IDENTIFICATION AND CHARACTERIZATION OF A UNIQUE SUBPOPULATION OF DOUBLE NEGATIVE SPLENIC T CELLS WHICH EXPRESS THE αβ T CELL RECEPTOR

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ABSTRACT

A unique class of T lymphocytes has been identified in the spleen of normal mice. This splenic T cell population, which expresses the αβ TCR associated with the CD3 complex yet lacks the CD4 and CD8 coreceptor molecules, constitutes approximately 0.1-0.3% of total nucleated cells and 17-22% of $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ cells in the normal murine spleen. Phenotypic analysis of splenic TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ (DNαβT) cells demonstrated the expression of Lyt-1, Pgp-1, ICAM-1 and the transferrin receptor whereas minimal to no B220 or YE1/19 expression was observed. Amongst splenic DNαβT cells there is a higher frequency of $V_{\beta 8}$ usage in comparison with mature SP T cells. Similar to TCR $\alpha\beta^+$ DN thymocytes, splenic DNαβT cells proliferate in the presence of IL-7 and this response is enhanced by the addition of IL-1. $CD4^+$ and $CD8^+$ SP T cells, in contrast do not respond to IL-7 alone or in combination with IL-1. Splenic DNαβT cells are considered to be mature based on the expression of the heat stable antigen. Furthermore, this splenic T cell subpopulation expresses a functional T cell receptor as suggested by their responsiveness to crosslinking of the TCR associated CD3 complex which is comparable to that of mature SP T cells. In contrast, splenic TCR $\gamma\delta^+$ DN T cells were nonresponsive to anti-CD3 crosslinking despite the expression of the TCR/CD3 complex. The addition of IL-1, however, appeared to restore the responsiveness of TCR $\gamma\delta^+$ DN towards the activation of the TCR/CD3 complex. The functional maturity of the splenic DNαβT cell population was confirmed by their ability to express cytokine specific mRNA. DNαβT cells constitutively express IFNγ message whereas IL-4 mRNA was induced by crosslinking of the TCR associated CD3 complex. SP T cells, in contrast, express messages for IFNγ and IL-4 as well as IL-2 only in response to anti-CD3 crosslinking. The above results support the hypothesis that splenic DNαβT cells represent a unique class of mature T cells which are related to the phenotypically similar cells found in the thymus yet are distinct from the classical type of mature T cell which expresses the SP phenotype.

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LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
bp	base pair
BSA	bovine serum albumin
C	constant region
Ca^{+2}	calcium ion
CD	cluster of differentiation
CD25	Interleukin-2 receptor α chain IL-2Rα
CD44	Pgp-1
CDR	complementarity determining region
cDNA	complementary DNA
CHO	chinese hamster ovary cells
CO_2	carbon dioxide
ConA	concanavalin A
CTL	cytotoxic T lymphocyte
D	diversity region
DAG	diacyl glycerol
DN	double negative
DN$\alpha\beta$T	$CD4^-$ $CD8^-$ HSA^- Mac-1^- TCR $\alpha\beta^+$ T cell
DP	double positive
EBV	Epstein Barr Virus
EDTA	ethylenediamine tetraacetic acid
FACS	flourescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FTOC	fetal thymic organ culture
GVHD	graft-versus-host disease
GVL	graft-versus-leukemia
HEPES	N-2-hydroxyethylpiperazine
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSA	heat stable antigen
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP_3	inositol 1,4,5-triphosphate
kb	kilobase
kd	kilodalton
LFA	lymphocyte function-associated antigen
MAb	monoclonal antibody
MAP-2K	microtubule-associated protein-2-kinase
2ME	2-mercaptoethanol
MHC	major histocompatibility complex

MLC	mixed leukocyte culture
MLR	mixed leukocyte reaction
Mls	minor lymphocyte stimulatory antigen
MLTC	mixed lymphocyte tumour cell culture
MNC	mononuclear cell
MRL-*lpr/lpr*	autoimmune lymphoproliferative murine strain
mRNA	messenger RNA
NaOH	sodium hydroxide
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
PI	phosphatidylinositol
PIP_2	phosphatidyl inositol 4,5-biphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PTK	protein tyrosine kinase
RAG	recombinase activation genes
RBC	red blood cell
SA-PE	streptavidin-phycoerythrin
SCA	stem cell antigen
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
SEB	staphylococcal enterotoxin B
SEM	standard error of mean
SLE	systemic lupus erythematosus
SP	single positive
SSC	saline sodium citrate buffer
SSPE	saline sodium phosphate EDTA buffer
TBE	Tris borate EDTA buffer
T_C	cytotoxic T cell
TCR	T cell receptor
TE	Tris EDTA
TGF	tumour growth factor
T_H	helper T cell
TN	triple negative
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
T_S	suppressor T cell
VLA	very late antigen

CHAPTER I

INTRODUCTION

Thymus-derived lymphocytes, otherwise referred to as T cells, are critical components of the immune system. T lymphocytes express an antigen receptor complex which is capable of recognizing foreign antigens when presented in association with the products of the self major histocompatibility complex. As a consequence of this recognition, T cells are activated to perform their effector functions, whether it be as regulator T cells which control the development and activation of effector T and B cells through the delivery of either helper or suppressor signals, or as effector T cells capable of destroying infected or abnormal cells. Thus T cells contribute to the ability of the immune system to provide a protection against foreign pathogens.

1.1 THE T CELL ANTIGEN RECEPTOR COMPLEX

The T cell antigen receptor complex consists of two alternate forms of the TCR, namely the αβ and γδ heterodimers associated with the CD3 complex and either CD4 or CD8 coreceptors.

1.1.1 T Cell Receptor

1.1.1 A) Identification of the T Cell Receptor

T cell receptor (TCR) αβ heterodimers were initially identified on human and mouse T cells with the use of monoclonal antibody (MAb) specific to individual cell lines (Allison *et al.* 1982; Haskins *et al.* 1983; Meuer *et al.* 1983b). Such clonotypic antibodies could block the interaction of T cells with their targets (Haskins *et al.* 1983; Lancki *et al.* 1983; Samelson *et al.* 1983; Staerz *et al.* 1984) as well as activate T cell clones (Kappler *et al.* 1983; Kaye *et al.* 1983) and were therefore used to biochemically characterized the antigen receptors. Subsequently, complementary DNAs (cDNAs) were isolated which were strikingly similar to those encoding immunoglobulins (Igs), rearranged specifically in T cells but not B cells, encoded the α and β chains identified serologically (Hedrick *et al.* 1984; Chien *et al.* 1984; Saito *et al.* 1984b) and transferred both antigen and major histocompatibility complex (MHC) specificity from one T cell to another in gene transfer studies (Dembic *et al.* 1986; Saito *et al.* 1987b). During the search for TCR α, the γ chain of the second heterodimer, γδ, was isolated (Saito *et al.* 1984a). A subsequent serological study demonstrated γ chain expression together with CD3 and a fourth polypeptide δ (Brenner *et*

al. 1986) whose isolation was facilitated by its location within the TCR α locus (Lindstein *et al.* 1987; Chien *et al.* 1987).

1.1.1 B) Structure of the T Cell Receptor

Human TCR α (43-49 kd) and β (38-44 kd) chains are heterogeneously charged glycoproteins with TCR α containing N-linked oligosaccharides of the complex type and TCR β containing both high mannose and complex N-linked glycan side chains attached to polypeptide backbones of 32 and 34 kilodalton (kd) respectively. The murine TCR $\alpha\beta$ heterodimer consists of 40 to 50 kd TCR α and β glycoproteins containing N linked oligosaccharides attached to polypeptide backbones of 28 and 32 kd respectively (Allison and Lanier, 1987; Toyonaga and Mak, 1987). The TCR $\gamma\delta$ heterodimer, composed of 55 kd (TCR γ) and 40 kd (TCR δ) proteins, was co-precipitated with anti-CD3 MAb from human $CD4^-$ $CD8^-$ $CD3^+$ T cell clones with natural killer (NK) activity but lacking reactivity with the MAb WT31 specific for a common epitope on human TCR $\alpha\beta$ chains (Brenner *et al.* 1987). The murine TCR γ protein is a 35 kd glycoprotein with a polypeptide backbone of 32 kd which is disulfide linked to a 45 kd TCR δ chain with a 31 kd polypeptide backbone (Chien *et al.* 1987; Pardoll *et al.* 1987b).

The primary amino acid (aa) sequences deduced from four TCR cDNA clones (Toyonaga and Mak, 1987) reveal an Ig-like organization with variable (V) and constant (C) regions linked by a short joining (J) segment. An additional diversity (D) segment has been identified on the TCR β and γ proteins only. V(D)J recombination is thought to be mediated by a recombinase encoded by the two genes RAG-1 and RAG-2 (Lieber *et al.* 1987; Schatz *et al.* 1989) whose expression correlates with those stages of thymocyte development associated with such recombination events, namely TCR^- and TCR^+ DP thymocytes (Turka *et al.* 1991). Although the number of hypervariable regions within the TCR sequences is controversial, diversity is evident in regions equivalent to the classic Ig hypervariable or complementarity determining regions (CDRs) which form principle points of contact with antigen. TCR regions equivalent to CDR1 and CDR2 are encoded within the V gene whereas the conjunction of V and J forms the CDR3 equivalent region. The latter region is thought to be responsible for antigen contact whereas the remainder of the TCR heterodimer interacts with the MHC molecule (Davis and Bjorkman, 1988; Chothia *et. al.* 1988; Claverie *et al.* 1989). TCR constant segments, varying in length from 138-179 aa, are divided into four functional regions, the first of which includes a cysteine residue thought to link two β sheets in the characteristic Ig fold (Davis and Bjorkman, 1988). An additional cysteine residue located in a separate exon may be involved in the interchain sulphydral bond. Distinct hydrophobic segments have been found in all four TCR chains which represent membrane spanning regions containing a

highly conserved lysine residue thought to interact with conserved negatively charged residues (aspartate or glutamate) in the transmembrane region of four of the CD3 polypeptides. Beyond the transmembrane regions, short cytoplasmic tails of varying length (5-12 aa) have been identified.

1.1.1 C) Generation of T Cell Receptor Diversity

Similar to B cells, T cells use several mechanisms for the generation of diversity in the antigen receptor including use of several gene segments (V,D,J) for variable region formation, random combinatorial joining of different germline gene segments, addition of N nucleotides not encoded in the genome to the junction between joined segments and use of D gene segment in all 3 translation reading frames. However, TCR genes lack three important features of Ig expression and diversity (Davis, 1990). First of all, unlike the differential splicing which allows either a membrane or secretory form of Ig to be produced depending on the stage of response, no secretory form of TCR has been identified reflecting the fact that T cells are engaged solely in cell-cell interactions. Furthermore, somatic hypermutation evident in Ig is not permissible in T cell receptors as it would be counterproductive to thymic selection, possibly promoting autoimmunity. In addition, isotype (heavy chain class) switching which enables an antibody V_HV_L combination to be expressed as a fusion protein with constant regions of different functional and localizational properties, has not been observed for the TCR and is thought to be unnecessary in the absence of secretory forms. Although TCR genes lack the above mechanisms and employ fewer V gene segments than do Ig, they are still considered to be more sophisticated than Ig in generating diversity particularily at the V-J interface equivalent to the CDR3 region of Igs (Davis and Bjorkman, 1988). Several unique TCR gene characteristics contribute to the diversity observed in this region including: 1) N region diversification observed for all four TCR polypeptides (Elliot *et al.* 1988) but only in heavy chain genes of Ig; 2) large numbers of TCR J region gene segments in α and β genes as opposed to the four J regions identified in mouse Ig heavy and κ chain loci; 3) flexibility of the position of 3' joining points of several Vαs, Vγs, Vδs; 4) the ability to use both D regions in the adult δ chain; 5) three different positions for N region diversification and 6) the ability to translate D region sequences in all three reading frames, although rare in Ig heavy chains, is common in TCR β and δ chains. The above features contribute to the highly diverse CDR equivalent region on the TCR which is considered to be the site of antigen contact (Davis and Bjorkman, 1988).

1.1.1 D) Association of TCR-CD3 complex with a Cytoplasmic Tyrosine Kinase

A member of the *src* family of protein tyrosine kinases (PTK) referred to as p59*fyn* has been shown to be associated with the TCR-CD3 complex based on the detection of low levels of the

protein tyrosine kinase (PTK) in TCR immunoprecipitates (Samelson *et al.* 1990). Two forms of *fyn*, the products of mutually exclusive splicing of alternative seventh exons, are expressed in either neuronal or hemopoietic cells (Cooke and Perlmutter, 1989). The expression of *fyn* late in thymocyte development correlates with the ability to respond to TCR mediated signals (Cooke *et al.* 1991). Studies in which the overexpression of *fyn* in a T cell hybridoma cell line (Davidson *et al* 1992) or in transgenic mice (Cooke *et al.* 1991) enhanced TCR signal transduction have suggested a possible role for this PTK in T cell activation but not development.

1.1.2 CD3 Complex

1.1.2 A) Identification of the CD3 Complex

The CD3 antigen was initially identified by immune precipitation with the MAb OKT3 as a 20 kd glycoprotein present on the surface of all human T lymphocytes (van Agthoven *et al.*1981). Subsequent studies demonstrated CD3 expression as a complex of the major 20 kd glycoprotein (CD3-δ), 20 kd non-glycoprotein (CD3-ε) and a 25-28 kd glycoprotein (CD3-γ) (Borst *et al.* 1983; Borst *et al.* 1984). Studies with an anti-TCR MAb have identified a murine TCR/CD3 complex consisting of five CD3-like proteins including 2 N-glycosylated proteins of 21 and 28 kd (CD3-γ and CD3-δ respectively), one 25 kd glycoprotein which may contain O-linked oligosaccharides (CD3-ε), 34 kd non-glycosylated homodimer not previously found in human cells by co-precipitation with anti-TCR or anti-CD3 MAbs (CD3-ζ) and an additional 21 kd non-glycosylated murine protein (CD3-η) often disulphide linked to CD3 ζ (Samelson *et al.* 1985a; Oettgen *et al.* 1986; Berkhout *et al.* 1987).

1.1.2 B) Structure of the CD3 Complex

With respect to the CD3 complex, γ and δ are glycoproteins whose core protein sizes are approximately 15 kd. Following glycosylation, the apparent molecular weight of γ is 21 kd in humans and 25 kd in mouse, whereas δ is 25-28 kd in humans and 21 kd in mouse. In contrast, the ε chain is non-glycosylated. Each with an N terminal extracellular domain, transmembrane segment and cytoplasmic domain, these three CD3 components are highly homologous, closely linked within 50-300 kb on human chromosome 11q23 and mouse chromosome 9 (Tunnacliffe *et al.* 1987) and are considered to belong to the Ig gene superfamily (Gold *et al.* 1987). In contrast to the TCR, a significant percentage of the primary structure of these CD3 components is located in the cytoplasmic domain and is thought to facilitate the role of CD3 in signal transduction. The ζ chain, on the other hand, is a 16 kd non-glycosylated protein with no sequence or structural

homology to either of the above three CD3 components or the TCR. Encoded by a gene found on human and mouse chromosome 1 (Baniyash *et al.* 1989), the ζ chain has a very short (6-9 aa) extracellular domain with the vast majority of the protein (113 aa) represented in the cytoplasmic segment. Although the majority of the ζ chain exists as a 32 kd homodimer, 5-20% is disulfide linked to a highly homologous 22 kd η protein forming a 38 kd heterodimer (Frank *et al.* 1990). The η chain is thought to be derived by alternate splicing of the ζ transcript (Jin *et al.* 1990) and lacks one of six possible tyrosine phosphorylation sites as well as a putative nucleotide binding site identified in CD3-ζ (Weissman *et al.* 1988). Interestingly, ζ expression is not restricted to T cells as it is also expressed on NK cells in association with CD16, the ligand binding subunit of FcγRIII (Anderson *et al.* 1989; Lanier, *e. al.* 1989).

1.1.2 C) Association of CD3 with the TCR

The T cell receptor complex consists of the disulfide-linked TCR noncovalently associated with the CD3 γ,δ and ε chains and the disulfide linked homo- or heterodimer containing the ζ chain. Evidence for such an association between the CD3 complex and the TCR heterodimers has been provided by numerous studies. First of all, MAb against TCR has been shown to co-precipitate CD3 and vice versa. Incubation of T cells with TCR or CD3 specific MAb leads to the co-modulation of both TCR and CD3 due to the apparent uptake of TCR-CD3 into multivesicular bodies. (Meuer *et al.* 1983; Zanders *et al.* 1983). Futhermore, restoration of cell surface TCR-CD3 complex expression has been observed following gene transfer of either TCR α or β cDNA (Ohashi *et al.* 1985; Saito *et al.* 1987a).

Studies of somatic cell mutants have shown that surface expression of the TCR complex is dependant upon the expression of the TCR α and β chains (Weiss and Stobo, 1984c; Ohashi *et al.* 1987; Saito *et al.* 1987a) as well as the ζ chains (Sussman *et al.* 1988; Weissman *et al.* 1989). Recently the requirement for the CD3 chains has been demonstrated by a reconstitution study of TCR in CHO cells (Carson *et al.* 1991).

TCR-CD3-ζ associations are thought to be mediated through hydrophobic transmembrane domains based on studies demonstrating the varying ability of different detergents in preserving the integrity of the TCR complex during solubilization (Weiss *et al.* 1986; Koning *et al.* 1990). Lysine and arginine residues within the transmembrane domain of the four TCR chains are thought to form salt bridges with the glutamic and aspartic acids located within the transmembrane regions of the CD3-γ and CD3-δ, ε and ζ chains respectively. Site-directed mutagenesis has identified residues lysine 126 and arginine 121 of the TCR α chain (John *et al.* 1989; Blumberg *et al.* 1990) and

lysine 290 of the TCR β chain (Morely *et al.* 1988) as playing an essential role in the proper assembly and expression of the TCR complex.

1.1.3 CD4 and CD8

CD4 and CD8 glycoproteins are nonpolymorphic members of the Ig gene superfamily expressed on two mutually exclusive subsets of mature T cells. Although most helper T cells (T_H) express CD4 whereas cytotoxic T cells (T_C) and suppressor T cells (T_S) cells express CD8, this correlation is not as strict as initially thought. Anti-CD8 MAb have been shown to inhibit $CD8^+$ cytotoxic and $CD8^+$ helper T cells specific for class I MHC antigen (Swain, 1981). Furthermore, CD4 expression has been demonstrated on certain cytolytic T cells specific for antigen presented in association with class II MHC proteins (Krensky *et al.* 1982a; Krensky *et al.* 1982b). Therefore CD4 and CD8 expression is more closely correlated with MHC specificity of the T cell than with function.

1.1.3 A) Structure of CD4

The human and murine CD4 molecule, also referred to as T4, Leu-3 or L3T4, is a 55 kd single chain glycoprotein with four extracellular domains of which the most distal bears a striking homology to the Ig light chain variable genes, a stretch of hydrophobic transmembrane residues and a highly conserved 40 aa cytoplasmic tail (Maddon *et al.* 1985; Littman and Gettner, 1987). Although considered to be a T cell specific marker, CD4 glycoprotein expression has been observed on monocytes, macrophages, Langerhan cells and brain cells.

1.1.3 B) Structure of CD8

Unlike CD4 which exists as a 55 kd monomer in both human and murine T cells, CD8 expression differs between the two species. In humans, the 34 kd CD8 molecule forms disulfide-linked homodimers and homomultimers on peripheral T cells (Snow and Terhorst, 1983) yet on thymocytes, in addition to homodimers, the CD8 polypeptide forms complexes with the 45 kd CD1 (T6) glycoprotein (Snow *et al.* 1985). In contrast, the murine CD8 homologue, both in the thymus and on peripheral T cells, consists of two chains, the 38 kd CD8 α (Lyt-2) chain or its corresponding 34 kd product of alternative splicing of the cytoplasmic tail, and the 30 kd β (Lyt-3) glycoprotein which are expressed as disulfide-linked αβ or αβ' heterodimers (Reilly *et. al.* 1980; Ledbetter *et al.* 1981; Ledbetter and Seamen, 1982). Whereas the 38 kd α chain of murine CD8 is thought to be the equivalent of the human CD8 molecule, the existence of a human Lyt-3

homologue has yet to be demonstrated. The human CD8 protein and the corresponding murine α chain of Lyt-2 both contain an amino terminal hydrophobic signal sequence followed by an Ig-like domain, a short extracellular hinge region, a hydrophobic transmembrane segment and a highly charged cytoplasmic domain (Littman *et al.* 1985; Sukhatme *et al.* 1985; Nakauchi *et al.* 1985).

1.1.3 C) Association of CD4 and CD8 with a Cytoplasmic Tyrosine Kinase

Early studies suggested that CD4 and CD8 might have a signal transduction function in addition to ligand binding capacity and it has been subsequently shown that the cytoplasmic domains of these two glycoproteins are indeed associated with the cytoplasmic tyrosine kinase p56 ^{lck} (Marth *et al.* 1985; Rudd *et al.* 1988; Veillette *et al.* 1988). Identified initially as a tyrosine kinase overexpressed in malignant lymphocytes, *lck* belongs to the src family of tyrosine kinases. In addition to an amino terminal region facilitating binding to cysteine residues in the cytoplasmic tails of the CD4 and CD8 coreceptor molecules, *lck* also contains a site for myristylation to allow association with the inner leaflet of the plasma membrane. Similar to other members of the src family kinases, *lck* also expresses a conserved carboxy terminus which includes two regulatory tyrosine residues, amino acid residues 505 and 394, which when phosphorylated inhibit and stimulate kinase activity respectively (Veillett *et al.* 1991).

1.2 T CELL DIFFERENTIATION

1.2.1 Thymocyte Ontogeny

Epithelial components of the murine embryonic microenvironment arise from the ectoderm and endoderm of the third pharyngeal cleft and pouch respectively at 10-11 days in ontogeny (Owen and Jenkinson, 1984) and subsequently organize into cortical and medullary stroma. By the 11th day of gestation, the thymus is first colonized by hemopoietic cells (Moore and Owen, 1967) which lack TCR, CD4 and CD8 expression (Bluestone *et al.* 1987) and represent the earliest intrathymic developmental stage. Southern blot analysis has shown that the majority of all TCR loci of these murine thymocytes remain in germline configuration up to day 13-14 when progressive rearrangement occurs. Although full length γ (V-J-C) and δ (V-D-J-C) mRNA are detected on day 14 of gestation, the majority of β messenger RNA (mRNA) at this time exists as 1 kb transcripts of incomplete D-J rearrangements with significant levels of full length 1.3 kilobases (kb) (V-D-J-C) β mRNA not observed until day 16 (Samelson *et al.* 1985). Full length α mRNA (V-J-C), on the other hand does not appear until day 17.

With respect to surface expression, CD3 is first detected on day 14-15 in association with TCR γδ (Pardoll *et al.* 1987). TCR αβ expression follows 2-3 days later as cells bearing this receptor increase in proportion until the time of birth on day 20 when they represent the major thymocyte population. Initial accessory molecule expression occurs on day 15-16 of gestation with the transient appearance of $CD3^-$ $CD4^-$ $CD8^+$ thymocytes (Ceredig *et al.* 1983; Kisielow *et al.* 1984). By day 17 however, essentially all accessory molecule-bearing thymocytes co-express both CD4 and CD8 and progessively increase in number until they represent 75-80% of the total thymocyte population shortly after birth. Although initially $CD3^-$, by day 18 approximately 5% of $CD4^+$ $CD8^+$ cells have begun to express low levels of TCR αβ. The first $CD4^+$ $CD8^-$ thymocytes begin to appear by day 17-18, followed shortly thereafter by an increase in the number of $CD4^-$ $CD8^+$ cells. In contrast to the double positive thymocytes, both subsets of single positive cells express high levels of TCR αβ.

Studies of human thymocyte ontogeny have revealed a sequence of TCR expression similar to that observed in the murine model. While approximately 10% of thymocytes from 9 1/2 week fetuses express TCR γδ, not until 10 weeks of gestation do TCR αβ expressing thymocytes appear and progressively increase in proportion concomitant with a decrease in number of TCR $\gamma\delta^+$ thymocytes (Haynes *et al.* 1988).

1.2.2 Classical T Cell Differentiation Pathway

From murine ontogeny studies, a basic outline of the stages of thymocyte development has evolved (Figure 1). More specifically, hemopoietic $CD3^-$ $CD4^-$ $CD8^-$ (triple negative) progenitors rearrange TCR genes with subsequent surface expression of TCR αβ and associated accessory molecules giving rise to TCR $\alpha\beta^+$ $CD4^+$ $CD8^+$ (double positive) thymocytes. Through positive and negative selection processes, these double positive thymocytes give rise to $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ single positive cells which emigrate from the thymus to become functional T cells. Finer phenotypic analyses have further subdivided the above stages into additional steps providing an increasingly more detailed pathway for T cell differentation.

1.2.2 A) Hemopoietic Stem Cell

The initial stage of T cell development involves the seeding of the thymus by multipotent stem cells derived from fetal liver and adult bone marrow. Such precursor cells, capable of reconstituting B

lymphoid, myeloid and erythroid lineages in addition to T lymphoid cells, have been purified from adult murine bone marrow (Sprangrude *et al.* 1988; Sprangrude and Scollay, 1990) and have been shown to express low levels of Thy-1 yet lack markers characteristic of other hemopoietic lineages. These multipotent stem cells also express stem cell antigen-1 (Sca-1), CD44 (Pgp-1), heat stable antigen (HSA) and MHC class I.

