression of the homing receptor MEL-14 (or L-selectin), CD44 and CD45RB (Budd *et al.*, 1987; Bradley *et al.*, 1992; reviewed in Bradley *et al.*, 1993). CD69 and the α chain of the IL-2R (CD25) are two other cell surface markers which are upregulated at an early stage following a productive TCR engagement. CD69 is even referred to as the early activation cell surface marker. On T cells, its expression is induced within 2 hours upon TCR ligation and disappears quickly from the cell surface in the absence of further signalling (Hara *et al.*, 1986; Testi *et al.*, 1989).

We analysed the CD3γ-deficient lymph node T cells for their expression of the CD69, CD25, CD44, L-selectin and CD45RB cell surface markers. Pools of lymph nodes derived from CD3γ-deficient mice or from the parental wild type mouse strains, 129 Ola and FVB, were processed into single cell suspensions. The cells were triple stained for CD4, CD8 and the cell surface markers referred above. Subsequently the cells were analysed by flow cytometry. Interestingly, as compared to wild type mice, T cells derived from CD3γ-deficient mice were found to comprise a relatively high percentage of cells positive for the expression of the CD69 and CD25 activation markers (Figure 4.2). Moreover, a relatively high fraction of the mutant T cells were found to display an effector/memory T cell phenotype. The percentage of T cells positive for CD44 or negative for the L-selectin cell surface marker is relatively high among the CD3γ-deficient T cells, as compared to the percentage observed among wild type mouse T cells.

We next examined the T cells for their DNA content. In agreement, with our data concerning the T cells resting/activated or memory phenotype, lymph node T cells

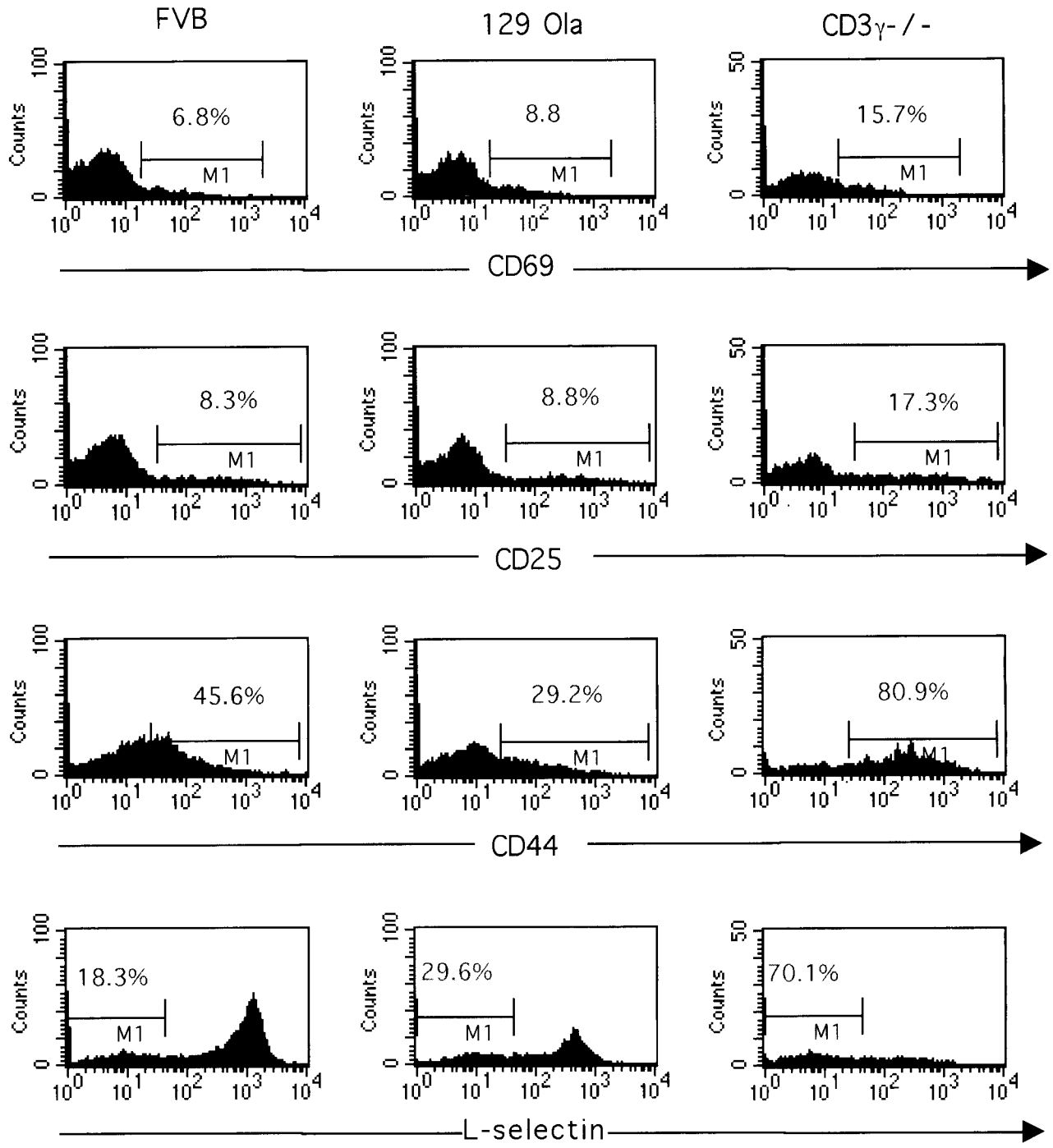

Figure 4.2: Lymph node T cells phenotype. Flow cytometry analysis of cell surface expression of CD69, CD25, CD44 and L-selectin markers among CD4+/CD8+ lymph node cells.

derived from the CD3γ-deficient mice were found to comprise a relatively high percentage of cells in the S/G2/M stages of the cell cycle. As compared to lymph node T cells from the wild type mouse strains, FVB and 129 Ola, a higher percentage of CD3γ-deficient T cells are undergoing proliferation (Figure 4.3).

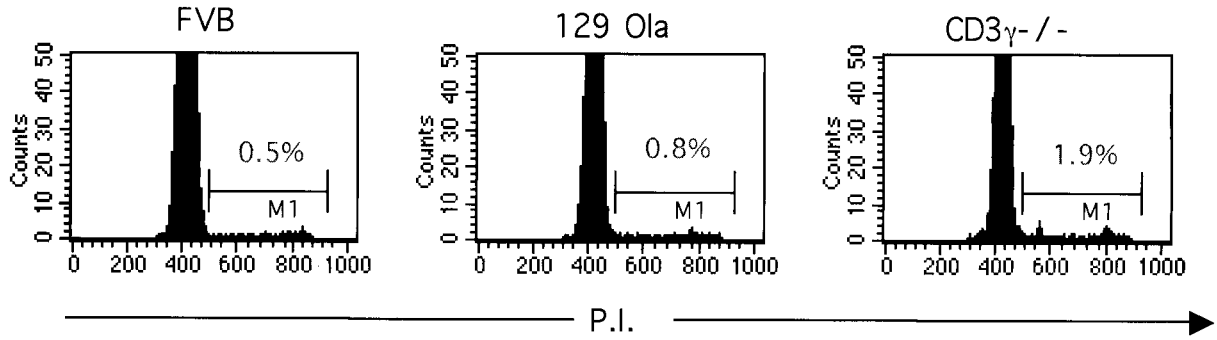

Figure 4.3: Cell cycle analysis of lymph node T cells. Flow cytometry analysis of DNA content, by propidium iodine staining, in CD4+/CD8+ lymph node cells. Shown is the percentage of T cells in G2/S/M stages of the cell cycle.

Altogether, the pattern of expression of the cell surface markers used in our analysis and the cell cycle status, strongly suggest that a relatively high fraction of the peripheral T cells of the CD3γ-deficient mice are not naive T cells, but rather represent activated/effector or memory T cells. Moreover, a separate analysis of $CD4^+$ and $CD8^+$ T cells revealed that this differential functional state mainly affects the $CD4^+$ T cells. Indeed, CD3γ-deficient $CD8^+$ T cells are nearly indistinguishable from their wild type counterparts in terms of the expression of the CD69 cell surface marker. In contrast, CD3γ-deficient $CD4^+$ T cells clearly comprise a higher percentage of cells positive for the cell surface expression of this marker (Figure 4.4). Curiously, a predominantly

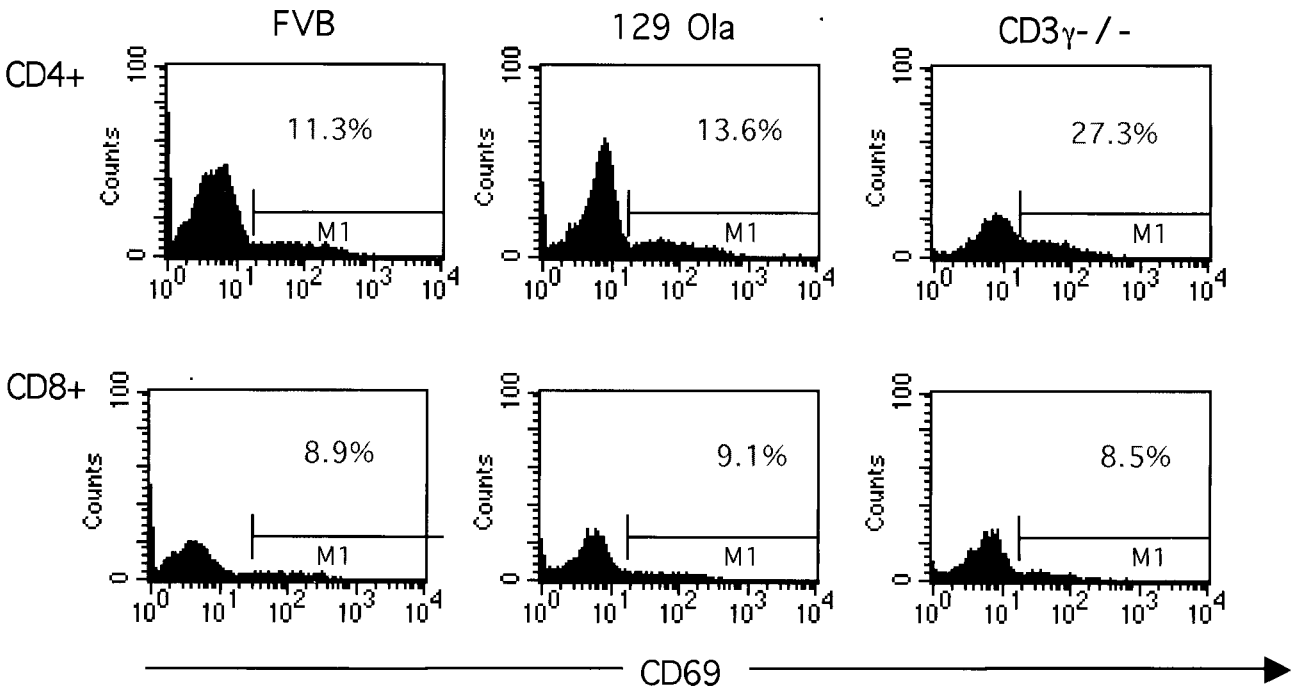

Figure 4.4: Differential activation state of CD4+ versus CD8+ lymph node T cells. Flow cytometry analysis of CD69 cell surface expression among CD4+ and CD8+ lymph node cells.

effector/memory phenotype has been previously observed among peripheral T cells derived from mice which lack the CD3δ chain, as well as from TCRζ/η-deficient mice (Lin *et al.*, 1997; Shores *et al.*, 1998; Dave *et al.*, 1997). Although they do not show the data, Dave and co-workers described the CD3δ-deficient peripheral T cells as mostly $CD45RB^{lo}$, L-$selectin^{lo}$ and $CD44^{hi}$ (Dave *et al.*, 1997). TCRζ/η-deficient T cells were shown to be uniformly $CD44^{hi}$ and L-$selectin^{lo}$ (Shores *et al.*, 1998). In contrast to the CD3γ-deficient T cells, T cells derived from mice lacking the TCRζ and TCRη chains are similar to wild type in terms of their expression of CD69 and CD25 cell surface markers (Lin *et al.*, 1997). In both CD3δ and TCRζ/η-deficient mice there is a severe blockage in T cell development from the DP to SP stage.

4.3 CD3γ-deficient T cells are defective in their ability to become activated upon anti-CD3 stimulation, but not by PMA treatment

As the CD3γ knock out mice, CD3γ-deficient patients have a reduced number of peripheral T cells. These patients have been reported to display an impairment in several functions associated with T cell activation (Arnaiz-Villena *et al.*, 1991; Perez-Aciego *et al.*, 1991; Arnaiz-Villena *et al.*, 1992). Nevertheless, the phenotype of the CD3γ-deficient mouse peripheral T cells suggests that a relatively large fraction of these cells are either activated or memory T cells. Therefore, we have examined the ability of T cells from CD3γ-deficient mice to become activated upon stimulation with anti-CD3ε mAb. T cells derived from CD3γ-deficient mice and from wild type parental mouse strains were triggered with plate-bound anti-CD3ε and analysed for their ability to upregulate the cell surface expression of the early activation cell surface marker CD69. As can be seen in Figure 4.5A, CD3γ-deficient T cells are clearly defective in their ability to upregulate CD69 cell surface expression upon TCR triggering by the anti-CD3ε mAb. While, after 20 hours of culture, 45% and 35% of FVB and 129 Ola mice derived T cells, respectively, express the CD69, only 10% of the CD3γ-deficient T cells do so. In contrast, incubation of the lymph node T cells with the phorbol ester PMA (1ng/ml) equally induces CD69 expression on CD3γ-deficient T cells, as on FVB and 129 Ola mice T cells (Figure 4.5B). Treatment with phorbol esters activates T cells in a TCR-independent fashion, by directly activating downstream components of the TCR signalling pathway (Truneh *et al.*, 1985 and reviewed in Berry and Nishizuka, 1990). Therefore, the observation that CD3γ-deficient T cells are defective in Tcell activation by stimulation with anti-CD3ε mAb, but not by PMA treatment, indicates that their defect in activation occurs at the TCR proximal level.

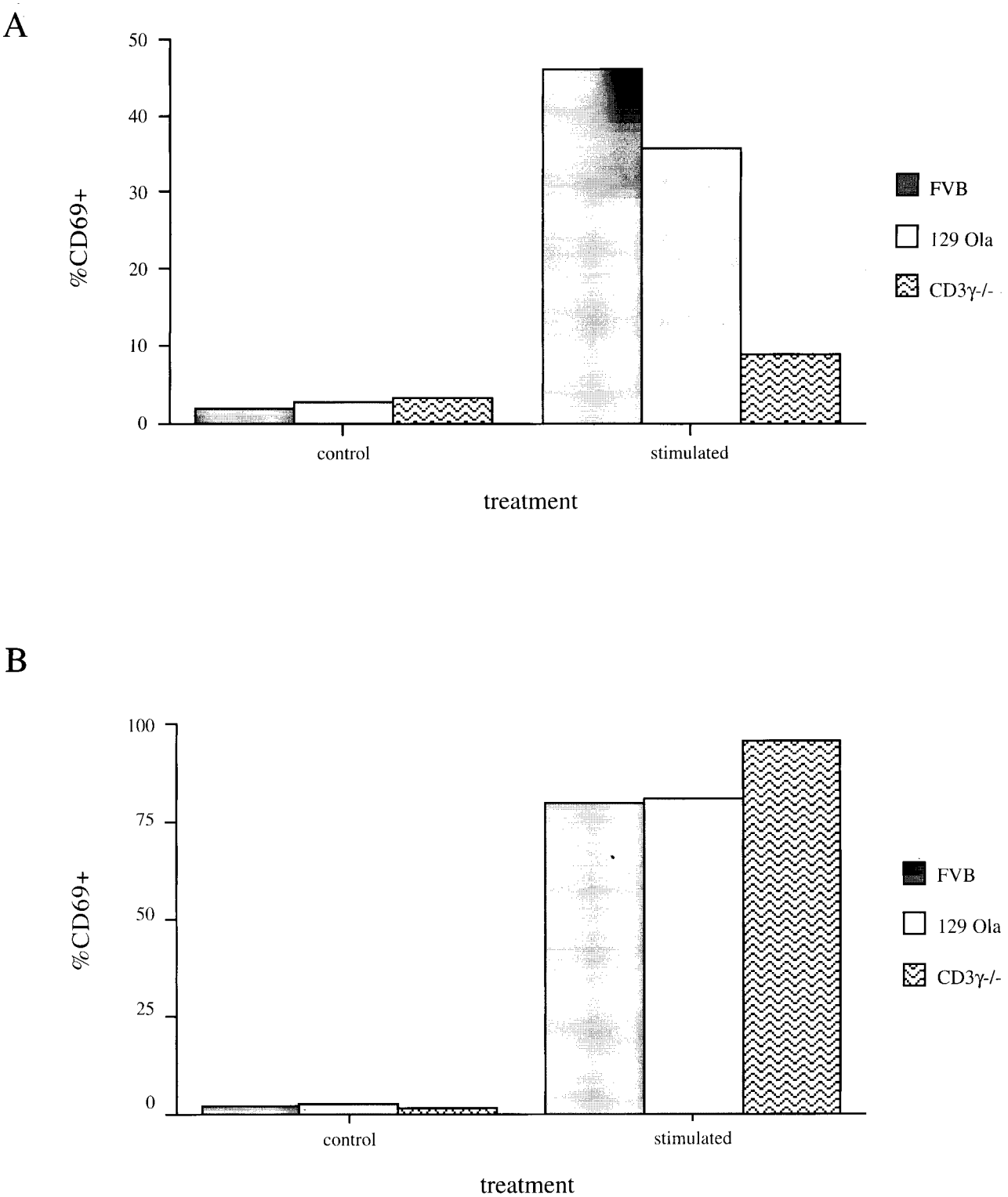

Figure 4.5: Ability of lymph node T cells to become activated. Lymph node cells enriched for T cells, by nylon wool passage, were stimulated with either 10μg/ml plate-bound anti-CD3 (A) or 1ng/ml PMA (B). Shown are the percentages of CD69+ cells among $CD4^+/CD8^+$ cells, after culture.

T cells derived from TCRζ/η-deficient mice have also been described as defective in terms of their ability to become activated upon TCR engagement, but not PMA treatment (Shores *et al.*, 1998). Nevertheless, it has been shown that low levels of cell

surface expressed TCRζ-deficient TCRs can still minimally signal when stimulated with high density plate-bound anti-CD3ε mAb, or high concentrations of antigen or superantigen (Wegener *et al.*, 1992; Hermans and Malissen, 1993). As the CD3γ-deficient T cells display low levels of cell surface TCR expression, when compared to FVB and 129 Ola mouse T cells, we hypothesised that their apparently defective ability to become activated by anti-CD3ε stimulation, could merely reflect the requirement for a higher concentration of the stimulating antibody. To test this hypothesis, peripheral T cells from the CD3γ-deficient mice and from FVB and 129 Ola mouse strains, were triggered with different concentrations of plate-bound anti-CD3ε, up to 100 μg/ml, and analysed for the expression of CD69. In support of our hypothesis, by increasing the concentration of anti-CD3ε, we could also increase the percentage of CD3γ-deficient T cells that are induced to express CD69 on their cell surface. Nevertheless, while 100% of FVB and 129 Ola mice T cells can be induced to express CD69, a fraction of CD3γ-deficient T cells remains negative for the expression of CD69, at the highest anti-CD3ε concentration tested (Figure 4.6).

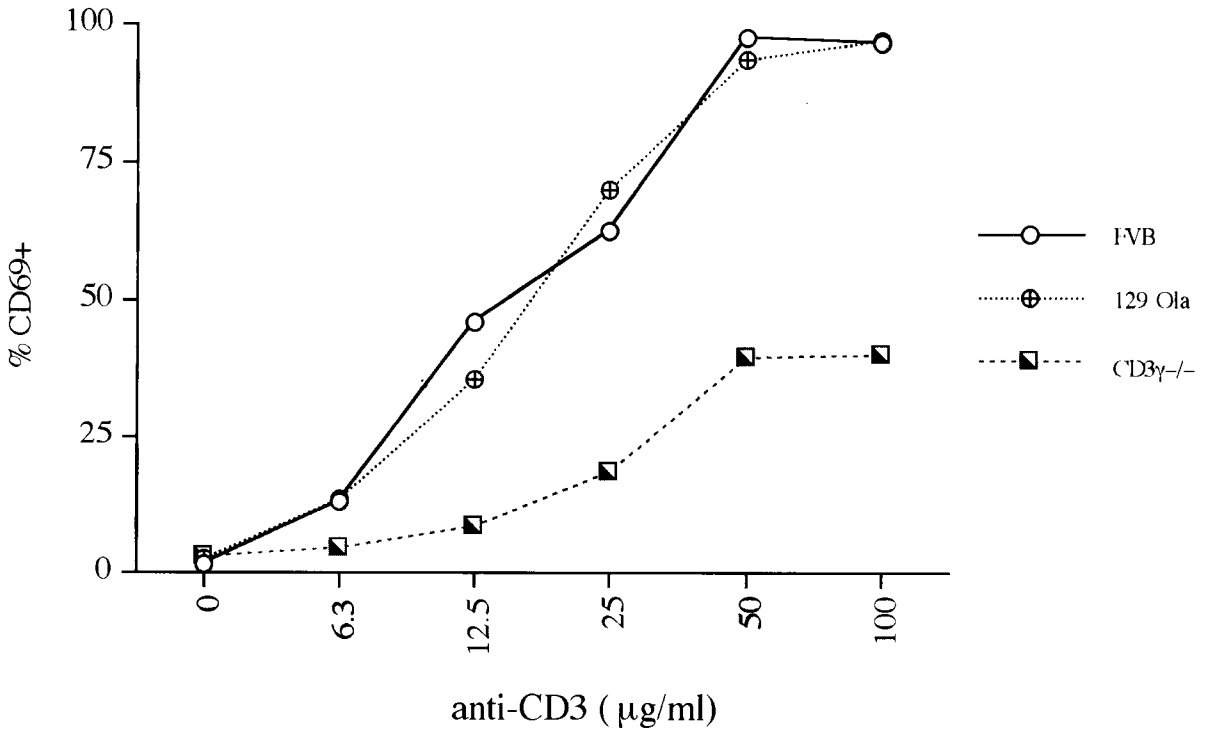

Figure 4.6: Ability of lymph node T cells to become activated upon TCR triggering with different doses of anti-CD3ε mAb. Lymph node cells enriched for T cells were stimulated with different doses of plate-bound anti-CD3ε mAb. Shown is the percentage of CD69+ cells among CD4+/CD8+ lymph node cells, after culture.