1.2.2 B) $CD4^{lo}$ $CD8^-$ $CD3^-$ thymocytes

Although the earliest intrathymic precursors were once thought to be contained within a small population of thymocytes expressing neither CD4 or CD8 accessory molecules ($CD4^-$ $CD8^-$, double negative thymocytes) (Fowlkes *et al.* 1985), even the most immature cells of this heterogenous group have already partially rearranged TCR γ and β genes (Pearse *et al.*1989). Recently, a minor population of lymphoid cells with both TCR and IgH genes in the germline state has been identified in the murine thymus (Wu *et al.* 1991). Referred to as "low CD4 precursors based on the expression of moderate levels of CD4, this novel population is thought to represent a newly defined stage in T cell differentiation linking the bone marrow derived stem cells which continuously colonize the thymus to the $CD4^-$ $CD8^-$ precursors engaged in TCR rearrangement. Although phenotypically similar to bone marrow hemopoietic stem cells which are also Thy-1^+ HSA^+ Pgp-1^{++} H-$2k^{++}$ Sca-1^+, the low CD4 precursors appear to be more mature in several respects including a higher proportion in the $S+G_2+M$ phases of cell cycle, more rapid progression to mature T cells following intrathymic transfer and the expression of Sca-2 (stem cell antigen-2) characteristically absent on purified bone marrow stem cells. Furthermore, unlike bone marrow stem cells, low CD4 precursors are not multipotent as suggested by the inability to form either myeloid or erythroid colonies in the spleen or in agar culture. Instead low CD4 precursors appear to be restricted to lymphoid lineage development although not exclusively to T cells. When transferred intravenously, this intrathymic precursor population is capable of producing both Thy-1^+ T cell as well as $B220^+$ B cell progeny in the bone marrow, spleen and lymph nodes. However, this low CD4 precursor population does display a preference for thymic seeding, giving rise to thymocytes of both TCR $\alpha\beta$ and $\gamma\delta$ lineages as well as thymic dendritic cells (Ardavin *et al.* 1993) in the irradiated hosts. In contrast to the $CD4^-$ $CD8^-$ population, low CD4 precursors are considered to be less mature with respect to reconstitution behavior and TCR gene rearrangement status. Therefore, low CD4 precursors are considered to be the earliest T-lineage precursor cells in the adult murine thymus.

1.2.2 C) Triple Negative Thymocytes

Following thymic seeding, low CD4 precursors cease expression of CD4 to enter the $CD3^-$ $CD4^-$ $CD8^-$ triple negative stage of development, characterized by extensive expansion and differentiation as cells begin to rearrange and express TCR genes in association with the accessory molecules CD4 and CD8. These triple negative (TN) thymocytes belong to a heterogenous group which can be further subdivided into subpopulations based on the expression of other surface markers. Progression through the developmental stages of this population is marked by the aquisition of high level Thy-1 and HSA expression, a reduction in CD44 (Pgp-1) levels and a transient expression of the low affinity IL-2 receptor α chain (CD25). Progressive increases in TCR γ and β gene rearrangement further signify maturation along this pathway.

The earliest cells in this pathway, which are $CD44^+$ $CD25^-$, have the slowest repopulation kinetics in irradiated thymi but demonstrate the greatest potential for expansion (Scollay *et al.* 1988). Few cells of this phenotype are in the active phases of the cell cycle. Although limited rearrangement of the TCR β genes has occured by this stage, no TCR α mRNA is detectable (Pearse *et al.* 1989). From the $CD44^+$ $CD25^-$ phenotype, these cell progress to a $CD25^+$ stage which can be further subdivided based on the expression of CD44. $CD44^+$ $CD25^+$ thymocytes are considered to be less mature than $CD44^-$ $CD25^+$ cells in several respects (Godfrey *et al.* 1993). $CD44^+$ $CD25^+$ cells are larger based on forward scatter, and are Thy-1^+ HSA^+ and c-kit^{hi} whereas $CD44^-$ $CD25^+$ thymocytes are significantly smaller and express higher levels of Thy-1 and HSA and are c-kit^{lo}. As both Thy-1 and HSA levels characteristically increase with TN maturation and c-kit expression is down regulated during the $CD25^+$ TN stage, the changes in expression levels of these markers suggest a more immature phenotype for the $CD44^+$ $CD25^+$ thymocytes. Furthermore, $CD44^+$ $CD25^+$ thymocytes have TCR β genes in the germline state whereas the $CD44^-$ $CD25^+$ counterparts are rearranged at the TCR β chain gene locus. This recent finding is, however, in contradiction with an earlier study (Pearse *et al.* 1989) which demonstrated partial TCR β gene rearrangement in the least mature TN subpopulation, $CD44^+$ $CD25^-$ thymocytes. The final subpopulation in the TN developmental pathway consists of $CD44^-$ $CD25^-$ thymocytes which demonstrate very rapid but short lived thymic reconstitution potential in irradiated recipients (Scollay *et al.*1988). When transplanted intrathymically into non irradiated hosts, $CD44^-$ $CD25^-$ thymocytes give rise to $CD4^+$ $CD8^+$ cortical cells within one day and to mature single positive cells following an additional 2-4 days (Petrie *et al.* 1990a). Following one day culture in simple medium, these cells spontaneously and rapidly acquire high levels of CD4 and CD8 (Wilson *et al.* 1989) accompanied by CD3 expression at a significantly slower rate (Nikolic-Zujic and Moore, 1989) and are therefore considered to be preprogramed to become $CD3^{lo}$ $CD4^+$ $CD8^+$ cortical

thymocytes. Thymocytes of the $CD44^-$ $CD25^-$ phenotype express completely rearranged TCR β genes and high levels of mature TCR β mRNA and are the only cell type in the TN population to express TCR α mRNA (Pearse *et al.* 1989). Although traditionally considered to be members of the TN population, mRNA and corresponding low levels of surface CD4 and CD8 expression have been observed for this $CD44^-$ $CD25^-$ population, therefore suggesting that the accessory molecule expression begins concurrent with the down regulation of IL-2Rα (Petrie *et. al.* 1990b) such that $CD44^-$ $CD25^-$ thymocytes, actually represent a transition stage between TN and $CD3^{lo}$ $CD4^+$ $CD8^+$ cells. On their way to becoming $CD4^+$ $CD8^+$ $CD3^{lo}$ cells, preprogramed $CD44^-$ $CD25^-$ thymocytes are thought to pass through a transient single positive (SP) stage- either as $CD3^-$ $CD4^-$ $CD8^+$ or $CD3^-$ $CD4^+$ $CD8^-$ (Matsumoto *et al.* 1989; Hugo *et al.* 1990) cells. Similar to their immediate precursors, $CD3^-$ $CD4^-$ $CD8^+$ and $CD3^-$ $CD4^+$ $CD8^-$ SP cells express low levels of CD4 and CD8 respectively (Nikolic-Zujic and Moore, 1989; Petrie *et al.* 1990b; Hugo *et al.* 1991). Evidence that both SP subsets represent similar stages of differentiation include similar phenotypes (MacDonald *et al.* 1988a; Shortman *et al.* 1989; Yagita *et al.* 1989; Hugo *et al.* 1991) and timing of appearance during *in vivo* and *in vitro* T cell ontogeny and during thymic reconstitution following cortisone treatment and irradiation (Hugo *et al.* 1990; Hugo *et al.* 1991; Matsumoto *et al.* 1989; Arase *et al.* 1990).

1.2.2 D) Double Positive Thymocytes

Approximately 80-85% of adult thymocytes are defined by coexpression of the accessory molecules, CD4 and CD8, and as such are the predominant cell type in the thymic cortex (Bluestone *et al.* 1987). Of this double positive (DP) population, 20% are large and medium sized dividing cells, whilst the remaining 80%, the typical small cortical thymocytes, are the noncycling products of this division. Interestingly, the majority of these DP cortical thymocytes are believed to die in the thymus as a result of a stringent thymic selection process involving positive selection for appropriate interaction with the self-MHC proteins of the antigen presenting system, and negative selection against reactivity with a variety of self antigens. However, a small proportion (approximately 3%) of DP thymocytes are not dead end cells but differentiate further to become mature SP medullary cells. Support for the direct precursor relationship of these DP thymocytes to mature SP cells is based on the observation of SP progeny following intrathymic transfer of $CD4^+$ $CD8^+$ TCR^{lo} blasts (Guidos *et al.* 1989). In addition, intrathymic expression of the self antigens Mls^a and I-E, recognized by $CD4^+$ SP T cells bearing TCR $V_{\beta 6}$ and $V_{\beta 17}$ respectively, eliminate not only $CD4^+$ but also $CD8^+$ SP cells (Kappler *et al.* 1987, MacDonald *et al.* 1988). Futhermore, the $CD8^+$ TCR $V_{\beta 6}{}^+$ and $V_{\beta 17}{}^+$ cells may be rescued by CD4 blocking monoclonal

antibodies during thymic development (Fowlkes *et al.* 1988, MacDonald *et al.* 1988a) therefore suggesting that all T cells must pass through a $CD4^+$ $CD8^+$ stage.

The DP population is thought to represent a stage in T cell development when TCR αβ is first expressed in association with the CD3 complex (Shortman *et al.* 1990). Low level expression of surface TCR-CD3 has indeed been observed on approximately 50% of DP thymocytes (Havran *et al.*1987, Crispe *et al.*1987), the majority of which are rapidly dividing blast cells (Shortman *et al.* 1991). Furthermore, a small proportion of DP thymocytes have been shown to express high levels of TCR-CD3 (Bluestone *et al.*1987, Havran *et al.* 1987, Blue *et al.*1987) and represent the nondividing small cortical thymocytes (Shortman *et al.* 1991). It is from this nondividing DP population that mature SP thymocytes are thought to differentiate based on kinetic data (Penit, 1986, Edgerton *et al.* 1990). The intermediate phenotype (Thy-1^{hi} HSA^{hi} $CD44^-$ $CD5^{hi}$ MEL-$14^{int/hi}$ LFA-1^{hi} and H-$2K^{lo/hi}$) (Hugo *et al.* 1991) and timing of appearance during *in vivo* and *in vitro* embryonic development and thymic reconstitution following cortisone treatment (Hugo *et al.* 1991a) provide further evidence that $CD4^+$ $CD8^+$ $CD3^{hi}$ thymocytes represent a transitional stage between the $CD4^+$ $CD8^+$ $CD3^{lo}$ thymocytes and mature SP medullary cells.

The importance of the TCR αβ during this stage of thymocyte development has been demonstrated in numerous studies. First of all, the expression of the TCR β chain genes is thought to play a key role in the progression of TN thymocytes to the DP stage based on studies of severe combined immunodeficient (SCID) mice whose T cell development is arrested at the TN stage. Crossing of SCID mice with those mice whose T cells express TCR consisting of the β chain only facilitates the progression of some TN thymocytes to the DP stage (Kishi *et al.* 1991; Groettrup *et al.* 1992). Furthermore, the arrest in thymocyte development at the TN stage observed in mice in which the recombinase activation genes *RAG-1* and *RAG-2* have been inactivated (Mombaerts *et al.*1992b; Shinkai *et al.* 1992) has been reversed by the crossing of *RAG-1* knockout mice with mice expressing the transgenic TCR β chain (Mombaerts *et al.* 1992a). Functional deletion of the TCR α chain, on the other hand, arrests thymocyte development at the DP stage (Philpott *et al.* 1992; Mombaerts *et al.* 1992a). Unlike the TCR β chain, termination of the TCR α chain gene rearrangements that occurs during the DP stage is not a consequence of the expression of productive α chain gene rearrangements on the other chromosome, but rather due to the termination of *RAG-1* and *RAG-2* expression following the engagement of the TCR in DP thymocytes during positive selection (Borgulya *et al.* 1992; Brandle *et al.*1992). TCR αβ also plays a key role in the thymic selection process which occurs during the DP stage of development. More specifically, appropriate interaction of TCR with self MHC expressed primarily on thymic epithelial cells facilitates the rescue of DP thymocyte from programmed cell death through a

process of positive selection whereas reactivity of TCR against self antigens expressed on bone marrow derived cells leads to clonal deletion via negative selection or tolerance.

Positive Selection

Post thymic T cell repertoire is thought to arise from a subset of DP thymocytes with TCR binding affinity for the particular MHC molecules expressed in the thymus via a process referred to as positive selection. Those DP thymocytes which express TCR with affinity for either class I or II self MHC on thymic epithelial cells, in the absence of nominal antigen, are rescued from preprogramed cell death to become either $CD8^+$ or $CD4^+$ SP cells respectively. Evidence in support of positive selection has been provided by studies of transgenic animals which have shown that the specificity of the TCR along with the particular MHC expressed by the animal both determine the fate of developing thymocytes. While T cell development is skewed towards the production of $CD8^+$ SP cells in transgenic animals that express both a class I MHC-specific TCR and the appropriate class I MHC (Teh *et al.* 1988; Sha *et al.* 1988b), $CD4^+$ SP cells selectively develop in transgenic animals expressing the class II MHC TCR and appropriate class II MHC (Berg, *et al.* 1989; Kaye *et al.*1989; Murphy *et al.* 1990). Furthermore, in transgenic mice expressing MHC molecules not recognized by the transgene-encoded TCR, thymocyte maturation from the DP stage to SP cells is blocked (Scott *et al.*1989; Sha *et al.* 1988a; Berg *et al.* 1989; Kaye *et al.* 1992). TCR-MHC interactions, however, are not the only requirement for successful positive selection. The importance of CD4/CD8-MHC interactions in positive selection have been demonstrated in studies where mice, treated from birth with MAb specific for either CD4 or CD8, failed to develop $CD4^+$ or $CD8^+$ SP thymocytes respectively (Zuniga-Pflucker *et al.* 1989, Ramsdell and Fowlkes, 1989, Zuniga-Pflucker *et al.*, 1990). Evidence for thymic epithelium as the source of MHC mediated positive selection has been provided by a study in which dGuo-treated fetal thymuses from A mice were grafted into thymectomized A X B F_1 recipients (Lo and Sprent, 1986). Although the thymuses were recolonized with bone marrow derived cells expressing both A- and B-type MHC molecules, the T cells developing in these chimeric thymuses displayed preferential restriction of A-type MHC antigens. Furthermore, a recent study has shown that intrathymic injection of a cloned thymic epithelium cell line expressing class I MHC induces developing thymocytes to recognize those MHC antigens expressed on the thymic epithelium cell line as self-MHC (Vukmanovic *et al.* 1992).

Two models have been proposed for the positive selection and commitment to the CD4 or CD8 lineage (Robey *et al.* 1990). The first model, referred to as the "instruction" model, suggests that TCR specificity dictates CD4 or CD8 expression via an instructive signal generated when

thymocytes engage self MHC. In this model, positive selection occurs during the double positive stage prior to commitment to either CD4 or CD8 lineages. During positive selection, a DP thymocyte whose TCR is specific for self class I MHC would, for example, receive a signal directing its differentiation to the CD8 lineage. Commitment to the CD4 lineage, on the other hand, would be directed by a different signal generated during selection by class II MHC. Consistent with this model are studies demonstrating the failure of the expression of CD8 coreceptors, encoded by a transgene expressed in all T cells, to rescue $CD4^+$ SP cells with MHC class I restricted receptors from cell death (Borgulya *et al.* 1991). In further support is a recent study demonstrating that when placed in culture, small $CD4^+$ $CD8^+$ TCR^{hi} thymocytes can differentiate further into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ thymocytes in the absence of thymic stromal cells indicating that this DP thymocyte population has already recieved signals allowing maturation to SP cells (Swat *et al.* 1992). The instructive model assumes that two different intracellular signals exist for those DP thymocytes being positively selected on class I versus class II MHC, although the nature of these instuctive signals is elusive. It is possible that the CD4 and CD8 molecules themselves are responsible for the differential signalling. Differences in the properties of $p56^{lck}$ when bound to CD4 as opposed to CD8, including the degree of autophosphorylation *in vitro* and dissociation following protein kinase C (PKC) activation, have indeed been observed (Hurley *et al.* 1989). Furthermore, a recent study demonstrating higher kinase specific activities of $p56^{lck}$ as well as $p59^{fyn}$ in DP thymocytes undergoing positive selection versus nonselecting thymocytes provides evidence for the role of tyrosine phosphorylation in the positive selection process (Carrea *et al.* 1992).

An alternate model, referred to as the "selection" model, suggests that the commitment to CD4 or CD8 lineages is stochastic and precedes positive selection. Therefore positive selection occurs during the single positive stage and requires the coengagement of TCR and CD4 or CD8. Those SP thymocytes with a mismatch between CD4 or CD8 expression and TCR specificity are unable to be positively selected and thus die within the thymus. Unlike the first model, the intracellular event for both MHC class I and II positive selection are similar in the selection model.

Negative Selection

Negative selection was initially proposed to provide an explanation as to how the developing immune system learns to distinguish self from non-self. The initial evidence that tolerance to self-antigens occurs during intrathymic development was provided by the demonstration that T cells maturing in fetal thymic organ culture (FTOC) are significantly more reactive against allogenic stimulator cells than against syngenic stimulator cells (Robinson and Owen, 1977). It was

subsequently shown that developing T cells are tolerant to self-antigens only in the context of thymically expressed MHC antigens (Dos Reis and Shevach, 1983; Groves and Singer, 1983; Rammensee and Bevan, 1984; Matzinger *et al.* 1984). Negative selection is thought to involve deletion rather than suppression or functional inactivation of self reactive clones based on studies demonstrating the absence of peripheral T cells expressing a particular V_β TCR product in mouse strains expressing the corresponding stimulating antigen. The C57BR mouse strain, for example, expresses the class II MHC molecule, I-E, and possesses a functional $V_\beta 17a^+$ gene yet has virtually no peripheral T cells or SP thymocytes, of either $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ phenotype, expressing $V_\beta 17a^+$ TCR (Kappler *et al.*1987). $V_\beta 17a^+$ DP thymocytes, however, are numerous in the C57BR strain suggesting that negative selection occurs during the DP stage of development (Kappler *et al.* 1987). DP thymocytes have indeed been shown to be rosetted around thymic stromal cells thought to be involved in negative selection (Kyewski *et al.* 1987). Furthermore, injection of anti-CD4 MAb into mice following irradiation and bone marrow reconstitution inhibited the deletion of $CD4^-$ $CD8^+$ cells expressing $V_\beta 17a$, confirming that deletion occurred at the DP stage and required the interaction of CD4 with MHC class II (Fowlkes *et al.*1988). Similar results have been obtained for $V_\beta 8.1$ and $V_\beta 6$ products which confer anti-Mls^a reactivity (Kappler *et al.* 1988; MacDonald *et al.* 1988). Evidence for the deletion of class I restricted TCR has been provided by studies of transgenic mice expressing both α and β TCR chains from a CTL clone reactive with male H-Y antigen + H-$2D^b$ (Kisielow *et al.* 1988). Significantly diminished numbers of SP thymocytes were observed in the transgenic male H-2^b mice than in their female littermates. Interestingly, those T cells which escaped the deletion process in the male mice expressed normal levels of the transgenic receptor but expressed low levels of the CD8 molecule. In a second transgenic model, T cells expressing a receptor reactive against the class I molecule L^d, were significantly diminished in number in L^{d+} mice (Sha *et al.* 1988a).

Evidence that negative selection by clonal deletion is primarily mediated by bone marrow derived cells residing in the thymus has been provided by numerous models including radiation chimeras (Ready *et al.*1984; von Boehmer and Schubiger, 1984, Widera *et al.* 1987; van Ewijk *et al.*1988), dGuo-treated thymic transplantations (Jenkinson *et al.* 1985), frog (Flajnik *et al.* 1985) and chick (Ohki *et al.* 1987) chimeras. These bone marrow derived cells include dendritic cells expressing both class I and II MHC antigens which are located in the corticomedullary and medullary regions of the thymus, as well as class I MHC expressing macrophages residing primarily in the medulla (Barclay and Mayrhofer, 1981; Jenkinson *et al.* 1981). Other thymic cells of hemopoietic origin may also participate in negative selection including B cells whose expression of self-antigens is thought to induce both clonal deletion and anergy (Inaba *et al.* 1991; Mazda *et al.* 1991; Iwabuchi *et al.* 1992). Thymic epithelial cells have also been implicated in negative selection. Recent

studies have demonstrated that the expression of foreign antigens on thymic epithelial cells in TCR-transgenic mice induce functional inactivation of the corresponding antigen specific T cells (Carlow *et al.* 1992; Schonrich *et al.* 1992; Husbands *et al.* 1992; Hoffman *et al.* 1992).

Clonal deletion of negatively selected DP thymocytes is thought to involve a form of programmed cell death referred to as apoptosis in which membrane blebbing is accompanied by nuclear and cytoplasmic shrinking and DNA fragmentation. Recent studies have been focused on elucidating the signals and genes involved in this apoptotic process. Although crosslinking of the surface protein *Fas* has been shown to transduce an apoptotic signal (Itoh *et al.* 1991), the *Fas* gene alone is not responsible for thymic negative selection since deletion of self reactive T cells is observed in lymphoproliferative (*lpr*) mice despite a mutation in the *Fas* gene (Sidman *et al.* 1992). Furthermore, the apoptosis-blocking *Bcl-2* gene alone is also not sufficient to control negative selection as suggested by studies of transgenic mouse lines expressing high *Bcl-2* protein levels in the T lineage (Sentman *et al.* 1991; Strasser *et al.* 1991; Siegel *et al.* 1992).

A question remains as to how DP thymocytes distinguish between positive and negative selection signals since both processes involve the interaction of TCR and CD4/CD8 accessory molecules with self MHC. The first model proposed to explain this paradox was based on receptor affinity (Sprent *et al.* 1988). This affinity model suggests that it is the qualitative differences in the avidity of the interaction between DP thymocytes and thymic stroma or bone marrow derived cells that determine whether positive or negative selection occurs. More specifically, if the avidity, which takes into consideration the density and affinity of the TCR-MHC interactions as well as those of the CD4 and CD8 accessory molecules and adhesion molecules, is low, positive selection occurs whereas high avidity leads to negative selection. Recent studies demonstrating that the fate of thymocytes expressing a particular transgene was altered from positive to negative selection following the introduction of a CD8 transgene (Lee *et al.* 1992; Robey *et al.* 1992) provide support for this model. An alternate model (Marrack *et al.* 1988; Marrack and Kappler, 1988) suggests that thymic selection is "compartmentalized" as a result of the different signals delivered by differential peptide, costimulatory molecule or adhesion molecule expression on thymic epithelial cells as opposed to bone marrow derived dendritic cells. More specifically, recognition of self peptides derived from epithelial cell proteins facilitates positive selection whereas recognition of self proteins derived from dendritic cell proteins is required for negative selection. This model, however, fails to explain why proteins would not be released from the epithelial cells to be taken up by the dendritic cells leading to the induction of tolerance to epithelial peptides as has been previously shown for minor histocompatibility antigens (von Boehmer and Hafen, 1986) and Mls (Pullen *et al.* 1988). Additional studies demonstrating negative selection by thymic epithelial cells

(Carlow *et al.* 1992; Schonrich *et al.*1992; Husbands *et al.* 1992; Hoffman *et al.*1992) and positive selection by thymic APCs under certain circumstances (Bix and Raulet, 1992) provide further evidence against this compartmentalization theory. A third model has been proposed (Finkel *et al.* 1991) based on the observed differential responses of DP thymocytes to ligation of the TCR. Those DP thymocytes susceptible to negative selection respond to ligation of TCR αβ as well as CD3-ε with mobilization of calcium (Ca^{+2}) which signals cell death. Those DP thymocytes resistant to tolerance, on the other hand, do not respond to TCR ligation with Ca^{+2} mobilization perhaps as a result of the uncoupling of the TCR to CD3 as suggested by the absence of ζ homodimers in the receptor complex.

1.2.2 E) Single positive thymocytes

DP thymocytes that progress beyond the thymic selection stage have continued upregulation of TCR-CD3 accompanied by loss of either CD4 or CD8 depending on whether class I or class II MHC restriction occured during selection. The final maturation process of these $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ SP thymocytes, which now reside in the medulla and constitute 10-15% of the adult thymus, is gradual and involves the loss of HSA and the acquisition of Qa-2 (Fowlkes and Pardoll, 1989). Further maturation occurs including altered signal transduction through the TCR-CD3 complex as well as the acquisition of antigen responsiveness and cortisone resistance.

1.2.2 F) Single positive T cells

Following the acquisition of functional maturity, SP $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ thymocytes are ready to leave the thymus to colonize peripheral lymphoid tissues including the lymph nodes and spleen as helper and cytotoxic/suppressor T cells respectively. Following activation by the presentation of foreign antigen in the context of the class II MHC, $CD4^+$ SP helper T cells respond by the secretion of soluble mediators which, for example, provide positive signals, to assist in the differentiation of B cells into antibody secreting plasma cells. $CD8^+$ SP T cells, in contrast, assume cytotoxic activity resulting in the destruction of cells presenting antigen in the context of class I MHC by direct cell contact.