The reduced level of TCR cell surface expression observed in CD3γ-deficient mice is reminiscent of the phenotype of T cells derived from CD3δ-deficient mice. In these mice, the absence of the CD3δ chain was also found to lead to a reduction in the level of

CD3ε cell surface expression (Dave *et al.*, 1997). In TCRζ/η-deficient mice, TCR expression is barely detectable on the few T cells that colonize their peripheral lymphoid organs (Malissen *et al.*, 1993; Liu *et al.*, 1993; Love *et al.*, 1993). These observed reductions in the level of TCR cell surface expression most likely reflect the requirement of the different chains as structural components of the TCR complex. While the CD3ε and the TCRζ chains have consistently been found to be absolutely required for TCR cell surface expression (Sussman *et al.*, 1988; Hall *et al.*, 1991; Kappes and Tonegawa, 1991), the same is not true for the CD3γ and -δ chains. Indeed, while in some instances, the TCR cell surface expression appears to be absolutely dependent on the presence of the CD3γ and CD3δ chains (Geisler, 1992; Buferne *et al.*, 1992). Other examples have been described where the absence of CD3δ or CD3γ reduces, but does not abolish the cell surface expression of a TCR complex (Kappes and Tonegawa, 1991; Perez-Aciego *et al.*, 1991). These last observations have led the authors to suggest the existence of two types of αβ TCR forms: those lacking CD3γ but having CD3δ, and those containing CD3γ instead of CD3δ (reviewed in Regueiro *et al.*, 1992). Nevertheless, an alternative hypothesis can be considered for the low TCR cell surface expression observed in the CD3γ-/-mice: the αβ TCR complex found in these mice may comprise two CD3δ chains, instead of a putative regular one CD3δ chain:one CD3γ chain composition. Such a TCR may assemble less efficiently or their cell surface expression may be more unstable.

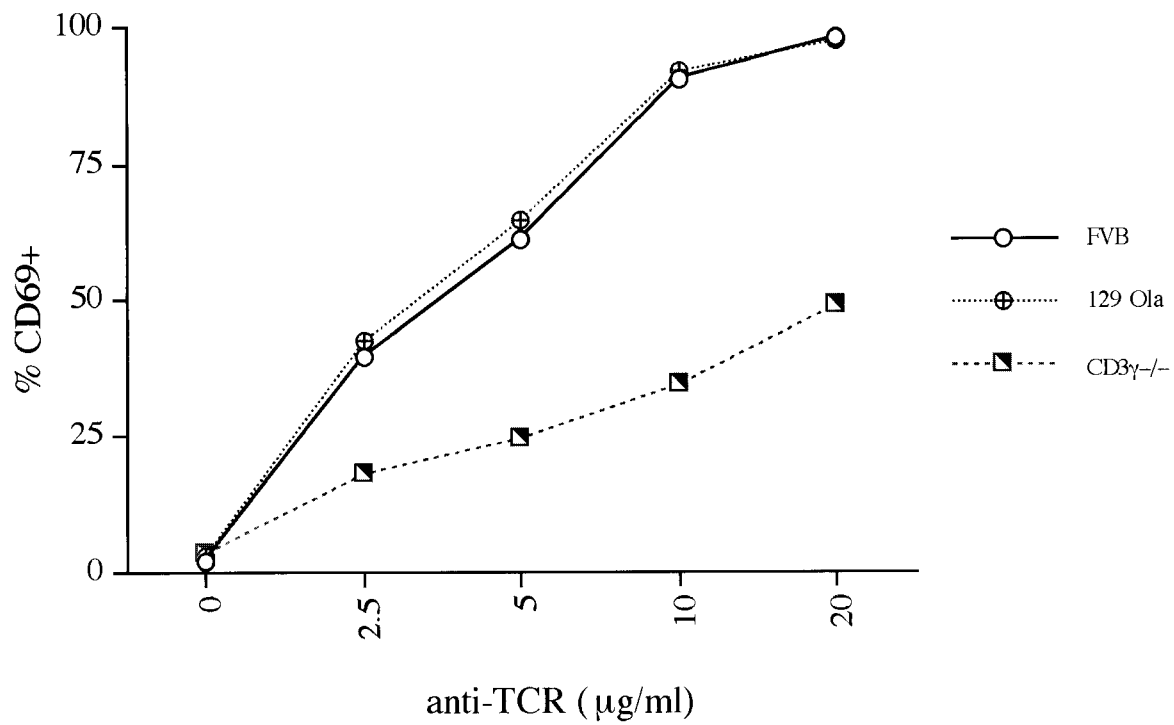

Figure 4.7: Ability of lymph node T cells to become activated upon TCR triggering with different doses of anti-TCRβ mAb. Lymph node cells enriched for T cells were stimulated with different doses of plate-bound anti-TCRβ mAb. Shown is the percentage of CD69+ cells among CD4+/CD8+ lymph node cells, after culture.

The 145.2C11 anti-CD3ε mAb recognizes both the CD3γε and the CD3δε dimers. Wild type αβTCRs are generally thought to comprise a TCRαβ heterodimer, a TCRζζ homodimer, a CD3γε dimer and a CD3δε dimer, in a stoichiometry of 1:1:1:1 (Koning *et al.*, 1990; de la Hera *et al.*, 1991; Hou *et al.*, 1994; Punt *et al.*, 1994a). According to this model, the anti-CD3ε mAb would bind two epitopes per TCR complex and therefore crosslink αβTCRs expressed on the cell surface. In CD3γ-deficient T cells, in addition to a low TCR cell surface expression, the CD3γ chain is not a component of the αβTCR complex. The exact composition of the mutant mice TCRs is not known. Two possible subunit compositions can be envisaged. The CD3γ-deficient αβTCR lacks a CD3γε dimer, but includes two CD3δε dimers or alternatively it does not include a CD3γε, but only one CD3δε dimer. According to our first hypothesis, the CD3γ-deficient αβTCR would retain two anti-CD3ε mAb binding epitopes per TCR. In contrast, according to our second hypothesis, the anti-CD3ε mAb would bind two epitopes per TCR on wild type TCRs, while it would bind only one epitope per TCR on CD3γ-deficient TCRs. Therefore, we have considered the possibility that differential TCR triggering could play a role in the relative inability of CD3γ-deficient T cells to become activated upon stimulation with anti-CD3ε mAb. In order to eliminate the variable presence/absence of the CD3ε/γ dimer, and to test the hypothesis that an altered number of epitopes per TCR may play a role in the differential ability of the T cells to become activated, we stimulated T cells with anti-TCRβ mAb (H57-597). As can be seen in Figure 4.7, stimulation with anti-TCRβ mAb induced the upregulation of CD69 expression on 100% of FVB and 129 Ola mice T cells. Stimulation of CD3γ-deficient T cells with anti-TCRβ resulted in a slight increase in the maximal percentage of CD69 expressing cells, as compared to the percentage observed upon stimulation with anti-CD3ε. Nevertheless, with anti-TCRβ as with anti-CD3ε stimulation, a considerable fraction of the CD3γ-deficient T cells does not upregulate CD69 cell surface expression. These results suggest that a differential number of mAb epitopes per TCR, if it occurs, is not the cause for the observed relative inability of CD3γ-deficient T cells to become activated upon TCR engagement.

The defective ability of the CD3γ-deficient T cells to become activated upon *in vitro* TCR engagement is not in agreement with the phenotype of T cells analysed directly *ex-vivo*. Indeed, as compared to wild type mice, peripheral T cells from the CD3γ-deficient mice appear to include a relatively high fraction of activated/memory T cells. We considered the possibility that upon *in vitro* TCR engagement, the mutant T cells undergo apoptosis rather than activation. However we did not detect any significant difference between the mutant and wild type T cells in terms of the percentage of dead cells upon culture (data not shown). Another possibility is that the mutant T cells can become activated *in vivo* because a defective TCR signalling is compensated by

costimulation. Under physiological conditions, suboptimal crosslinking of the TCR is not sufficient to induce a productive T cell response, as it requires co-stimulatory signals provided by the APCs (Jenkins and Schwartz, 1987; Jenkins *et al.*, 1988; Mueller *et al.*, 1989). Interactions between CD28 on T cells and B7-1 or B7-2 on APCs can generate crucial costimulatory signals for T cell activation (reviewed in Linsley and Ledbetter, 1993; June *et al.*, 1994). Triggering of CD28 activates cellular PTKs whose substrates include proteins that are known to also become phosphorylated upon TCR signalling (Nunes *et al.*, 1994). A synergistic effect is clear with respect to the activation of the PI3K, as costimulation of the TCR and CD28 is required for an optimal activation of this enzyme. CD28 has the ability to recruit the PI3K to the plasma membrane via an association between the SH2 domains of the enzyme p85 regulatory subunit and the tyrosine phosphorylated cytoplasmic domain of CD28 (Page *et al.*, 1994; Truitt *et al.*, 1994). In addition, CD28 has been shown to generate signals that synergize with TCR signalling to activate the MAP kinase JNK1 (Su *et al.*, 1994).

Given this background, we tested the hypothesis that T cell costimulation with anti-CD28 is capable of circumventing a putative defective TCR signalling and lead to the full activation of CD3γ-deficient T cells. Purified peripheral T cells from the CD3γ-deficient mice and from FVB and 129 Ola mouse strains, were triggered with anti-TCRβ either without or with additional anti-CD28 mAb. After 20 hours of incubation, the cells were harvested, triple stained for CD69, CD4 and CD8 and analysed by flow cytometry. As shown in Figure 4.8, virtually all wild type mice derived T cells become CD69$^+$ with or

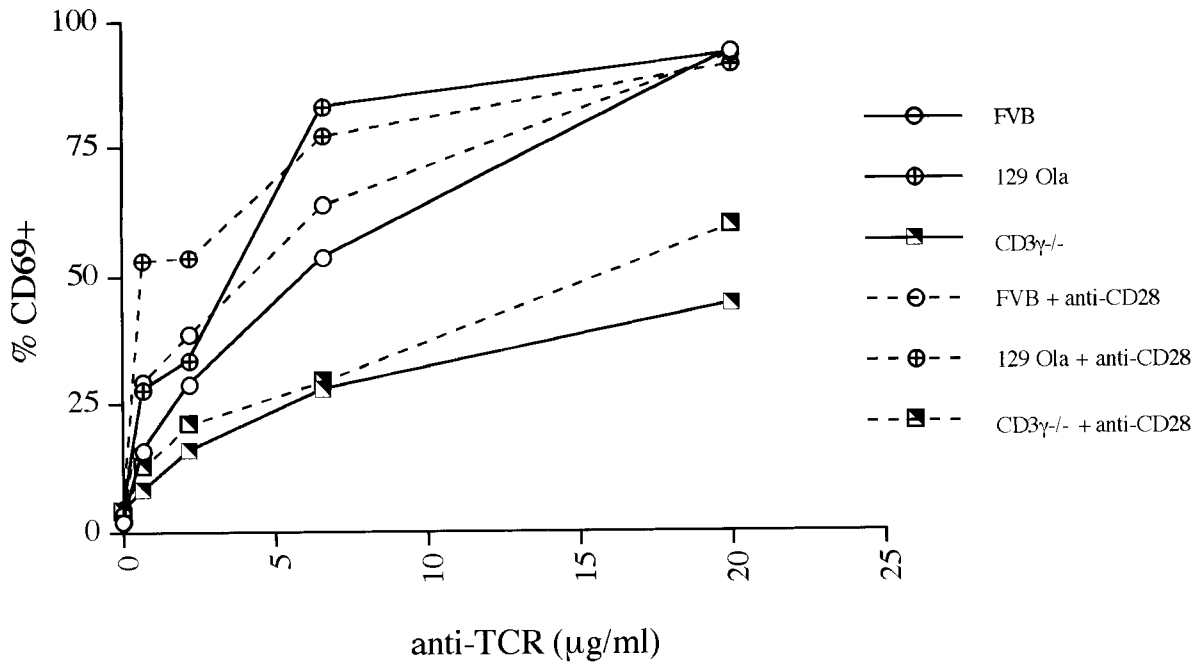

Figure 4.8: Ability of lymph node T cells to become activated upon TCR-CD28 co-stimulation. Purified lymph node T cells were stimulated with different doses of plate-bound anti-TCRβ with or without co-stimulation by plate-bound anti-CD28. Shown is the percentage of CD69$^+$ cells after culture.

without CD28 costimulation. In contrast, CD28 costimulation leads to an increase in the percentage of CD3γ-deficient T cells that upregulated CD69 expression, as compared to mutant T cells stimulated only through their TCRs. Nevertheless, a fraction of CD3γ-deficient T cells still remains CD69- negative upon costimulation with anti-CD3ε and anti-CD28. A separate analysis of CD4+ and CD8+ T cells also revealed a differential ability of these cells to become activated upon TCR engagement (Figure 4.9). Indeed, the percentage of CD4+ CD3γ-deficient T cells which are induced to upregulate CD69 cell surface expression is approximately 10% higher, as compared to the percentage of mutant CD8+ T cells which become CD69+ upon anti-TCR and anti-CD28 co-stimulation.

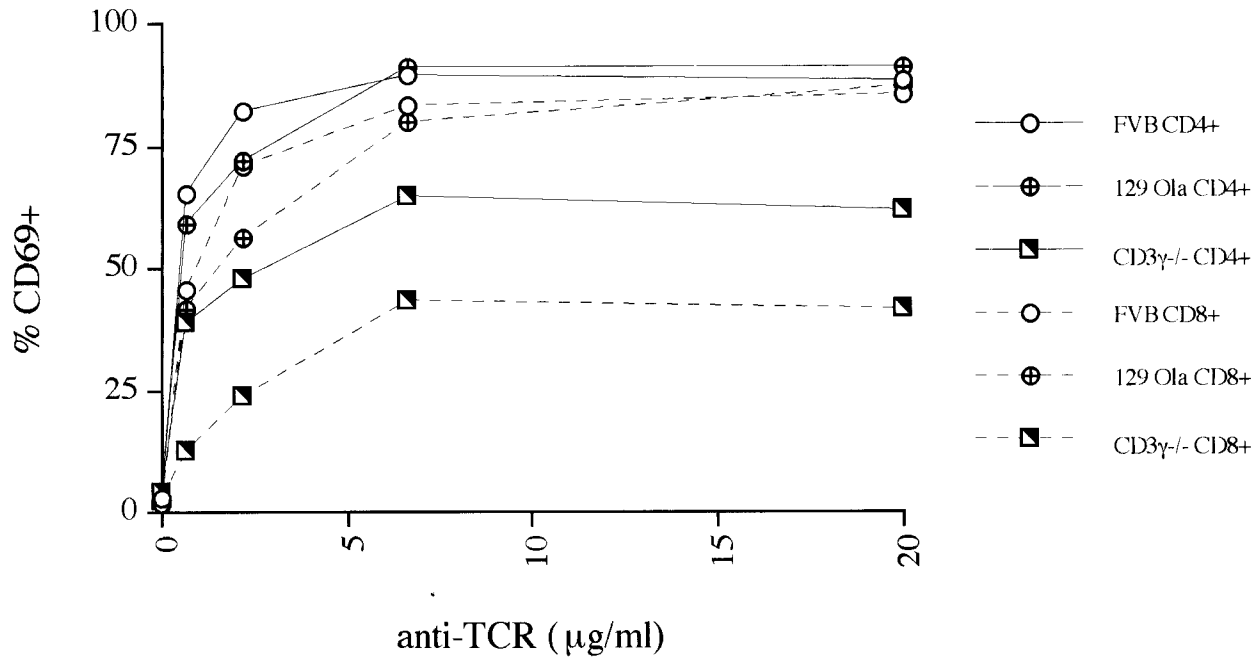

Figure 4.9: Differential ability of CD4+ and CD8+ T cells to become activated upon TCR triggering. Purified lymph node T cells with stimulated with different doses of plate-bound anti-TCRb with or without CD28 co-stimulation by plate-bound anti-CD28. Shown is the percentage of CD69+ cells among CD4+ and CD8+ T cells, after culture.

4.4 CD3γ-deficient T cells are defective in their ability to undergo activation-induced cell death

On mature resting peripheral T cells, engagement of the TCR leads to T cell activation, interleukin-2 production and entry into the cell cycle. However, strong TCR stimulation of cycling T cells in the periphery can also lead to cell death by apoptosis, a phenomenon which has been designated by activation-induced cell death (Jones *et al.*, 1990; Rocha and von Boehmer, 1991; reviewed in Kabelitz *et al.*, 1993 and Lenardo *et al.*, 1995). Apoptosis is an active process by which unnecessary or harmful cells are self-

eliminated in multicellular organisms (reviewed in Steller, 1995 and Nagata, 1997). Likewise, activation-induced cell death is thought to control the expansion of antigen-activated T cells after an immune response and to contribute to the establishment of peripheral T cell tolerance through the deletion of unwanted self-reactive T cells (reviewed in Green and Scott, 1994 and Lenardo *et al.*, 1995). Several members of the growing families of the TNF (tumour necrosis factor) and TNFR (tumour necrosis factor receptor) have been shown to be involved in mediating the final stages of programmed cell death or apoptosis (reviewed in van Parijs and Abbas, 1996; Wong and Choi, 1997 and Winoto, 1997). Experimental evidence strongly suggests that the TNF family member CD95/Fas and the TNFR family member CD95L/FasL are the most crucial mediators of apoptosis in peripheral T cells. Indeed, it has been shown that stimulation through the TCR complex results in the upregulation of CD95/Fas and CD95L/FasL, and that subsequent binding of CD95/Fas by its ligand results in the direct activation of a cascade of proteases that finally lead to T cell death (reviewed in Wong and Choi, 1997 and Winoto, 1997). Interestingly, it has been shown that the processes of T cell activation and T cell activation-induced cell death can be differentially affected upon TCR deficiencies. This has been clearly shown with an experimental system comprising a TCRβ-deficient Jurkat T cell line, reconstituted with either a wild type TCRβ chain or with a TCRβ chain mutated in its transmembrane region. This mutation resulted in a deficient association of the TCRζ chain with the rest of the TCR complex. Using this system, it was shown that upon TCR engagement, TCRβ-deficient Jurkat T cells transfected with the mutated TCRβ chain, have the ability to upregulate the activation cell surface markers CD69 and CD25 and to produce and secrete interleukin-2, but are resistant to undergo activation-induced cell death. This resistance was found not to be due to generalized lower levels of calcium influx or activation of protein tyrosine kinases. Instead, the observed resistance was found to be based on a defective induction of CD95L/FasL (Rodriguez-Tarduchy *et al.*, 1996). Other studies have also suggested that the cytoplasmic domain of the TCRζ chain plays an important role in Fas-dependent T cell death. Crosslinking of chimeric receptors containing a TCRζ cytoplasmic domain was shown to result in induction of apoptosis in a transfected murine T cell hybridoma (Vignaux *et al.*, 1995; Combadiere *et al.*, 1996). In contrast, T cells derived from TCRζ-deficient mice reconstituted with a TCRζ transgene without its cytoplasmic domain, were shown to be deficient in their ability to undergo activation-induced cell death, while they were as able to produce IL-2 upon TCR engagement, as T cells from mice reconstituted with the full length TCRζ chain (Combadiere *et al.*, 1996). Further analysis has revealed that the three different ITAMs present on the cytoplasmic tail of the TCRζ chain contribute both quantitatively and qualitatively in promoting activation-induced cell death. Indeed, the ability of chimeric

receptors to induce apoptosis upon triggering was found to be dependent on their number of intact TCRζ ITAMs. A chimeric receptor with all three TCRζ ITAMs intact was more effective in inducing apoptosis than a receptor with two TCRζ ITAMs, which in turn was more effective in apoptosis induction than a chimeric receptor with only one intact TCRζ ITAM. Moreover, the same study shows evidence for qualitative differences between the different TCRζ ITAMs: it was found that the absence of the membrane proximal TCRζ ITAM results in more severe impairment in apoptosis induction than the absence of any one of the remaining ITAMs. When this analysis was extended to all the ITAMs present in a TCR complex, i.e., the three ITAMS on the TCRζ chains and the ITAMs present on the CD3ε, γ and δ chains, further support was obtained to the notion that the different ITAMs are qualitatively distinct in their ability to contribute to apoptosis. Using chimeric receptors containing each one of these ITAMs, in the context of a CD3ε cytoplasmic sequence, it was found that the membrane proximal ITAM of the TCRζ chain (Z1) , induces substantial apoptosis, whereas the TCRζ membrane distal ITAM (Z3) and the CD3ε ITAM triggers very low but detectable levels of apoptosis. The Z2 ITAM, the CD3γ and the CD3δ ITAMs were found to induce negligible levels of apoptosis (Combadiere *et al.*, 1996).

Given the physiological importance of activation-induced cell death, we investigated how the absence of the CD3γ chain affects this process. We tested the ability of CD3γ-deficient T cells to undergo activation-induced cell death, as compared to T cells derived from the parental wild type mouse strains, FVB and 129 Ola. Purified lymph node T cells were driven into an activation-induced cell death susceptible state, by incubation with PMA and ionomycin, followed by incubation with IL-2. Stimulation with PMA and ionomycin was chosen over stimulation with an anti-TCR antibody, since we previously showed the CD3γ-deficient T cells have a defect in their susceptibility to undergo T cell activation upon stimulation with anti-CD3ε or anti-TCRβ, but not upon stimulation with PMA. This regimen induced proliferation in both mutant and wild type T cells, as judged by their morphological appearance and by DNA content (data not shown). Viable proliferative T cells were purified on a lymphoprep density gradient and subsequently analysed for their ability to undergo TCR-mediated activation-induced cell death. This was done by triggering the cells with different concentrations of anti-CD3ε mAb. As a control for the ability of the T cells to undergo activation-induced cell death, regardless of the signalling capacities of the TCR complex, the proliferating T cells were cultured in the presence of PMA and Ionomycin. As shown in Figure 4.10, CD3γ-deficient T cells are clearly defective in their ability to undergo activation-induced cell death upon stimulation with anti-CD3ε mAb, as compared to T cells derived from the parental wild type mice. In contrast, CD3γ-deficient T cells are as susceptible, as wild type T cells, in undergoing

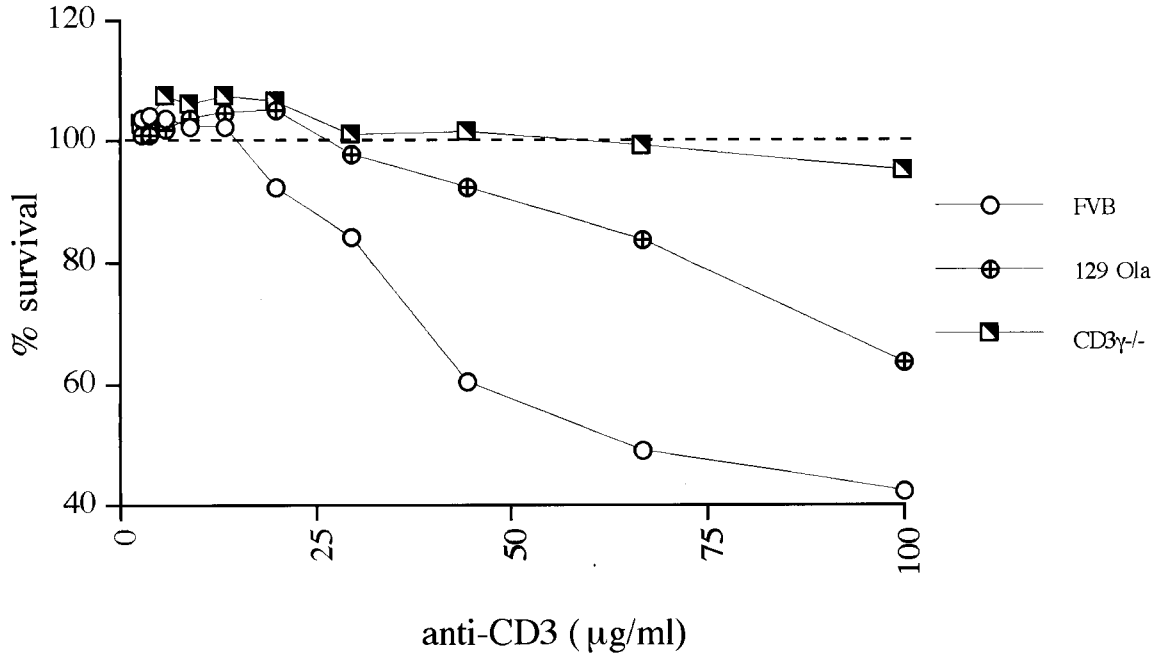

Figure 4.10: Ability of lymph node T cells to undergo activation induced cell death. Viable pre-activated T cells, by two days incubation with PMA+IM followed by two days incubation with IL-2, were stimulated with different doses of plate-bound anti-CD3ε mAb. Upon overnight culture the cells were analysed by flow cytometry for the percentage of alive cells. Shown is the percentage of cell survival considering the percentage of alive cells in control cultures, i.e., unstimulated cells, as 100% survival.

apoptosis upon treatment with PMA and Ionomicin. This treatment provoked the death of nearly all T cells of both wild type mouse and CD3γ-deficient mouse T cells (data not shown).

4.5 TCR signalling on CD3γ-deficient T cells is defective at the TCR proximal level

CD3γ-deficient T cells, not only display relatively low levels of TCR expression on their cell surfaces, but they also express TCRs devoid of a CD3γ chain. The low level of TCR cell surface expression, clearly supports the notion that the CD3γ chain is an important structural element for the assembly of the TCR complex. Nevertheless, the CD3/TCRζ components of the αβTCR complex are known to be required both for TCR assembly and for TCR signal transduction. Since the CD3γ-deficient TCRs are also devoid of a specific ITAM, the ITAM present on the cytoplasmic tail of the CD3γ chain, we questioned whether the observed defective abilities of the mutant T cells to undergo activation and activation-induced cell death could be due not only to the low TCR cell surface expression but also to an altered signalling through the CD3γ-ITAM-deficient TCR.

There is considerable debate whether the ITAMs present on the different CD3/TCRζ chains of the TCR simply quantitatively contribute to signalling through the TCR or whether each ITAM has a unique signalling function. Indeed, while the different ITAMs have in common the two tyrosine residues spaced 9 to 11 amino acid residues apart with isoleucine or leucine residues positioned three residues carboxy-terminal of each tyrosine, there is considerable variation among the amino acid residues that flank the tyrosine and leucine/isoleucine residues (Reth, 1989). Due to this significant sequence variation, it has been hypothesized that the different ITAMs could perform unique signalling function, by binding different intracellular signalling proteins. In support of this notion, experiments using synthetic phosphopeptides corresponding to the individual CD3/TCRζ ITAMs have revealed that the different ITAMs bind differentially to signalling proteins such as Shc, Grb2, ZAP-70 or the p85 regulatory subunit of the PI3K (Johnson *et al.*, 1995; Osman *et al.*, 1996; de Aos *et al.*, 1997; Ottinger *et al.*, 1998). Additional experiments support the notion of non-redundant ITAMs. Using TCRζ-deficient T cells, it has been shown that the CD3-ε, -δ or -γ are unable to substitute the TCRζ chain in T cell activation via triggering of the Thy-1, Ly-6 or CD2 molecules (Moigeon *et al.*, 1992; Wegener *et al.*, 1992). Moreover, as already referred to in this thesis, the TCRζ membrane-proximal ITAM was found to be particularly effective in promoting activation-induced cell death, as compared to the other TCRζ ITAMs or to the ITAMs present on the CD3ε, γ or δ chains (Combadiere *et al.*, 1996). Comparison of chimeric receptors, containing the cytoplasmic domains of either the TCRζ or CD3ε chain, also revealed differences in their signalling abilities. Crosslinking of these chimeric receptors was shown to result in distinct signalling outcomes as detected by analysis of the pattern of induced protein tyrosine phosphorylation and intracellular free calcium mobilization (Letourneur and Klausner, 1992; Jensen *et al.*, 1997). Nevertheless, with the exception of the role of TCRζ in activation-induced cell death, most studies in this area suggest that the different ITAMs are mostly redundant and additive in terms of the final biological outcome upon receptor triggering. The same TCRζ and CD3ε chimeric receptors, which were found to promote differential patterns of protein tyrosine phosphorylation, were also found to be equally capable of eliciting early and late activation events (Letourneur and Klausner, 1992; van Oers *et al.*, 1998). Chimeric receptors comprising either the TCRζ or CD3ε cytoplasmic domains were both found capable of promoting DN to DP thymocyte differentiation (Shinkai *et al.*, 1995). Using TCRζ/η-deficient mice, it was found that a TCRζ chain lacking its cytoplasmic domain was nearly as capable as the full-length TCRζ chain transgene in promoting DP to SP thymocyte transition (Shores *et al.*, 1994). Both of these studies also provided evidence for a role of the different ITAMs in signal amplification. Indeed, it was found that

crosslinking of the chimeric receptor containing the cytoplasmic domain of the TCRζ, and therefore three TCRζ ITAMs, induced a greater degree of thymocyte proliferation, as compared to crosslinking of the chimeric receptor containing the cytoplasmic domain of CD3ε. DP to SP thymocyte transition was also found to be more efficient in the presence of a full-length TCRζ chain, than in the presence of a TCRζ chain lacking one or more of its ITAMs. Another study has provided clear evidence for a role of the different ITAMs in signal amplification. It showed that signalling, as detected by inductive protein tyrosine phosphorylation, calcium mobilization and ZAP-70 recruitment and phosphorylation, is increased in cells expressing a chimeric receptor containing three copies of the membrane-proximal TCRζ ITAM, as compared to a chimeric receptor containing only one copy of the same membrane-proximal TCRζ ITAM. Nevertheless, this study also provides evidence for a unique function of the different ITAMs. It shows that even chimeric receptors containing three copies of the membrane-proximal TCRζ ITAM, are not as effective as receptors containing all three different ITAMs of the TCRζ chain, in signal transduction (Irving *et al.*, 1993). In summary, there is experimental evidence supporting both a qualitative and quantitative role of the different ITAMs in TCR signalling.

Many of the studies on the distinct roles of the CD3/TCRζ ITAMs have been performed using T cell hybridomas or lymphomas. Yet, there is also experimental evidence suggesting that data obtained using these systems may not necessarily reflect the *in vivo* situation. For instance, while the TCRζ cytoplasmic domain was found to be capable of mediating activation of T cell hybridomas and lymphomas (Irving and Weiss, 1991; Letourneur and Klausner, 1991, 1992; Romeo and Seed, 1991; Eshhar *et al.*, 1992; Romeo *et al.*, 1992a,b), the cytoplasmic domain of the TCRζ chain was found to be incapable of triggering activation of primary naive resting T cells (Brocker and Karjaleinen, 1995). Furthermore, most of these studies are focused on the TCRζ and CD3ε ITAMs, while little investigation has been done on a putative unique signalling function of the CD3δ or CD3γ chain ITAMs.

We, therefore, investigated whether the absence of the CD3γ chain leads to an altered signalling through the TCR complex. For this purpose, we investigated TCR signalling at the level of protein tyrosine phosphorylation, which is one of the earliest detectable biochemical events upon TCR engagement. As can be seen in Figure 4.11, CD3γ-deficient T cells appear essentially indistinguishable from their wild type counterparts, concerning the pattern of phosphoproteins induced upon TCR crosslinking. Nevertheless, it is clear that TCR triggering on CD3γ-deficient T cells leads to a general lower level of phosphoproteins, as compared to wild type mouse T cells.

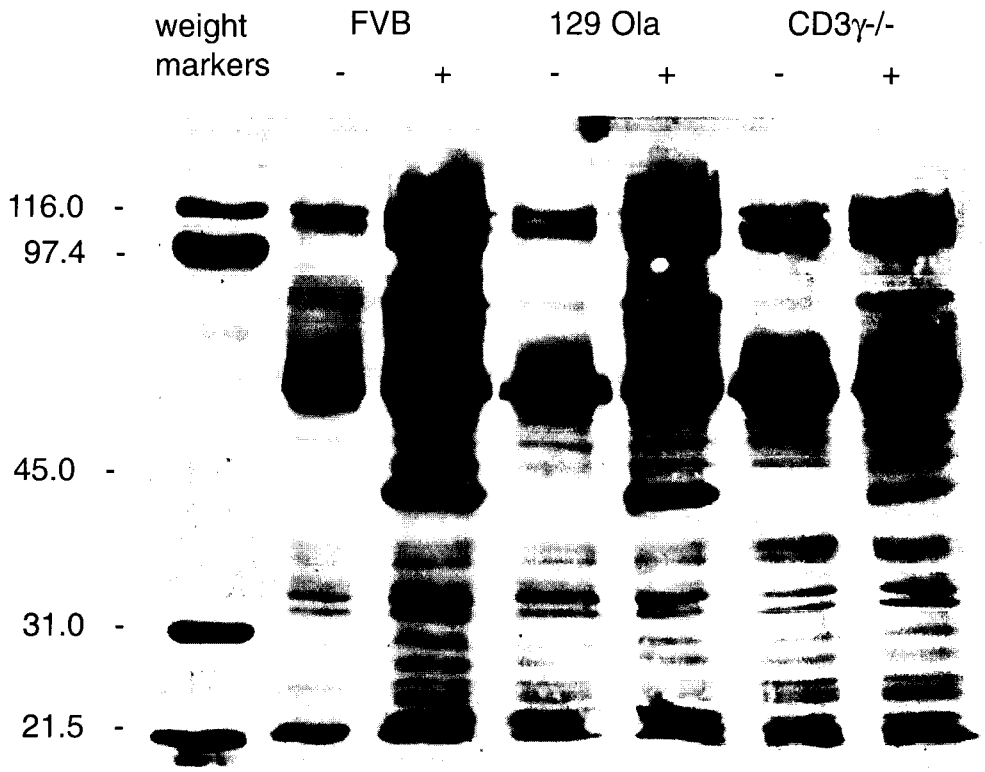

Figure 4.11: TCR proximal signalling: pattern of tyrosine phosphorylated proteins induced upon TCR triggering. Purified lymph node T cells were incubated with anti-CD3ε, followed by antibody crosslinking for 2.5 minutes. Whole cell lysates were analysed by western blot using the anti-phosphotyrosine antibody 4G10.

Taken together, our data suggest that the observed defective abilities of the CD3γ-deficient T cells to undergo T cell activation or activation-induced cell death are due to quantitatively rather than qualitatively distinct TCR signalling. This weak signalling upon TCR engagement is most likely a consequence of the low levels of TCR cell surface expression observed on CD3γ-deficient T cells. Indeed, it has been shown that a certain threshold number of TCR complexes must be triggered in order to enable T cell activation to occur (Viola and Lanzavecchia, 1996; Valitutti *et al.*, 1996; reviewed in Lanzavecchia *et al.*, 1999). Although we have not determined the number of TCRs per CD3γ-deficient T cell, we speculate that a fraction of the mutant T cells do not express a sufficient number of TCR complexes on their cell surface, to undergo activation or activation-induced cell death upon TCR triggering. Furthermore, in agreement with the notion that the CD3γ ITAM does not possess unique signalling functions, it has been shown that a CD3δ/CD3γ deficient T cell clone, when reconstituted with a full-length CD3δ chain and a CD3γ chain lacking its cytoplasmic domain, recovers its cytotoxic function, upon TCR engagement (Luton *et al.*, 1997a). In addition, subsequently to the work described here, our group has shown that mice with a CD3γ chain lacking its ITAM sequence do not display any apparent defect in T cell development (unpublished results).

4.6 CD3γ-deficient T cells are unable to down-regulate their TCRs in response to phorbol esters

Upon TCR engagement or treatment with phorbol esters, T cells not only become activated, but they also internalize their TCRs. The phenomenon of receptor internalization is not a unique feature of the TCR. Indeed, receptor internalization has been described for nutrient binding receptors such as the low density lipoprotein receptor (LDLR), the transferrin receptor (TfR), the mannose 6-phosphate/insulin-like growth factor II receptor (Man-6-IGF-IIR) and the lysosomal acid phosphatase (LAP) (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Peters *et al.*, 1990; Canfield *et al.*, 1991; Eberle *et al.*, 1991). Also other signal transducing receptors, such as the epidermal growth factor receptor (EGFR), the interleukin 2 receptor (IL-2R) and the insulin receptor (IR) are internalized upon ligand binding (Beguinot *et al.*, 1985; Robb and Greene, 1987; Smith and Jarett, 1988; Hatakeyama *et al.*, 1989; Smith *et al.*, 1991; Duprez *et al.*, 1992). While, internalization of nutrient binding receptors is obviously fundamental in delivering nutrients to the cell, the role of receptor internalization in the case of signal transducing receptors, such as the TCR, is less clear. Internalization of these type of receptors is mostly thought to diminish their ability to signal in a negative feedback fashion.

As referred above, the TCR is internalized upon engagement by ligands such as anti-TCR antibodies, peptide/MHC complexes and superantigens, as well as upon T cell treatment with phorbol esters (Cantrell *et al.*, 1985; Viola and Lanzavecchia, 1996). Some distinctions have been found concerning the mechanisms of ligand versus phorbol ester induced TCR internalization. Phorbol ester induced TCR downregulation has been found to be strictly dependent on a motif present on the cytoplasmic tail of the CD3γ chain, the phosphoserine-dependent di-leucine motif (Dietrich *et al.*, 1994). Experimental evidence indicates that the mechanism of phorbol ester induced TCR internalization involves (i) PKC activation, (ii) phosphorylation of S126 amino acid residue on the CD3γ chain and (iii) recognition of the di-leucine motif by molecules involved in receptor internalization. Indeed, it has been demonstrated that phosphorylation of S126 on the CD3γ chain is required for TCR down-regulation (Davies *et al.*, 1987; Dietrich *et al.*, 1994). Also the relevance of the CD3γ chain phosphoserine residue in rendering the di-leucine motif active and accessible to adaptor molecules has been clearly established. In completely assembled TCR, the CD3γ di-leucine motif is not accessible for adaptor proteins, unless phosphorylated. In contrast, in chimeric monomeric molecules, comprizing the CD3γ cytoplasmic domain, the motif is constitutively active independently of phosphorylation, and these chimeras are rapidly internalized (Sussman *et al.*, 1988; Letourneur and Klausner, 1992; Dietrich and Geisler, 1998). Another feature which characterizes phorbol

ester induced TCR internalization is that it does not involve TCR degradation and allows the TCR to recycle back to the cell surface upon dephosphorylation of the CD3γ chain (Minami *et al.*, 1987b; Ruegg *et al.*, 1992; Dietrich *et al.*, 1998). A di-leucine motif is also found in the cytoplasmic domain of the CD3δ chain, but this chain lacks a serine residue corresponding to the CD3γ S126 (Krissansen *et al.*, 1986). The role of di-leucine-mediated or PKC-mediated TCR internalization is not known. The TCR is in a constant process of internalization and recycling, at a rate that has been shown to be dependent upon the basal level of CD3γ phosphorylation (Dietrich *et al.*, 1998). Therefore, it has been suggested that the CD3γ phosphoserine-dependent di-leucine motif plays a role in this process of TCR recycling and in setting the activation threshold of the T cell (Dietrich *et al.*, 1998).

Ligand-induced TCR down-regulation is thought to be involved in the control of ongoing immune responses and in the establishment of tolerance towards self-antigens. Compared to phorbol ester-induced TCR downregulation, the mechanism of ligand-induced TCR downregulation is less well known. TCR down-regulation, upon ligand binding, has been shown to occur via clathrin coated pits and to involve receptor degradation (Luton *et al.*, 1994; Valitutti *et al.*, 1997; Luton *et al.*, 1997b). The identity of the TCR components or motifs involved is not known. Ligand-induced TCR downregulation has been shown to be independent of the CD3γ phosphoserine di-leucine motif, as well as of another putative internalization motif present on this chain, the tyrosine-based motif (Lauritsen *et al.*, 1998). Nevertheless, one study provided evidence suggesting that the CD3γ chain may play a role in ligand-induced TCR downregulation. A CD3γ-δ-deficient T cell clone was found to be able to down-regulate its TCR upon reconstitution with a cytoplasmic truncated CD3δ chain and a full-length CD3γ chain, while it could not do so upon reconstitution with cytoplasmic truncated CD3δ and CD3γ chains (Luton *et al.*, 1997b). While ligand-induced TCR downregulation has been shown to be independent of PKC activity, there are conflicting results concerning the involvement of protein tyrosine kinases. One study has suggested that ligand-induced TCR down-regulation is independent of PTKs, by showing that this process of internalization is not affected by inhibitors of PTKs. Yet, other studies have suggested the opposite, as PTK inhibitors were shown to affect the kinetics or final level of TCR down-regulation by anti-TCR (Thuillier, 1991; Luton *et al.*, 1994). Also conflicting results have been obtained concerning the role of Lck in ligand-induced TCR internalization. While transient transfection of an active form of Lck has been shown to lead to rapid TCR internalization, suggesting a role for Lck in TCR down-regulation (D'Oro *et al.*, 1997), Lck-deficient T cells were found to be fully susceptible to ligand-induced TCR internalization, suggesting that Lck is not required in this process (Strauss and Weiss, 1992).

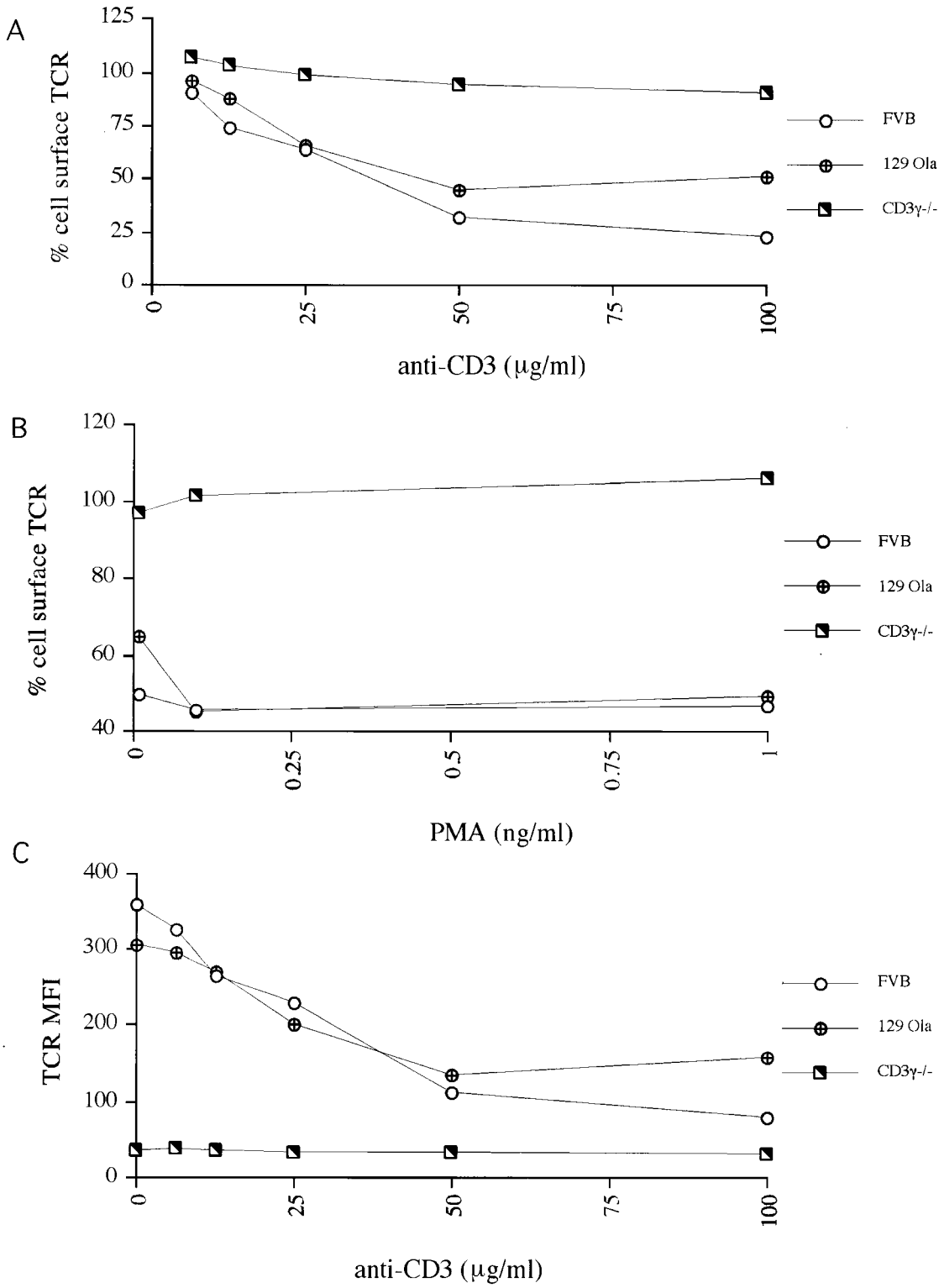

Figure 4.12: Ability of lymph node T cells to down-regulate their TCR expression level. T cells were stimulated with different doses of plate bound anti-CD3ε (A and C) or different doses of PMA (B). Shown is the percentage of TCR remaining on the cell surface, considering the TCR MFI on control cultures as 100% (A and B) and the TCR MFI (C).