1.2.3 Alternate T Cell Differentiation Pathways

Although the majority of peripheral T cells express the TCR αβ-CD3 complex along with either CD4 or CD8 co-receptors, a distinct T cell population has been identified in a number of peripheral organs which express CD3 associated with either TCR αβ or γδ heterodimers yet lack

both CD4 and CD8. TCR $\gamma\delta^+$ DN T cells have been identified in such peripheral tissues as lymph nodes, spleen (Cron *et al.* 1988), skin (Stingl *et al.* 1987) and intestinal epithelia. DN T cells expressing the TCR $\alpha\beta$ have been recently identified in normal human peripheral blood (Shivakumar *et al.* 1989; Quaratino *et al.*1991), as well as murine bone marrow (Sykes, 1990; Levitsky *et al.* 1991), lymph nodes (Guidos *et al.* 1989a), spleen (Levitsky *et al.* 1991; Prud'homme *et al.* 1991) and liver (Seki *et al.* 1991; Ohteki *et al.* 1992). Although thymic equivalents of these two peripheral $CD3^+$ DN T cell populations have been identified (Lew *et al.* 1986; Nakanishi *et al.* 1987; Fowlkes *et al.* 1987) , neither TCR $\alpha\beta^+$ or $\gamma\delta^+$ DN thymocytes are thought to follow the conventional T cell differentiation pathway that gives rise to mature SP T lymphocytes.

1.2.3 A) TCR $\alpha\beta^+$ DN Lineage

TCR $\alpha\beta^+$ double negative (DN) thymocytes constitute 2-30% of the thymic $CD4^-$ $CD8^-$ subpopulation depending on the mouse strain studied and display a $CD3^+$ HSA^- $CD5^+$ $CD25^-$ $CD44^-$ $B220^-$ Qa-2^+ NK1.1^+ phenotype (Fowlkes *et al.* 1987; Howe and MacDonald, 1988; Wilson *et al.* 1988a,b; Levitsky *et al.* 1991). This thymic subpopulation is thought to be composed of late stage differentiated cells based on studies demonstrating the lack of HSA expression, the relatively late appearance in FTOC and during ontogeny, occuring between birth and day 5 of life in the mouse (Fowlkes *et al.* 1987) and reaching plateau levels between 6 to 10 weeks of age (Takahama *et al.* 1991) as well as lack of precursor activity following intrathymic transfer (Crispe *et al.* 1987). In addition, TCR $\alpha\beta^+$ DN thymocytes express predominantly the products of a single V_β gene family, namely $V_{\beta 8}$, in a frequency twofold higher than in SP T cells (Fowlkes *et al.* 1987; Papiernik and Pontoux, 1990; Takahama *et al.* 1991). It was therefore suggested that TCR $\alpha\beta^+$ DN thymocytes may be derived from DP thymocytes which, due to potential self reactivity, have escaped clonal deletion by the downregulation of surface expression of their CD4 and CD8 determinants. Evidence in support of this hypothesis was provided by a study of TCR transgenic mice in which the majority of thymocytes and T cells in male mice expressing the transgenic anti-male TCR were of the DN phenotype and expressed high levels of the transgenic TCR but were nonetheless tolerant to male self antigen (Teh *et al.* 1989). A comparison of TCR^+ HSA^- DN thymocytes expressing $V_{\beta 8.1}$ in Mls-1^a and Mls-1^b mice demonstrated similar proportions of this thymic subpopulation in the two strains (Egerton and Scollay, 1990). $V_{\beta 8.1}{}^+$ mature T cells, in contrast, undergo clonal deletion in Mls-1^a mice suggesting an apparent escape of the $V_{\beta 8.1}{}^+$ DN thymocytes from this selection process. The observed reactivity of $V_{\beta 8.1}{}^+$ with Mls-1^a in association with not only class II MHC but also class I MHC suggested that the TCR $V_{\beta 8.1}{}^+$ DN thymocytes may be derived from thymic DP

cells which had downregulated both CD4 and CD8 expression following interaction with both class I and II MHC. Furthermore, the demonstration of demethylation of DNA sequences 5' to the CD8α gene implied that TCR $\alpha\beta^+$ DN thymocytes are derived from a cell population that had at one time expressed the CD8α gene locus (Wu *et al.* 1990; Takahama *et al.* 1991). Cytosine residues of developmentally regulated genes are often demethylated at the time of first expression and progressive demethylation of several CCGG sites has been observed as T cells develop from immature TN through the DP stage to peripheral SP T lymphocytes (Carbone *et al.* 1988). However, the demethylation pattern observed was distinct from that of thymic or peripheral $CD8^+$ T cells, suggesting that TCR $\alpha\beta^+$ DN thymocytes are not derived from SP thymocytes or peripheral T cells that have returned to the thymus and downregulated CD8 expression. Instead the CD8α demethylation pattern was considered as further evidence in support of a DP origin for the TCR $\alpha\beta^+$ DN thymocyte population.

Recent evidence however suggests that TCR $\alpha\beta^+$ DN thymocytes may not be derived from thymic DP cells but represent a distinct T cell lineage which has branched off from the classical T cell differentiation pathway prior to the DP stage. First of all, in conflict with the suggestion that TCR $\alpha\beta^+$ DN thymocytes were derived from the down regulation of CD4 and CD8 molecules on potentially self reactive DP thymocytes was the demonstration that the frequency of TCR $\alpha\beta^+$ DN thymocytes expressing various anti-self TCR including either Mls-1^a reactive $V_{\beta 6}$ and $V_{\beta 8,1}$ or I-E reactive $V_{\beta 11}$ and $V_{\beta 17a}$ were not increased in those strains expressing their putative self antigen but were either unaffected or significantly reduced (Takahama *et al.* 1991). Although $V_{\beta 8}$ overexpression appeared to be independent of the developmental appearance of the basic thymocyte repertoire, TCR $\alpha\beta^+$ $V_{\beta 8}^+$ DN thymocytes were also susceptible to deletion by the introduction of the super-antigen staphylococcal enterotoxin B (SEB) into the neonatal differentiation environment. Secondly, following intrathymic transfer of DP thymocytes, no detectable levels of $CD3^+$ DN cells were obtained although SP progeny were observed (Guidos *et al.* 1989b). With respect to the association of CD8α demethylation with a potential DP thymic origin for TCR $\alpha\beta^+$ DN thymocytes, it has been recently shown that the proportion of the thymic subpopulation referred to as TN based on FACS analysis actually express low levels of CD8α (Nikolic-Zugic *et al.* 1989). Furthermore, CD8α methylation has indeed been observed in a portion of the TN thymocytes and may correspond to the $CD8^{lo}$ subset (Wu *et al.* 1990). Support for this hypothesis has been provided by the study of $CD4^{-/lo}$ $CD8^{lo}$ thymocytes which were isolated by anti-CD8α MAb coated plates but were identified by FACs analysis as lacking both CD4 and CD8 (Nikolic-Zugic and Moore, 1989). Although in short term culture, the majority of $CD4^{-/lo}$ $CD8^{lo}$ thymocytes spontaneously differentiated into thymic DP cells, a significant number of these cells maintained their DN phenotype and expressed CD3. Therefore demethylated CD8α genes observed in TCR

$\alpha\beta^+$ DN thymocytes may be reminiscent of a TN phenotype during a previous step in development. Recent studies have provided support for the TN origin of the TCR $\alpha\beta^+$ DN thymocytes (Vissinga *et al.* 1991; Suda and Zlotnik, 1991; Suda and Zlotnik, 1993). Those TN thymocytes which express CD25 were shown to contain precursor cells for all TCR^+ subsets found within the thymus including thymic $CD3^+$ DN cells as well as DP and SP thymocytes. When $CD25^+$ TN thymocytes were cocultured in IL-7, a proportion of this thymic population acquired the expression of CD3. Of these $CD3^+$ DN thymocytes, the majority of those which expressed the TCR $\alpha\beta$ were HSA^- $CD44^+$ except for approximately 10% which were HSA^- $CD44^{-/lo}$. TCR $\gamma\delta^+$ DN thymocytes were also included in the $CD3^+$ DN population and consisted of three major subsets: HSA^+ $CD44^-$, HSA^- $CD44^-$ and HSA^- $CD44^+$ cells. When HSA^+ $CD44^-$ $CD3^+$ DN thymocytes were cocultured with interleukin-7 (IL-7), a reduction in HSA expression was observed for both TCR $\alpha\beta^+$ and $\gamma\delta^+$ subsets whereas stimulation with IL-7 and anti-CD3 gave rise to the HSA^- $CD44^+$ phenotype. When $CD3^+$ DN thymocytes were subdivided based on the expression of either of the two types of TCR and subsequently stimulated with IL-7 +/- anti-CD3, the majority of TCR $\alpha\beta^+$ HSA^- $CD44^-$ DN cells acquired CD44 with IL-7 alone whereas TCR $\gamma\delta^+$ HSA^- $CD44^-$ DN thymocytes required the addition of anti-CD3 to the culture conditions suggesting that the former may have already acquired a TCR mediated signal and were committed to become $CD44^+$ prior to isolation. This preprogrammed differentiation was further supported by the detection of both complete and incomplete TCR β mRNA in the $CD25^+$ TN population. $CD3^+$ DN thymocytes obtained from IL-7 stimulated cultures of $CD25^+$ TN cells were also shown to undergo differentiation similar to that of freshly isolated $CD3^+$ HSA^+ $CD44^-$ DN thymocytes when cocultured with IL-7 and anti-CD3. Therefore it has been proposed that $CD25^+$ TN thymocytes represent a stage in T cell development when those cells destined to become $CD3^+$ DN branch off from the classical differentiation pathway to acquire either TCR $\alpha\beta$ or $\gamma\delta$ and differentiate from HSA^+ $CD44^-$ via HSA^- $CD44^-$ to the HSA^- $CD44^+$ phenotype. It has also been shown that positive selection of TCR $\alpha\beta^+$ DN thymocytes may occur during the transition from HSA^+ to HSA^- phenotype based on $V_{\beta 8.2}$ overexpression in the latter population only.

As mentioned earlier, peripheral equivalents of thymic TCR $\alpha\beta^+$ DN cells, referred to as DN$\alpha\beta$T cells, have been identified in a number of tissues including the bone marrow, peripheral blood, lymph nodes, liver and spleen. Whether these peripheral DN$\alpha\beta$T cells are derived from the phenotypically similar thymocyte population or are a result of an extrathymic differentiation pathway is the subject of current research. Evidence of a thymic origin for at least some of these peripheral DN$\alpha\beta$T cells has been provided by the detection of a minor population of $Thy\text{-}1^+$ $CD3^+$ DN T cells in the lymph nodes and peripheral blood of the irradiated mice following the intrathymic injection of either adult or fetal $CD4^-$ $CD8^-$ thymic precursors (Guidos *et al.* 1989).

Similar $CD5^+$ HSA^- phenotype as well as similar frequencies of $V_{\beta 8}$ usage were demonstrated between the peripheral DN$\alpha\beta$T cells of the manipulated mice and their putative TCR $\alpha\beta^+$ DN thymocytes precursors. Furthermore, although a significant proportion of DN$\alpha\beta$T cells identified in the bone marrow and spleen of normal mice have been shown to express the NK1 surface antigen, there is a virtual absence of $NK1^+$ TCR $\alpha\beta^+$ cells in either of the two tissues in C57B10 nude mice (Sykes, 1990; Levitsky *et al.* 1991). However, following fetal thymic engraftment into nude mice, reconstitution of the peripheral $NK1^+$ TCR $\alpha\beta^+$ populations occured therefore providing support for the thymic origin of the bone marrow and splenic $NK1^+$ DN$\alpha\beta$T cells which are also distinct from the thymic independent NK cells.

Not all peripheral DN$\alpha\beta$T cells are thought to be of a thymic origin but rather are the products of an extrathymic differentiation pathway. A significantly higher proportion of bone marrow T cells express the TCR $\alpha\beta^+$ DN phenotype than in either the thymus or spleen suggesting that the bone marrow is a potential site for extrathymic differentiation (Sykes, 1990; Palathumpat *et al.* 1992b). Support for the potential extrathymic origin of the bone marrow DN$\alpha\beta$T cells was provided by the observation of the apparent *in vitro* conversion of TCR $\alpha\beta^-$ $CD4^-$ $CD8^-$ bone marrow cells to the TCR $\alpha\beta^+$ DN phenotype (Palathumpat *et. al.* 1992b). Although TCR rearrangement, as detected by PCR analysis of genomic DNA amplified with $V_{\beta 7}$ and $J_{\beta 2}$-$C_{\beta 2}$ primers, was not observed in freshly isolated TCR $\alpha\beta^-$ DN marrow cells, β chain gene rearrangement associated with the conversion of this marrow subset to the TCR $\alpha\beta^+$ phenotype was observed following 48 hr incubation in tissue culture medium suggesting an apparent maturation of bone marrow T cells in the absence of a thymic microenvironment. In a second study, IL-3 was shown to induce the generation of DN$\alpha\beta$T cells with predominant $V_{\beta 2}$ gene usage from $CD4^+/CD8^+$ depleted bone marrow of both normal and nude BALB/c mice (Kubota *et al.* 1992). The absence of negative selection of normal bone marrow DN$\alpha\beta$T cells expressing the self reactive $V_{\beta 5}$ receptors in BALB/c mice was also considered to be consistent with the putative lack of dependence of this bone marrow subpopulation on the presence of a mature thymus (Palathumpat *et al.* 1992b). Studies in which the deletion of T cells expressing self reactive Vβ receptors did not occur in either nude mice or neonatally thymectomized mice (Hodes *et al.* 1989; Jones *et al.* 1990) have implicated a requirement for the development of a mature thymic microenvironment in this selection process. If the hypothesis suggesting an extrathymic origin for bone marrow DN$\alpha\beta$T cells were true then one would expect the proportion of DN$\alpha\beta$T cells in the bone marrow in athymic nude mice to be similar to that in euthymic mice. Two studies have demonstrated that the proportion of $NK1.1^+$ TCR $\alpha\beta^+$ T cells actually decrease in the bone marrow of nude mice in comparison to normal mice (Sykes, 1990; Levitsky *et al.* 1991). The majority of bone marrow $NK1.1^+$ TCR $\alpha\beta^+$ cells, however, have been shown to express CD4 and CD8 and therefore the

reduction of this population in nude mice can not necessarily be extrapolated to the DN subset. A subsequent study has demonstrated, however, that although the percentage of typical TCR$\alpha\beta^+$ $CD4^+$ and $CD8^+$ SP T cells was markedly reduced in the spleens of nude mice, the proportion of DN$\alpha\beta$T cells in the bone marrow of this athymic murine strain was indeed similar to that of normal adult mice (Palathumpat *et. al.* 1992b). Similarily, the percentage of splenic DN$\alpha\beta$T cells are also not reduced in nude mice unlike the mature SP T cells of this tissue. In addition, the spleens of neonatal thymectomized mice also demonstrate a similar percentage of DN$\alpha\beta$T cells and a significantly reduced number of typical SP T cells in comparison to the normal adult spleen. Furthermore, during the early recovery period following total lymphoid irradiation, the ratio of splenic DN$\alpha\beta$T cells to typical SP T cells was significantly elevated. These results suggest that DN$\alpha\beta$T cells may be thymic independent and may be present in the spleen prior to the emigration of typical T cells from the thymus. Whether these splenic DN$\alpha\beta$T cells are derived from the phenotypically similar bone marrow cells or have undergone differentiation within the spleen itself has yet to be determined. A recent study has shown that production of DN$\alpha\beta$T cells could be induced in the spleens of both normal and nude mice by the lymphokine IL-3 (Kubota *et al.* 1992)

The liver has been proposed as an alternate site for extrathymic differentiation of DN$\alpha\beta$T cells. While a single peak pattern of bright TCR $\alpha\beta$ and $V_{\beta 8}$ expression was observed in intestinal epithelia as well as mononuclear cells (MNC) of the lymph nodes, spleen and blood of normal mice, a two peak pattern, more specifically intermediate and bright expression of both TCR $\alpha\beta$ and V_β was detected in the liver (Seki *et al.* 1991). Further characterization of such intermediate hepatic cells revealed that although a large proportion of $V_{\beta 8}^+$ T cell with intermediate TCR also expressed either CD4 or CD8, a significant percentage of $V_{\beta 8}^+$ DN cells with an intermediate TCR were also present. Although the intermediate TCR $\alpha\beta$ cells constitute a small proportion of cells in the liver of young mice (6-8 wks), this population has been shown to significantly expand as a consequence of aging with DN$\alpha\beta$T cells becoming a predominant phenotype in the older mice (60 wks) (Ohteki *et al.* 1992). At older ages when the thymus is characteristically involuted, the extrathymic pathway was in its most activated state and the apparent intermediate TCR progeny of this pathway began to appear in the periphery, first in the peripheral blood and then eventually in the spleen and lymph nodes. Confirmation that the liver was a site of extrathymic differentiation was provided by the demonstration of only intermediate TCR cells including DN$\alpha\beta$T cells in the liver of congenitally athymic nude mice. Furthermore, an age dependent increase in the proportion of this hepatic population was also observed in the nude mice.

1.2.3 B) TCR $\gamma\delta^+$ DN Lineage

A second population of $CD3^+$ DN T cells which express the alternate TCR γδ heterodimers has been identified in numerous tissues including the thymus, lymph nodes, spleen, blood, skin, female reproductive organs, tongue, lung and liver. Although both express the $CD3^+$ DN phenotype, TCR $\gamma\delta^+$ DN T cells and DNαβT cells are two distinct T cell lineages which arise from a common precursor cell. Divergence of these two lineages is thought to occur relatively late in intrathymic development just prior to CD4 and CD8 expression (Petrie *et al.* 1992). Recent studies have shown that following incubation with IL-7, $CD25^+$ TN thymocytes acquire CD3 expression associated with either the αβ or γδ TCR heterodimers (Vissinga *et al.* 1991; Suda and Zlotnik, 1991). IL-7 was also associated with the loss of HSA expression whereas the addition of anti-CD3 MAb induced a $CD44^+$ phenotype (Suda and Zlotnik, 1993). Whether DN thymocytes commit to the γδ or αβ lineage depends on the activation of a γ chain silencer which acts to silence in-frame as well as out-of-frame rearranged γ genes leading to the development of TCR $\alpha\beta^+$ T cells (Heilig and Tonegawa, 1986; Bonneville *et al.* 1989). Following expression of the γδ heterodimers, TCR $\gamma\delta^+$ DN thymocytes are thought migrate to peripheral organs without the acquisition of either CD4 or CD8 accessory molecules.

TCR $\gamma\delta^+$ T cells can be divided into several subpopulations based on differences in appearance in ontogeny, thymic dependence, V gene usage, junctional variability and tissue localization. Those TCR $\gamma\delta^+$ T cells which disseminate in epithelia, more specifically, the V5 subpopulation in the epidermis of skin (Asarnow *et al.* 1988) as well as the V6 subset in the mucosal surfaces of the tongue and, vagina and uterus (Itohara *et al.* 1990) are both generated from precursors in the fetal thymus (Havran and Allison, 1988; Ito *et al.* 1989). Shortly after birth, the majority of thymic γδ cells express the Vγ4 and Vγ1 genes (Bluestone *et al.* 1991). Whereas the V4 subset circulate through the blood as well as lymph nodes and spleen, $V\gamma1^+$ cells reside in skin and intestinal epithelia in addition to the spleen. A minority of TCR $\gamma\delta^+$ T cells arising from the postnatal thymus express either Vγ2 or Vγ7 genes. However, not all TCR $\gamma\delta^+$ T cells are thymic dependent. One TCR $\gamma\delta^+$ subset, thought to arise in the intestinal epithelia shortly after birth (Bandeira *et al.* 1991), has been characterized by predominant Vγ1 and Vγ7 usage, multiple Vγ chain expression and high junctional diversity. Furthermore, similar to thymic independent αβ T cells, the majority of cells from this γδ subset express CD8α homodimers but lack Thy-1 (Guy-Grand *et al.* 1991). Support for the thymic independence of the V7 T cells has been provided by the observation of this γδ subset in the intestines of athymic nude mice as well as lethally irradiated mice reconstituted with syngeneic bone marrow (Bandeira *et. al.* 1991; Guy-Grand *et al.* 1991). It has been

suggested that the order of appearance in ontogeny may be due to preprogrammed rearrangement of the specific V_γ and V_δ gene segments (Raulet *et al.* 1991).

1.3 T CELL ACTIVATION

Interaction of the TCR with degraded fragments of antigen presented in the context of MHC is considered to be the central event in the initiation and propagation of immune responses toward foreign antigens. Crystallographic analysis of several class I MHC molecules has revealed N-terminal domains consisting of two long parallel α helices supported on a platform of β strands creating a groove in which the site of antigen binding is contained (Bjorkman *et al.* 1987; Madden *et al.* 1991). An extra density representing an antigen fragment has indeed been detected within this groove. Although a high resolution structure of the class II MHC molecule has not yet been determined, similarities to the class I MHC structure are expected based on sequence comparisons (Brown *et al.* 1988). The peptide within the MHC groove is thought to interact with the CDR3 loops of the TCR whereas as the flanking α helices of the MHC molecule contact TCR CDR1 and CDR2 loops (Davis and Bjorkman, 1988). Also important for T cell recognition and activation is the interaction of MHC on APC with CD4 and CD8 accessory molecules. Initial evidence for the participation of CD4 and CD8 in T cell function was provided by studies demonstrating the inhibition of TCR mediated responses to class II or class I restricted antigens by antibodies directed against either CD4 (Nakayama *et al.* 1979; Dialynas *et al.* 1983) or CD8 (Reinherz *et al.* 1981; Hollander, 1982). Therefore it was proposed that CD4 and CD8 were involved in the augmentation of T cell recognition by binding to monomorphic determinants on class II and class I MHC molecules respectively. The subsequent identification of a lymphocyte specific tyrosine kinase $p56^{lck}$ noncovalently associated with the cytoplasmic tails of CD4 and CD8 as well as the demonstration of an increase in tyrosine phosphorylated substrates in mature T cells following CD4 or CD8 crosslinking (Veillette *et al.* 1989; Luo and Sefton, 1990) suggested a possible role for these accessory molecules in the TCR signal transduction pathway (Veillette *et al.* 1988; Barber *et al.* 1989; Turner *et al.* 1990).

As a consequence of occupancy of the T cell receptor by antigens presented in the context of MHC, two signal transduction pathways, namely protein tyrosine kinase and phospholipase C (PLC) pathways, are activated giving rise to a cascade of intracellular biochemical changes including protein phosphorylations, cytoplasmic alkalinization and fluxes in ions and cyclic nucleotides. Furthermore TCR signal transduction activates a regulated cascade of sequential gene activation events, including the induction of expression of IL-2 and its corresponding receptor which ultimately lead to the proliferation and aquisition of effector functions by the T cells.

1.3.1 TCR Signal Transduction Pathway

Two signal transduction pathways have been associated with TCR stimulation by antigens, mitogen lectins or anti-TCR and anti-CD3 MAb, namely the activation of the protein tyrosine kinase and phospholipase C pathways.

1.3.1 A) Protein Tyrosine Kinase Pathway

The first signalling event thought to occur following activation of the TCR involves an increase in the tyrosine phosphorylation of several proteins (June *et al.* 1990a). With the use of anti-phosphotyrosine MAb to immunoblot lysates of stimulated cells, an increase in tyrosine phosphorylation activity was observed within 5 sec. following TCR activation and prior to the detection of increased PLC activity. Furthermore, tyrosine phosphorylation appears to be required for the activation of the PLC pathway based on studies demonstrating the inhibition of PLC activation and subsequent events following cellular stimulation by tyrosine kinase inhibitors (Mustelin *et al.* 1990; June *et al.* 1990b).

Although the TCR lacks intrinsic PTK function, interactions with several cytoplasmic PTKs have been documented. The two *src* family PTKs *fyn* and *lck* have, for example, been implicated in this inital signalling event. TCR does indeed coimmunoprecipitate with *fyn* although the stoichiometry of this association appears to be low (Samelson *et al.* 1990). Furthermore, overexpression of *fyn* in transgenic mice (Cooke *et al.* 1991) renders T cells hyper-responsive to TCR stimulation whereas a hyporesponse was observed for those mice expressing the kinase defective form suggesting therefore a possible role for *fyn* in TCR mediated signal transduction. The second *src* family candidate, *lck*, is directly associated with the cytoplasmic tail of the CD4 and CD8 coreceptors (Veillette *et al.* 1988; Barber *et al.* 1989; Turner *et al.* 1990). Similar to *fyn*, hypersensitivity to TCR stimulation has also been observed following the overexpression of the activated form of *lck* in a T cell hybridoma (Abraham *et al.* 1991). Furthermore, a defect in signalling through the TCR in T cell mutants which lack *lck* expression can be restored by reexpression of the PTK (Strauss and Weiss, 1992; Karnitz *et al.* 1992). As members of the *src* family PTKs, both *fyn* and *lck* share a *src* homology (SH)-2 domain capable of binding phosphotyrosine residues (Koch *et al.* 1991) and a carboxy-terminal tyrosine residue, which when dephosphorylated is activated (Cantley *et al.* 1991). CD45 is thought to be the phosphatase responsible for the activation of both *lck* and *fyn* as suggested by the *in vitro* activation of these PTKs by CD45 (Mustelin *et. al.* 1989; Mustelin *et al.* 1992) and the increased phosphorylation of

lck on tyrosine 505 in CD45$^-$ mutants (Ostergarrd *et al.* 1989). Recently, a third possible candidate for PTK activity in TCR signal transduction has been identified. Found not only in T cells but also NK cells, ZAP-70 has been shown to associate with the TCR subsequent to receptor stimulation (Chan *et al.* 1992). An association of ZAP-70 with either *fyn* or *lck* has been suggested by the apparent requirement of the expression of these two *src* family PTKs for the interaction of ZAP-70 with ζ in cotransfection studies with COS cells. An additional PTK implicated in TCR signalling is the IL-2 inducible T cell kinase *Itk* (Siliciano *et al.* 1992). Unlike the *src* family PTKs, ZAP-70 and *Itk* lack the negative regulatory carboxy terminal tyrosine residues and the inner membrane anchoring-amino-terminal myristoylation sites (Desiderio, 1993).

A number of substrates for tyrosine phosphorylation following TCR stimulation have been identified. Phosphorylation of the TCR ζ chain, for example, has been observed following TCR stimulation (Samelson *et al.* 1986). Murine and human ζ chains include either 6 or 7 potential sites for tyrosine phosphorylation respectively and immunoprecipitation analyses have identified several forms of phospho-ζ, suggesting a complex pattern of phosphorylation (Klausner *et al.* 1987; Irving, and Weiss, 1991). The association of CD4 with TCR is thought to be a requirement for effective tyrosine phosphorylation of the ζ chains possibly due to the delivery of the PTK *lck* to the receptor (Dianzani *et al.* 1992). Other phosphoproteins which have been identified include vav (Margolis *et. al.* 1992), valosin containing protein (Egerton *et al.* 1992a), ezrin (Egerton *et al.* 1992b) and CD5 (Davies *et al.* 1992). The ability of PTK inhibitors to block TCR induced PLC activity has provided an additional substrate for tyrosine phosphorylation. TCR stimulation induces tyrosine phosphorylation of PLC-γ1 which can be blocked by PTK inhibitors (Weiss *et al.* 1991).

1.3.1 B) Phospholipase C Pathway

Activation of the phosphatidylinositol (PI) pathway was the first TCR-regulated signal transduction event to be identified (Imboden and Stobo, 1985). Activation of PLC by tyrosine phosphorylation following TCR stimulation leads to the hydrolysis of the phosphodiester bond of the membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) (Rhee *et al.* 1989). The end products of this reaction, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) function as second messengers facilitating the mobilization of cytoplasmic free calcium $[Ca^{+2}]_i$ and activation of protein kinase C (PKC) (Berridge and Irvine, 1989). Activation of this PI pathway is thought to contribute to the production of IL-2 based on studies demonstrating the ability of phorbal ester and calcium ionophore reagents, which activate PKC and increase $[Ca^{+2}]_i$ respectively, to induce IL-2 production and T cell proliferation (Weiss *et al.* 1984b; Truneh *et al.* 1985) while agents which

block PKC activation and $[Ca^{+2}]_i$ increase inhibit production of this lymphokine (Weiss *et al.* 1984a; Nel *et al.* 1987). Furthermore, elements within the regulatory region of the IL-2 gene have been identified as being responsive to activation of PKC and increases in $[Ca^{+2}]_i$ (Crabtree, 1989).

Although the mechanisms for the transduction of the initial biochemical signals, namely PKC activation and increased $[Ca^{+2}]_i$, to the nucleus remain to be elucidated, a number of potential intermediate targets have been identified. Activation of the *ras* family of G proteins occurs within minutes of TCR stimulation as well as in the presence of phorbal esters suggesting that an apparent dependence on PKC activation (Downward *et al.* 1992). Inhibition of *ras*-mediated GTP hydrolysis is thought to be involved in the TCR activation of this G protein. Recent studies have suggested a role for *ras* in the regulation of TCR induced transcription of the IL-2 gene. While IL-2 promoter activity was enhanced by the expression of an activated form of *ras*, this activity was significantly inhibited by the dominant negative form of *ras*, Ha-rasN17 (Rayter *et al.* 1992).

Additional downstream events dependent on PKC activation include the stimulation of a number of serine/threonine protein kinases. Phosphorylation on serine/threonine residues and subsequent increase in activity has been observed for both the c-*raf* proto-oncogene as well as the ribosomal protein S6 kinase in response to TCR crosslinking or treatment with phorbal esters (Siegel *et al.* 1990; Calvo *et al.* 1992). Furthermore microtubule-associated protein-2-kinase (MAP-2K) is activated within minutes of TCR stimulation and is dependent on both a serine/threonine kinase such as PKC as well as a tyrosine kinase such as *lck* (Nel *et al.* 1990). It has been proposed that *raf* can activate the MAP kinases which in turn stimulate the 90 kDa S6 kinase (Howe *et al.* 1992). Therefore these three kinases may function via a single pathway which may be involved in lymphokine gene expression since studies have demonstrated that MAP kinases accumulate in HeLa cell nuclei following serum stimulation and are capable of phosphorylating a number of transcription factors (Chen *et al.* 1992).

Inositol 1,4,5-triphosphate mediated increase in $[Ca^{+2}]_i$ is thought to be involved in lymphokine gene expression via the activation of calmodulin regulated enzymes. A calcium/calmodulin dependent serine/threonine phosphatase, calcineurin, has been identified as a result of an investigation into the action of the immunosuppressant drugs cyclosporin A and FK506. Both the overexpression of calcineurin or the expression of the activated form have been shown to enhance IL-2 gene inducibility (O'Keefe *et al.* 1992; Clipstone and Crabtree, 1992). Therefore the increase in $[Ca^{+2}]_i$ following activation of the PI pathway may be involved in the stimulation of calcineurin phosphatase activity, the substrate for which may be the cytosolic component of the transcription factor, NF-AT, which regulates the expression of the IL-2 gene (Flanagan *et al.* 1992).

1.3.1 C) IL-2 Gene Activation

Following the activation of the TCR signalling pathway, T cells proliferate and differentiate to effector functions. The critical event involved in this response is the production of the T cell growth inducing cytokine, IL-2. Activation of IL-2 gene expression is thought to involve a sequential series of transcriptional events based on the requirement for protein synthesis and the delay in the initiation of transcription. Induction of IL-2 promoter activity by immediate-early genes is thought to be one such event. The induction, within minutes of TCR activated signal transduction, of transcription of several immediate-early genes involved in the regulation of T cell proliferation and lymphokine expression including *c-fos* and *c-myc* has indeed been observed. It is the products of such immediate-early genes which are considered to facilitate the production and activity of factors required for lymphokine transcription. Post-translational modifications involving serine/threonine kinases and phosphatases may also regulate the activity of the transcription factors required for IL-2 gene expression. One transcription factor associated with IL-2 expression, AP-1, is composed of the *c-fos* and *c-jun* protein families and is subject to regulation by phosphorylation (Hunter and Karin, 1992). In addition, promoter activation by NF-AT, AP-3 and NF-κB may be blocked by treatment with cyclosporin A and FK506 which inactivate the serine/threonine phophatase, calcineurin (Schreiber and Crabtree, 1992).

Expression of the IL-2 gene produces a 15.5 kDa glycoprotein consisting of 133 aa organized in anti-parallel α helices (Bazan, 1992) which recognizes three different forms of the IL-2 receptor with differing affinities. Three critical IL-2 receptor subunits have been identified, namely IL-2Rα, IL-2Rβ and IL-2Rγ. Identified by the anti-Tac MAb, IL-2Rα is a 55 kDa glycosylated protein (Leonard *et al.* 1984). A second 70/75 kDa IL-2 binding protein, referred to as IL-2Rβ was identified by crosslinking with radiolabelled IL-2 (Tsudo *et al.* 1986). Recently, a third IL-2 receptor subunit, the 64 kDa γ chain has been identified (Takeshita *et al.* 1992). When expressed alone, neither IL-2Rα nor IL-2Rβ demonstrate significant binding to IL-2. In contrast, intermediate binding affinity has been observed when both IL-2Rβ and IL-2Rγ are coexpressed, such as on large granular lymphocytes. Cells which express all three subunits as a noncovalently associated complex, including activated T cells, demonstrate high affinity binding. Two alternative signalling pathways have been associated with the occupation of the IL-2 receptor (Shibuya *et al.* 1992). The first pathway involves the induction of the *c-myc* gene via an unknown mechanism and enables T cells to enter the S phase of cell cycle. In the alternate pathway, *c-fos* and *c-jun* induction occurs following activation of cytoplasmic protein tyrosine kinases. Following the production of IL-2, T cells are induced to proliferate as well as differentiate to effector function. More specifically, IL-2

augments the production of cytokines involved in T cell functions including IL-4 and IFNγ. Furthermore, IL-2 facilitates the differentiation of $CD8^+$ SP T cells into cytotoxic effector cells.

1.3.2 Accessory Receptor Signalling Pathways

Although TCR signal transduction plays a key role in the activation of several transcription factors involved in lymphokine gene expression, TCR mediated signals on their own are not sufficient to induce lymphokine production and in fact often induce anergy. An increasing number of accessory receptors are now thought to also be involved in T cell activation.

One possible accessory receptor providing assistance to the TCR during T cell activation is the 44 kDa glycoprotein member of the Ig supergene family, CD28 (June *et al.* 1990c). Approximately 50% of $CD8^+$ T cells and virtually all $CD4^+$ T cells express this costimulatory molecule. CD28 is thought to play a significant role in T cell activation although the signals delivered by this accessory molecule appear to be distinct from those provided by the TCR itself. Activation of CD28 has been shown to enhance proliferation as well as IL-2 production by anti-TCR or mitogen stimulated T cells in the absence of enhanced phospholipid hydrolysis. Furthermore, this CD28-induced expression of IL-2 is resistent to cyclosporin A suppression. CD28 crosslinking does however induce tyrosine phosphorylation activity (Vandenberghe *et al.* 1992). In addition to the upregulation of IL-2, CD28 activation has been shown to increase mRNA levels and secretion of other lymphokines including IL-3, IFNγ, TNF-α, GM-CSF and lymphotoxin (Thompson *et al.* 1989). Therefore the primary role of CD28 appears to be the regulation of the amount of lymphokine produced by T cells via an increase in mRNA half-life. In the absence of CD28, mRNA is labile and subject to rapid degradation such that little if any is secreted. Activation of CD28 leads to the stabilization and enhanced translation of lymphokine mRNA. A cell surface ligand for CD28, referred to as B7/BB1 was identified initially on activated B cells and subsequently on activated monocytes and dendritic cells (Linsley *et al.* 1990; Freeman *et al.* 1989; Larsen *et al.* 1992). Cocculturing of B7-transfected chinese hamster ovary cells (CHO) or B7-Ig fusion protein with suboptimally stimulated T cells leads to increased proliferation and expression of IL-2 mRNA (Linsley *et al.* 1991) Furthermore, addition of MAb specific for either B7 or CD28 inhibit T cell responses including alloantigen-induced T cell proliferation and cytotoxicity (Azuma *et al.* 1992).

Additional accessory receptors implicated in T cell activation include lymphocyte function-associated antigen-1 (LFA-1) and intracellular adhesion molecule-1 (ICAM-1) which are required for the adhesion between T cells and their corresponding antigen presenting cell (APC). Several of

the adhesion molecules including CD2, CD44, LFA-1 and very late antigen-4 (VLA-4) are also capable of mediating transmembrane signalling events. MAb specific for HSA expressed on murine B cells has been shown to suppress the costimulatory activity of the APC although the identity of the ligand for HSA on T cells remains to be identified (Liu *et al.* 1992).

1.4 T CELL FUNCTION

Although both $CD4^+$ and $CD8^+$ SP T cells have been shown to perform key helper, suppressor and cytotoxic roles, there is increasing evidence to suggest that both subpopulations of $CD3^+$ T DN cells, namely TCR $\alpha\beta^+$ and $\gamma\delta^+$ DN T cells, also contribute to the immune response to foreign antigen.

1.4.1 $CD4^+$ SP Helper T Cells

Following the recognition of foreign antigen presented in the context of class II MHC, $CD4^+$ SP T cells acquire the ability to regulate both B and T cell activity through the delivery of "helper" signals. The $CD4^+$ SP population, however, is heterogeneous and can be further subdivided based on the pattern of cytokine secretion following receptor activation. Two major subsets of $CD4^+$ SP helper T cells have been identified, namely T_H1 and T_H2 which mediate distinct immune responses. Recently it has been shown that the cytokine environment is a major determinant of which type of T_H cell is activated during an immune response. More specifically, interferon γ (IFNγ) drives naive $CD4^+$ SP T cells towards a T_H1 phenotype (Scott, 1991) whereas Il-4 plays a key role in the differentiation of the T_H2 subset (Seder *et al.* 1992). T_H1 cells have been shown to produce IL-2 and IFNγ whereas T_H2 cells secrete IL-4 and IL-5. Other lymphokines including GM-CSF and IL-3 are the products of both types of helper T cells. Of the lymphokine signals delivered by $CD4^+$ SP T cells, IL-4, IL-5 and IFNγ are known to have selective activities on the induction of specific Ig isotypes. While IL-4, selectively enhances IgG_1 and IgE responses to LPS (Coffman *et al.* 1986; Snapper and Paul, 1987), IL-5 and IFNγ preferentially enhance polyclonal B cell IgA (Coffman *et al.* 1987) and IgG_{2a} (Snapper and Paul, 1987) responses to the antigen respectively. Furthermore the ability of T_H1 and T_H2 cells to induce IgG_{2a} and IgG_1 responses respectively in TNP specific B cells has been confirmed (Stevens *et al.* 1988). IL-2, in contrast has been shown to play a significant role in the activation and differentiation of mature cytotoxic T lymphocyte (CTL) effector cells from their precursors (Nabholz and MacDonald, 1983).

1.4.2 $CD8^+$ SP Cytotoxic T Cells

The effector function of $CD8^+$ SP T cells involves the killing of target cells which bear antigen in the context of class I self MHC. Following recognition of antigen by the TCR, intracellular signals result in the induction of possibly several different cytotoxic mechanisms. Two major alternatives for $CD8^+$ SP T cell effector function include target cell death triggered either by the loss of plasma membrane integrity and therefore ion imbalance due to perforin induced pore formation (Ortaldo *et al.* 1992) or by CTL mediated signalling of target cell receptors leading to cell disintegration (Berke, 1991). Recently, the *Fas* gene has been implicated in CTL mediated triggering of programmed target cell death (Rouvier *et al.* 1993).

1.4.3 SP Suppressor T cells

Suppressor T cells (T_S) are heterogeneous group of T lymphocytes which are thought to include both conventional and modified Th1, Th2 and CTL subsets as well as T cells specialized for suppression. A number of mechanisms have been proposed for T cell mediated immunosuppression including the release of inhibitory lymphokines including tumour growth factor-β (TGF-β), IFNγ and IL-10, secretion of soluble antigen-specific suppressor factors encoded at least in part by TCR α and/or β chain genes, cytolysis and delivery of a non-cytolytic negative signal via direct contact with the target cell (Murphy, 1993).

1.4.4 TCR $\alpha\beta^+$ DN T Cells

Based on the late appearance in ontogeny, the absence of precursor activity and the ability to respond to lectins and anti-TCR αβ MAb (Ceredig *et al.* 1987), TCR $\alpha\beta^+$ DN T (DNαβT) cells are considered to be a mature population of T cells. Although the role of DNαβT cells in the immune response has yet to be definitively established, a number of possible functions have been proposed.

One potential role for DNαβT cells in the immune response is that of a suppressor function. Numerous studies have demonstrated that normal adult bone marrow cells are capable of suppressing the proliferative response towards mitogens of normal spleen cells as well as the mixed leukocyte reaction (MLR) regardless of the stimulator and responder strain combinations employed. Characterization of the bone marrow cells capable of mediating this suppressive

function has revealed a "null" lymphocyte function, more specifically Thy-1$^-$ Ig$^-$ Mac-1$^-$ (Weingenberg *et al.* 1984; Holda *et al.* 1986) and a similarity in phenotype and function to natural suppressor cells identified in the neonatal mice or adult mice exposed to total lymphoid irradiation or suffering from chronic graft versus host disease (GVHD) (Oseroff *et al.* 1984; Maier *et al.* 1986). When murine bone marrow cells were further fractionated by Percoll density gradients, those cells comprising the low density fraction were also found to be enriched for natural suppressor activity and were capable of inhibiting MLR (Benveniste *et al.* 1990; Palathumpat *et al.* 1992b). Furthermore, this low density fraction of murine bone marrow cells was also capable of inhibiting GVHD (Palathumpat *et al.* 1992a) Interestingly, the low density bone marrow fraction was found to be enriched in cells with the TCR $\alpha\beta^+$ CD4$^-$ CD8$^-$ phenotype. When bone marrow DN$\alpha\beta$T cells were further purified by sorting, inhibition of MLR as well as GVHD was again observed. The ability to inhibit both MLR and GVHD has also been observed with cloned cell lines of the DN$\alpha\beta$T phenotype derived from the spleens of neonatal or adult mice recovering from either irradiation or bone marrow transplantation (Hertel-Wulff *et al.* 1984; Schwadron *et al.* 1985; Strober *et al.* 1987; Sykes *et al.* 1990a). Furthermore, suppression of acute GVHD was detected in the low density fraction of normal murine spleen cells and was correlated with the depletion of typical SP T cells and the enrichment of DN$\alpha\beta$T cells (Palathumpat *et al.* 1992a). Lethal GVHD suppression was also performed by DN$\alpha\beta$T cells obtained from the spleens of Mls-1^b mice following immunization with Mls-1^a cells (Bruley-Rosset *et al.* 1990). In further support of a suppressor like function for DN$\alpha\beta$T cells, consistent increases in splenic CD3$^+$ CD4$^-$ CD8$^-$ cells have been demonstrated in animals receiving GVH-inducing inocula (Abraham *et al.* 1992). The percentage of this splenic subpopulation was further enhanced when the recipients of the allogeneic inocula were treated with IL-2. Early administration of IL-2 following bone marrow transplantation has indeed been shown to provide potent prophylaxis against GVHD mortality without preventing alloengraftment or reducing graft versus leukemia effects (Sykes *et al.* 1990b,c).

A role for DN$\alpha\beta$T cells in cytotoxic response and allogeneic tumour rejection has also been suggested. CTL with the TCR $\alpha\beta^+$ CD4$^-$ CD8$^-$ phenotype were generated in mixed lymphocyte tumour cell culture (MLTC) spleen cells from anti-CD8 MAb treated B6 mice (Mieno *et al.* 1991). It was suggested that when CD8$^+$ cells were blocked by anti-CD8 MAb, CD4$^+$ cells were capable of mediating tumour rejection by the induction of DN$\alpha\beta$T cells. Similarily, rejection of an IA$^+$ variant line of FBL-3 leukemia in a CD8 depleted syngeneic B6 mice was also mediated by DN$\alpha\beta$T cells as well as CD4$^+$ CTL generated by the activation of CD4$^+$ helper cells (Yoshimura *et al.* 1993). Furthermore, IL-2 dependent T cell lines of the DN$\alpha\beta$T phenotype with non-MHC-restricted cytolytic activity against tumour targets, syngeneic and allogeneic fibroblasts and

phytohemagglutinin (PHA) blasts from allogeneic donours have been developed from the peripheral blood of a patient with combined immunodeficiency and tissue injury resembling GVHD who had a massive expansion of this cell type (Brooks *et al.* 1990). When activated by mitogens or phorbol myristate acetate (PMA) and IL-1, these cell lines were capable of producing IFNγ as well as tumour necrosis factor-α (TNF-α) and -β.

A number of studies have also implicated DNαβT cells with the development of autoimmune disease.A marked expansion of DNαβT cells has been observed in the peripheral blood of patients with the autoimmune disease systemic lupus erythematosus (Shivakumar *et al.* 1989). Along with $CD4^+$ SP T cells, DNαβT cells are responsible for the induction of oligoclonal B cell production of highly cationic Ig anti-DNA autoantibodies. In addition, TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ $B220^+$ Pgp-1^+ cells of which a high percentage are $V_{\beta}8^+$, have been shown to accumulate in the massively enlarged lymph nodes and spleen of the autoimmune lymphoproliferative MRL-*lpr/lpr* mice (Wofsy *et al.* 1984; Nemanzee *et al.* 1985). Strains which carry the mutant autosomal recessive gene, *lpr*, spontaneously develop a severe and rapidly progressive autoimmune disease characterized by the production of a variety of autoantibodies, immune complex-mediated glomerulonephritis and vasculitis in addition to the massive lymphadenopathy (Andrews *et al.* 1978; Theofilopoulos and Dixon, 1981). The thymus is thought to play a role in the acceleration of this disorder based on studies which have shown that neonatal thymectomy reduces lymphadenopathy and the autoimmune syndrome (Steinberg *et al.* 1980) whereas a recurrence of the lymphadenopathy occurs following thymic grafting into the neonatal thymectomized *lpr* mice irrespective of the thymic genotype (Theofilopoulos *et al.* 1981). It has been recently shown that the liver is a major site for the proliferation of the abnormal αβ-T cell population based on the demonstration by *in vivo* [^{3}H] tritiated thymidine injection of hepatic DNαβT cell migration to the periphery with subsequent lymphadenopathy (Ohteki *et al.* 1990). Furthermore, the abnormal αβ-T cells in *lpr* mice are thought to be a counterpart of the normal T cells in the liver based on similarities with respect to the TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ Pgp-1^+ phenotype, intermediate TCR expression and $V_{\beta}8$ expression (Seki *et al.* 1991). In a subsequent study it was shown that the increased expansion in the liver of DNαβT cells is a common feature of other autoimmune mouse strains including C3H/HeJ-*gld/gld*, BxSB, NOD, MRL-+/+ and NZB/W F_1 mice (Masuda *et al.* 1991). Furthermore, the accumulation of this abnormal DNαβT cell population was also age dependent. Additional evidence for a potential role of DNαβT cells in the development of autoimmune disease was provided by a study in which the neonatal thymectomy of CBA/H mice resulted in a marked transient increase in $CD3^+$ $CD4^-$ $CD8^-$ cells in the peritoneal cavity which selectively overexpressed the products of the TCR $V_{\beta}11$ gene family (Andreu-Sanchez *et al.* 1991). Intraperitoneal inoculation of an IL-2 vaccinia virus construct which provides high titers of

human IL-2 *in vivo*, induced the conversion of this DN population to either $CD4^+$ or $CD8^+$ SP T cells and provoked autoimmune manifestations including anti-DNA antibodies, rheumatoid factors and interstitial nephritis. An apparent capacity for autoreactivity has also been demonstrated in splenic DN$\alpha\beta$T cells of normal mice (Prud'homme *et al.* 1991). Although DN$\alpha\beta$T cells were unresponsive in MLC, following activation with either concanavalin A (Con A) or anti-TCR $\alpha\beta$ antibodies this splenic subpopulation acquired CD4 expression and high reactivity in both syngeneic and allogeneic mixed leukocyte culture (MLC) which could be blocked by anti-CD4 antibodies.

1.4.5 TCR $\gamma\delta^+$ T Cells

The $\gamma\delta$ T cell receptor has been shown to recognize a diverse selection of antigens. Such antigens include both classical and non-classical MHC molecules (Matis *et al.* 1987; Bonneville *et al.* 1989b), bacterial antigens including mycobacterial heat shock proteins (Born *et. al.* 1990b) and superantigens such as staphylococcal enterotoxins (Rust *et al.* 1990), and numerous self antigens including the β_2-microglobulin-associated human CD1 molecules (Porcelli *et al.* 1989), cell surface immunoglobulin (Wright *et al.* 1989) and autologous heat shock proteins (Born *et al.* 1990a).

The ability of TCR $\gamma\delta^+$ T cells to display specificity for conventional MHC antigens has been suggested by the overlap in specificity thought to occur between TCR $\gamma\delta$ and TCR $\alpha\beta$ cells as a result of the interspersal of V_α and V_δ elements in the germline (Raulet, 1989) as well as by the detection of known V_α elements which are rearranged to DJ_δ to encode functional $\gamma\delta$ receptors (Elliot *et al.* 1988). TCR $\gamma\delta^+$ T cells specific for both conventional class I (Bluestone *et al.* 1988; Rivas *et al.* 1989) and class II MHC molecules (Matis *et al.* 1989; Rellahan *et al.* 1991) have indeed been isolated. Not all TCR $\gamma\delta^+$ T cells are thought to be restricted by conventional MHC molecules as suggested by an essentially normal development of TCR $\gamma\delta^+$ T cells observed in either MHC class II or β_2-microglobulin deficient mice (Correa *et al.* 1992; Bigby *et al.* 1993). Alternatively, TCR $\gamma\delta^+$ T cells may display specificity for non-classical MHC antigens. Human TCR $\gamma\delta^+$ cells isolated from peripheral blood have been shown, for example, to be specific for CD1 molecules (Porcelli *et al.* 1989). These non-MHC linked CD1 antigens demonstrate limited homology to class I MHC molecules and are expressed in noncovalent association with β_2 microglobulin. Furthermore, Lam *et al.* (1990) have isolated human TCR $\gamma\delta^+$ cell lines specific for autologous non-classical human leukocyte antigen (HLA)-like molecules serologically related to HLA B and C. Whether non-classical class I MHC molecules play a prominent role in antigen presentation to TCR $\gamma\delta^+$ cells, however, remains to be determined.