As reviewed above, the CD3γ chain has been found to be absolutely required for phorbol ester-induced TCR down-regulation and one study suggested that it may, as well, be required for ligand-induced TCR down-regulation. Since all these studies were

performed using either T cell lines or chimeric molecules, we decided to investigate how the absence of the CD3γ chain affects phorbol ester and ligand-induced TCR down-regulation in the context of primary T cells. For that purpose purified lymph node T cells derived from the CD3γ-deficient mice and from the wild type parental mouse strains, FVB and 129 Ola, were stimulated either with different concentrations of plate-bound anti-CD3ε mAb or with different concentrations of the phorbol ester PMA. The cells were subsequently stained for TCRβ and analysed by flow cytometry for the TCRβ mean fluorescence intensity (MFI). In Figure 4.12A and B it is shown how stimulation with different concentrations of anti-CD3ε or PMA affects the fraction of TCRs which remain on the T cell surface, as compared to untreated cells. The fraction of TCRs remaining on the T cell surface was determined after the MFI of TCRβ staining, regarding the TCRβ MFI of control T cells as 100%. The ability of wild type mouse T cells to down-regulate their TCRs in response to TCR triggering or PMA treatment is obvious, as at the highest concentrations of these reagents, their TCRβ MFI is 60% to 40% of the TCRβ MFI on untreated cells. In agreement with the described role of the CD3γ chain in phorbol ester induced TCR down-regulation, T cells from the CD3γ-deficient mice were found to be unable to down-regulate their TCRs in response to PMA treatment (Figure 4.12B). Whether the CD3γ-deficient T cells are able or unable to down-regulate their TCRs in response to anti-CD3ε stimulation was less clear. Indeed, while in some experiments CD3γ-deficient T cells appear to be capable of down-regulating their TCRs in response to anti-CD3ε stimulation, in other experiments, it appears to be the opposite. The likely explanation for these conflicting results relies on the low levels of TCR cell surface expression which characterize the CD3γ- deficient T cells. Indeed, if we consider the TCRβ MFI, not as a percentage of the initial TCRβ MFI, but as the TCRβ MFI itself, it is revealed that even upon TCR down-regulation, the TCRβ MFI of wild type mouse T cells do not reach the values observed for the CD3γ-deficient T cells (Figure 4.12C). The low levels of TCR cell surface expression on CD3γ-deficient T cells severely impair a conclusive analysis of their ability to down-regulate their TCRs.

CHAPTER 5

RESULTS AND DISCUSSION ON THE ROLE OF CD3γ-CHAIN ON POSITIVE AND NEGATIVE SELECTION

Contents

5.1 Introduction

Phenotypic analysis of thymocytes derived from mice deficient for the different components of the TCR complex has provided valuable information on which are the essential TCR components in T cell development beyond the β-selection and positive/negative selection checkpoints. Indeed, according to these analysis, the TCRβ and CD3ε chains are absolutely required for DN to DP thymocyte transition (Mombaerts *et al.*, 1992b; Malissen *et al.*, 1995; DeJarnette *et al.*, 1998). The TCRα chain is not required for DN to DP thymocyte transition, but it is absolutely necessary for T cell development beyond the DP thymocyte stage (Mombaerts *et al.*, 1992b). The CD3δ chain is dispensable for the DN to DP thymocyte transition, while it is required for thymocyte maturation beyond the DP stage (Dave *et al.*, 1997). The lack of a TCRζ chain leads to a partially defective DN to DP transition and a severely defective DP to SP thymocyte transition (Love *et al.*, 1993; Liu *et al.*, 1993; Malissen *et al.*, 1993).

Phenotypic analysis of CD3γ-deficient thymocytes has revealed that the lack of the CD3γ chain leads to a deficient transition between the DN and the DP thymocyte developmental stages (Haks *et al.*, 1998). Nevertheless, based on the thymocyte cell surface expression of the CD4 and CD8 co-receptors, it is not clear whether or not the lack of the CD3γ chain also affects DP to SP thymocyte transition. In contrast to thymocytes derived from TCRα, TCRζ or TCRδ-deficient mice, which display none or nearly undetectable SP thymocytes, CD3γ-deficient thymocytes clearly include a small percentage of SP thymocytes. The percentage of SP thymocytes among CD3γ-deficient thymocytes is reduced, as compared to wild type mice values. Yet, the ratio of DP to SP thymocytes in the CD3γ-deficient mice is nearly indistinguishable from its counterpart ratio among wild type thymocytes (Figure 5.1). While this

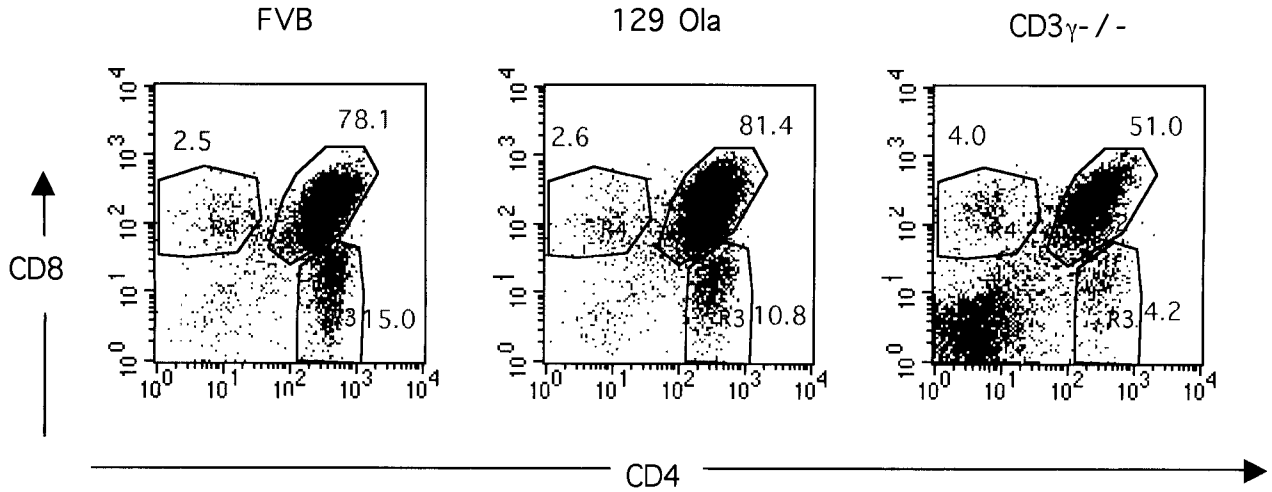

Figure 5.1: Thymocyte phenotype. Flow cytometry analysis of ex-vivo thymocytes for the relative representation of DN, DP and SP cells among wild type and CD3γ-deficient mouse thymocytes.

ratio could suggest that the CD3γ chain is not required for DP to SP thymocyte maturation, other hypotheses may be considered. The unchanged DP/SP ratio could be a result of impaired DP to SP maturation and deficient DN to DP transition. Alternatively, the lack of the CD3γ chain may differentially affect positive and negative selection. For instance, the CD3γ-deficiency may more strongly inhibit negative selection and allow the further maturation of a relatively higher fraction of DP thymocytes. In order to investigate a putative role of the CD3γ chain on DP to SP thymocyte maturation we performed a more extensive phenotypic analysis of CD3γ-deficient thymocytes. In addition, we tested the ability of the mutant DP thymocytes to undergo both positive and negative selection, upon *in vitro* TCR triggering. The data obtained will be presented in this chapter.

5.2.1 CD3γ-deficient DP thymocytes comprise a relatively reduced fraction of cells undergoing or capable of undergoing positive selection

DN thymocytes which have undergone a productive TCRβ gene rearrangement progress to the DP stage, with a concomitant burst in cell proliferation (Hoffman *et al.*, 1996, reviewed in Fehling and von Boehmer, 1997). At the DP thymocyte stage, cell proliferation comes to an end and the cells start to rearrange their TCRα genes. Following completion of a productive TCRα gene rearrangement, the DP thymocytes express low level of αβ TCRs on their cell surface and are subjected to the second major TCR-mediated checkpoint in T cell development, positive/negative selection (reviewed in Kisielow and von Boehmer, 1995; Guidos, 1996).

The newly generated DP thymocytes have an average life span of 3-4 days (Shortman *et al.*, 1991; reviewed in Kisielow and von Boehmer, 1995) and express a highly diverse αβ TCR repertoire. Depending on the ability of their αβTCRs to interact with thymic MHC/peptide complexes, they progress to further maturation (they are positively selected), are induced to undergo cell death (they are negatively selected) or die by neglect. As a result of the random nature of the TCRβ and TCRα rearrangements, only a reduced fraction of the DP thymocyte will display on their cell surface αβTCRs with an appropriate specificity that allows further maturation. Indeed, it has been estimated that only approximately 5% of DP thymocytes progress to the SP stage (Huesman *et al.*, 1991; Egerton *et al.*, 1990; Zerrahn *et al.*, 1997; van Meerwijk *et al.*, 1997).

Although it has been estimated that approximately 90% of DP thymocytes die by neglect and 5% die by negative selection (van Meerwijk *et al.*, 1997), *ex-vivo* thymocytes comprise a negligible fraction of dead cells. Thymocytes undergoing programmed cell death appear to be rapidly phagocytosed by thymic macrophages (Surh and Sprent, 1994). Therefore, DP thymocytes undergoing negative selection *in vivo* have been difficult to identify and characterise

phenotypically. In contrast to the negatively selected DP thymocytes, several cell surface and intracellular markers can be used to identify DP thymocytes which have undergone positive selection.

Positive selection operates on DP thymocytes characterized by a low expression of αβTCR and CD5. The earliest detectable phenotypic changes upon positive selection include the upregulation of expression of the cell surface markers CD69, CD5 and αβTCR, as well as upregulation of Bcl-2 and downregulation of RAG-1, RAG-2, TdT and pTα expression (reviewed in Guidos, 1996). DP thymocytes which are $TCR^{hi,}$ in particular, have been shown to represent an intermediate developmental stage between the TCR^{lo} DP and the SP thymocytes. Intrathymic adoptive transfer studies have shown that SP thymocytes arise more rapidly from purified TCR^{hi} DP thymocytes than from TCR^{lo} DP thymocytes (Petrie *et al.*, 1993). The origin of αβTCR cell surface upregulation upon positive selection has been reported to be due to an increased rate of TCRα synthesis (Kosugi *et al.*, 1992) and to an increased stability of the TCRα chain in the E.R. (Kearse *et al.*, 1994b).

In order to investigate how the absence of the CD3γ chain affects the ability of the DP thymocytes to undergo positive selection *in vivo*, we analysed the phenotype of DP thymocytes from CD3γ-deficient mice, with respect to their percentage of TCR^{hi}, $CD69^{+}$ and $CD5^{hi}$ cells. In some of the experiments we used the wild type parental strains FVB and 129 Ola, as controls. In other experiments, also (FVB x 129 Ola) F1 mice were used as controls. Both controls were required, since the CD3γ-deficient mice have been produced in a mixed FVB and 129 Ola genetic background and we considered the possibility that the outcome of a mixed genetic background may differ from the genetic background of either of the parental mouse strains. Nevertheless, F1, FVB and 129 Ola mouse thymocytes were found not to be significantly different in terms of their percentage of DP thymocytes (Figure 5.1 and data not shown). Also data obtained from experiments using either FVB and 129 Ola or F1 mouse thymocytes did not differ significantly. Therefore we will only present the results derived from experiments using the CD3γ-deficient parental mouse strains as controls.

As previously shown (Figure 5. 1), the percentage of DP cells among FVB and 129 Ola mouse thymocytes was found to be approximately two fold higher, as compared to the average percentage of DP cells observed among the CD3γ-deficient thymocytes. When analysed for the expression of CD69, the percentage of CD3γ-deficient DP thymocytes positive for this cell surface marker was found to be relatively lower, as compared to the percentage observed among DP thymocytes derived from FVB and 129 Ola mice (Figure 5.2A). Indeed, the percentage of $CD69^{+}$ cells among the CD3γ-deficient mouse DP thymocytes was typically 1.5 to 2-fold lower, as compared to their wild type counterpart. Similarly, the percentage of CD3γ-deficient mouse DP thymocytes displaying a high expression of the CD5 cell surface marker was found to be relatively low, as compared to that percentage observed among DP thymocytes derived from

wild type mice (Figure 5.2B). The percentage of $CD5^{hi}$ cells among the mutant DP thymocytes was typically 2 to 3-fold lower, as compared to the percentage observed among DP thymocytes derived from wild type mice. Therefore, the pattern of CD69 and CD5 expression suggests that the CD3γ-deficient mouse DP thymocytes are relatively deficient in their ability to undergo positive selection. In agreement with this notion, also the percentage of TCR^{hi} cells among CD3γ-deficient DP thymocytes was found to be relatively lower, as compared to the wild type (Figure 5.2C).

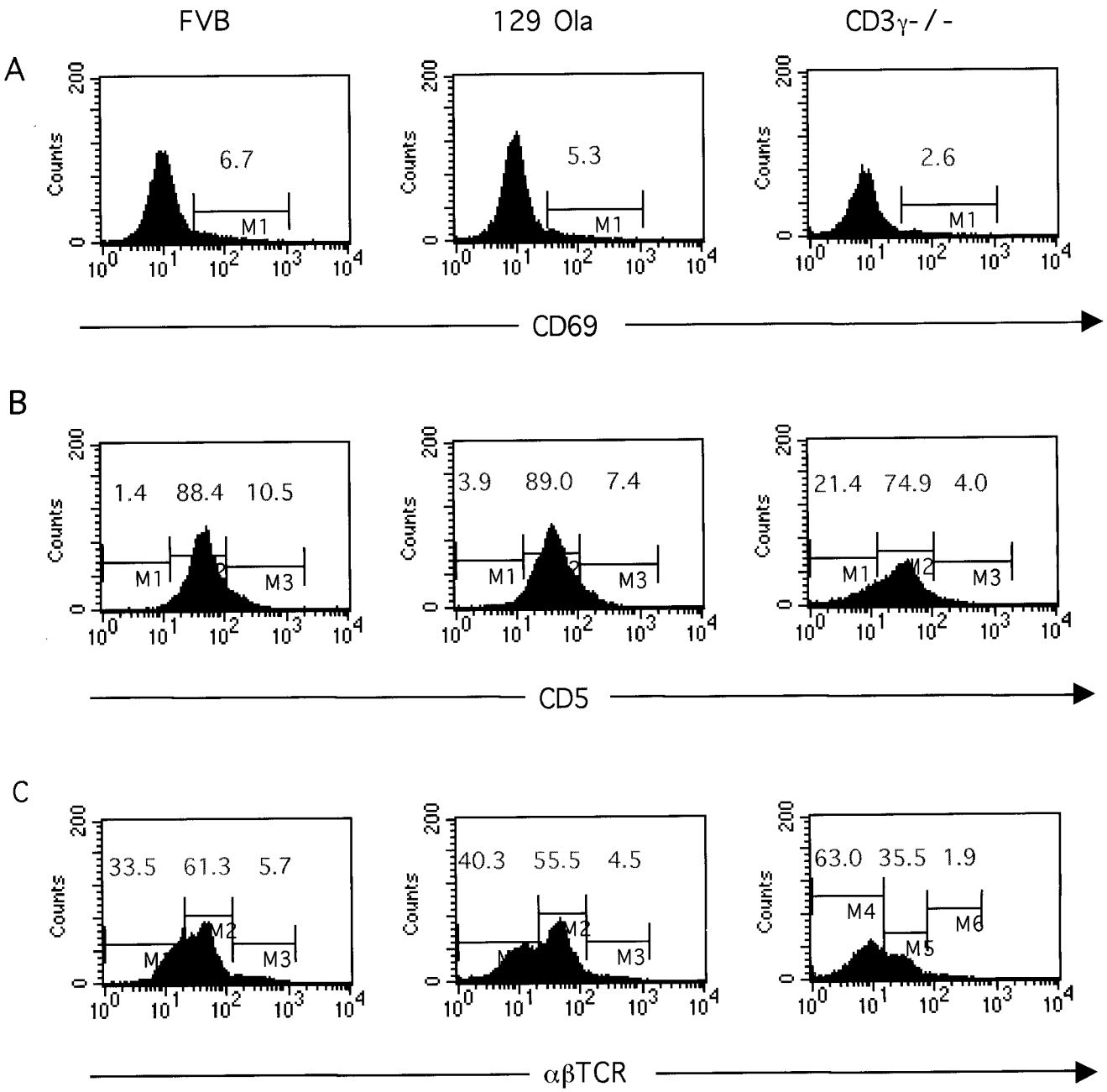

Figure 5.2: Analysis of *ex-vivo* DP thymocytes for the expression of the CD69 (A), CD5 (B) and αβTCR (C) cell surface markers.

Interestingly, as compared to wild type mouse cells, the DP thymocytes derived from the mutant mice were also found to display a relatively low percentage of TCR^{lo} cells and a concomitant increase in TCR^- cells. Similarly, while the majority of DP thymocytes derived from the wild type mice expressed intermediate levels of CD5 on their cell surface, 10 to 20% of the CD3γ-deficient mouse DP thymocytes were found to be $CD5^{lo}$ (Figures 5.2B and C). Therefore, the pattern of expression of TCR and CD5 suggests that in the CD3γ-deficient mice, the DN to DP thymocyte transition is not only quantitatively deficient, but also qualitatively different. A deficient upregulation of CD5 expression upon DN to DP transition in the CD3γ-deficient mice is consistent with experimental evidence suggesting that pre-TCR signalling is involved in CD5 up-regulation (Azzam *et al.*, 1998). The altered pattern of TCR expression among the CD3γ-deficient mouse DP thymocyte differs from the pattern observed among DP thymocytes derived from TCRζ- or CD3δ-deficient mice. Indeed, while the DP thymocyte from CD3γ-deficient mice appear to comprise distinct TCR^-, TCR^{lo} and TCR^{hi} subpopulations, DP thymocytes derived from CD3δ-deficient mice appear to comprise only a dominant TCR^- and a reduced TCR^{lo} population (Dave *et al.*, 1997). In TCRζ-deficient mice, DP thymocytes appear to be uniformly negative for αβTCR cell surface expression (Love *et al.*, 1993; Liu *et al.*, 1993; Malissen *et al.*, 1993).

The pattern of TCR expression among the CD3γ-deficient DP thymocytes is reminescent of the pattern observed for DP thymocytes derived from pTα-deficient mice. Albeit more severely altered, also in mice lacking the pTα component of the pre-TCR, the DP thymocytes comprise an abnormal high percentage of TCR^- cells. This has been shown to be due to the fact that in pTα-deficient mice, many DN cells transit to the DP stage in the absence of β-selection, i.e., many DP thymocytes lack a functional TCRβ chain (Buer *et al.*, 1997). The CD3γ chain, as the pTα chain, is required for an efficient DN to DP thymocyte maturation (Haks *et al.*, 1998). Therefore, the pattern of TCR expression among the CD3γ-deficient DP thymocytes suggests that also in the CD3γ-deficient mice, a fraction of DN cells mature to the DP stage in the absence of β-selection. In order to test this hypothesis, the CD3γ-deficient DP thymocytes will have to be analysed for the presence of intracellular TCRβ chains and for the presence of productive TCRβ gene rearrangements.

The relatively low percentage of TCR^{lo} cells among DP thymocytes derived from CD3γ-deficient mice implies that, as compared to wild type mice, a relatively low percentage of the mutant DP thymocytes are apt to undergo positive selection. Therefore, it is not surprising that the fraction of CD3γ-deficient DP thymocytes displaying phenotypic changes associated with positive selection is relatively low in these mice. Nevertheless, the ratio of DP to SP thymocytes in the CD3γ-deficient mice is nearly indistinguishable from the ratio observed among thymocytes derived from wild type mice. This observation, which suggests that DP to SP thymocyte transition is undisturbed in mice lacking the CD3γ chain, is apparently in

contradiction with the demonstrated relatively low percentage of mutant DP thymocytes undergoing positive selection. Yet, as mentioned above, the seemingly undisturbed ratio of DP to SP thymocytes in the CD3γ-deficient mice may be explained by a combined deficient DP to SP transition and DN to DP thymocyte maturation. Another possible explanation for this apparent contradiction is that the lack of CD3γ chain not only affects the ability of the DP thymocytes to mature to the SP stage, but also affects the fate of the newly differentiated SP thymocytes.

5.2.2 CD3γ-deficiency appears to favor the further maturation of newly positively selected thymocytes

In addition to the commonly used CD4 and CD8 co-receptors, sequential maturational stages on T cell development can be defined by the differential cell surface expression of TCR and CD69. Based on the expression of these markers, all thymocytes can be grouped into five subpopulations of increasing maturity: $TCR^{-}CD69^{-}$, $TCR^{lo}CD69^{-}$, $TCR^{lo}CD69^{+}$, $TCR^{hi}CD69^{+}$ and $TCR^{hi}CD69^{-}$ (Barthlott *et al.*, 1997). In this sequence of thymocyte maturation, upon positive selection, CD69 upregulation precedes the upregulation of TCR and, shortly after positive selection the expression of the CD69 cell surface marker is gradually downregulated (reviewed in von Boehmer, 1994). We therefore analysed T cell development in CD3γ-deficient mice using this combination of cell surface markers, in order to search for possible abnormalities among post-positive selection thymocytes, i.e., for abnormalities in the ratios of $TCR^{lo}CD69^{+}$ to $TCR^{hi}CD69^{+}$ or $TCR^{hi}CD69^{+}$ to $TCR^{hi}CD69^{+}$ cells. Nevertheless, the TCR and CD69 markers did not define subpopulations among the CD3γ-deficient mouse thymocytes as clearly as among thymocytes derived from wild type mice (data not shown). This is likely due to an altered TCR expression among the CD3γ-deficient mouse thymocytes.

Following positive selection and the upregulation of the CD69 expression, thymocytes undergo a gradual downregulation of not only CD69, but also the HSA marker (reviewed in von Boehmer, 1994). Since the vast majority of thymocytes are HSA^{+}, we considered that by analysing CD3γ-deficient thymocytes for their pattern of expression of TCR and HSA, we could identify the most mature thymocyte subpopulations. Excluding DN thymocytes from our analysis, four thymocyte subpopulations of increasing maturity are defined based on the differential expression of TCR and HSA: $TCR^{-}HSA^{+}$, $TCR^{lo}HSA^{+}$, $TCR^{hi}HSA^{+}$ and $TCR^{hi}HSA^{-}$.

We identified all four subpopulations among both the wild type thymocytes and the CD3γ-deficient thymocytes (Figure 5.3). This analysis provided three interesting pieces of information on how the absence of CD3γ chain affects T cell development beyond the DN stage.

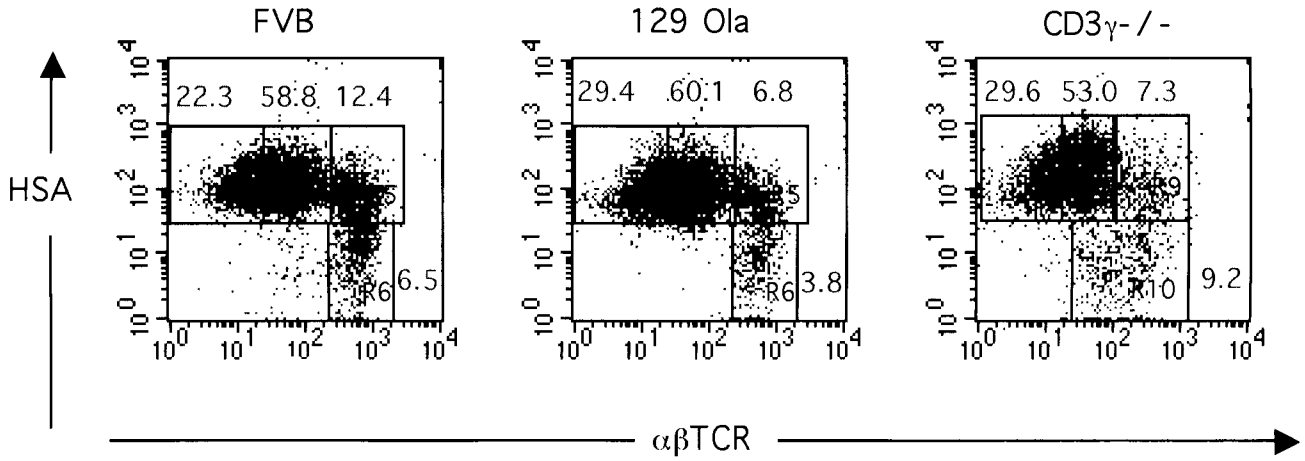

Figure 5.3: Analysis of ex-vivo DP+SP thymocytes for the relative representation of cell populations defined by the differential expression of αβTCR and HSA.

First, the ratio of the $TCR^{lo}HSA^{+}$ to $TCR^{hi}HSA^{+}$ among CD3γ-deficient thymocytes was found to be nearly indistinguishable from the wild type counterpart ratio. Secondly, the ratio of the $TCR^{hi}HSA^{+}$ to the $TCR^{hi}HSA^{-}$ subpopulations was clearly altered among the CD3γ-deficient mouse thymocytes. Among wild type mice derived thymocytes, the percentage of $TCR^{hi}HSA^{+}$ thymocytes was typically 2 to 4-fold higher as compared to the percentage of $TCR^{hi}HSA^{-}$. In contrast, in CD3γ-deficient thymocytes, these two subpopulations were either equally represented or the percentage of the most mature $TCR^{hi}HSA^{-}$ cells was superior to the percentage of the $TCR^{hi}HSA^{+}$ subpopulation. Finally, we found that, among the CD3γ-deficient mouse thymocytes, the $TCR^{hi}HSA^{+}$ to $TCR^{hi}HSA^{-}$ transition is accompanied by downregulation of the TCR expression level, while among wild type thymocytes, the TCR expression level on the $TCR^{hi}HSA^{+}$ and the $TCR^{hi}HSA^{-}$ subpopulations is nearly indistinguishable. Altogether, this phenotypic analysis suggests that the lack of CD3γ chain has no major effect on the ability of TCR^{lo} DP thymocytes to undergo positive selection. In addition, it strongly suggests that the CD3γ-deficiency favours the further maturation of newly positively selected thymocytes. Nevertheless, it is not clear whether completion of thymocyte maturation in CD3γ-deficient mice involves an abnormal down-regulation of TCR expression level, or whether a fraction of thymocytes mature without passing through a TCR^{hi} thymocyte stage.

5.3. CD3γ-deficient DP thymocytes are relatively defective in their ability to upregulate positive selection cell surface markers, upon *in vitro* TCR triggering

Analysis of the ability of DP thymocytes to undergo positive selection and maturation to the SP stage, by phenotypic analysis of *ex-vivo* thymocytes is hampered by the dynamic nature

of the T cell developmental process. This type of analysis does not allow to distinguish whether an altered ratio between two certain thymocyte subpopulations is due to an altered differentiation of one or the other subpopulation. In order to circumvent this drawback, we investigated the ability of CD3γ-deficient DP thymocytes to undergo positive selection *in vitro*. Although TCR cross-linking *in vitro* does not lead to complete differentiation of DP thymocytes to the SP stage, suggesting that additional TCR-engagements or others are required for full SP thymocyte differentiation, it has been shown that TCR crosslinking of TCR^{lo} DP thymocytes and DP cell lines *in vitro* induces some early hallmarks of positive selection, including down-regulation of RAG, TdT and pre-TCR expression, up-regulation of CD69, TCR, CD5 and Bcl-2 expression (Kearse *et al.*, 1995b; Groves *et al.*, 1995).

We therefore tested the ability of CD3γ-deficient DP thymocytes to undergo positive selection by stimulating unsorted thymocytes with plate-bound anti-TCR and anti-CD28 antibodies. Their ability to undergo positive selection, as compared to wild type DP thymocytes, was monitored through the percentage of DP thymocytes that up-regulated the cell surface expression of CD5 and CD69. Although others have reported that a combination of TCR and CD28 triggering leads to DP thymocyte apoptosis (Punt *et al.*, 1994b; Kishimoto and Sprent, 1997), in our experience, CD28 co-stimulation merely leads to an increase in the percentage of DP thymocytes displaying a phenotype of positively selected cells. Thymocyte stimulation with either anti-TCR alone, or co-stimulation with anti-TCR and anti-CD28 did not lead to any significant increase in the percentage of dead cells, as compared to unstimulated thymocytes (data not shown).

DP thymocytes derived from the CD3γ-deficient mice were found to have the ability to up-regulate the expression of both CD69 and CD5 cell surface markers upon *in vitro* stimulation (Figure 5.4 and data not shown). As expected, the percentage of CD3γ-deficient DP that was induced to become $CD69^{+}$ or $CD5^{hi}$ was relatively low, as compared to the percentage observed among FVB and 129 Ola wild type counterparts. Indeed, the percentage of induced $CD69^{+}$ or $CD5^{hi}$ cells among mutant DP thymocytes was tipically 1.3- to 2-fold lower, as compared to the percentage observed among stimulated wild type DP thymocytes. Interestingly, the percentage of TCR^{lo} cells among CD3γ-deficient DP thymocytes has been shown to be approximately 1.5-fold lower, as compared to the percentage of wild type DP thymocytes which are TCR^{lo}. Our data strongly suggest that, as compared to wild type mice derived thymocytes, a reduced fraction of CD3γ-deficient DP thymocytes is apt to undergo positive selection. In addition, they suggest that CD3γ-deficient TCR^{lo} DP thymocytes are as able as their wild type counterparts to be positively selected.

In our just described experiment we have tested the ability of DP thymocytes to undergo positive selection, by stimulating unsorted thymocytes. We considered that by using unsorted thymocytes, however the experiment would have two major drawbacks. Although unlikely, we

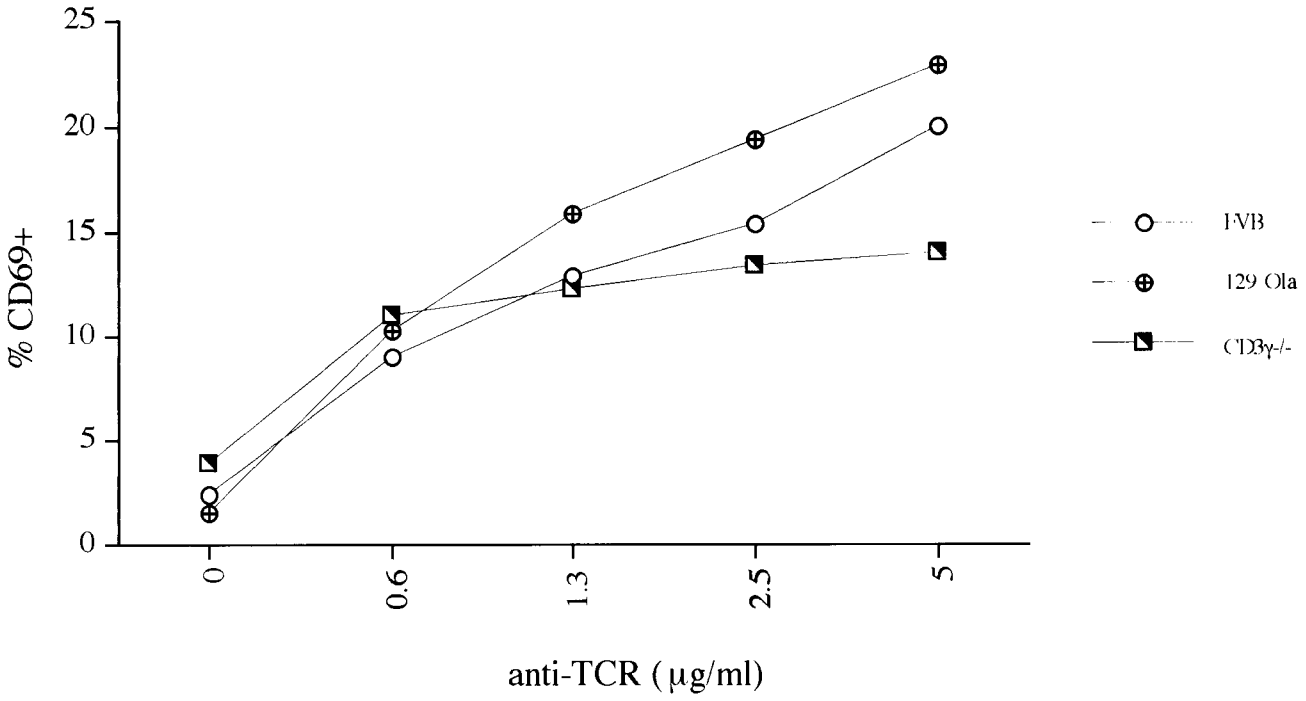

Figure 5.4: Ability of DP thymocytes to undergo positive selection *in vitro*. Unsorted thymocytes were co-stimulated with different doses of plate-bound anti-TCRβ and with 10μg/ml anti-CD28. After overnight culture, DP thymocytes were analysed, by flow cytometry, for the expression of CD69.

could not be certain whether DN to DP or DP to SP thymocyte transition will occur during the overnight culture. More importantly, as reported by others, we found that *in vitro* TCR triggering leads to a down-regulation of the CD4 and CD8 co-receptors (data not shown). This implies that, after culture, it was often difficult to identify the DP compartment among the co-cultured DN and SP thymocytes. In order to be absolutely certain that our analysis only included DP thymocytes, we performed the same assay as describe above, using sorted DP thymocytes. In agreement with the data from our experiments using unsorted cells, CD3γ-deficient DP thymocytes were found to be relatively incapable of up-regulating CD69 or CD5 upon *in vitro* TCR and CD28 co-triggering, as compared to DP thymocytes derived from wild type mice (Figures 5. 5 A and B). Upon culture, the percentage of CD3γ-deficient DP thymocytes that were induced to up-regulate the CD69 or CD5 cell surface expression was typically 2-fold reduced as compared to percentage observed among DP thymocytes derived from wild type mice.

Our analysis on the ability of the CD3γ-deficient DP thymocytes to undergo *in vitro* positive selection is hampered by the fact that the mutant and their wild type counterpart DP thymocytes are considerably different. Namely, they differ in terms of the percentage of TCR^{lo} cells, i.e., cells capable to respond to the TCR triggering. Nevertheless, our data strongly suggest that the absence of the CD3γ chain has no major effect on the ability of TCR^{lo} DP thymocytes to undergo positive selection. In order to further clarify this issue, the CD3γ-deficient mice have been crossed with TCR transgenic mice. These TCR transgenic CD3γ-deficient mice are currently being analysed by our group.

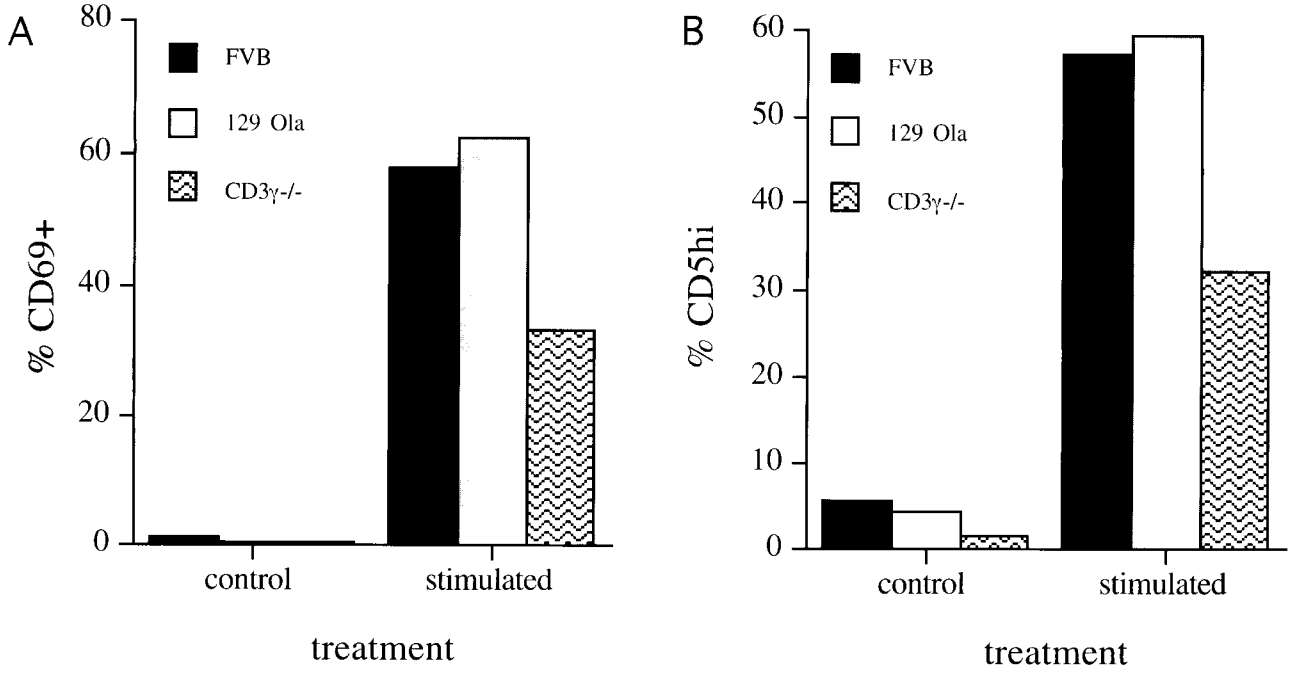

Figure 5.5: Ability of DP thymocytes to undergo positive selection *in vitro*. Sorted DP thymocytes were co-stimulated with plate-bound anti-TCRβ (5μg/ml) and anti-CD28 (10μg/ml). After overnight culture, thymocytes were analysed, by flow cytometry, for the upregulation of CD69 (A) and CD5 (B).

5.4 CD3γ-deficient DP thymocytes are relatively defective in their ability to undergo cell death, upon *in vitro* TCR triggering

Positive and negative selection appear to operate on thymocytes at the same stage of development (reviewed in Kisielow and von Boehmer, 1995; Jameson and Bevan, 1998; Amsen and Kruisbeek, 1998). It is not clear how TCR engagement can lead to such opposite cellular fates, as further maturation (positive selection) or cell death (negative selection). Nevertheless, it is generally accepted that the affinity/avidity of the interaction between the TCRs on DP thymocytes and peptide/MHC complexes on thymic stromal cells is a crucial factor in this process (reviewed in Ashton-Rickardt and Tonegawa, 1994 and Jameson *et al.*, 1995). Low affinity/avidity interactions are thought to lead to positive selection, while high affinity/avidity interactions lead to negative selection.

The absence of the TCRζ or CD3δ αβTCR components has been shown to affect the ability of DP thymocytes to undergo not only positive selection, but also negative selection (Lin *et al.*, 1997; Shores *et al.*, 1997; Dave *et al.*, 1997; Dave *et al.*, 1998). Our data suggest that the absence of the CD3γ chain has no major effect on the ability of the TCR^{lo} DP thymocytes to undergo positive selection. Nevertheless, they do not exclude the possibility that the CD3γ-deficient DP thymocytes are defective in their ability to undergo negative selection.

We have shown that the TCR expression level on the CD3γ-deficient TCR^{lo} DP thymocytes is relatively lower, as compared to the levels observed among wild type mice derived DP thymocytes (Figure 5.2C). The avidity of the TCR-peptide/MHC is thought to

depend, at least, on the affinity of the TCR to a particular peptide/MHC complex and on the densities of the TCR and its ligand. Therefore, we considered the possibility that the relatively low levels of TCR expression on CD3γ-deficient thymocytes would lead to a relative inability of these mutant DP thymocytes to undergo high avidity TCR engagements, and therefore, to undergo negative selection. According to this hypothesis, the absence of the CD3γ chain would differentially affect the ability of the mutant DP thymocytes to undergo positive and negative selection. A more pronounced inhibition of negative selection would favour the DP to SP transition. Moreover, this would provide a possible explanation for the apparently contradictory undisturbed DP/SP ratios and the relatively low fraction of "selectable" CD3γ-deficient DP thymocytes, as compared to their wild type counterparts. Indeed, the fraction of DP thymocytes which mature to the SP stage is likely to depend on a balance between the percentage of DP thymocytes which are able to become positively selected, and the percentage which undergoes negative selection. Indeed, it has been estimated that half to two thirds of thymocytes able to undergo positive selection are negatively selected (Ignatowicz *et al.*, 1996; van Meerwijk *et al.*, 1997). In order to test this hypothesis, we decided to investigate the ability of the CD3γ-deficient DP thymocytes to undergo negative selection.

Thymocytes derived from the CD3γ-deficient mice are characterized by considerable interindividual variation, in terms of both cell numbers and the percentage of the different thymocyte subpopulations. One possible explanation for this variability is the fact that the CD3γ-deficient mice have been produced in a mixed FVB and 129 Ola mice genetic background (Haks *et al.*, 1998). As a consequence, every single mutant mouse is likely to have a unique genetic background. It also implies that *in vivo* experiments would be severely hampered by the lack of an appropriate control. We considered therefore that it would be preferable to perform our tests *in vitro*. Surprisingly, induction of DP thymocyte apoptosis *in vitro*, through TCR engagement, proved to be more difficult than expected. It has been reported that a combination of TCR and CD28 triggering leads to DP thymocyte apoptosis (Punt *et al.*, 1994b; Kishimoto and Sprent, 1997). Nevertheless, according to our experience, DP thymocyte stimulation with plate-bound anti-TCR or co-stimulation with plate-bound anti-TCR and anti-CD28 only led to an up-regulation of positive selection cell surface markers. Thymocyte stimulation with a high dose (100μg/ml) of plate-bound anti-CD3ε, alone or in combination with anti-CD28, did not induce any significant DP thymocyte apoptosis either. We next tested the possibility of inducing DP thymocyte apoptosis by culturing the cells in the presence of soluble anti-TCR or anti-TCR + anti-CD28 antibodies with or without an additional soluble anti-hamster cross-linker antibody. Once again, the percentage of DP thymocytes that was induced to die was negligible (data not shown). A well established system for studying *in vitro* negative selection consists of culturing TCR transgenic thymocytes together with APCs and peptide (Swat *et al.*, 1991; Vasquez *et al.*, 1992). Since, at the time of this study, TCR transgenic CD3γ-deficient mice were not available

yet, we could not perform this type of assay. Finally, we found that we could induce DP thymocyte apoptosis, by culturing the thymocytes in the presence of soluble anti-TCR and B7.1 transfected cells of the P815 mastocytoma cell line. P815 cells transfected with I-E have been previously shown to be able to induce TCR transgenic DP thymocyte apoptosis, in the presence of peptide (Amsen and Kruisbeek, 1996). These cells express high levels of Fc receptors (CD16). Therefore, P815 cells are thought to induce DP thymocyte deletion by cross-linking the thymocyte bound anti-TCR antibodies and by engagement of the CD28 molecule.

We tested the ability of the CD3γ-deficient mouse DP thymocytes to undergo cell death, upon overnight culture in the presence of B7-transfected P815 cells and different concentrations of anti-TCR. The fraction of DP thymocytes to undergo cell death was determined based on the percentage of alive DP thymocytes, among treated cells, and the percentage of their counterparts, among untreated thymocytes. As compared to wild type thymocytes, the percentage of CD3γ-deficient DP thymocytes capable of undergoing cell death was typically 1.5-2-fold reduced (Figure 5.6). Interestingly, a similar difference was observed concerning the ability of CD3γ-deficient DP thymocytes to undergo *in vitro* positive selection, and the percentage of mutant DP thymocytes which are TCR^{lo}, i..e, capable of responding to either positive or negative selection inducing signals. Our results therefore suggest that the absence of the CD3γ chain does not differentially affect the ability of the CD3γ-deficient DP thymocytes to undergo positive or negative selection. Moreover, they suggest that, as for positive selection, the absence of the CD3γ chain has no major effect on the ability of TCR^{lo} DP thymocytes to undergo negative selection.