TCR $\gamma\delta^+$ T cells are thought to play a key role in infectious diseases. T cells expressing the $\gamma\delta$ heterodimers have been shown to accumulate in numerous tissues following bacterial infection including in the peritoneal cavity of mice infected with *Listeria monocytogenes* (Hiromatsu *et al.* 1992), in the draining lymph nodes of mycobacteria infected mice (Janis *et al.* 1989), and in the skin lesions of patients infected with the tuberculosis form of leprosy (Modlin *et. al.* 1989). Protozoan infections have also been associated with TCR $\gamma\delta^+$ T cell accumulation including that observed in the spleens of mice infected with *Trypanosoma cruzi* and *Plasmodium chabaudi* (Minoprio *et al.* 1989) as well as in the skin lesions of patients with cutaneous leischmaniasis (Falini *et al.* 1989). TCR $\gamma\delta^+$ T cell accumulation has also been demonstrated in viral infections including the lungs of mice infected intranasally with influenza virus (Eichelberger *et al.* 1991), and in the blood of human immunodeficiency virus (HIV) infected acquired immunodeficiency syndrome (AIDS) patients (Autran *et. al.* 1989; De Paoli *et al.* 1991) and during the acute phase of Ebstein Barr virus (EBV) infection (De Paoli *et al.* 1990).

An involvement of TCR $\gamma\delta^+$ T cells in immune surveillance against cancer has been suggested by the ability of this T cell subset to kill tumour cells following activation with IL-2. Subsets of TCR $\gamma\delta^+$ cells have also been shown to recognize EBV transformed B cells (Hacker *et al.* 1992) as well as superantigen expression on Burkitt lymphoma cells (Sturm *et al.* 1990). Furthermore, autologous tumour cells have been recognized by TCR $\gamma\delta^+$ cells obtained from patients suffering from Burkitt lymphoma as well as from acute lymphoblastic leukemia in complete remission (Bensussan *et al.* 1989). Class II MHC dependent lysis of autologous tumour cells has also been shown to be mediated by TCR $\gamma\delta^+$ T cell lines derived from tumour infiltrating lymphocytes (Zocchi *et al.* 1990).

TCR $\gamma\delta^+$ T cells have also been implicated in a number of pathological immune responses. Although TCR $\gamma\delta^+$ cells of the V1 subset represent a relatively minor percentage of circulating $\gamma\delta$ cells in healthy individuals, significant numbers of these cells have been detected in the synovial fluid of patients with rheumatoid arthritis (Holoshitz *et al.* 1989). While Vδ1 cells have been shown to accumulate both in intestinal lesions of patients with coeliac disease (Rust *et al.* 1992) as well as in the blood of a patient with the type 1 autoimmune polyglandular syndrome associated with aplastic anemia (Hara *et al.* 1990), an expansion of Vδ2 cells has been observed in the blood of an atopic dermatitis patient (De Paoli *et al.* 1990) as well as in brain tissue from multiple sclerosis patients (Hvas *et al.* 1992).

TCR $\gamma\delta^+$ T cells have also been shown to accumulate in a number of immunodeficiency disorders including ectodermal dysplasia syndrome, bare lymphocyte syndrome, partial DiGeorge syndrome, common variable immunodeficiency (Seki *et al.* 1989), Wiskott Aldrich syndrome (Morio *et al.* 1990) and ataxia telangiectasia (Carbonari *et al.* 1990).

1.5 Thesis Objectives

Although thymic maturation involves the aquisition of the TCR along with the CD4 and CD8 co-receptors, not all thymocytes are thought to follow this conventional T cell pathway. Indeed subpopulations of thymocytes which express either the αβ or γδ TCR heterodimers yet lack CD4 and CD8 have been identified and are thought to be equivalent to the phenotypically similar cells residing in a number of peripheral organs. Furthermore both TCR $\alpha\beta^+$ and $\gamma\delta^+$ DN T cells are thought to be functionally mature and their accumulation in tissues have been correlated with pathological immune responses including several autoimmune disorders.

The objective of this thesis was to characterize the TCR $\alpha\beta^+$ DN (DNαβT) cell population identified in the spleen of normal mice. Splenic DNαβT cells were characterized with respect to their surface phenotype and preferential usage of V_β TCR genes. Furthermore, the ability of splenic DNαβT cells to respond in the presence of cytokines, MAb which crosslink the TCR as reagents which bypass the TCR and activate intracellular events was also assessed. Finally the function of this splenic DNαβT cell population was also studied specifically with respect to lymphokine production. The purpose of these studies was to provide support for the hypothesis that splenic DNαβT cells represent a unique class of mature T cells which are related to the phenotypically similar cells found in the thymus and are distinct from single positive mature T cells.

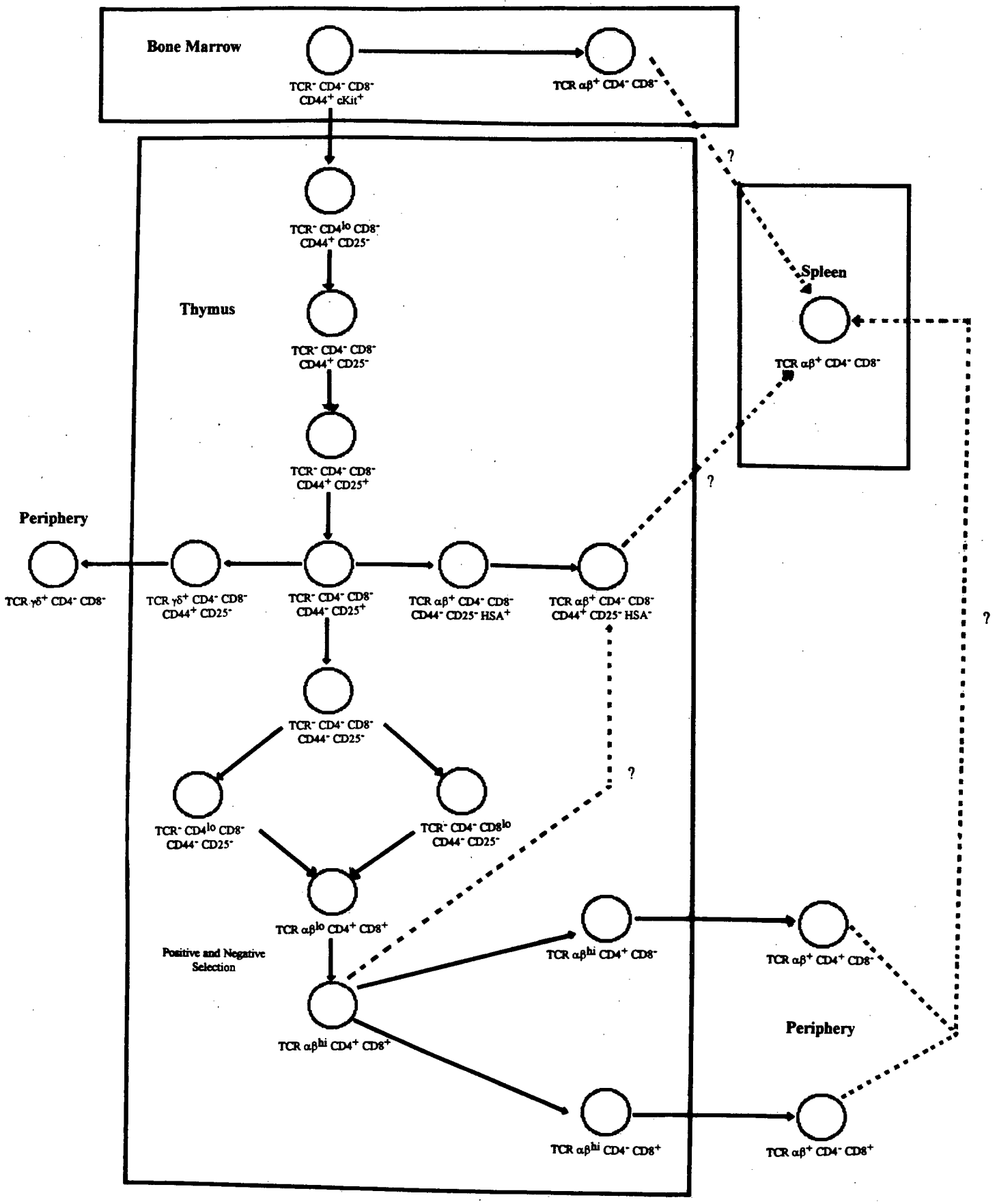

Figure 1 Schematic overview of the thymic differentiation pathway.

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CHAPTER II

MATERIALS AND METHODS

2.1 ANIMALS

Inbred BALB/c mice were obtained from the Jackson Laboratories, Bar Harbor, ME. and maintained in the Animal Facility of the B.C. Cancer Research Center.

2.2 ANTIBODIES

Hybridoma lines 53-6.72 (Ledbetter and Herzenberg, 1979) producing MAb to CD8, M1/70 (Springer *et al.* 1979) producing MAb to the α unit of Mac-1 , M1/69 (Springer *et al.* 1978) producing MAb to heat stable antigen, KJ16 producing MAb to $V_{\beta 8.1}$ and $V_{\beta 8.2}$ of the TCR αβ (Haskins *et al.* 1984), RG7/9.1 HLK (Springer *et. al.*1982) producing MAb to rat κ light chain were obtained from the American Type Culture Collection (Rockville, MD). Hybridoma lines GK1.5 (Dialynas *et al.* 1984) and 145-2C11 (Leo *et al.* 1987) were used to produce MAb to CD4 and CD3 respectively. Undiluted supernatants of overgrown cultures were used as the source of MAb. MAb RG7/9.1 and 145-2C11 were also purified and conjugated with fluorescien isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO) or biotinylated (Sigma). Biotinylated anti-TCR αβ and TCR γδ and FITC-conjugated anti-TCR γδ were obtained from Pharmingen (San Diego, CA).

2.3 ISOLATION OF T CELL POPULATIONS

2.3.1 DN splenic T cell subpopulations

An outline of the procedure for the isolation of splenic T cell subpopulations is provided in Figure 2. Wire mesh was used to obtain single cell suspensions, in Hanks balanced salt solution supplemented with 5% heat inactivated fetal calf serum (FCS), of total splenic T cells from 5-6 week old BALB/c mice. Contaminating red blood cells (RBC) were lysed in 2X ammonium chloride solution (0.16 M Tris NH_4Cl pH 7.2) and washed in Hanks 5% FCS. $CD4^+$ $CD8^+$ HSA^+ and Mac-1^+ cells were depleted using a modified indirect panning method (Takei, 1988). Briefly, cells were incubated with a mixture of anti-CD4, anti-CD8, anti-HSA and anti-Mac-1

MAb (1 ml/spleen for each of the undiluted hybridoma supernatants) on ice for 45 min. The cells were then washed and incubated twice for 40 min. at 4°C on plastic plates precoated with purified mouse anti-rat Igκ antibody, RG7/9.1 (0.1 mg/ml in 10 mM Tris pH 8.0) for 1 hr at room temperature followed by phosphate buffered saline (PBS) 5% FCS for 1 hr at 4°C. Nonadherent cells were restained on ice for 30 min. with CD4, CD8, HSA, and Mac-1 antibodies followed by FITC-conjugated RG7/9.1. Propidium iodide (1 μg/ml, 5 min. on ice) was added to stain dead cells. Positively stained cells were removed by sorting with a single beam flow cytometer (FACStar, Becton, Dickinson, Sunnyvale, CA) based on gating set by a negative control population of total nucleated spleen cells incubated with FITC-conjugated RG7/9.1 on ice for 30 min. For further purification of $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ (DN) splenic T cell subpopulations, cells were stained for 30 min. on ice with either biotinylated TCR αβ recognized by phycoerythrin-conjugated streptavidin (SA-PE) or FITC-conjugated TCR γδ and CD3 antibodies. TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ cells (DNαβT cells) were isolated either by positively sorting for TCR $\alpha\beta^+$ DN cells or by negatively sorting for TCR $\gamma\delta^-$ DN cells. TCR $\gamma\delta^+$ $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ cells, similarly, were isolated either by positively sorting for TCR $\gamma\delta^+$ DN cells or by negatively sorting for TCR $\alpha\beta^-$ DN cells. In order to isolate TCR $\alpha\beta^{+/-}$ DN, gating was determined from a negative control population of total nucleated spleen cells incubated with FITC-conjugated RG7/9.1 and SA-PE for 30 min. on ice. The negative control used for setting the gates in the isolation of TCR$\gamma\delta^{+/-}$ DN and $CD3^{+/-}$ DN cells consisted of total nucleated spleen cells incubated with biotinylated RG7/9.1 for 30 min. on ice followed by SA-PE for 30 min. on ice. To reassess the purity following sorting, all cell populations were restained with the antibodies used in the isolation procedure and analysed by flourescence activated cell sorter (FACS).

2.3.2 Mature splenic T cells

Mature T cells were isolated from splenic cell suspension by passage over nylon wool column (Julius *et al.* 1973). Nylon wool was boiled for 10 min. in 25 mM ethylenediamine tetraacetic acid (EDTA) pH 8.0, washed twice with distilled H_2O and dried overnight at 37°C in preparation for packing into the barrel of 6 ml plastic syringes (1g nylon wool per syringe) and subsequent autoclaving. The sterile packed syringe barrels were equilibrated overnight at 37°C with RPMI 1640 media 5% FCS. Nylon wool columns were loaded with 1 ml cell suspension in warm RMPI 1640 5% FCS from the spleens of 5-6 week old BALB/c mice (1 spleen/column). Following a 1 hr incubation at 37°C, the columns were washed slowly with RPMI 1640 5% FCS and the resulting effluent was collected for 45 min. The nonadherent cells were resuspended in 0.16 M Tris NH_4Cl pH 7.2 for depletion of contaminating RBCs.

2.3.3 DN thymocytes subpopulations

The procedure for the isolation of DN thymocytes and their subpopulations was similar to that for splenic DN T cell subpopulations.

2.4 PHENOTYPIC ANALYSIS

For phenotypic analysis, sorted populations were incubated with the specific antibody for 30 min. on ice. Propidium iodide was added prior to reanalysis to stain dead cells.

2.4.1 Splenic DN T cells

In order to determine the proportion of splenic DN T cells which express either the αβ γδ TCR associated with the CD3 complex, sorted splenic $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ (DN) T cells were stained with either biotinylated anti-TCR αβ recognized by SA-PE or FITC-conjugated anti-TCR γδ or anti-CD3.

2.4.2 Splenic $CD3^-$ DN T cells

In order to further characterize the phenotype of splenic $CD3^-$ DN T cells, sorted splenic $CD3^-$ $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ T cells were stained with either of the MAb specific for Thy-1, Pgp-1, T220, Lyt-1, YE1/19 (PCI), YE1/22 (4F2), LFA-1, ICAM-1 and B220 recognized by FITC conjugated RG7/9.1.

2.4.3 Splenic DNαβT cells

In order to further characterize splenic DNαβT cells, sorted splenic $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ TCR αβ T cells were stained with Pgp-1 recognized by FITC conjugated RG7/9.1 or with B220, YE1/19, YE1/9, ICAM-1 and Lyt-1 recognized by FITC conjugated goat anti-rat antibody. In order to determine whether splenic DNαβT DNαβT cells overexpress TCR $V_{\beta 8}$ genes, splenic DN cells as well as nylon wool purified mature splenic T cells were stained with either anti-TCR $V_{\beta 8}$ antibody KJ16 or anti-TCR αβ MAb recognized by FITC conjugated RG7/9.1.

2.5 PROLIFERATION ASSAY

In order to determine the cytokine concentrations which would give optimal stimulation, sorted DN thymocytes and spleen cells were assessed in triplicate for their proliferative response to varying concentrations of either IL-7 or IL-1 in wells previously coated for 1 hour at 4°C with 145-2C11 anti-CD3 antibody respectively. Optimal proliferative responses were obtained with 10 ng/ml IL-7 and 200 U/ml IL-1 in the presence of immobilized anti-CD3. Sorted subpopulations of DN splenic T cells and thymocytes as well as nylon wool non-adherent splenic T cells were stimulated in triplicate in round bottom microtiter wells in either 0.1 ml (if $\geq 5.0 \times 10^4$ cells/well) or 0.05 ml (if $< 5.0 \times 10^4$ cells/well) of RPMI 1640 medium containing 5% FCS and 50 μM 2-mercaptoethanol (2-ME) with either 10 ng/ml IL-7 (Genzyme, Boston, MA) or 200 U/ml IL-1 (Biogen, Geneva, Switzerland) or a combination of both. Cells were also cultured with or without 200 units/ml IL-1 in wells previously coated with 145-2C11 anti-CD3 antibody for 1 hr at 4°C. Furthermore, cells were stimulated with either 10 ng/ml PMA or 600 nM ionomycin or a combination of both. As a negative control, cells were incubated in media alone. Due to the rarity of the splenic DNαβT cell population and therefore the minimal and often variable number of cells obtained from the purification procedure, the number of cells used per well often varied between experiments from 1.4×10^4 to 1.0×10^5 cells per well in order to provide triplicate wells for each culture condition including negative and positive controls. All cultures were incubated for 72 hr at 37°C in a humidified atmosphere of 5% CO_2 and subsequently pulsed with 50 μl ^{3}H-thymidine (1μCi) for 6 hr. Cells were then harvested and counted by a liquid scintillation counter. For each of the cell populations and stimulating conditions studied, the results given in Tables I, II and III are representative of at least three separate experiments. The standard error of means (SEM) given are calculated based on triplicate samples of the representative experiment. Standard error of means comparing the three experiments for each set of cell populations and stimulating conditions were not calculated as the number of cells/well were not consistent between experiments.

2.6 LYMPHOKINE mRNA EXPRESSION

2.6.1 Stimulation of splenic T cells

Sorted splenic TCR $\alpha\beta^+$ DN, TCR $\gamma\delta^-$ DN, $CD3^-$ DN and nylon column purified mature T cells were stimulated for 16 hr at 37°C in round bottom microtiter wells (1×10^5 cells/well) in 100 μl of RPMI 1640 medium containing 5% FCS and 50 μm 2-ME with either 10 ng/ml IL-7 or 200 U/ml IL-1 or a combination of both. As mentioned previously, the concentrations of IL-7 and IL-1

used were those identified as providing optimal stimulation, as assessed by ^{3}H-thymidine incorporation, of thymic DN T cells and splenic DN T cells in wells previously coated with anti-CD3 antibodies respectively. Cells were also cultured in round bottom microtiter wells previously coated with 145-2C11 anti-CD3 antibody for 1 hr at 4°C. As a negative control, cells were incubated in media alone. All cultures were incubated for 16 hr at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2). A minimum of three seperate experiments were performed in which the splenic subpopulations were stimulated under the conditions outlined above.

2.6.1 RNA extraction

Total cellular RNA from sorted splenic DN subpopulations and nylon column purified mature T cells was isolated by the acid-phenol method (Chomczynski and Sacchi, 1987) either directly following purification or after 16 hr stimulation. Cells were washed 2 X in phosphate buffered saline (PBS) and the pellet was dissolved in 0.1 ml of solution D (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol). Following this 10 µl of 1 mg/ml yeast transfer RNA (tRNA), 10 µl of 2 M sodium acetate pH 4.0, 100 µl water saturated phenol and 40 µl of chloroform:isoamyl alcohol (49:1) were added. The solution was thoroughly mixed and microfuged at 14,000 g for 10 min. at 4°C. The aqueous layer was mixed with 100 µl isopropanol, incubated at -20°C for 90 min and microfuged at 14,000 g for 15 min. at 4°C. The pellet was resuspended in 100 µl of solution D followed by 10 µl of 2 M sodium acetate pH 4.0, 100 µl of water saturated phenol, 40 µl chloroform:isoamyl alcohol (49:1). The solution was thoroughly mixed and microfuged at 14,000 g for 10 min. at 4°C. The aqueous layer was mixed with 100 µl isopropanol and incubated at -20°C for 90 min and microfuged at 14,000 g for 15 min at 4°C. The pellet was resuspended in 100 µl of distilled water, to which was added 10 µl of 3 M sodium acetate pH 6.6 and 220 µl ethanol. The solution was microfuged for 10 min. at 4°C and the pellet was resuspended in 200 µl of 75% ethanol and centrifuged for 10 min. at 4°C. The supernatant was removed and the pellet was dried in a vacuum centrifuge.

2.6.2 cDNA synthesis

Extracted RNA was converted to cDNA by reverse transcription using random hexamers as follows. The pellet obtained from RNA extraction was resuspended in 16.5 µl of distilled water to which was added 6 µl of 5 X buffer (250 mM tris HCl pH 8.3, 300 mM potassium chloride, 15 mM magnesium chloride and 50 mM dithiothreitol), 3 µl of 1 mg/ml bovine serum albumin (BSA),

1 µl of 40 U/µl RNase, 1 µl of 100 ng/µl random hexamers, 1.5 µl dNTP (10 mM each of dATP, dGTP, dCTP and dTTP), and 1 µl of 200 U/µl Moloney Murine Leukemia Virus reverse transcriptase. The solution was incubated at 40°C for 1 hr.

2.6.3 Polymerase Chain Reaction

Cytokine specific fragments in the cDNA samples were amplified by the polymerase chain reaction (PCR) as follows. To 5 µl of cDNA was added 26.75 µl of distilled water, 5µl of 10 X reaction buffer (500 mM potassium chloride, 100 mM tris HCl pH 8.3, 15 mM magnesium chloride, 0.1% gelatin), 8 µl dNTP (1.25 mM each of dATP, dGTP, dCTP, dTTP), 2.5 µl of 20 µM 5' oligonucleotide primer, 2.5 µl of 20 µM 3' oligonucleotide primer and 0.25 µl of 5 U/µl Taq polymerase. Specific 21-mer oligonucleotide primers for the cytokines IFNγ, IL-2, IL-4 and IL-6 as well for the positive control, β actin, were used. Sequences for the oligonucleotide primers is included in Figure 3. Cytokine and actin specific DNA fragments were amplified by 30 cycles of PCR. Each cycle consisted of denaturation for 1 min. at 94°C, annealing for 2 min. at 50°C and extension for 3 min. at 72°C.

2.6.7 Agarose Gel Electrophoresis

The amplified DNA (15 µl) was loaded onto 1% agarose mini-gels and electrophoresed in tris borate EDTA (TBE) buffer (89 mM Tris, 89 mM borate, 25 mM EDTA pH 8.3) at 80 volts for 1.5 hr. Ethidium bromide was added to both the gel and the running buffer at a concentration of 500 ng/ml. Molecular weights were determined with a λ HindIII molecular weight standard. The gels were visualized and photographed under ultra violet light. Expected sizes of amplified fragments of β actin, IFNγ, IL-2, IL-4 and IL-6 were 540, 460, 502, 399 and 638 base pairs (bp) respectively.

2.6.8 Southern Blotting

The agarose gel containing the PCR amplified fragments was layered on top of a 3M Whatman filter paper saturated with 0.4 M sodium hydroxide (NaOH). The ends of the filter paper were in contact with the 0.4 M NaOH solution. A piece of Zeta Probe (Bio-rad Laboratories; Mississauga, Ontario), cut to the size of the gel, was presaturated with 0.4 M NaOH and placed on top of the gel. Two pieces of Whatman paper were layered on top of the membrane followed by approximately 10 cm of paper towels and then a glass plate and weight. The dry paper towels draw the 0.4 M NaOH solution upward by capillary action and provide a unidirectional transfer of

the DNA onto the membrane. The transfer was allowed to proceed overnight following which the membrane was rinsed in 6X saline sodium citrate buffer (SSC) (20 X SSC: 3 M NaCl, 0.15 M sodium citrate pH 7.0).

2.6.9 Prehybridization

The membrane was incubated at 60°C for 2 hr in a prehybridization solution consisting of 3X saline sodium phosphate EDTA buffer (SSPE) (20 X SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 7.4), 1% sodium dodecyl sulphate (SDS), 0.5% Blotto (Carnation Instant Skim Milk) and 0.5 mg/ml denatured salmon sperm DNA.

2.6.10 Oligolabelled Probe

The IFNγ cDNA (Clontech Laboratories, Palo Alto, CA) was radiolabelled by the following oligolabelling procedure (Feinberg and Vogelstein, 1983). Briefly, 20 ng of IFNγ DNA was added to 200 ng of randomly generated hexanucleotides in a final volume of 14 µl. The solution was incubated at 95°C for 3 min. followed by 1 min. on ice. To this solution was added 2 µl of 10X HLB buffer (0.5 M HEPES pH 6.9, 0.1 M $MgCl_2$, 0.06 M 2-mercaptoethanol), 2 µl of a nucleotide mixture (dATP, dGTP, dTTP, 2.5 mM each), 5 µl [α-^{32}P]-dCTP and 1 unit of Kienow polymerase (Pharmacia). The solution was then incubated at room temperature for 2 hr and the radiolabelled IFNγ DNA was subsequently purified using a Nick Column. Briefly, the solution was added to a Nick column pre-equilibrated with 3 ml of tris EDTA (TE) (10 mM Tris pH 8.0, 1 mM EDTA). Two 400 µl aliquots of TE were passed through the column, the second of which was collected, boiled for 3 min. and then stored on ice.

2.6.11 Hybridization and Washing

The membrane was removed from the prehybridization solution and transferred to the hybridization mixture consisting of 3X SSPE, 10% SDS, 0.5% Blotto and 0.5 mg/ml denatured salmon sperm DNA to which the oligolabelled probe was then added. The membrane was incubated overnight at 60°C and then washed in each of the following three solutions for 15 min. at room temperature; 2X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS; and 0.1X SSC, 0.1% SDS. The final wash of the membrane was in prewarmed 0.1 X SSC, 1% SDS at 55°C for 1 hr. The membrane was then dried at room temperature for 15 min. and exposed to Kodak XAR film at -70°C for 1 hr.

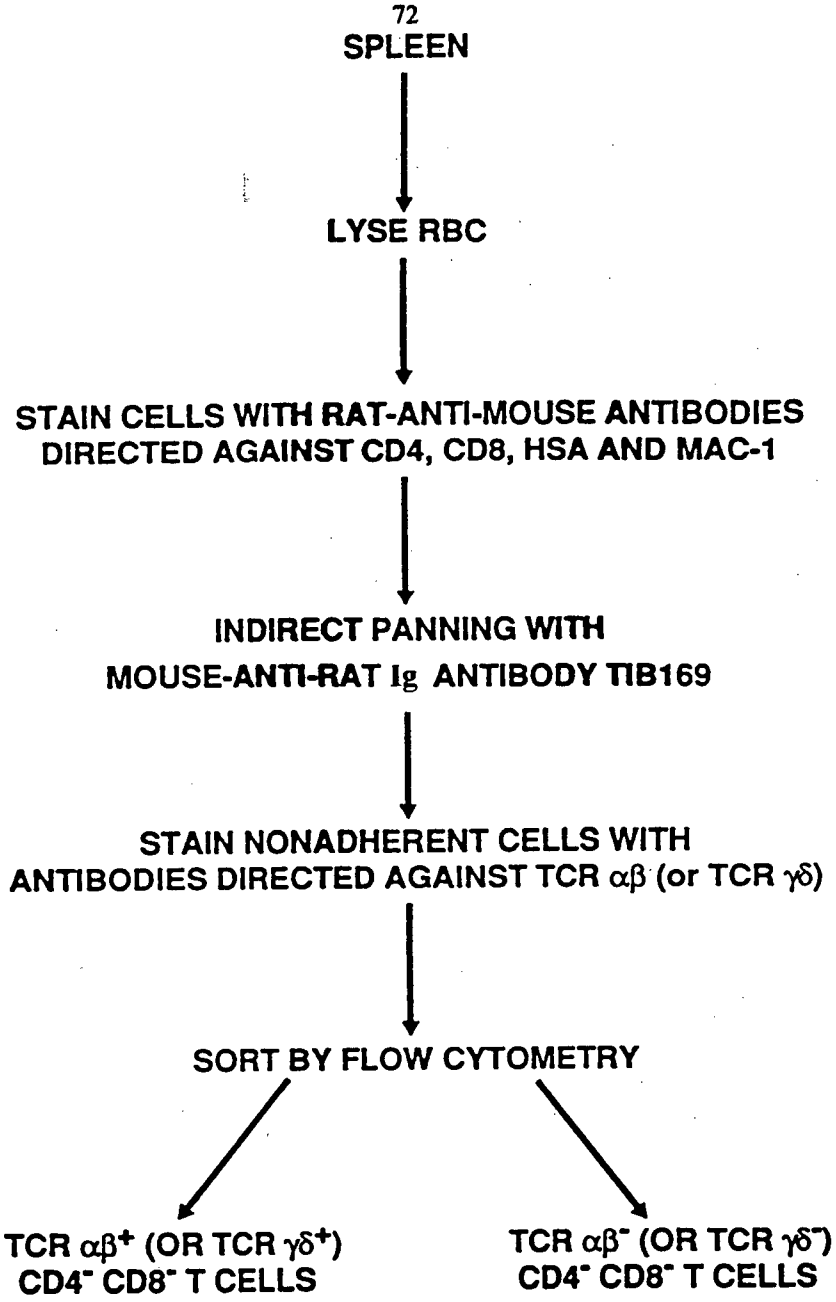

Figure 2 Procedure for the isolation of spleen cell subpopulations. Total spleen cells depleted of contaminating RBC were incubated with the rat-anti-mouse MAb specific for CD4, CD8, HSA and Mac-1. Positively stained cells were removed by two rounds of panning on mouse-anti-rat MAb coated plates. Nonadherent cells were restained with the above MAb and sorted by FACS. To further purify DNαβT cells, panned cells were also stained with MAb towards the TCR αβ prior to sorting into TCR $\alpha\beta^+$ and $\alpha\beta^-$ DN populations. This procedure was also used to purify DN cells expressing the alternate TCR γδ by including MAb towards this TCR in the staining step prior to sorting into TCR $\gamma\delta^+$ and $\gamma\delta^-$ DN subpopulations.

a) β Actin	5' primer	5'-GTG GGC CGC TCT AGG CAC CAA-3'
	3' primer	5'-CTC TTT GAT GTC ACG CAC GAT TTC-3'
b) IFNγ	5' primer	5'-GTG GGC CGC TCT AGG CAC CAA-3'
	3' primer	5'-CGA CTC CTT TTC CGC TTC CTG AG-3'
c) IL-2	5' primer	5'-ATG TAC AGC ATG CAG CTC GCA TC-3'
	3' primer	5'-GGC TTG TTG AGA TGA TGC TTT GAC A-3'
d) IL-4	5' primer	5'-ATG GGT CTC AAC CCC CAG CTA GT-3'
	3' primer	5'-GCT CTT TAG GCT TTC CAG GAA GTC-3'
e) IL-6	5' primer	5'-ATG AAG TTC CTC TCT GCA AGA GAC T-3'
	3' primer	3'-CAC TAG GTT TGC CGA GTA GAT CTC-5'

Figure 3 PCR cytokine oligonucleotide primers. Sequences for the 5' and 3' oligonucleotide primers used in the PCR amplification of a) β actin (Alonso *et al.* 1986), b) IFNγ (Gray and Goeddel, 1983) c) IL-2 (Kashima *et al.* 1985), d) IL-4 (Otsuka *et al.* 1987) and e) IL-6.

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CHAPTER III

RESULTS

3.1 IDENTIFICATION OF SPLENIC DNαβT CELLS

For the detection of splenic DNαβT cells, total nucleated spleen cells from young adult BALB/c mice were stained with MAb to CD4, CD8, HSA and Mac-1 recognized by FITC-conjugated anti-rat Ig κ (RG7/9.1) and biotinylated anti-TCR αβ recognized by SA-PE. MAb against CD4 and CD8 were used to remove mature SP splenic T cells. HSA was a convenient marker for this study as it is widely distributed on murine leukocytes including immature thymocytes (Crispe and Bevan, 1987), most B cells (Bruce *et al.* 1981, Takei *et al.* 1981) and the majority of myeloid cells (Bruce *et al.* 1981), yet is absent on mature peripheral T cells (Crispe and Bevan, 1987) and thymic DNαβT cells (Ceredig *et al.* 1987). Since initial FACS analysis revealed the presence of Mac-1$^+$ cells in the splenic TCR αβ$^+$ CD4$^-$ CD8$^-$ HSA$^-$ population, anti-Mac-1$^+$ antibody was included in subsequent studies. Based on two colour FACS analysis, DNαβT cells constituted only 0.1-0.3% of total nucleated spleen cells (Fig. 4). Following two rounds of panning, DNαβT cells represented approximately 1.4-2.5% of panned CD4$^-$ CD8$^-$ HSA$^-$ Mac-1$^-$ (DN) cells (Fig. 4). To further enrich for DNαβT cells, panned CD4$^-$ CD8$^-$ HSA$^-$ Mac-1$^-$ cells were subsequently isolated by FACS sorting. Reanalysis of the isolated cells (Fig.5) suggested that of the 0.7-1.1% of total splenic cells which expressed the DN phenotype, 17-22% were TCR αβ$^+$ whereas 21-25% were TCR γδ$^+$. Approximately 52-60% of DN spleen cells expressed the CD3 complex. The phenotype of the remaining CD3$^-$ DN was assessed (Fig. 6) in the hope of finding a distinct marker that could be used to deplete this population during the purification procedure. The remaining CD3$^-$ cells expressed significant levels of Pgp-1 (93.1%), T200 (99.8%) and LFA-1 (99.8%) whereas expression of ICAM-1, YE1/19 (PCI), YE1/22 (4F2), Lyt-1, B220 and surface Ig was minimal to absent. The CD3$^-$ DN population also consists two distinct populations based on either the expression or lack of expression of Thy-1.

3.2 CHARACTERIZATION OF SPLENIC DNαβT CELLS

3.2.1 Phenotypic analysis of Splenic DNαβT cells

Following identification, splenic DNαβT cells were then characterized with respect to their phenotype. As mentioned previously, splenic DNαβT cells lack the expression of HSA, a marker

characteristically found on immature thymocytes. Furthermore, when DNαβT were assessed with a number of MAb following isolation by panning and FACS sorting (Fig. 7), this splenic population was shown to express Lyt-1 (95.1%), Pgp-1 (90.9%), ICAM-1 (79.9%) and the transferin receptor (71.2%). Minimal to no expression of B220 (9.0%) and YE1/19 (plasma cell antigen) (18.0%) was observed.

3.2.2 Determination of $V_{\beta 8}$ Usage by Splenic DNαβT Cells

Thymic DNαβT cells are known to express an unusual TCR repertoire with preferential usage of $V_{\beta 8}$ genes (Fowlkes *et al.* 1987; Papiernik and Pontoux, 1990; Takahama *et al.* 1991). In order to determine whether splenic DNαβT share this characteristic $V_{\beta 8}$ skewing, $CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$ spleen cells were analysed (Fig. 8), following purification by panning and FACS sorting, with MAb KJ16 which recognizes T cell receptors bearing either $V_{\beta 8.1}$ or $V_{\beta 8.2}$ (Haskins *et al.* 1984). Approximately 11% of the DN spleen cells were $KJ16^+$ whereas 23% were TCR $\alpha\beta^+$. Therefore the frequency of $V_{\beta 8}$ usage amongst splenic DNαβT cells was approximately 48%. $V_{\beta 8}$ usage in DNαβT cells was then compared to that in mature SP T cells isolated by a nylon wool column method (Julius *et al.* 1973). In order to confirm that nylon wool purified cells were indeed SP T cells, the phenotype of this population was assessed. As can be seen in Fig. 9, the majority (94.5%) of nylon wool purified spleen cells do indeed express either CD4 or CD8 and are $Thy\text{-}1^+$(93.7%) HSA^- and $Mac\text{-}1^-$ (61%). Although 61% of nylon wool purified spleen cells are HSA^- $Mac\text{-}1^-$, approximately 39% of cells expressed low levels of either of these markers which may be accounted for in part by the presence of contaminating B cells. It has been previously shown that approximately 5-10% of nonadherent cells obtained following passage through a nylon column are indeed B cells (Julius *et al.* 1973). Furthermore a subpopulation of cells of the monocyte/macrophage lineage has been shown to express the CD4 molecule. In contrast to DNαβT cells, approximately 21% of nylon wool purified spleen T cells were $KJ16^+$ while 92% were TCR $\alpha\beta^+$. Therefore only 22% of the TCR $\alpha\beta^+$ T cells expressing either CD4 or CD8 used members of this $V_{\beta 8}$ family.

3.2.3 Proliferative Response of Splenic DNαβT cells to Cytokines

Further characterization of this splenic DNαβT cell population included an assessment of the response to antigen independent stimulation. Since $CD4^-$ $CD8^-$ thymocytes, including those which express the TCR αβ have been shown to respond to IL-7 in the absence of additional co-mitogen (Suda *et al.* 1990, Vissinga *et al.* 1992), the effects of this cytokine on splenic DNαβT cells were examined. Initially $CD4^-$ $CD8^-$ HSA^- spleen cells, isolated by panning and sorting, were cultured

with purified recombinant IL-7 for 72 hr and the proliferative response was assessed by ^{3}H-thymidine incorporation. As indicated in Table I, CD4$^-$ CD8$^-$ HSA$^-$ spleen cells were directly responsive to IL-7 and this responsiveness is comparable to that observed for CD4$^-$ CD8$^-$ thymocytes. However, when this population was further purified by the removal of Mac-1$^+$ cells, the response to IL-7 was significantly lower. The addition of IL-1, a known macrophage product, apparently restored this response. In order to confirm that the IL-7 responsive cells are indeed T cells and not other cells which may be present in the CD4$^-$ CD8$^-$ HSA$^-$ Mac-1$^-$ population such as pre-B cells, DN spleen cells were sorted into CD3$^+$ and CD3$^-$ subpopulations and were studied. As shown in Table I, only those cells expressing the CD3 complex were responsive to IL-7, and this response was enhanced by the addition of IL-1. Since the CD3 complex is co-expressed on T cells with the T cell receptor, the CD3$^+$ DN population was further subdivided into those DN cells expressing either the αβ or γδ TCR. Both of these TCR expressing T cell populations were stimulated by IL-7 and this response was enhanced by IL-1. In order to determine whether the proliferative response was due to the activation of the TCR-CD3 complex by the antibodies used in the purification procedure, both populations were sorted negatively. DNαβT cells, sorted negatively as TCR γδ$^-$ DN, proliferated in the presence of IL-7 and this response was significantly enhanced by IL-1. Reanalysis of TCR γδ$^-$ DN cells following sorting confirmed the presence of DNαβT cells (25-33%) (Fig. 10). Similarly, minimal change in the proliferative response was observed when TCR γδ$^+$ DN cells were sorted negatively as TCR αβ DN. Therefore the proliferative response observed for both TCR expressing populations was not influenced by the anti-TCR antibodies. Unlike DNαβT cells, the proliferation of nylon wool purified splenic T cells in the presence of IL-7 was minimal and was not influenced by the addition of IL-1.

3.2.4 Stimulation of Splenic DNαβT cells by anti-TCR crosslinking

Splenic DNαβT cells, which are HSA$^-$, are thought to be mature T cells since the loss of HSA expression is closely associated with the maturation of T lymphocytes (Crispe and Bevan, 1987). In order to assess the functional maturity of DNαβT cells, the effects of TCR cross-linking on this splenic subpopulation were determined by ^{3}H-thymidine incorporation assay. Initially, splenic DN T cells were isolated by panning followed by FACS sorting and were incubated in wells pre-coated with MAb against CD3. As shown in Table II, splenic DN T cells proliferate vigorously in the presence of immobilized anti-CD3, a response comparable to that of nylon wool purified mature SP T cells. When DN spleen cells were further divided into CD3$^+$ and CD3$^-$ subpopulations, only those DN cells expressing the TCR and therefore associated CD3 complex were responsive to anti-CD3 crosslinking. When the CD3$^+$ DN population was further subdivided into those expressing either the αβ or γδ TCR, an interesting difference was observed. DNαβT cells, sorted either

positively as TCR $\alpha\beta^+$ DN or negatively as TCR $\gamma\delta^-$ DN, vigorously proliferated in the presence of immobilized anti-CD3, a response comparable to that of nylon wool purified mature SP T cells. Although still capable of proliferating when cultured with immobilized anti-CD3 MAb, negatively sorted TCR $\gamma\delta^-$ DN were significantly less responsive than their positively sorted counterparts. This may be due to the fewer number of DN$\alpha\beta$T cells within the TCR $\gamma\delta^-$ DN population which, unlike the positively sorted subset, also contains $CD3^-$ DN cells which comprise approximately 67-75% of this negatively sorted population. In contrast, TCR $\gamma\delta^+$ DN cells, sorted either positively as TCR $\gamma\delta^+$ DN or negatively as TCR $\alpha\beta^-$ DN, were non-responsive to CD3 crosslinking. Interestingly, when this TCR $\gamma\delta^+$ subset is purified without the depletion of Mac-1^+ contaminating cells, TCR $\gamma\delta^+$ $CD4^-$ $CD8^-$ HSA^- cells appear to be capable of responding to crosslinking of the TCR associated CD3 complex. This restoration of responsiveness to anti-CD3 crosslinking is particularly evident when TCR $\gamma\delta^+$ cells, sorted negatively as TCR $\alpha\beta^-$ $CD4^-$ $CD8^-$ HSA^-, are not depleted of Mac-1^+ cells. Furthermore, the addition of IL-1 to TCR $\gamma\delta^+$ DN cells cultured with immobilized anti-CD3 appears to facilitate the response to TCR crosslinking thus suggesting a requirement for the presence of Mac-1^+ cells such as monocytes and macrophages and their IL-1 product for the antigen dependent activation of TCR $\gamma\delta^+$ DN subpopulation. The response of DN$\alpha\beta$T cells to anti-CD3 was also enhanced, by the addition of IL-1. Similar to their splenic counterparts, TCR $\alpha\beta^+$ DN thymocytes also respond to anti-CD3, although not to the same extent, and this response is enhanced by IL-1.

3.2.5 Stimulation of Splenic DN$\alpha\beta$T Cells by PMA and Ionomycin

Further analysis of the activation pathway (Table III) involved in the stimulation of DN$\alpha\beta$T cells revealed that this spleen cell population is responsive to PMA but not ionomycin alone. When cocultured in the presence of both PMA and ionomycin, DN$\alpha\beta$T cells vigorously proliferate, a response comparable to that observed in the presence of immobilized anti-CD3. In order to determine whether the antibodies used in the purification procedure influenced this response, DN$\alpha\beta$T cells were sorted negatively as TCR $\gamma\delta^-$ DN and assessed. As shown in Table 3, DN$\alpha\beta$T cells still respond to PMA and ionomycin but not PMA alone when sorted negatively as TCR $\gamma\delta^-$ DN. The phorbal ester PMA and the calcium ionophore ionomycin bypass the TCR to directly influence PKC and $[Ca^{+2}]_i$.

3.2.6 Cytokine mRNA expression by splenic DN$\alpha\beta$T cells

Since splenic DN$\alpha\beta$T cells are thought to be functionally mature, the capacity for cytokine production in response to stimulation was assessed by PCR amplification of cytokine specific

messages (Fig. 11). Therefore RNA was extracted from purified spleen cell populations either directly following sorting or after 16 hr incubation in wells precoated with anti-CD3 or containing media, IL-7 and/or IL-1. The extracted RNA was converted to cDNA by reverse transcription and cytokine specific messages were amplified by 30 cycles of PCR. Interestingly, IFNγ message was detected in freshly isolated DNαβT cells prior to stimulation. Following 16 hr incubation with either immobilized anti-CD3, or IL-7 alone or in combination with IL-1, IFNγ message detected but at lower levels. In the absence of stimulatory factors, IFNγ was not detected in cultured DNαβT cells. Southern hybridization (Fig. 12) confirmed the specificity of the amplified sequence. The pattern of IFNγ mRNA expression in TCR $\gamma\delta^-$ DN cells (Fig. 11) was similar to that of the positively sorted DNαβT cell population indicating that the anti-TCR antibodies used in the purification procedure had no influence on cytokine expression. Although only 30% of TCR $\gamma\delta^-$ DN cells were TCR $\alpha\beta^+$ (Fig. 10), the remaining 70% of cells which expressed neither TCR type were not responsible for the expression of the IFNγ message as indicated by the absence of cytokine specific bands for the $CD3^-$ DN.

In addition to IFNγ message, splenic DNαβT cells sorted either positively or negatively expressed IL-4 specific mRNA (Fig. 11) in response to anti-CD3. However, IL-4 message was not detected at Day 0 or following culture with IL-7 and/or IL-1 or with media alone. Furthermore, splenic DNαβT cells expressed neither IL-2 or IL-6 messages under the conditions tested.

Similar to DNαβT cells, nylon wool purified mature SP T cells also express mRNA specific for IFNγ and IL-4 in response to anti-CD3 crosslinking (Fig. 11). Unlike DNαβT cells, however, significant IFNγ mRNA was not detected in mature SP T cells following isolation but prior to stimulation, or after 16 hr incubation with immobilized anti-CD3 or IL-7 with or without IL-1. Although a very faint band corresponding to IFNγ mRNA can be seen for freshly isolated SP T cells this may be attributed to the presence of a small proportion of activated T cells expressing IFNγ mRNA within this normal splenic T cell population. Furthermore, a small proportion of DNαβT cells contained within the nylon wool purified T cell population may have also contributed to the expression of IFNγ messages detected by this sensitive assay. In addition, anti-CD3 stimulated mature SP T cells but not DNαβT cells also express IL-2 message.

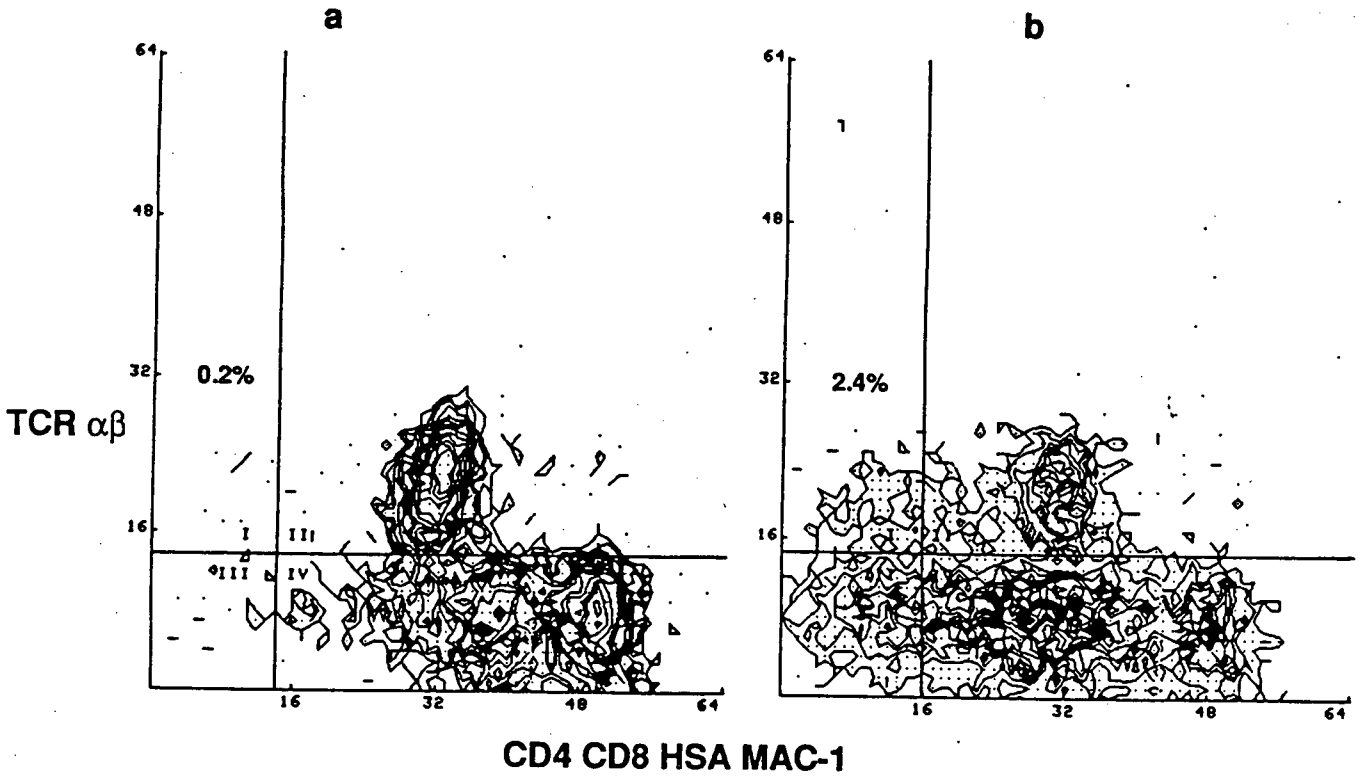

Figure 4 Identification of DNαβT cells in the spleen of normal mice. Spleen cells obtained from 5-6 week old BALB/c mice following RBC depletion (a) and following a subsequent two rounds of panning to remove those cells stained with the rat anti-mouse MAb against CD4, CD8, HSA and Mac-1 using plastic plates precoated with anti-rat Igκ (b) were stained with MAb to CD4, CD8, HSA and Mac-1 recognized by FITC-conjugated anti-rat Igκ (RG7/9.1) and biotinylated anti-TCR αβ followed by PE-conjugated streptavidin (SA-PE). Gates were set based on the negative control population of total nucleated spleen cells stained with both FITC-conjugated RG7/9.1 and SA-PE. DNαβT cells constitute 0.1-0.3% of total nucleated spleen cells and 1.4-2.5% of panned splenic T cells based on the results of three experiments.