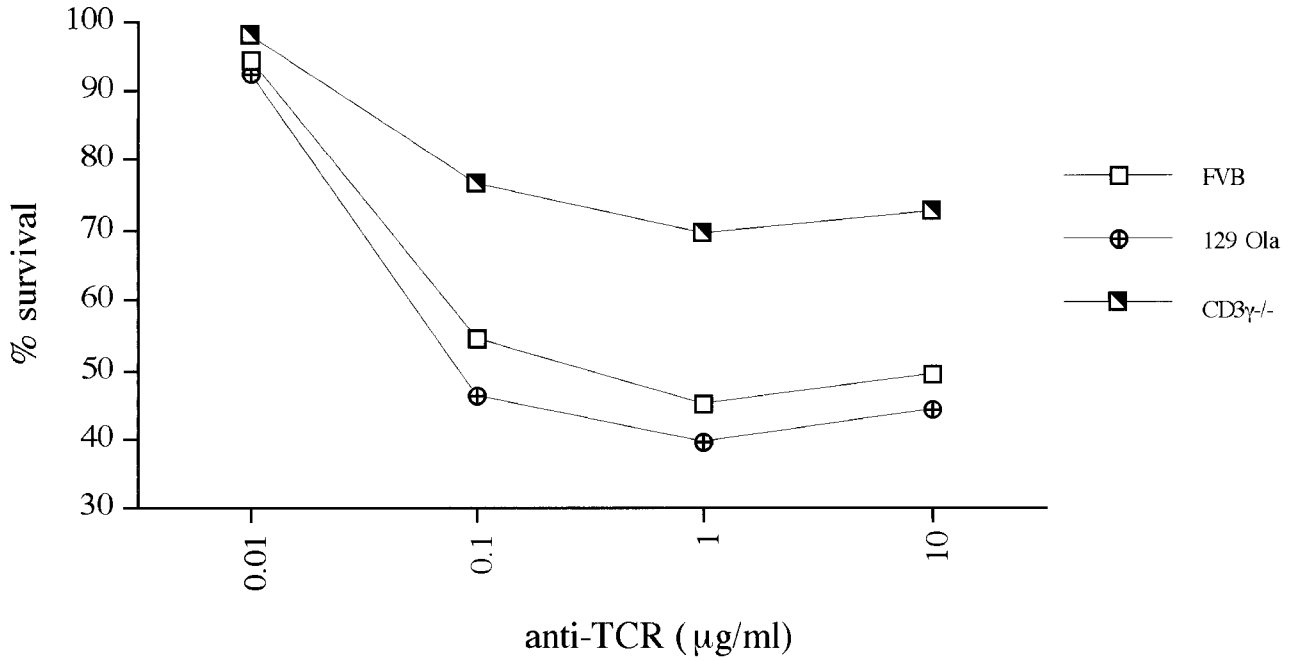

Figure 5.6: Ability of DP thymocytes to undergo negative selection *in vitro*. Unsorted thymocytes were co-cultured with P815-B7.1 cells in the presence of different doses of soluble anti-TCRβ. After overnight culture, thymocytes were analysed, by flow cytometry, for the percentage of alive DP cells. Shown is the percentage of surviving DP thymocytes, considering the percentage of alive DP cells among unstimulated cultures as 100%.

According to our data, the absence of the CD3γ chain equally affects the fraction of mutant DP thymocytes capable of undergoing positive or negative selection. Interestingly, at suboptimal TCR stimulation, the ability of the CD3γ-deficient DP thymocytes to undergo positive or negative stimulation, as compared to wild type thymocytes, did appear to be differentially affected. Indeed, at low doses of anti-TCR stimulation, the percentage of CD3γ-deficient DP thymocytes to undergo CD69 or CD5 upregulation was nearly indistinguishable from the percentage observed among wild type mouse derived DP thymocytes (Figure 5.4). In contrast, the CD3γ-deficient DP thymocytes were found to be relatively resistant to undergo TCR induced cell death upon stimulation with suboptimal doses of anti-TCR. This apparent differential ability of the CD3γ-deficient DP thymocytes to undergo positive or negative selection, at suboptimal TCR stimulation, may result from the different nature of the *in vitro* assays employed to mimic the two selection processes. Alternatively, it suggests that the absence of the CD3γ-chain alters the balance of positive selection over negative selection induced by low density peptide/MHC ligands.

5.5 CD3γ-deficient peripheral T cells do not display overt signs of autoreactivity

Peripheral T cells from TCRζ/η-deficient mice have been shown to overtly react to self-peptide/self-MHC complexes upon restoration of normal surface density of their TCRs (Lin *et al.*, 1997). Achievement of a normal TCR density was accomplished through the fusion of TCRζ/η-deficient peripheral T cells with BW5147 TCRα-/β- thymoma cells. The reactivity of these hybridomas towards syngeneic spleen cells led the authors to suggest that the absence of the TCRζ/η chains may imply that only thymocytes displaying TCRs with high affinity for self peptide/MHC complex mature and accumulate in the periphery. T cell reactivity depends on the number of TCR complexes which are engaged (Viola and Lanzavecchia, 1996; Valitutti *et al.*, 1996; reviewed in Lanzavecchia *et al.*, 1999). This suggests that self-reactivity upon restoration of normal TCR expression levels does not necessarily imply self-reactivity *in vivo*. Nevertheless, the reactivity of peripheral T cell derived hybridomas does provide information on the TCR specificity of thymocytes able to complete their maturational program. Therefore, we considered to use this same methodology to analyse the reactivity of CD3γ-deficient peripheral cells. In contrast to the TCRζ/η-/- mice, the CD3γ-deficient mice have been produced on a mixed genetic background. As a consequence, we could not use pools of peripheral CD3γ-deficient T cells in our experiments. Given the limited number of peripheral T cells per individual CD3γ-deficient mouse, our intended T cell-BW5157 fusions proved to be impracticable.

Subsequently, we chose a more simple experimental approach. We analysed the reactivity of primary CD3γ-deficient mouse peripheral T cells against autologous versus allogeneic spleen cells. Also this experimental approach was limited by the reduced T cell numbers, and therefore we could not perform dose responses or response kinetics. Nevertheless, our data does suggest that the absence of the CD3γ chain does not lead to any abnormal peripheral T cell reactivity towards self peptide/self-MHC complexes (Figure 5.7). Indeed, CD3γ-deficient peripheral T cells were found to be as unreactive towards autologous spleen cells, as their counterpart wild type peripheral T cells. Moreover, in agreement with their defective ability to undergo activation upon anti-TCR/CD3 stimulation *in vitro* (Figure 4.6), CD3γ-deficient T cells were found to be relatively deficient in their ability to proliferate in response to allogeneic spleen cells (Figure 5.7). Further clarification on the role of the CD3γ chain in positive versus negative selection absolutely requires an experimental system with a TCR of known specificity and with the possibility of manipulating the concentration and nature of selecting peptides.

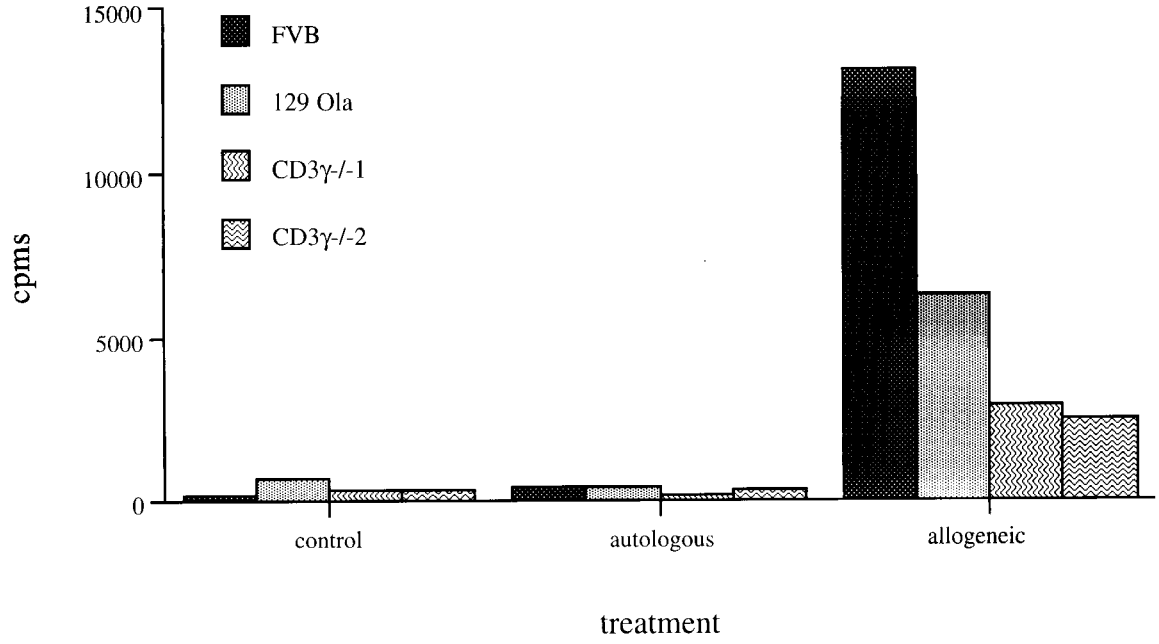

Figure 5.7: Analysis of the ability of thymocytes to undergo negative selection by testing the reactivity of lymph node T cells towards self-MHC/self-antigen. Purified lymph node T cells from individual mice were cultured alone and in the presence of autologous or allogeneic irradiated spleen cells. After three days of culture, cell proliferation was measured by [3H] thymidine incorporation.

5.6 CD3γ-deficient SP thymocytes are relatively defective in their ability to undergo cell death, upon *in vitro* TCR triggering

Our data indicate that the absence of the CD3γ chain equally reduces the percentage of mutant DP thymocytes capable of undergoing either positive or negative selection, as compared to wild type thymocytes. Also the percentage of TCR^{lo} cells, i.e., "selectable" cells, among the

CD3γ-deficient DP thymocytes is reduced, as compared to their wild type counterparts. Therefore, as compared to wild type mouse thymocytes, we expected a lower fraction of the mutant DP thymocytes to mature to the SP stage and a concomitant higher DP to SP ratio among the CD3γ-deficient thymocytes. Nevertheless, this is not what we observe. The ratio of DP to SP cells among the CD3γ-deficient mouse thymocytes is nearly indistinguishable from their wild type counterpart. One possible explanation for these apparently contradictory observations is that in the CD3γ-deficient mouse, low density peptide/MHC ligands favour positive over negative selection, as compared to wild type thymocytes. Another possible explanation is that the absence of the CD3γ chain affects the fate of newly generated SP thymocytes.

In addition to the DP thymocytes, it has been demonstrated that newly generated SP thymocytes are susceptible to undergo negative selection (Pircher *et al.*, 1989; MacDonald and Lees, 1990; Guidos *et al.*, 1990; Kishimoto and Sprent, 1997; reviewed in von Boehmer, 1990). Therefore, we hypothesised that the CD3γ-deficient SP thymocytes are relatively resistant to undergo TCR-mediated cell death, as compared to wild type thymocytes. In order to test our hypothesis, we investigated the ability of the mutant and wild type SP thymocytes to undergo cell death upon TCR engagement, *in vitro*. As expected, the CD3γ-deficient SP thymocytes were found to be relatively deficient in their ability to undergo TCR-mediated cell death, as compared to SP thymocytes derived from wild type mice (Figure 5.8). We attribute this relative resistance to the relatively low levels of TCR cell surface expression observed among CD3γ-deficient SP thymocytes, as compared to their wild type counterpart SP thymocytes.

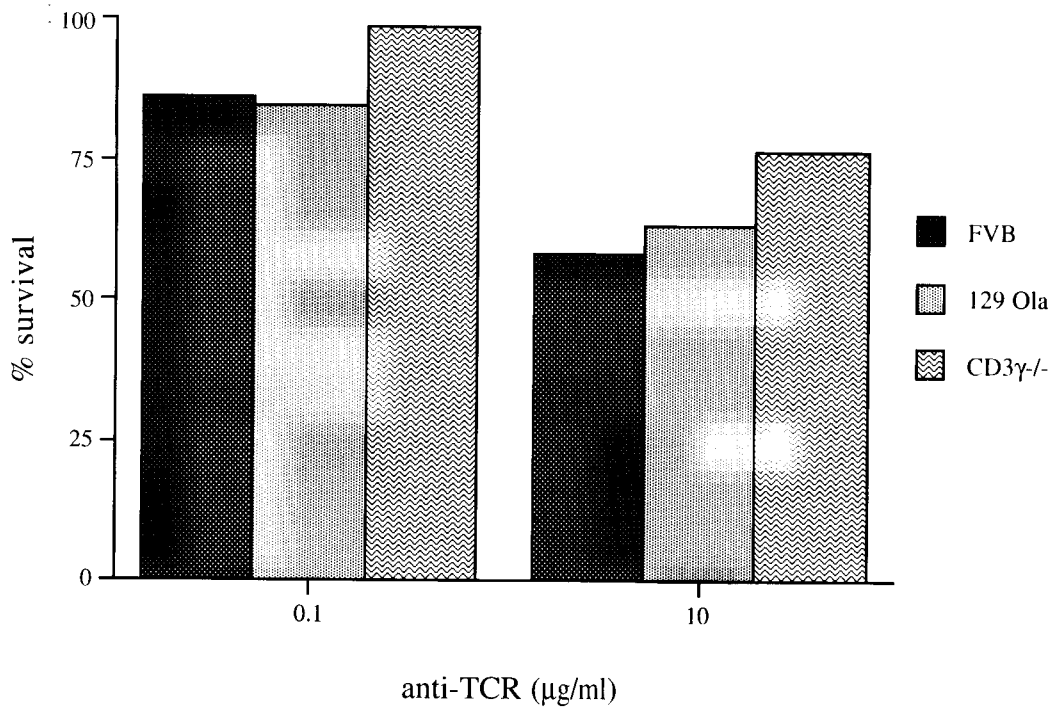

Figure 5.8: Ability of SP thymocytes to undergo cell death upon *in vitro* TCR triggering. Unsorted thymocytes were co-cultured with P815-B7.1 cells in the presence of different doses of soluble anti-TCRβ. Shown is the percentage of SP thymocytes that survived upon overnight culture in the presence of 0.1 and 10 µg/ml of anti-TCRβ. Percentage of survival was determined based on the percentages of alive SP thymocytes in control and treated cultures.

We cannot definitively exclude the possibility that the absence of the CD3γ chain affects the TCR repertoire on the DP thymocytes which are allowed to complete their maturational program. Nevertheless, our data suggest that the absence of the CD3γ chain has no major effect on the ability of the mutant TCR^{lo} DP thymocytes to undergo positive or negative selection. Moreover, they suggest that the relatively low percentage of "selectable" cells, among the CD3γ-deficient DP thymocytes, is compensated by a relatively high resistance of mutant SP thymocytes to undergo TCR-mediated cell death.

CHAPTER 6

RESULTS AND DISCUSSION ON THE ROLE OF CD3γ-CHAIN ON T CELL LINEAGE COMMITMENT

Contents

6.1 Introduction

Positively selected DP thymocytes down-regulate either the CD4 or CD8 co-receptors and differentiate to the CD8 SP or CD4 SP stage, respectively (reviewed in Fink and Bevan, 1995). It is well established that there is a high correlation between MHC recognition and the lineage of the generated SP thymocytes. Most DP thymocytes bearing class II MHC-specific TCRs retain the expression of CD4, the class II MHC co-receptor, whereas those bearing class I MHC-specific TCRs retain expression of CD8, the class I MHC co-receptor (reviewed in Robey and Fowlkes, 1994; Fowlkes and Schweighoffer, 1995; Kisielow and von Boehmer, 1995; Guidos, 1996; Marrack and Kappler, 1997). Several models have been proposed to explain how MHC recognition dictates T cell lineage commitment. Nevertheless, the exact mechanism involved in this process is not known (reviewed in von Boehmer, 1996).

Phenotypic analysis of the CD3γ-deficient mouse thymocytes has revealed that the lack of the CD3γ chain leads to an altered CD4 SP/CD8 SP ratio. Indeed, while among wild type mouse thymocytes, the CD4 SP/CD8 SP ratio is typically approximately 3:1, among the CD3γ-deficient thymocytes, this ratio has been show to be approximately 1:1 (Haks *et al.*, 1998). Therefore, we investigated how the lack of the CD3γ chain may affect the ratio of CD4/CD8 SP thymocytes. We considered two major possible explanations, not mutually exclusive, for the altered CD4 SP/CD8 SP ratio observed among the CD3γ-deficient thymocytes: (i) the lack of the CD3γ chain leads to an altered T cell lineage commitment at the DP to SP thymocyte transition and (ii) the lack of the CD3γ chain influences the cellular fate of newly generated CD4 and/or CD8 SP thymocytes.

6.2 CD3γ-deficiency affects CD4 SP thymocytes differentiation

Upon positive selection, CD69 is rapidly up-regulated and subsequently gradually down-regulated (reviewed in von Boehmer, 1994). Therefore, we considered that, by analysing the CD3γ-deficent SP thymocytes for their percentage of $CD69^+$ cells, we might get some insight on how the lack of CD3γ chain affects SP thymocytes differentiation. As shown in Figure 6.1A, the percentage of $CD69^+$ cells among CD3γ-deficient CD4 SP thymocytes was found to be relatively reduced, as compared to the counterpart percentage observed among wild type thymocytes. This indicates that, as compared to control mouse thymocytes, a relatively reduced fraction of the mutant CD4 SP thymocytes are newly generated cells. These findings might indicate that the lack of the CD3γ chain affects the generation of CD4 SP thymocytes. However, this analysis does not allow us to distinguish whether the low percentage of newly generated cells among the CD3γ-deficient CD4 SP is due to a reduced generation of these cells or whether it is due to increased survival or accumulation of the mutant CD4 SP thymocytes, as compared to wild type CD4 SP thymocytes.

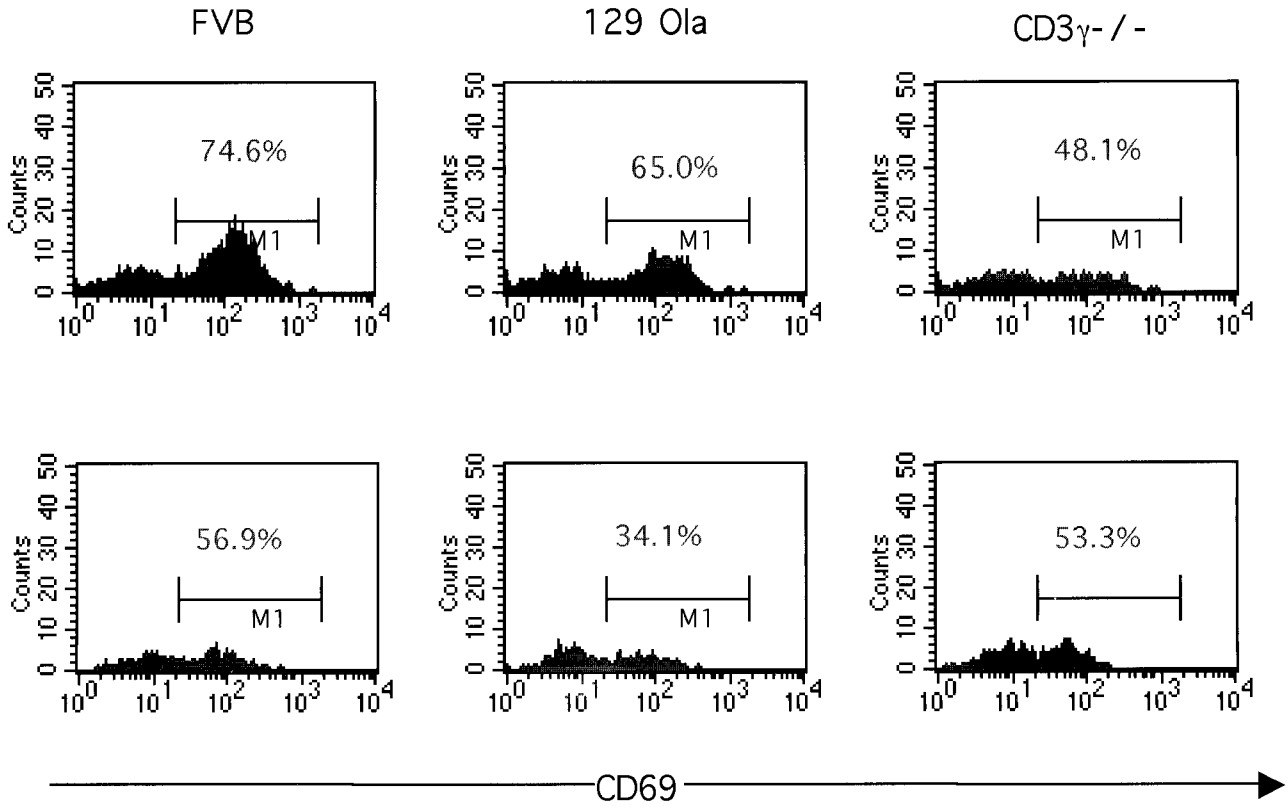

Figure 6.1: Analysis of ex-vivo CD4 SP (A) and CD8 SP (B) thymocytes for the expression of the CD69 cell surface marker.

In contrast to CD4 SP thymocytes, the percentage of $CD69^+$ cells among the CD3γ-deficient CD8 SP thymocytes was found to be nearly indistinguishable from wild type CD8 SP thymocytes (Figure 6.1B). This suggests that CD8 SP generation is not affected by the lack of the CD3γ chain. Nevertheless, several studies have shown that, upon positive selection and concomitant CD69 upregulation, CD8 SP generation is delayed, as compared to CD4 SP thymocyte generation (Swat *et al.*, 1994; Lundberg and Shortman, 1994; Kydd *et al.*, 1995; Lundberg *et al.*, 1995; Lucas *et al.*, 1995). Moreover, it has been shown that early positively selected thymocytes, i.e., $TCR^{lo}CD69^+$ thymocytes, include CD4 SP, but not CD8 SP cells (Barthlott *et al.*, 1997). Therefore, concerning the CD8 SP thymocytes, the percentage of $CD69^+$ cells may not reflect newly generated cells.

As hypothesised above, the altered ratio of CD4 SP/CD8 SP observed among CD3γ-deficient thymocytes may also be due to an altered cellular fate of newly differentiated CD4 SP or CD8 SP thymocytes. The lack of the CD3γ chain may differentially affect proliferation, survival or accumulation of newly generated CD4 SP and CD8 SP thymocytes. We therefore investigated each of these three possibilities. We attempted to analyse the SP thymocytes for their proliferative state, either by analysing their DNA content or their ability to incorporate BrdU. Both approaches proved to be technically impracticable. Analysis of the cellular DNA content, by propidium iodine staining, was not compatible with a CD4 and CD8 staining.