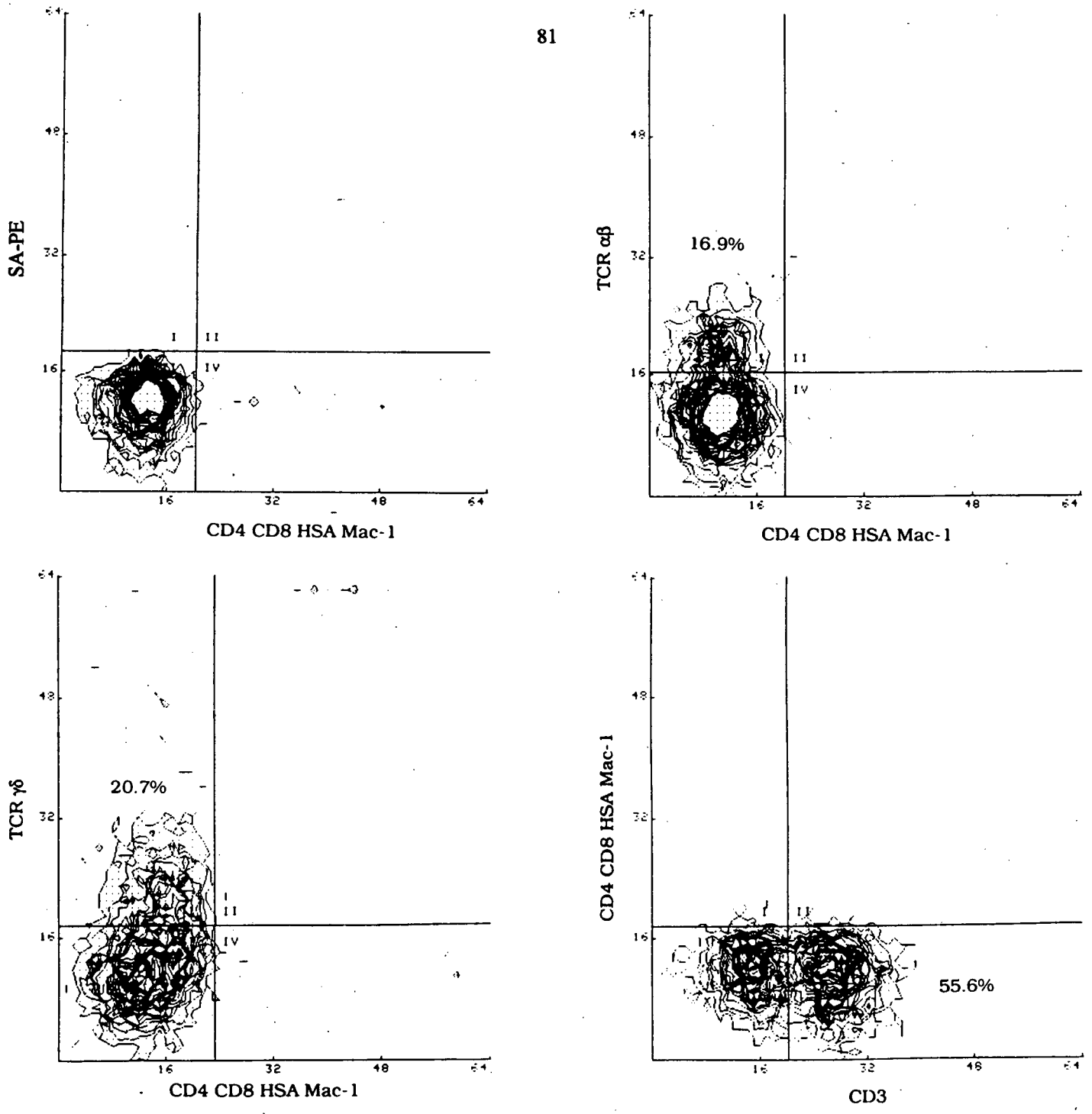

Figure 5 Phenotypic analysis of splenic DN cells. Nucleated spleen cells were depleted of $CD4^+$ $CD8^+$ HSA^+ Mac-1^+ cells by an indirect panning method followed by FACS sorting. Following FACS sorting, isolated DN cell were restained with the above MAb recognized by FITC-conjugated mouse anti-rat Ig MAb RG7/9.1 to assess purity as well as with SA-PE. Sorted DN cells were also stained with either biotinylated anti-TCR αβ or anti-TCR γδ plus SA-PE or FITC-conjugated anti-CD3 and analysed by FACS. Of the sorted DN spleen cells, 17-23% were TCR $\alpha\beta^+$, 21-25% were TCR $\gamma\delta^+$ and 52-60% were $CD3^+$ based on the results of three experiments.

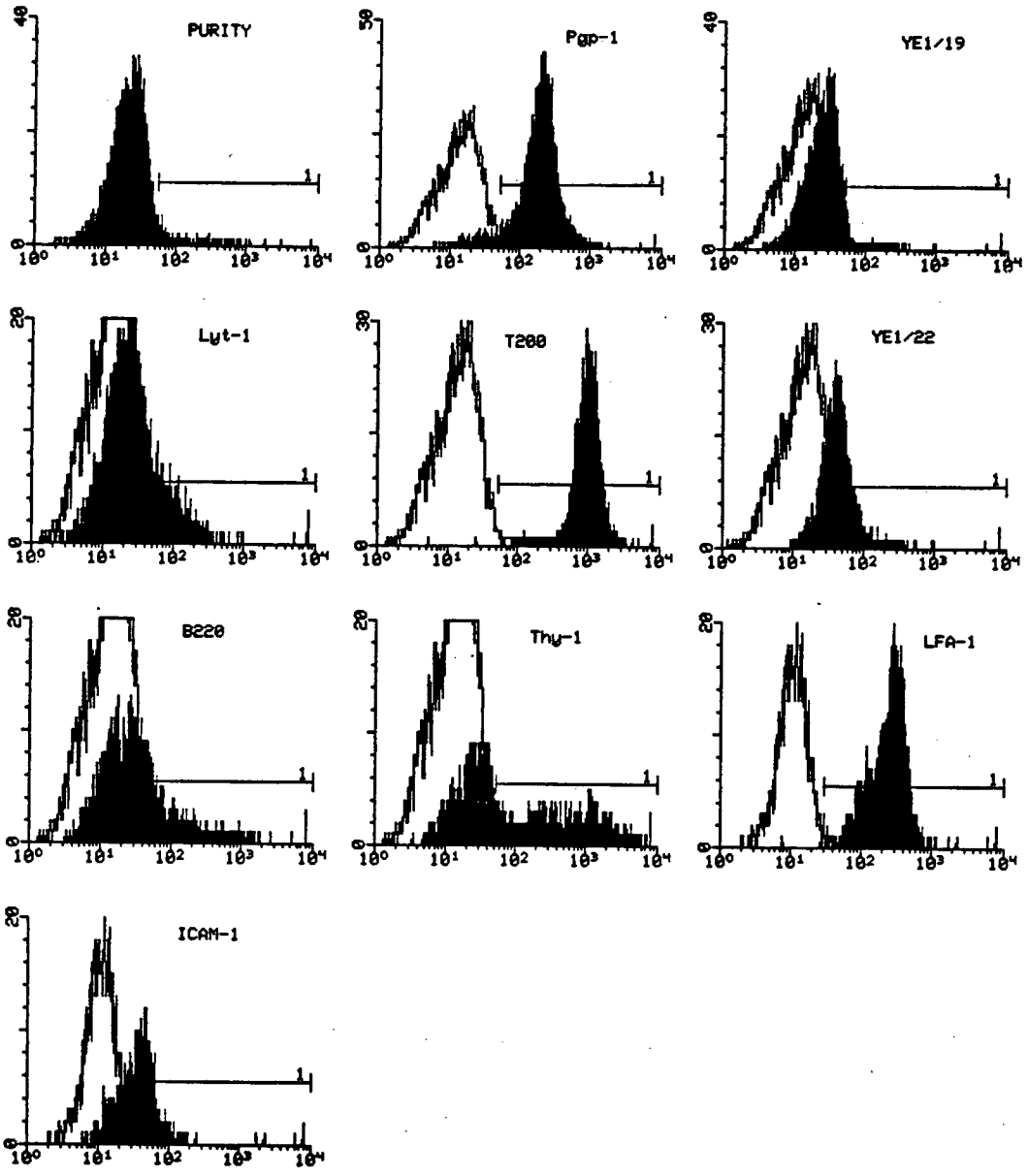

Figure 6 Phenotypic analysis of CD3$^-$ DN spleen cells. The purification procedure used to isolate CD3$^-$ DN spleen cells involved 2 rounds of indirect panning to remove those cells stained with rat anti-mouse MAb against CD4, CD8, HSA and Mac-1 using plastic plates precoated with anti-rat Igκ MAb. Panned cells were then restained with MAb against CD4, CD8, HSA and Mac-1 recognized by FITC-conjugated anti-rat IgκMAb as well as with FITC-conjugated anti-CD3MAb and the resulting negatively stained cells (CD4$^-$ CD8$^-$ HSA$^-$ Mac-1$^-$ CD3$^-$ or CD3$^-$ DN) were then isolated by FACS. Purified CD3$^-$ DN spleen cells were then stained by a number of MAb including Pgp-1, YE1/19, Lyt-1, T200, YE1/22, B220, Thy-1, LFA-1 and ICAM-1 recognized by FITC-conjugated anti-rat Igκ and analysed by FACS. Isolated CD3$^-$ DN cells were also restained with the same MAb used in the purification procedure (MAb against CD4, CD8, HSA, and Mac-1 recognized by mouse anti-rat Ig MAb RG7/9.1 and FITC-conjugated anti-CD3 MAb) in order to assess purity. In order to quantify positive staining, gates were set based on the negative control population of sorted cells stained with the FITC-conjugated second antibody (superimposed). The percentage of DN cells staining positively for the above MAb are indicated in brackets as follows: Pgp-1$^+$ (93.1%), YE1/19$^+$ (3.8%), Lyt-1$^+$ (18.7%), T200 (99.8%), YE1/22$^+$ (27.7%), B220$^+$ (24.9%), Thy-1$^+$ (47.1%), LFA-1$^+$ (99.8%) and ICAM-1 (61.7%).

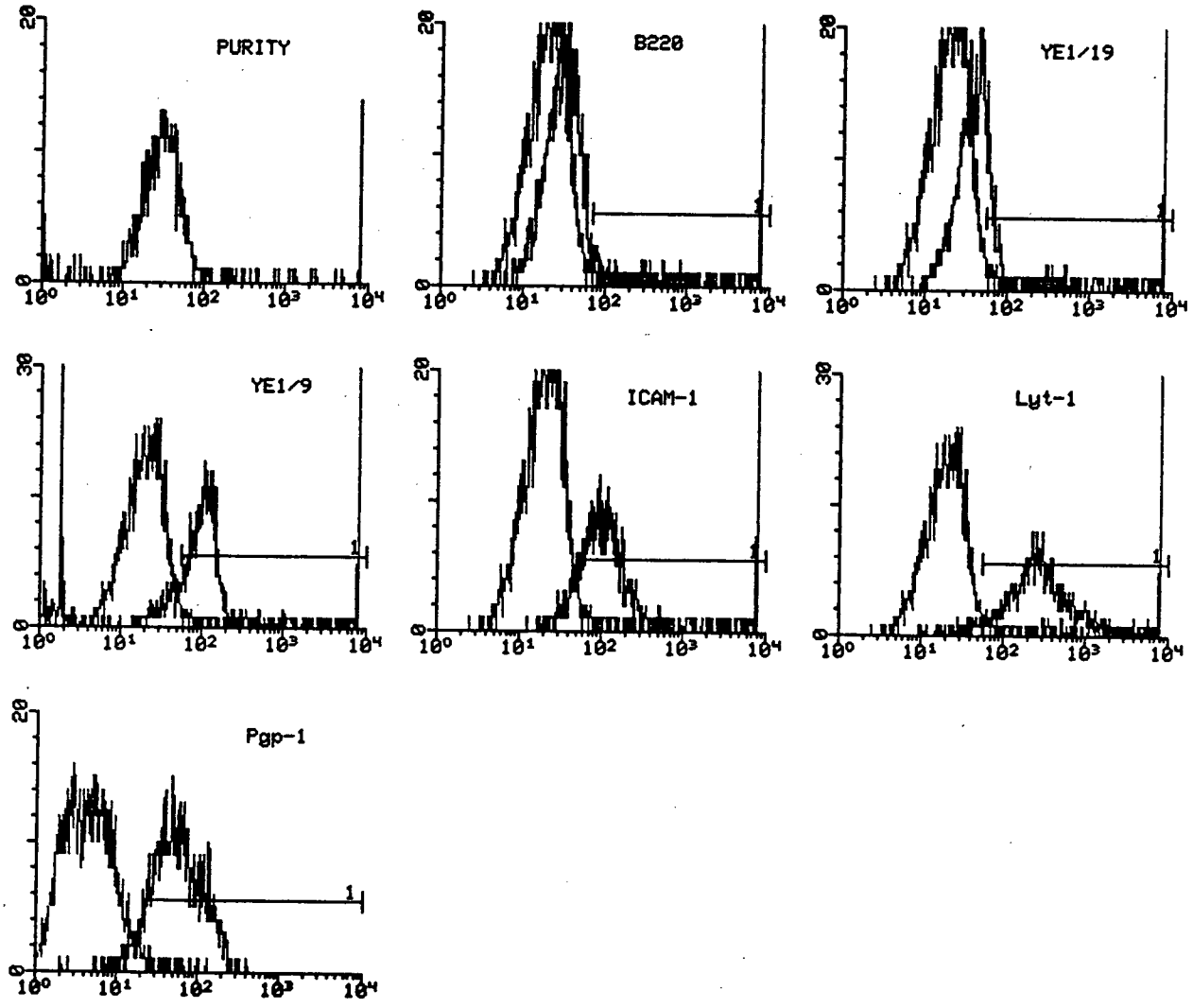

Figure 7 Phenotypic analysis of splenic DNαβT cells. The purification procedure used to isolate splenic DNαβT cells involved 2 rounds of panning to remove those cells stained with the rat anti-mouse MAb against CD4, CD8, HSA and Mac-1 using plastic plates precoated with anti-rat Igκ. Panned cells were then restained with MAb against CD4, CD8, HSA and Mac-1 recognized by FITC-conjugated anti-rat Igκ as well as biotinylated anti-TCR αβ recognized by SA-PE and those cells of the DNαβT cell phenotype (TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ HSA^- Mac-1) were isolated by FACS. Purified splenic DNαβT were stained by a number of MAb including B220, YE1/19, YE1/9, ICAM-1 and Pgp-1 recognized by FITC-conjugated anti-rat IGκ. Isolated DNαβT cells were also restained with the same MAb used in the purification procedure (MAb against CD4, CD8, HSA and Mac-1 recognized by mouse anti-rat Ig MAb and biotinylated anti-TCR αβ recognized by SA-PE) in order to assess purity. In order to quantify positive staining, gates were set based on the negative control population of sorted cells stained with the FITC-conjugated second antibody (superimposed). The percentage of DNαβT cells staining positively for the above MAb are indicated in brackets as follows: $B220^+$ (9.0%), $YE1/19^+$ (18.0%), $YE1/9^+$ (71.2 %), $ICAM\text{-}1^+$ (79.9 %), Lyt-1 (95.1 %) and $Pgp\text{-}1^+$ (90.9 %).

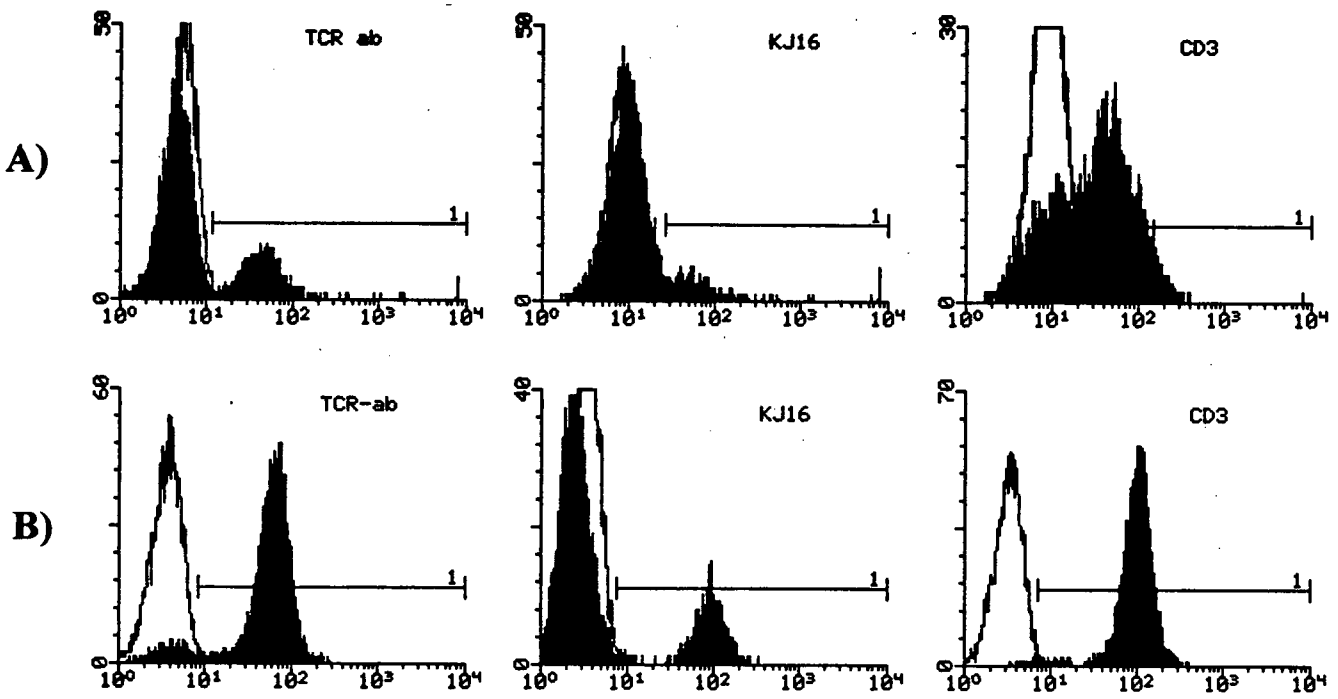

Figure 8 Determination of $V_{\beta 8}$ usage by splenic DN$\alpha\beta$T cells. A) DN ($CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$) spleen cells were isolated by two rounds of panning followed by sorting by FACS to deplete those cells stained by the rat anti-mouse MAb against CD4, CD8, HSA and Mac-1 recognized by FITC-conjugated anti-rat Igκ MAb. The purity of the sorted population was confirmed by FACS analysis of the cells restained with the above MAb. B) Mature SP (TCR $\alpha\beta^+$ $CD4^+$ $CD8^-$ or TCR $\alpha\beta^+$ $CD4^-$ $CD8^+$) splenic T cells were isolated by passage of nucleated spleen cells through nylon wool columns and subsequent collection of nonadherent cells. Sorted DN spleen cells or nylon wool nonadherent splenic T cells were then stained with either biotinylated anti-TCR $\alpha\beta$ recognized by SA-PE, anti-TCR $V_{\beta 8}$ (KJ16) recognized by FITC-conjugated anti-rat Igκ or FITC-conjugated anti-CD3 MAb. In order to quantify positive staining, gates were set based on the negative control population of sorted cells stained with SA-PE as well as second antibody. DN spleen cells analysed by FACS were 11.0% $KJ16^+$, 23.0% TCR $\alpha\beta^+$ and 57.6% $CD3^+$. Approximately 21% of nylon wool purified mature spleen T cell were $KJ16^+$ whereas 92.4% were TCR $\alpha\beta^+$ and 98.9% were $CD3^+$.

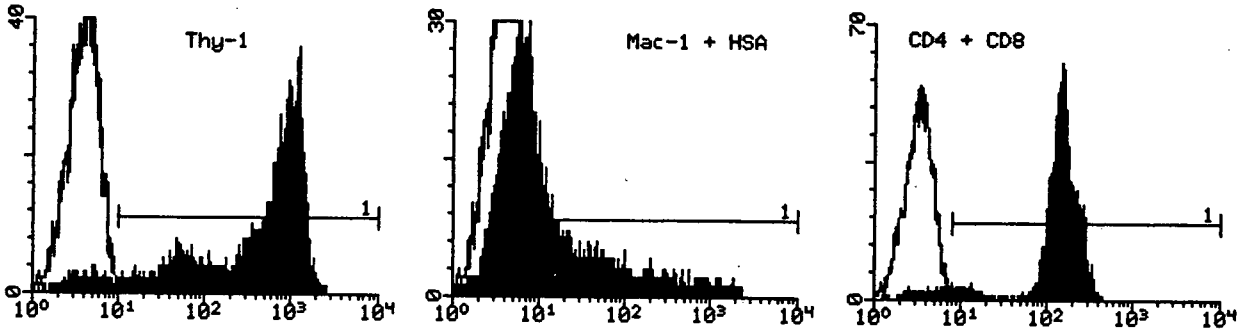

Figure 9 Confirmation that nylon wool purified cells are indeed SP T cells. Nylon wool purified T cells were restained with either Thy-1 or HSA and Mac-1 or CD4 and CD8 MAb recognized by FITC-conjugated anti-rat Igκ. In order to quantify positive staining, gates were set based on the negative control population of nylon wool purified spleen cells stained with the FITC conjugated second antibody (superimposed). Approximately 93.7% of nylon wool purified spleen cells were Thy-1^+ whereas 39.0% were HSA^+ and/or Mac-1^+ and 94.4 % were $CD4^+$ and/or $CD8^+$.

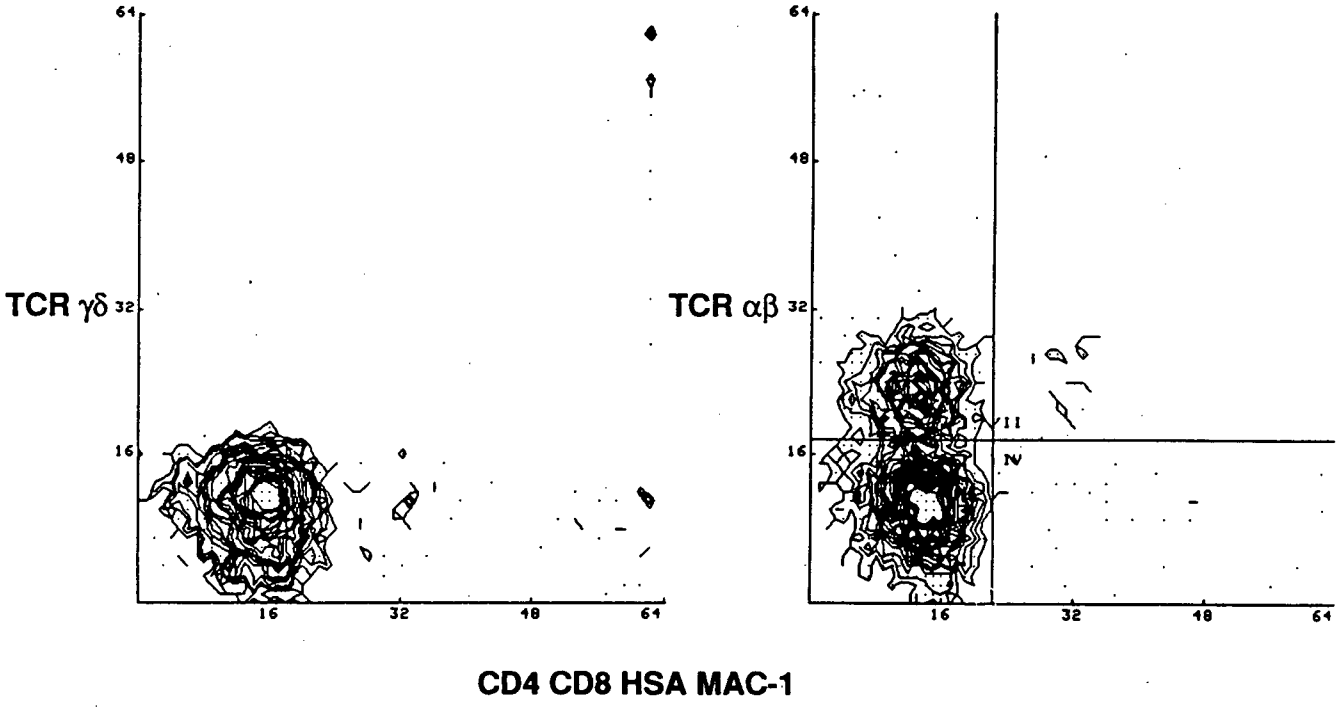

Figure 10 Presence of DNαβT cells in the TCR γδ⁻ DN spleen cell population. The purification of splenic TCR γδ⁻ DN cells involved 2 rounds of panning to deplete those cells stained by rat anti-mouse MAb against CD4, CD8, HSA and Mac-1 on plastic plates precoated with anti-rat Igκ MAb. Panned cells were then restained with the above rat anti-mouse MAb recognized by FITC-conjugated anti-rat Igκ MAb as well as biotinylated anti-TCR γδ MAb recognized by SA-PE and the resulting negatively stained cells (TCR γδ⁻ CD4⁻ CD8⁻ HSA⁻ Mac-1⁻ or TCR γδ⁻ DN) were isolated by cell sorting. Sorted TCR γδ⁻ DN cells were then restained with the above antibodies to assess purity as well as with biotinylated anti-TCR αβ MAb recognized by SA-PE and analysed by FACS. Approximately 25-33% of TCR γδ⁻ DN spleen cells expressed TCR αβ based on the results of three experiments.