Likewise, analysis of cellular proliferation, *in vivo*, via incorporation of BrdU administered either in drinking water or through intraperitoneal injections was not possible. This was so because, surprisingly, BrdU administration was found to provoke the death of CD3γ-deficient mice. Interactions with other drugs used in other mouse facility may be a problem, and application of the BrdU technology waits further preliminary experiments.

6.3. A differential ability of CD4 SP/CD8 SP thymocytes to undergo TCR-mediated cell death may constitute a contributing factor for the altered CD4/CD8 T cell ratio observed in CD3γ-deficient mice

We next analysed whether the lack of the CD3γ chain differentially affects the ability of SP thymocytes to undergo cell death upon TCR triggering. This was done by analysing the ability of CD4 SP and CD8 SP thymocytes to die upon overnight culture in the presence of P815 cells and different doses of anti-TCR antibody. CD3γ-deficient CD4 SP thymocytes were consistently found to be relatively more susceptible to undergo TCR-mediated cell death, as compared to their counterpart CD8 SP thymocyte (Figure 6.2). This suggests that newly differentiated CD3γ-deficient CD8 SP thymocytes are more prone to escape TCR-mediated cell death and to complete their maturational program. Therefore, our data support the notion that a differential ability of CD4 SP versus CD8 SP thymocytes to undergo TCR-mediated cell death may constitute a contributing factor for the altered CD4/CD8 T cell ratio observed among CD3γ-deficient T cells. Nevertheless, we could not determine whether this differential ability to undergo cell death constitutes a unique feature of CD3γ-deficient thymocytes or whether it occurs also among wild type thymocytes. Indeed, the low percentage of CD8 SP

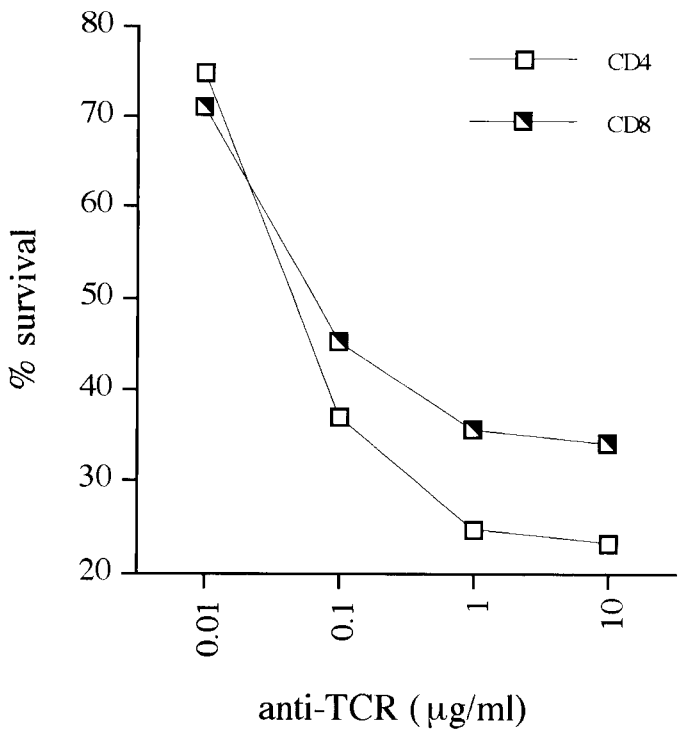

Figure 6.2: Differential ability of CD4 SP and CD8 SP CD3γ-deficient mouse thymocytes to undergo cell death upon *in vitro* TCR triggering. Unsorted thymocytes were co-cultured with P815-B7.1 cells in the presence of different doses of soluble anti-TCRβ. Shown is the percentage of CD4 SP and CD8 SP thymocytes that survived upon overnight culture. Percentage of survival was determined based on the percentages of alive CD4/CD8 SP thymocytes in control and treated cultures.

cells and the partial CD4/CD8 co-receptor down-regulation on DP thymocytes made it extremely difficult to identify CD8 SP cells among stimulated wild type mouse thymocytes. In the future, this technical problem may be circumvented by analysing TCR-mediated cell death at an earlier time point, using DNA fragmentation as a marker for induced apoptosis.

6.4 CD3γ-deficient CD4 SP thymocytes are more resistant to dexamethasone-induced cell death than wild type

Glucocorticoids, produced both by the adrenal gland and within the thymus, have complex effects on the development of thymocytes (reviewed in Vacchio *et al.*, 1998). Steroids are able to induce apoptosis in thymocytes *in vitro* in concentrations similar to those in circulation. In this respect, DP thymocytes have been found to be extremely sensitive to glucocorticoids-induced apoptosis, whereas SP thymocytes have been found to be relatively resistant (Screpanti *et al.*, 1989; reviewed in Scollay *et al.*, 1984 and Cohen, 1992). Given the ability of glucocorticoids to induce thymocyte cell death, we tested whether, as compared to wild type mouse derived thymocytes, the CD3γ-deficient altered CD4 SP/CD8 SP ratio may reflect a differential susceptibility of the mutant SP thymocytes to undergo cell death in response to glucocorticoid treatment. In order to test this hypothesis, we analysed to ability of mutant CD4 SP and CD8 SP thymocytes to die upon overnight culture in the presence of different concentrations of the synthetic glucocorticoid dexamethasone.

As compared to wild type thymocytes, CD3γ-deficient CD4 SP thymocytes were consistently found to be relatively resistant to dexamethasone induced cell death (Figure 6.3). In contrast, we did not observe a consistent differential ability of the mutant CD8 SP thymocytes to undergo dexamethasone-mediated cell death. In some experiments, the CD3γ-deficient CD8 SP thymo-cytes appeared to be relatively more susceptible to undergo cell death, as compared to their wild type counterpart. In other experiments, however, susceptibility of the mutant CD8 SP thymocytes to undergo dexamethasone induced cell death appeared to be indistinguishable from the susceptibility of their wild type counterpart CD8 SP thymocytes. Nevertheless, the CD3γ- deficient CD8 SP thymocytes were never found to be relatively resistant to dexamethasone-induced cell death (data not shown). Therefore, our data suggest that glucocorticoid-induced cell death does not contribute to the altered CD4 SP/CD8 SP ratio observed among the CD3γ-deficient mouse thymocytes. Indeed, our results are the opposite of what would be expected in case the mutant CD4 SP/CD8 SP was caused by a differential susceptibility of SP thymocytes to glucocorticoid-induced cell death. The observed consistent resistance of the CD3γ-deficient CD4 SP thymocytes is likely to reflect the already reported reduced fraction of newly generated cells among the mutant CD4 SP thymocytes, as compared to their wild type mouse counterpart thymocytes.

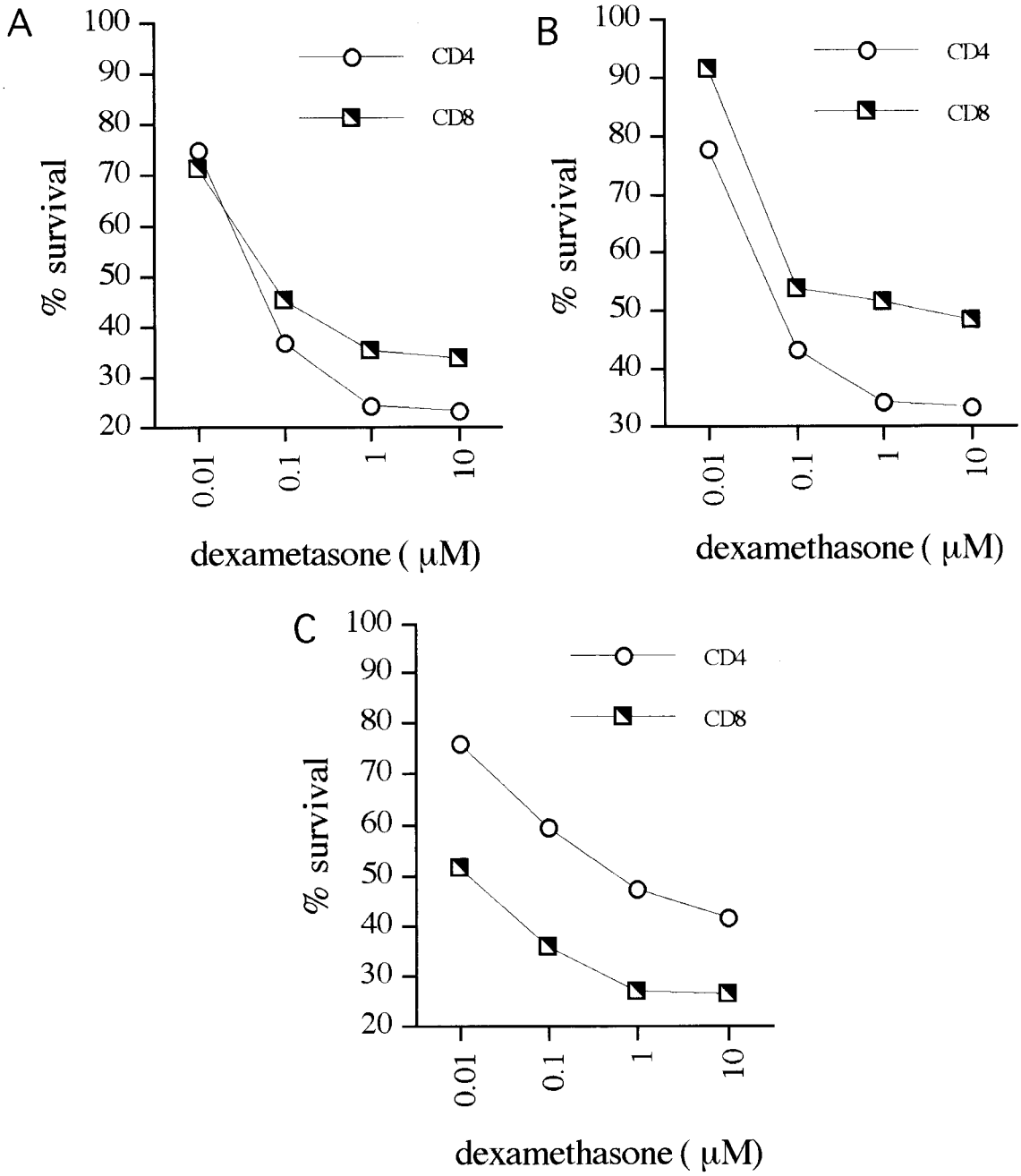

Figure 6.3: Differential ability of CD4 SP and CD8 SP thymocytes to undergo cell death in response to dexamethasone treatment. Unsorted thymocytes derived from FVB (A), 129 Ola (B) and CD3γ-deficient (C) mice were cultured in the presence of different doses of dexamethasone. Shown is the percentage of CD4 SP and CD8 SP thymocytes that survived upon overnight culture. Percentage of survival was determined based on the percentages of alive CD4/CD8 SP thymocytes in control and treated cultures.

6.5 CD3γ-deficiency inhibits CD4 SP differentiation

We next investigated whether the altered CD4 SP/CD8 SP ratio, among the CD3γ-deficient thymocytes, is due to an altered T cell lineage commitment at the DP to SP transition, or whether it is due to a preferential accumulation of CD8 SP thymocytes. Our first approach to this investigation was to analyse thymocytes derived from newborn CD3γ-deficient mice. We reasoned that, if the lack of the CD3γ chain affects T cell lineage commitment, thymocytes from

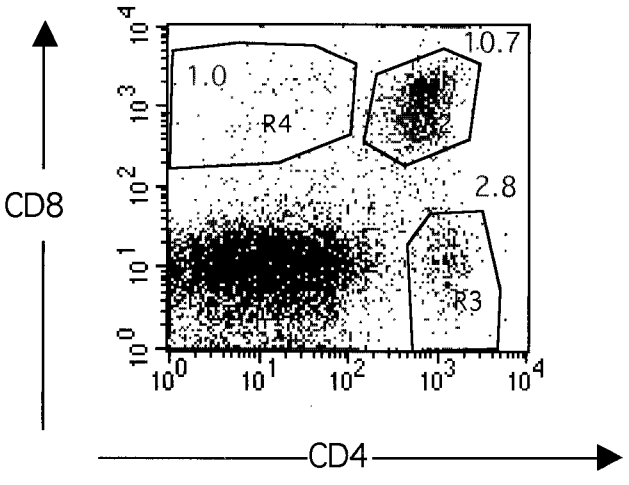

Figure 6.4: Analysis of CD3γ-deficient CD4 SP/CD8 SP thymocyte ratio, early in ontogeny. Shown is a representative example of the relative representation of DN, DP, CD4 SP and CD8 SP thymocyte subsets at day 4 of age.

newborn mice would already display an altered ratio of CD4 SP to CD8 SP thymocytes. We therefore analysed thymocytes derived from CD3γ-deficient and wild type mice, from their day of birth until the first week of age, for the percentages of CD4 SP and CD8 SP cells. In contrast to the altered CD4 SP/CD8 SP ratio, among adult mouse thymocytes, we found that newborn CD3γ-deficient mouse thymocytes comprise a higher percentage of CD4 SP, as compared to CD8 SP thymocytes (Figure 6.4). This observation, apparently, indicates that the absence ot the CD3γ chain has no major effect on T cell lineage commitment at the DP to SP thymocyte transition, but it affects the accumulation of CD4 SP versus CD8 SP thymocytes. Nevertheless, the higher CD4 SP/CD8 SP ratio among newborn CD3γ-deficient mouse thymocytes may be due to the fact that CD4 SP differentiate before CD8 SP thymocytes (Kydd *et al.*, 1995; Lundberg *et al.*, 1995; Lucas *et al.*, 1995; Swat *et al.*, 1994; Lundberg and Shortman, 1994). We therefore took yet another experimental approach to investigate whether the lack of the CD3γ chain affects T cell lineage commitment at the DP to SP thymocyte transition. We analysed the early positively selected $TCR^{hi}HSA^{+}$ thymocytes for their CD4 versus CD8 phenotype (see Figure 5.3). This analysis revealed that the percentage of CD4 SP cells, among the mutant $TCR^{hi}HSA^{+}$ thymocyte population, is reduced. In contrast, the percentage of CD8 SP cells among the CD3γ-deficient $TCR^{hi}HSA^{+}$ thymocyte population was nearly indistinguishable from the counterpart percentage among wild type mouse thymocytes

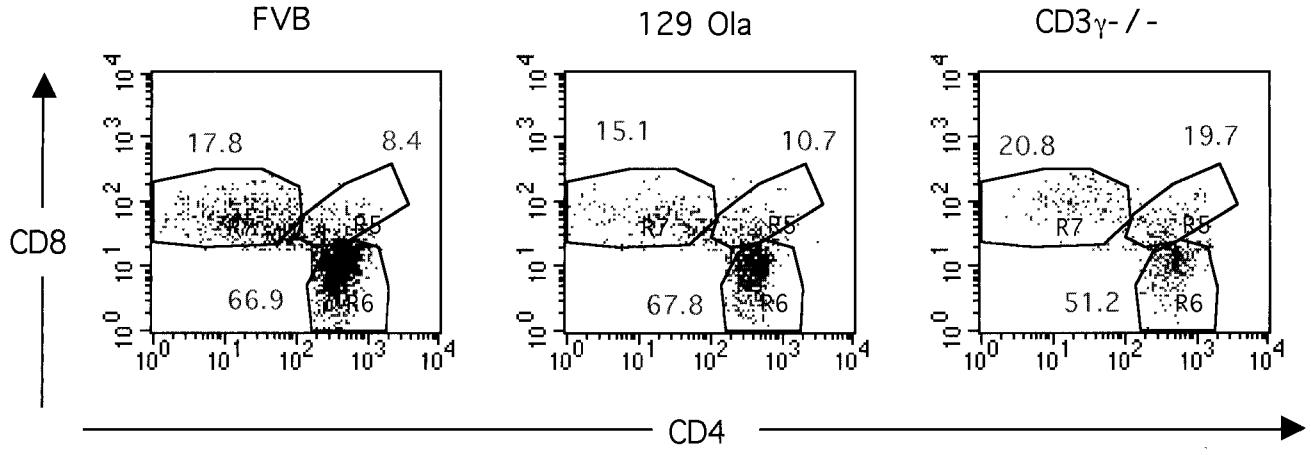

Figure 6.5: Flow cytometry analysis of the relative representation of DP, CD4 SP and CD8 SP cells among $TCR^{hi}HSA^{+}$ thymocytes.

(Figure 6.5). This suggests that the absence of the CD3γ chain inhibits the differentiation of CD4 SP cells, but has no effect on the differentiation of CD8 SP thymocytes. An inhibition of CD4 SP cell differentiation in the CD3γ-deficient mice is in agreement with the already reported relatively lower percentage of $CD69^+$ cells among the mutant CD4 SP thymocytes, as compared to the counterpart percentage among wild type mouse thymocytes (Figure 6.1A).

We found that the percentage of CD8 SP cells, among the mutant $TCR^{hi}HSA^+$ population, is nearly indistinguishable from the counterpart percentage among wild type mouse thymocytes. This observation suggests that the lack of the CD3γ chain does not affect CD8 SP thymocyte differentiation. Nevertheless, we also found that the percentage of DP cells among the CD3γ-deficient $TCR^{hi}HSA^+$ population is relatively increased, as compared to that same percentage among the wild type mouse thymocytes. Since the differentiation of CD8 SP cells is delayed, as compared to the differentiation of CD4 SP thymocytes (Kydd *et al.*, 1995; Lundberg *et al.*, 1995; Lucas *et al.*, 1995; Swat *et al.*, 1994; Lundberg and Shortman, 1994), we hypothesised that the majority of the $TCR^{hi}HSA^+$ DP cells are CD8 SP thymocytes precursors, i.e., that these DP cells are cells in the process of down-regulating the CD4 co-receptor. In order to test our hypothesis, we sorted the $TCR^{hi}HSA^+$ thymocyte population and performed a co-receptor reexpression assay. The sorted cells were treated with trypsin, in order to remove the CD4 and CD8 co-receptors from their cell surface, and subsequently incubated overnight at 37 ^{0}C to allow the re-expression of the synthetized co-receptor(s). In agreement with the hypothesis, upon co-receptor re-expression, we observed a reduction in the percentage of DP cells and an increase in the percentage of CD8 SP cells, as compared to control thymocytes (Figure 6.6). This suggests that the lack of the CD3γ chain favours the differentiation of CD8 SP thymocytes.

Our data suggest that CD3γ-deficiency affects T cell lineage commitment at the DP to SP thymocyte transition. The lack of the CD3γ chain promotes CD8 SP differentiation and inhibits CD4 SP thymocyte differentiation. An altered CD4/CD8 T cell ratio has also been observed in CD3δ-deficient and TCRζ/η-deficient mice (Malissen *et al.*, 1993; Ohno *et al.*, 1993; Liu *et al.*, 1993; Dave *et al.*, 1997). Numerous models have been proposed to explain T cell lineage commitment (reviewed in chapter 2). According to one of these models, the DP thymocytes integrate two distinct signals, one provided by the TCR, and the second provided by the activation of Lck. Moreover, it proposes that it is the relative ratio of these two signals that lead to distinct differentiation decisions in the thymus. CD8 SP differentiation occurs when TCR engagement is combined with reduced Lck activation and CD4 SP differentiation occurs when TCR engagement is combined with a stronger Lck signal (Basson *et al.*, 1998) . It is not clear which is the nature of the TCR derived signal, as opposed to Lck activation. Nevertheless, we can envisage that engagement of TCRs lacking the CD3γ chain may lead to a relatively reduced recruitment of Lck, as compared to wild type TCR complexes. According to this notion, we expect that thymocytes derived from the CD3γ ITAM-deficient mice will display the same

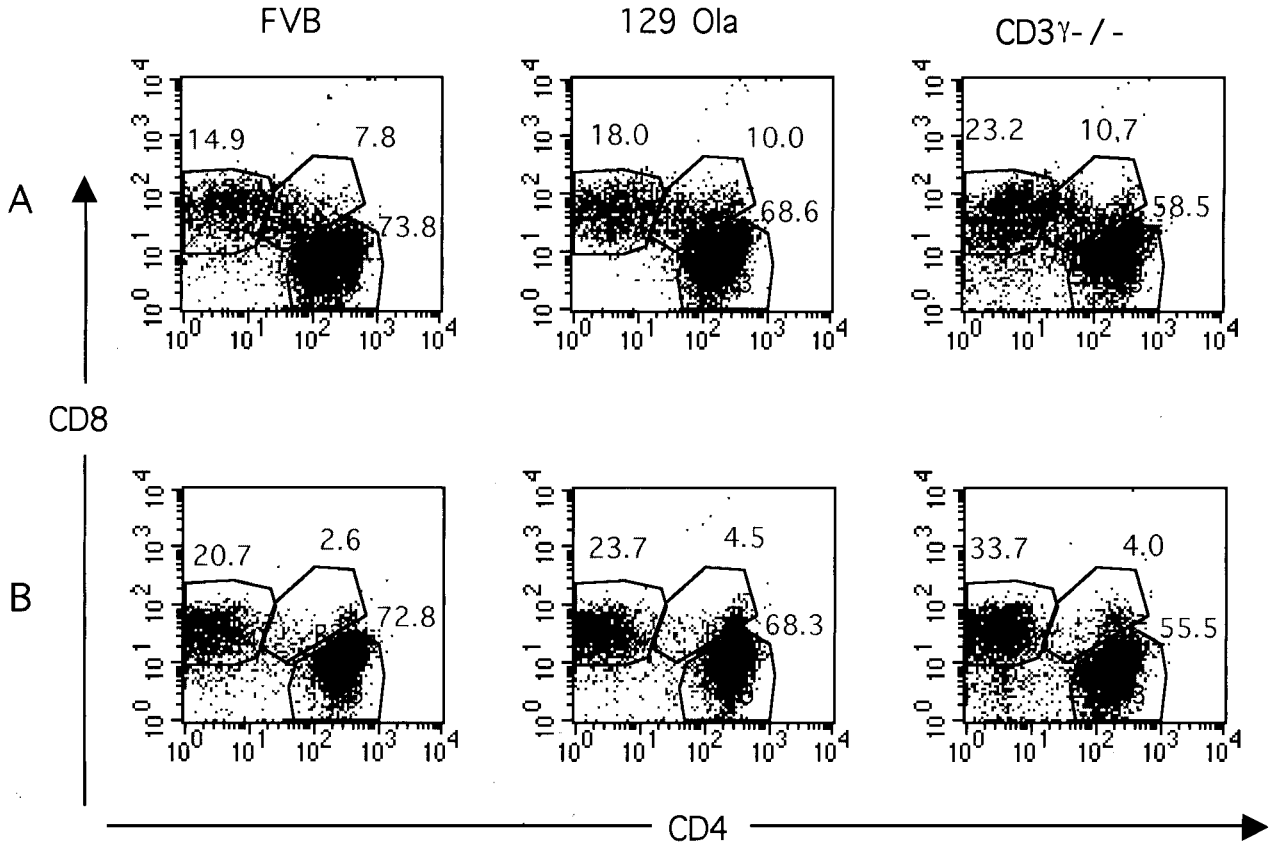

Figure 6.6: Analysis of T cell lineage commitment at the $TCR^{hi}HSA^{+}$ T cell developmental stage, by a co-receptor re-expression assay. $TCR^{hi}HSA^{+}$ thymocytes were sorted and treated with either 0.02% EDTA (A) or 0.02% EDTA + trypsin (B). Treatment with trypsin removed CD4 and CD8 co-receptors from the thymocyte cell surface. The cells were subsequently washed and incubated at 37 ^{0}C in supplemented IDMM, to allow re-expression of CD4/CD8 co-receptors on cells treated with trypsin. After overnight culture, all cells were analysed for the expression of CD4 and CD8 co-receptors.

altered CD4 SP/CD8 SP ratio, as observed among thymocytes from the CD3γ-deficient mice. The CD3γ ITAM deficient mice are currently being analysed by our group.

Another model has proposed that CD8 SP differentiation is highly dependent on the affinity of the TCR for a particular peptide/MHC class I complex. While, CD4 SP differentiation is less peptide specific, but it involves many low-affinity interactions with a diversity of MHC class II ligands (Goldrath *et al.*, 1997). The CD3γ-deficient TCR^{lo} DP thymocytes display a relatively reduced TCR cell surface expression level, as compared to wild type thymocytes. It is therefore possible that this lower TCR cell surface expression leads to a reduction in the number of TCR-peptide/MHC class II interactions, and concomitantly to a selective reduction in the number of DP thymocytes which mature to the CD4 SP stage. In support of this notion, it has been shown that a decreased expression of TCR complexes lacking the CD3δ cytoplasmic domain preferentially impairs the development of CD4 SP thymocytes (Dave *et al.*, 1999). According to this model and to the referred study, the differentiation of CD8 SP thymocytes would be expected to remain unaltered in CD3γ-deficient mice. Nevertheless, our data suggests that the lack of the CD3γ chain favours CD8 SP thymocyte differentiation. An

additional possible explanation for this observation, which does not exclude the previous ones, is that the absence of an appropriate signal for CD4 SP differentiation increases the probability of the CD3γ-deficient DP thymocytes to encounter an appropriate peptide/MHC class I complexes. Further clarification on how the absence of the CD3γ chain affects T cell lineage commitment will require the analysis of T cell development on an MHC class I and/or MHC class II-deficient background. It will also require mice that not only lack the CD3γ chain, but also express a TCR of known specificity.

CHAPTER 7

CONCLUDING REMARKS

CD3γ-deficient mice have previously been shown to display a profound blockage in T cell development at the DN to DP thymocyte transition (Haks *et al.*, 1998). In the present work, we have begun to investigate how the absence of the CD3γ chain affects T cell development beyond the DP stage and how it affects the functionality of peripheral T cells.

We found that the lack of the CD3γ chain indeed affects the DN to the DP transition, not only quantitatively, but also qualitatively. CD3γ-deficient DP thymocytes exhibit a reduced percentage of $CD5^{int}$ and TCR^{lo} cells, as compared to wild type thymocytes. DP thymocytes derived from the CD3γ-deficient mice were also found to be relatively deficient in their ability to undergo either positive or negative selection. Nevertheless, we found that the differential ability of CD3γ-deficient versus wild type DP thymocytes to undergo positive/negative selection closely matches their differential percentage of TCR^{lo} cells. Therefore, we suggest that the lack of the CD3γ chain has no major effect on the ability of TCR^{lo} DP thymocytes, i.e., "selectable" cells, to undergo either positive or negative selection.

Although the CD3γ-deficient DP thymocytes were found to comprise a reduced percentage of TCR^{lo} cells, the ratio of DP/SP thymocytes in these mice is nearly indistinguishable from wild type thymocytes. These two observations suggest that the lack of the CD3γ chain affects the fate of newly differentiated SP thymocytes. In support of this notion, we found that the CD3γ-deficient SP thymocytes are relatively resistant to undergo TCR-mediated cell death, as compared to wild type mouse SP thymocytes.

Another interesting feature which characterises the T cell compartment of CD3γ-deficient mice is their decreased CD4/CD8 ratio. This altered ratio has its origin in the thymus and we have shown evidence suggesting that it originates mostly at the DP to SP thymocyte transition. Indeed, we showed that, in the CD3γ-deficient mice early positively selected thymocytes comprise a reduced percentage of CD4 SP cells and an increased percentage of cells committed to the $CD8^+$ T cell lineage, as compared to wild type mouse thymocytes. In addition to an altered T cell commitment at the DP to SP transition, a differential susceptibility to undergo TCR-mediated cell death may also contribute to the altered ratio of CD4/CD8 T cells in the CD3γ-deficient mice.

CD3γ-deficient peripheral T cells were found to be relatively defective in their ability to undergo either activation or activation induced cell death, as compared to wild type T cells. Moreover, we showed evidence strongly suggesting that this relative inability to undergo TCR-mediated responses reflects the relatively low levels of TCR cell surface expression on CD3γ-deficient T cells, as compared to wild type mouse T cells. Indeed, T cells from the CD3γ-deficient mice are as able as wild type mouse T cells in undergoing activation in response to phorbol esters treatment. TCR signalling on CD3γ-deficient T cells was shown to be quantitatively deficient at one of the most TCR proximal levels, the induction of a cascade of protein tyrosine phosphorylations.

In summary, the work presented here demonstrates that the lack of the CD3γ chain affects T cell differentiation beyond the DP thymocyte stage, as well as the ability of peripheral T cells to responde upon TCR engagement. This indicates that the CD3γ chain is not only a fundamental component of the pre-TCR (Haks *et al.*, 1998), but that it is also essential for the proper assembly and functioning of the TCR *in vivo*. Our data strongly suggest that the defective ability of the CD3γ-/- peripheral T cells to responde upon TCR triggering is basically due to their low TCR cell surface expression. I.e., our data suggest that the CD3γ chain is mostly required as a structural component of the TCR. Nevertheless, the hypothesis of a qualitative contribution of the CD3γ chain to the proper functioning of the TCR is not excluded. To clarify this issue, the ability of peripheral T cells derived from mice which express a CD3γ chain lacking its ITAM motif is currently being investigated. Our work, together with the work presented by others (Dave *et al.*, 1997), also raises the issue of the differential role of the CD3γ and CD3δ chains as components of the mature TCR form. Both chains are generally considered to be components of the TCR in a 1:1 ratio, and the lack of either the CD3γ or CD3δ chains leads to a reduction of the TCR cell surface expression on peripheral T cells. Nevertheless, T cell maturation beyond the DP stage is severely blocked in CD3δ-deficient (Dave *et al.*, 1997), but not in the CD3γ-/- mice. This suggests that besides an upregulation of TCR expression, positive selection may be accompanied by an alteration in the TCR composition. Another possible interpretation is that at the DP thymocyte stage, but not in the periphery, the CD3γ and CD3δ chains provide the TCR with distinct abilities to signal. Finally, the observation that the lack of the CD3γ chain affects $CD4^+$ versus $CD8^+$ T cell lineage commitment, turns the CD3γ-deficient mice in a new animal model for the investigation of the still largelly unknown mechanisms which regulate T cell lineage choice.

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Sumário

A diferenciação intratímica dos linfócitos T αβ efectua-se sob o controle de duas formas de TCR (receptor dos linfócitos T): o TCR αβ maduro e o pre-TCR. O TCR αβ é composto por um heterodímero TCRβ-TCRα associado não-covalentemente às sub-unidades monomórficas CD3-ε, -γ, -δ e TCRζ. O pre-TCR é composto por um heterodímero TCRβ-pTα e por elementos do complexo CD3/TCRζ. As sub-unidades CD3/TCRζ são necessárias para a formação do TCR e a transmissão de sinais. O papel específico das diferentes sub-unidades CD3/TCRζ como elementos estruturais e/ou de transmissão de sinal é actualmente objecto de intensa investigação. A este respeito, foi recentemente demonstrado que a cadeia CD3γ é um componente essencial do pre-TCR. Ratinhos deficientes para esta sub-unidade apresentam um bloqueio no desenvolvimento dos linfócitos T ao nível da transição DN (duplas negativas) a DP (duplas positivas). No entanto, uma fracção dos timócitos destes ratinhos tem a capacidade de completar o programa de diferenciação e povoar os órgãos linfóides periféricos. Os linfócitos T maduros destes ratinhos CD3γ-/- são caracterizados por uma redução de cerca de 10-vezes da expressão de superfície do TCR, assim como por uma alteração da razão de células $CD4^+$ para $CD8^+$.

Neste trabalho, nós analisámos como a ausência da cadeia CD3γ afecta a transição dos timócitos DP a SP e a funcionalidade dos linfócitos T periféricos. Para isso, realizámos uma análise fenotípica aprofundada dos timócitos CD3γ-/-, dando particular ênfase a marcadores de selecção positiva em timócitos DP. Analisámos a capacidade dos timócitos DP CD3γ-/- para ser positiva ou negativamente seleccionados por ligação do TCR *in vitro*, usando o CD69 e o CD5 como marcadores de selecção positiva, e a apoptose como indicador de selecção negativa. Para uma maior compreensão em como a ausência da cadeia CD3γ-/- afecta a escolha da linhagem T, realizámos uma análise fenotípica dos timócitos SP. Também analizámos a susceptibilidade dos timócitos CD3γ-/- CD4 SP e CD8 SP a sofrerem apoptose após ligação do TCR ou tratamento com dexametasona, e a escolha de linhagem T ao nível dos timócitos DP. Os linfócitos T periféricos CD3γ-/- foram analizados em termos do seu fenótipo naive/activado ou de memória e em termos da sua capacidade de activação ou morte induzida por activação aquando da ligação do TCR *in vitro*. A capacidade dos linfócitos T mutantes para responder à ligação do TCR foi também analisada a um nível mais próximo do TCR, a indução de uma cascata de fosforilações de tirosinas. Finalmente, testámos a capacidade dos linfócitos T periféricos CD3γ-/- para reduzirem a expressão do TCR após ligação ao TCR ou por tratamento com esters de phorbol, visto que a cadeia CD3γ contém um motivo envolvido na internalização do TCR.

Nós demonstrámos que a ausência da cadeia CD3γ afecta a maturação dos timócitos DN a DP, não só quantitativa mas também qualitativamente. Demonstrámos que os timócitos DP CD3γ-/- incluem uma menor fracção de células com baixa expressão de TCR ou expressão intermédia de CD5, comparativamente aos timócitos controle. No que respeita à capacidade dos

timócitos CD3γ-/- para sofrer selecção positiva, uma análise fenotípica revelou que a ausência desta cadeia do TCR provoca uma redução na percentagem de células DP positivamente seleccionadas. Demonstrámos também que os timócitos DP CD3γ-/- são deficientes na sua capacidade de ser quer positiva quer negativamente seleccionados por ligação do TCR *in vitro*. No entanto, a redução na percentagem de timócitos DP CD3γ-/- capazes de ser positiva ou negativamente seleccionados é semelhante à redução na percentagem de timócitos DP TCR^{lo}. Esta observação sugere que a ausência da cadeia CD3γ não inibe fortemente a capacidade dos timócitos DP TCR^{lo} ou "seleccionavéis" para responder à ligação do TCR. Além disso, dados da nossa análise fenotípica de timócitos *ex-vivo* sugerem que uma vez positivamente seleccionados, os timócitos deficientes para a cadeia CD3γ têem uma maior probabilidade de completar o seu programa de maturação. No que respeita à escolha de lineagem T, os nossos dados sugerem fortemente que a ausência da cadeia CD3γ inibe preferencialmente a diferenciação de timócitos DP em CD4 SP. Na periferia, demonstrámos que os linfócitos T CD3γ-/- são deficientes na sua capacidade de responder com activação ou apoptose por activação à ligação do TCR *in vitro*. Demonstrámos também que esta deficiente capacidade de responder à ligação do TCR é quantitativa e não qualitativa. Não observámos nenhuma diferença clara, entre os ratinhos CD3γ-/- e os controlo, no que respeita ao padrão de proteínas que são fosforiladas nos seus resíduos de tirosina aquando de ligação do TCR. Finalmente, de acordo com os dados de outros, verificámos que os linfócitos T deficientes para a cadeia CD3γ não são capazes de internalizar os seus TCRs em resposta ao tratamento com esters de phorbol.

Summary

αβ T cell development in the thymus is under the control of two forms of TCR, the mature αβ TCR and the pre-TCR. The αβ TCR comprizes a TCRβ-TCRα heterodimer non-covalently associated with the monomorphic subunits CD3-ε, -γ, -ε and TCRζ. The pre-TCR is minimally composed of a TCRβ-pTα heterodimer and elements of the CD3/TCRζ complex. The CD3/TCRζ subunits are required for TCR assembly and signal transduction. The specific role of the different CD3/TCRζ subunits as structural and/or signalling components is currently the subject of extensive investigation. In this respect, it has recently been shown that the CD3γ chain is an essential component of the pre-TCR. Mice lacking this subunit display a blockage in T cell development at the DN to DP transition. Nevertheless, in these mice some thymocytes do complete their maturational program and populate the peripheral lymphoid organs. Furthermore, the mature T cells from the CD3γ-/- mice are characterized by an approximately 10-fold reduced TCR cell surface expression and by an altered ratio of $CD4^+$ to $CD8^+$ T cells.

In the present study we investigated whether the lack of the CD3γ chain affects DP to SP thymocyte transition and the functionality of peripheral T cells. For that purpose, we performed an extensive phenotypic analysis of CD3γ-deficient thymocytes with special emphasis on positive selection markers on DP thymocytes. We analysed the CD3γ-/- DP thymocytes for their ability to undergo either positive or negative selection upon TCR engagement *in vitro*, using CD69 and CD5 upregulation as a readout for positive selection, and apoptosis as a readout for negative selection. In order to clarify how the lack of the CD3γ chain affects T cell lineage commitment, we performed a phenotypic analysis of SP thymocytes. We also analysed the susceptibility of CD3γ-deficient CD4 SP and CD8 SP thymocytes to undergo cell death upon TCR triggering or treatment with dexamethazone, and T cell lineage commitment at the DP thymocyte stage. The peripheral CD3γ-/- T cells were analysed with respect to their naive/activated ou memory phenotype and their ability to undergo either activation or activation induced cell death upon TCR triggering *in vitro*. The ability of the mutant T cells to respond to TCR engagement was also analysed at the TCR proximal level, the induction of a cascade of protein tyrosine phosphorylations. Finally, we tested that ability of the CD3γ-deficient peripheral T cells to down-regulate their TCR cell surface expression upon TCR triggering or treatment with phorbol esters, since the CD3γ chain has been previously shown to contain a TCR internalization motif.

We found that the lack of the CD3γ chain affects DN to DP thymocyte transition, not only quantitatively but also qualitatively. CD3γ-/- DP thymocytes were found to comprise a smaller fraction of cells with a low expression of TCR or an intermediate expression of CD5, as compared to wild type thymocytes. Concerning the ability of the CD3γ-deficient thymocytes to undergo positive selection, phenotypic analysis revealed that the absence of the CD3γ chain

leads to a reduction in the percentage of DP cells which have undergone positive selection. CD3γ-/- DP thymocytes were also found to be deficient in their ability to undergo either positive selection or negative selection upon TCR triggering *in vitro*. Nevertheless, the reduction in the percentage of CD3γ-/- DP thymocytes capable of undergoing either positive or negative selection was found to closely match their reduced percentage of TCR^{lo} cells. This suggests that the lack of the CD3γ chain has no major inhibitory effect on the ability of TCR^{lo}, or "selectable" DP thymocytes, to respond to TCR triggering. Furthermore, data from our phenotypic analysis of *ex-vivo* thymocytes suggest that once positively selected, CD3γ-deficient thymocytes are more prone to complete their maturational program. Concerning T cell lineage commitment, our data strongly suggest that the lack of the CD3γ chain preferentially inhibits the differentiation of DP into CD4 SP thymocytes. Peripheral CD3γ-/- T cells were found to be defective in their ability to undergo either activation or activation-induced cell death upon TCR triggering *in vitro*. This deficient ability to respond to TCR engagement was also found to be quantitative rather than qualitative. We did not found any clear difference, between CD3γ-deficient and wild type mice, concerning the pattern of proteins which are phosporylated on their tyrosine residues upon TCR engagement. Finally, in agreement with data from others, CD3γ-deficient T cells were found to be unable to downregulate their TCR cell surface expression in response to treatment with phosbol esters.

Summaire

Le développement des cellules T αβ dans le thymus s'effectue sous le contrôle de deux formes de TCR (récepteur des cellules T): le TCR αβ mature et le pré-TCR. Le TCR αβ est composé d'un hétérodimère TCRβ-TCRα associé non covalamment aux sous-unités monomorphiques CD3-ε, -γ. -δ et TCRζ. Le pré-TCR est composé d'un hétérodimère TCRβ-pTα et des éléments du complexe CD3/TCRζ. Les sous-unités CD3/TCRζ son nécessaires pour l'assemblage du TCR et la tranmission du signal. Le rôle spécifique des différentes sous-unités CD3/TCRζ dans la structure et/ou la transmission du signal fait actuellement l'objet de nombreuses recherches. A cet égard, il a été récemment montré que la chaîne CD3γ est un composant essentiel du pré-TCR. Les souris déficientes pour cette sous-unité présentent un blocage dans le développement T au niveau de la transition DN (double négatifs) à DP (double positifs). Cependant, certains thymocytes de ces souris terminent leur programme de maturation et peuplent les organes lymphoides périphériques. De plus, les cellules T matures de ces souris CD3γ-/- sont caractérisées par une réduction d'environ 10 fois de l'expression membranaire du TCR ainsi que par une modification du ratio cellules $CD4^+$:$CD8^+$.

Dans ce travail, nous avons étudié si l'absence de la chaîne CD3γ affectait la transition DP-SP des thymocytes et la fonctionalité des cellules T périphériques. Pour cela, nous avons réalisé une analyse phénotypique approfondie des thymocytes CD3γ-/-, avec une emphase particulière sur les marqueurs de sélection positive des thymocytes DP. Nous avons analysé l'aptitude des thymocytes CD3γ-/- DP à réaliser la sélection positive ou négative après engagement du TCR *in vitro*, en analysant l'augmentation d'expression de CD69 et CD5 comme marqueurs de la sélection positive et l'apoptose comme marqueur de la sélection négative. Dans le but de comprendre comment l'absence de la chaîne CD3γ affect l'engagement dans la différenciation T, nous avons réalisé une analyse phénotypique des thymocytes SP. Nous avons également étudié la susceptibilité des thymocytes CD3γ-/- CD4 SP et CD8 SP à la mort cellulaire provoquée par l'engagement du TCR ou le traitement à la dexaméthasone, ainsi que l'engagement dans la différenciation T au stade DP. Les cellules T périphériques CD3γ-/- ont été analysées quant à leur phénotype naif/activé ou mémoire et leur aptitude à l'activation ou la mort cellulaire induite par l'activation après engagement du TCR. La capacité des cellules T mutées à répondre à l'engagement du TCR a été également analysée au niveau proximal du TCR, à savoir l'induction de phosphorylation de protéines. Enfin, nous avons testé l'aptitude des cellules T périphériques CD3γ-/- à réguler négativement l'expression de leur TCR à la surface cellulaire après engagement du TCR ou traitement avec des esters de phorbol, puisqu'il a déjà été montré que la chaîne CD3γ contient un motif d'enternalisation du TCR.

Nous avons montré que l'absence de la chaîne CD3γ affecte la transition DN-DP non seulement quantitativement mais aussi qualitativement. Les thymocytes CD3γ-/- comprennent

une plus petite fraction de cellules avec une expression faible du TCR ou intermédiaire de CD5, comparativement aux thymocytes normaux. Concernant l'aptitude des thymocytes CD3γ-/- à réaliser la sélection positive, les analyses phénotypiques ont montré que l'absence de la chaîne CD3γ conduit à une réduction du pourcentage de cellules DP sélectionées positivement. Nous avons également montré que les thymocytes CD3γ-/- sont déficients dans leur capacité à réaliser la sélection positive ou négative après engagement du TCR. Cependant la réduction du pourcentage des thymocytes CD3γ-/- DP aptes à réaliser la sélection positive ou négative correspond au pourcentage réduit de cellules TCR^{lo}. Ceci suggère que l'absence de chaîne CD3γ n'a pas d'effet majeur sur la capacité des thymocytes TCR^{lo}, ou "sélectionnables", à répondre à l'engagement du TCR. De plus, les données de nos analyses phénotypiques de thymocytes *ex-vivo* suggèrent qu'une fois sélectionnées positivement, les thymocytes CD3γ-/- ont plus tendance à terminer leur programme de maturation. En ce qui concerne l'engagement dans la voie de différenciation T, nos données suggèrent fortement que l'absence de la chaîne CD3γ inhibe préférentiellement la différenciation des thymocytes DP en CD4 SP. Nous avons montré que les cellules T CD3γ-/- périphériques sont déficientes dans leur aptitude à l'activation ou la mort cellulaire induite par l'activation après engagement du TCR *in vitro*. Cette déficience à répondre à l'engagement du TCR est quantitative plutôt que qualitative. Nous n'avons pas trouvé de nette différence entre les souris normales et les souris CD3γ-/- concernant le profil des protéines qui sont phosphorylées au niveau de résidus Tyrosine après engagement du TCR. Enfin, en accord avec d'autres données, les cellules T CD3γ-/- ne sont pas capables de réguler négativement l'expression membranaire de leur TCR en réponse à un traitement aux esters de phorbol.

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