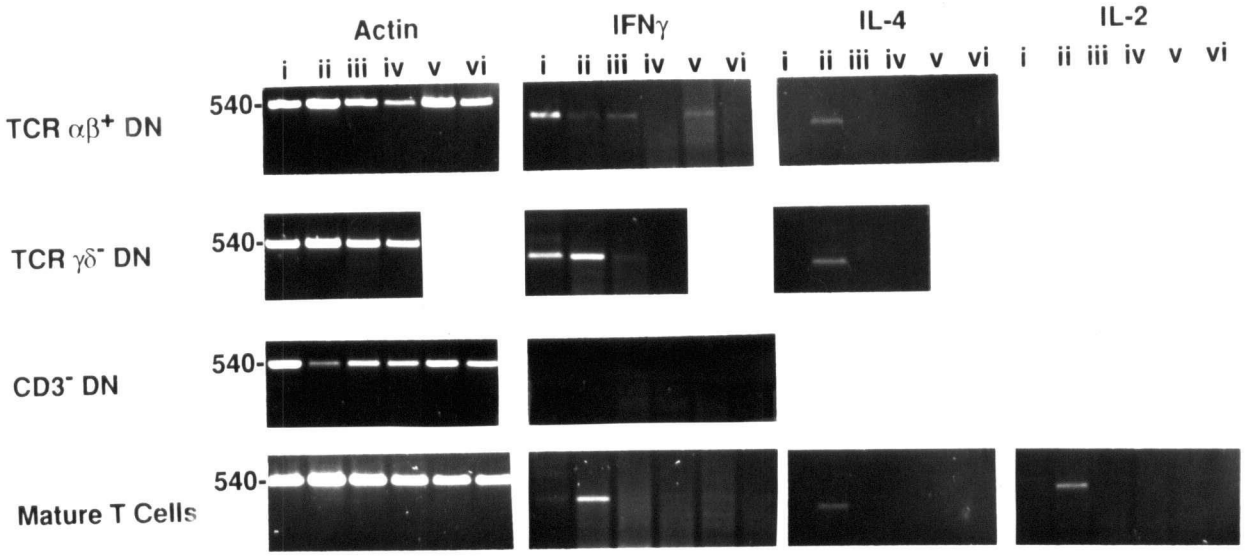

Figure 11 Lymphokine mRNA expression. Total cellular RNA was isolated from 1 X 10^5 sorted TCR $\alpha\beta^+$ DN, TCR $\gamma\delta^-$ DN, $CD3^-$ DN and nylon column purified mature spleen T cells following purification (i), or 16 hr incubation in round bottom microtitre wells with immobilized anti-CD3 MAb (ii), IL-7 + IL-1 (iii), media (iv), IL-7 (v) or IL-1 (vi). RNA was converted to cDNA and cytokine specific DNA fragments were amplified by 30 cycles of PCR with 21-mer oligonucleotide primers for IFNγ, IL-4, IL-2 and IL-6. PCR amplification of actin specific sequences was used as a positive control. Expected sizes of amplified fragments of actin, IFNγ, IL-2, IL-4 and IL-6 were 540, 460, 502, 399 and 638 bp respectively. The amplified DNA was separated by 1% agarose gel electrophoresis and stained with ethidium bromide.

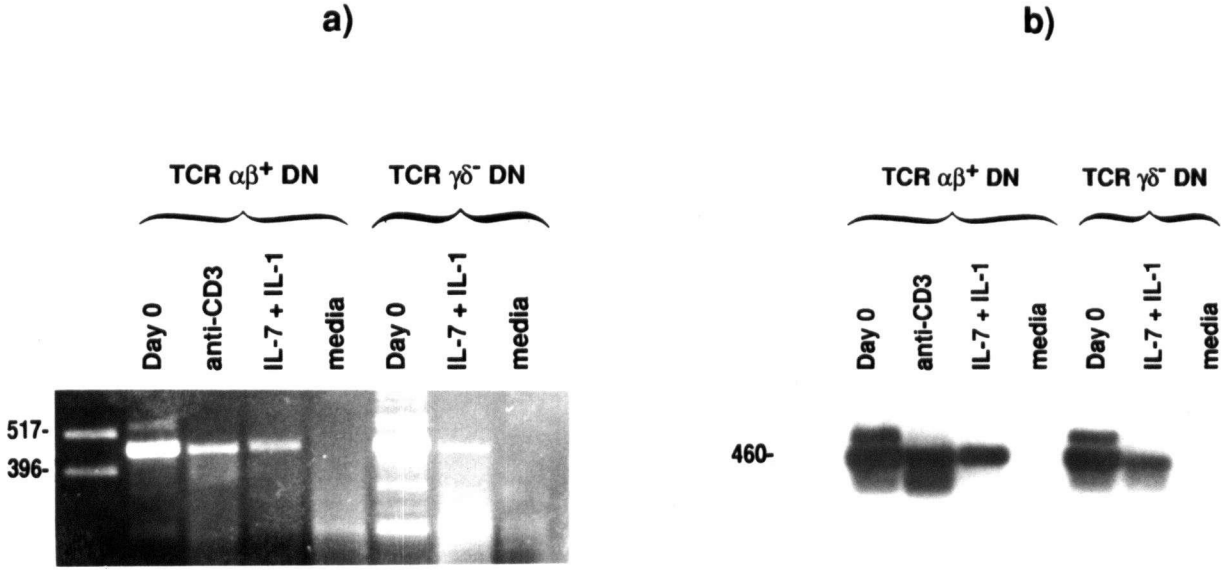

Figure 12 Confirmation of the specificity of the IFNγ message by Southern hybridization. Amplified DNA fragments were separated by 1% gel electrophoresis and transferred to a membrane by Southern blotting, followed by hybridization with labeled IFNγ cDNA probe and autoradiography.

TABLE I

CYTOKINE STIMULATION OF SPLEEN CELL SUBPOPULATIONS

Cell Type	# Cells/Well	^{3}H-Thymidine Incorporation (cpm)			
		Media	IL-7	IL-1	IL-7 + IL-1
Total Spleen	3.0×10^4	65 ± 26	197 ± 32	103 ± 40	182 ± 34
Splenic $CD4^- CD8^- HSA^-$	4.2×10^4	18 ± 2	11550 ± 2920		
Splenic DN*	5.0×10^4	61 ± 19	2952 ± 143	45 ± 15	8986 ± 882
$CD3^+$ DN	2.5×10^4	112 ± 4	7583 ± 665		
$CD3^-$ DN	2.5×10^4	198 ± 31	328 ± 46		
TCR $\alpha\beta^+$ DN	3.0×10^4	60 ± 24	2707 ± 285	43 ± 13	3648 ± 73
TCR $\gamma\delta^-$ DN	4.2×10^4	35 ± 1	2272 ± 229	74 ± 10	7068 ± 524
TCR $\gamma\delta^+$ DN	3.0×10^4	70 ± 6	6626 ± 175	161 ± 89	19611 ± 2588
TCR $\alpha\beta^-$ DN	5.0×10^4	45 ± 9	2471 ± 175	45 ± 6	10324 ± 519
DN Thymocytes	3.0×10^4	59 ± 9	12660 ± 211	1967 ± 521	25759 ± 2558
Nylon Wool Purified T Cells	5.0×10^4	130 ± 11	778 ± 41	118 ± 20	1044 ± 233

* $CD4^- CD8^- HSA^-$ Mac-1^-

** T cell populations were incubated in either media alone (RPMI 1640 + 5% FCS + 50 μm 2-ME) or in the presence of either 10 ng/ml IL-7 or 200 U/ml IL-1 or a combination of both for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 and subsequently pulsed with 1 μCi H^3-thymidine before being harvested and counted by a liquid scintillation counter. Each stimulation assay was performed a minimum of three times and the results shown are representative data from a single experiment.

TABLE II

ACTIVATION OF SPLEEN CELL SUBPOPULATIONS BY ANTI-CD3 CROSSLINKING

Cell Type	# Cells/Well	^{3}H-Thymidine Incorporation (cpm) Media	anti-CD3	IL-1	anti-CD3 + IL-1
Total Spleen	3.0×10^4	235 ± 32	40535 ± 5465		
Nylon Wool Purified T Cells	5.0×10^4	130 ± 11	38468 ± 1601		
Splenic DN*	5.0×10^4	67 ± 33	36536 ± 3889		
$CD3^+$ DN	2.5×10^4	112 ± 4	56319 ± 13104		
$CD3^-$ DN	2.5×10^4	198 ± 31	58 ± 24		
TCR $\alpha\beta^+$ DN	2.16×10^4	41 ± 11	57747 ± 7954	55 ± 17	130931 ± 6412
TCR $\gamma\delta^-$ DN	3.0×10^4	21 ± 6	7490 ± 3044	48 ± 8	12483 ± 1546
TCR $\gamma\delta^+$ DN	1.9×10^4	17 ± 5	38 ± 9	180 ± 135	10794 ± 1793
TCR $\alpha\beta^-$ DN	3.0×10^4	11 ± 7	218 ± 106	38 ± 24	17915 ± 2399
TCR $\gamma\delta^+$ $CD4^-$ $CD8^-$ HSA^-	1.0×10^5	36 ± 6	4444 ± 648		
TCR $\alpha\beta^-$ $CD4^-$ $CD8^-$ HSA^-	1.0×10^5	262 ± 34	67687 ± 8698		
TCR $\alpha\beta^+$ DN Thymocytes	2.2×10^4	23 ± 4	6831 ± 801	130 ± 54	67299 ± 7876

*$CD4^-$ $CD8^-$ HSA^- $Mac\text{-}1^-$

*T cell populations were incubated in either media alone (RPMI 1640 + 5% FCS + 50μm 2-ME) or with or without 200 U/ml IL-1 in wells previously coated with 145-2C11 anti-CD3 antibody for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 and subsequently pulsed with 1 μCi H^3-thymidine before being harvested and counted by a liquid scintillation counter. Each stimulation assay was performed a minimum of three times and the results shown are representative data from a single experiment.

TABLE III

ACTIVATION OF SPLENIC DN$\alpha\beta$T CELLS BY PMA AND IONOMYCIN

^{3}H-thymidine Incorporation (cpm)	TCR $\alpha\beta^+$ DN*	TCR $\gamma\delta^-$ DN**
media	26 ± 14	21 ± 6
anti-CD3	35168 ± 16258	7490 ± 3044
IL-1	133 ± 58	48 ± 8
anti-CD3 + IL-1	54974 ± 11973	12483 ± 1546
PMA	5094 ± 2172	84 ± 13
ionomycin	173 ± 116	53 ± 10
PMA + ionomycin	76872 ± 24274	47841 ± 10624

*TCR $\alpha\beta^+$ DN 1.4 X 10^4 cells/ well

**TCR $\gamma\delta^-$ DN 3.0 X 10^4 cells/well

***T cell populations were incubated with either media alone (RPMI 1640 + 5% FCS + 50μm 2-ME), with or without 200 U/ml IL-1 in wells previously coated with 145-2C11 anti-CD3 antibody, or in the presence of either 10 ng/ml PMA ± 600 nM ionomycin for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 and subsequently pulsed with 1 μCi H^3-thymidine before being harvested and counted by a liquid scintillation counter. Each stimulation assay was performed a minimum of three times and the results shown are representative data from a single experiment.

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CHAPTER IV

CONCLUSIONS AND DISCUSSION

A unique class of T lymphocytes has been identified in the spleen of normal mice. This novel T cell population, which expresses the αβ TCR/CD3 complex yet lacks the CD4 and CD8 coreceptor molecules as well as HSA and Mac-1, constitutes 0.1-0.3% of total nucleated spleen cells. Of the approximately 0.7-1.1% of nucleated spleen cells which lack CD4 and CD8, 17-22% express the TCR αβ. Furthermore, of the 52-60% of DN spleen cell which express the CD3 complex, approximately 30% are of the TCRαβ$^+$ lineage whereas approximately 3·7% express the alternate TCR γδ heterodimers. Phenotypic analysis of splenic DNαβT cells demonstrated the expression of Lyt-1, Pgp-1, ICAM-1 and the transferrin receptor whereas minimal to no B220 and YE1/19 expression was observed. Amongst splenic DNαβT cells there is a high frequency of $V_{\beta 8}$ usage (approximately 48%) . This DNαβT cell population proliferates in the presence of IL-7 and this response is enhanced by the addition of IL-1. Furthermore, splenic DNαβT cells are responsive to MAb which crosslink the TCR associated CD3 complex as well as reagents which bypass the TCR to activate intracellular events. Expression of cytokine mRNA, either constitutively as for IFNγ or following TCR activation as for IL-4, suggests an apparent capacity for lymphokine production by this splenic DNαβT cell population.

Although thymocytes of the DNαβT phenotype have been the subject of extensive studies, only recently has attention been turned towards their peripheral counterparts. DNαβT cells have been identified in normal human peripheral blood (Shivakumar *et al.* 1989; Quaratino *et al.* 1991; Niehues *et al.* 1994) as well as murine bone marrow (Sykes, 1990; Levitsky *et al.* 1991), lymph nodes (Guidos *et al.* 1989; Huang and Crispe, 1992) and liver (Seki *et al.* 1991; Ohteki *et al.* 1992). As shown in this thesis, DNαβT cells do exist in the spleen of normal mice and constitute 0.1 to 0.3% of total nucleated spleen cells. The existence of splenic DNαβT cells has since been confirmed by several other studies. According to Prud'homme *et al.* (1991), less than 1% of murine spleen cells were of the TCR αβ$^+$ CD4$^-$ CD8$^-$ phenotype. Similar to the results shown in Figure 7, this splenic DNαβT cell population expressed significant levels of Ly-1 as well as Thy-1 whereas only minimal expression of B220, Mac-1, J11d and Ia was observed. In a second study, Levitsky *et al.* (1991) reported that approximately 0.5 to 2.0% of B cell and macrophage depleted spleen cells isolated from C57BL6 mice were of the TCR αβ$^+$ CD4$^-$ CD8$^-$ phenotype. Furthermore, this splenic DNαβT cell population was also shown to express a 39 kDa surface molecule referred to as NK1, initially considered to be specific for NK cells (Sentman *et al.* 1989). Palathumpat *et al.* (1992) demonstrated that the percentage of DNαβT cells increased from 4 to 13

% when spleen cells isolated from the C57BL/K9 strain were further purified on a Percoll density gradient into fractionated low density cells. Although unsuccessful at the isolation of DNαβT cells in the spleens of normal BALB/c and B6 mice, Kubota *et al.* (1992) demonstrated that IL-3 induced the generation of DNαβT cells from the $CD4^+/CD8^+$ T cell-depleted spleen as well as bone marrow of both normal and nude BALB/c mice in primary short term cultures. A significant proportion of these IL-3 induced splenic as well as bone marrow DNαβT cells were shown to express the products of $V_{\beta 2}$ genes. Freshly isolated splenic DNαβT cells, on the other hand, showed preferential usage of $V_{\beta 8}$ genes as demonstrated in Figure 8 and since confirmed by Zlotnik *et al.* (1992).

Splenic DNαβT cells are considered to be functionally mature as suggested by the absence of expression of the heat stable antigen, a marker characteristically found on immature thymocytes yet absent on mature peripheral T cells (Crispe and Bevan, 1987). In addition, splenic DNαβT cells express a functional TCR which can be activated using either anti-TCRαβ MAb or the lectin Concanavalin-A (Con-A) (Prud'homme *et al.* 1991), or by crosslinking of the TCR associated CD3 complex with anti-CD3 MAb (Table II). It is possible that TCR mediated activation of this splenic T cell subset involves signal transduction through the phosphoinositol (PI) pathway as suggested by the responsiveness of splenic DNαβT cells to the combination of PMA and ionomycin (Table III). PMA is a phorbol ester which activates PKC whereas ionomycin is a calcium ionophore which induces an increase in $[Ca^{+2}]_i$, both of which are key events in the PI second messenger pathway. Furthermore splenic DNαβT cells are considered to be functionally mature based on their capacity to produce cytokines. As shown in Figure 11, splenic DNαβT cells expressed IFNγ and IL-4 specific mRNA but neither IL-2 nor IL-6 messages in response to crosslinking of the TCR associated CD3 complex. A recent study has confirmed both IFNγ and IL-4 production as well as TNF-α synthesis following anti-CD3 crosslinking by splenic DNαβT cells with the use of *in vitro* bioassays (Zlotnik *et al.* 1992). Furthermore, IL-2 production was not observed under this condition. Unlike SP T cells which proliferate following IL-2 receptor expression and production of the corresponding cytokine as a consequence of activation of the PI pathway (Weiss *et al.* 1984; Truneh *et al.* 1985), the absence of IL-2 production following TCR-CD3 activation of splenic and thymic DNαβT cells suggests that the activation of this T cell population may involve an alternate cytokine signalling pathway such as IL-4. To further elucidate the role of IL-4 in TCR signalling of DNαβT cells, additional studies are required including the determination of whether MAb directed against IL-4 are capable of blocking the proliferative response of this T cell population to TCR-CD3 activation. Recently, however, Prud'homme *et al.* (1991) have detected not only IL-4 but also IL-2 production by splenic DNαβT cells following activation either by anti-TCR αβ MAb or Con A, as determined by the IL-2/IL-4 sensitive cell lines CTLL-2 and CT4S. The absence of

CD4 and CD8 expression by DNαβT cells further suggests that the TCR signalling pathway employed in this T cell subpopulation may differ from that of classical SP T cells whose CD4 or CD8 coreceptors have been shown to play a role in T cell activation including signal transduction via an associated cytoplasmic tyrosine kinase $p56^{lck}$ (Veillette *et al.* 1988) as well as augmentation of T cell recognition by binding to monomorphic determinants on class II and class I MHC molecules respectively (Hollander, 1982; Biddison *et al* 1984). In order to further elucidate the mechanism of TCR signalling in DNαβT cells additional studies could therefore include the assessment of $p56^{lck}$ expression and protein-tyrosine kinase activity in this T cell population and the possible association of this particular member of the *src* family with alternative membrane bound molecules.

Splenic DNαβT cells may represent a population of previously activated T cells. As shown in Figure 7, this splenic subpopulation expresses significant levels of Pgp-1, an adhesion marker previously shown to be upregulated on activated lymphocytes (Budd *et al.* 1987). In addition, approximately 70% of splenic DNαβT cells express the transferrin receptor, recognized by the MAb YE1/9, which has been previously shown to be expressed on essentially all proliferating cells including activated T lymphocytes (Takei, 1983; Trowbridge and Omary, 1982; Sutherland *et al.* 1981). Furthermore, approximately 80% of splenic DNαβT cells were shown to express ICAM-1, the 86-114 kd glycosylated ligand for LFA-1. Although ICAM-1 is typically expressed at low levels on resting peripheral blood lymphocytes as well as nonhematopoietic cells, the expression of this adhesion molecule is upregulated following activation in the presence of cytokines including IFNγ, IL-1 and TNF (Dustin *et al.* 1986; Dustin *et al.* 1988). Phenotypically similar cells isolated from human peripheral blood have also been shown to express markers consistent with previous antigen encounter and activation including CD45RO and HLA-DR respectively (Brooks *et al.* 1993). Therefore the previous activation of splenic DNαβT may provide an explanation as to why this T cell subset constitutively expresses IFNγ mRNA (Figure 11). IFNγ specific mRNA was detected in freshly isolated DNαβT cells and expression of this cytokine mRNA was dependent only on the viability of this splenic subpopulation. Being previously activated may also enable splenic DNαβT cells to proliferate in response to IL-7 in the absence of added comitogens (Table I). SP T cells, on the other hand, do not proliferate in response to IL-7 alone (Table I) but require the addition of either PMA or ConA to IL-7 cultures (Morrisey *et al.* 1989; Chazen *et al.* 1989). Welch *et al.* (1989) have previously shown that memory ($CD45^-$) T cells were significantly more responsive to IL-7 than unprimed ($CD45^+$) T cells. The question remains as to the phenotype of the splenic DNαβT cells prior to their activation and thus the origin of this unique T cell population.

A number of characteristics of splenic DNαβT cells suggest that this peripheral T cell population may be related to the subset of DN thymocytes which also express the TCRαβ and have been shown to constitute 2-30% of the thymic DN subpopulation depending on the mouse strain studied (Fowlkes *et al.*1987; Howe and MacDonald, 1988; Wilson *et al.* 1988; Levitsky *et al.* 1991). Indeed the similarities in surface phenotype extend beyond the expression of the TCR αβ and lack of CD4 and CD8 coreceptors. Splenic DNαβT cells and their thymic counterparts both lack expression of the HSA, a marker characteristically found on immature thymocytes yet absent on mature peripheral T cells (Crispe and Bevan, 1987). Furthermore, a high percentage (90.9 %) of splenic DNαβT cells express Pgp-1 (CD44) (Figure 5), a marker also identified on the majority of TCR $\alpha\beta^+$ DN thymocytes (Suda and Zlotnik, 1993). Both the loss of HSA and subsequent expression of Pgp-1 have been shown to occur following the acquisition of the TCR/CD3 complex at a stage when TN thymocytes branch off from the classical differentiation pathway to become $CD3^+$ DN T cells. Others have shown that splenic DNαβT cells and their thymic counterparts also share the expression of NK1.1, a marker originally thought to be expressed exclusively on the natural killer cell lineage (Levisky *et al.* 1991). Recently it has been shown that the majority of thymic DNαβT cells express CD38 (Zlotnik and Bean, 1994). Unlike the majority of murine thymocytes which are $CD38^-$, approximately 80% of those thymocytes which express CD38 are of the $CD4^-$ $CD8^-$ TCR $\alpha\beta^+$ phenotype with the remaining 20% of $CD38^+$ cell being $CD8^+$. It has therefore been suggested that the CD38 molecule may be a unique marker of thymic DNαβT cells. Future studies demonstrating CD38 expression on splenic DNαβT cells would therefore provide further support for a developmental relationship between this population and the phenotypically similar thymocytes.

$V_{\beta 8}$ overexpression observed in splenic DNαβT cells is also a characteristic of thymic TCR $\alpha\beta^+$ DN cells. Approximately 48% of splenic DNαβT cells in BALB/c mice expressed either $V_{\beta 8.1}$ or $V_{\beta 8.2}$, whereas the frequency of $V_{\beta 8}$ usage amongst nylon wool purified SP T cells was only 22% (Figure 8). Similarly, TCR $\alpha\beta^+$ DN thymocytes have also been shown to predominantly express the products of the $V_{\beta 8}$ genes in at least a twofold higher frequency than in SP T cells (Fowlkes *et al.* 1987; Ceredig *et al.* 1987; Papiernik and Pontoux, 1990; Takahama *et al.* 1991; Zlotnik *et al.* 1992; Suda and Zlotnik, 1993). This $V_{\beta 8}$ overexpression was only observed after the first 5 weeks of life and appeared to be superimposed on the developmental appearance of other TCR $\alpha\beta^+$ $V_{\beta 8}^-$ DN thymocytes. Although the $V_{\beta 8}$ family consists of three closely related genes, namely $V_{\beta 8.1}$, $V_{\beta 8.2}$ and $V_{\beta 8.3}$, the disproportionately high frequency of $V_{\beta 8}^+$ thymocytes has been attributed to the overexpression primarily of $V_{\beta 8.2}$. In contrast, approximately 20% of SP thymocytes and peripheral T cells express $V_{\beta 8}$. The bias of TCR $\alpha\beta^+$ DN thymocytes and splenic T cells towards $V_{\beta 8}$ expression suggests a possible role for specific selection processes,

whether positive or negative, in the shaping of the TCR repertoire of this population. Unlike HSA$^-$ TCR $\alpha\beta^+$ DN thymocytes, whose $V_{\beta 8.2}$ usage was more than three fold compared to SP thymocytes in BALB/c mice, $V_{\beta 8.2}^+$ thymocytes of the HSA$^+$ TCR $\alpha\beta^+$ DN phenotype have been shown to be markedly reduced to proportions comparable to that of SP thymocytes (Suda and Zlotnik, 1993). As HSA$^-$ TCR$\alpha\beta^+$ DN thymocytes are considered to be the progeny of their HSA$^+$ precursors, it was therefore suggested that the $V_{\beta 8.2}$ overexpression observed for HSA$^-$ TCR $\alpha\beta^+$ DN thymocytes was as a result of positive selection with the HSA$^+$ TCR $\alpha\beta^+$ DN subset regarded as a preselection population. A recent study has suggested that the repertoire of TCR $\alpha\beta^+$ DN thymocytes may be influenced by positive selection events. Bix *et al.* (1993) observed a more than ten fold reduction in frequency of $V_{\beta 8.2}^+$ DN thymocytes in class I MHC deficient (β_2 microglobulin$^-$ or β_2m^-) mice relative to the β_2m^+ strain. Furthermore, overexpression of $V_{\beta 8}$ amongst TCR $\alpha\beta^+$ DN thymocytes was shown to be dependent on class I MHC expression by hematopoietic cells as opposed to thymic epithelial cells as required for selection of SP T cells. Analysis of mice deficient for class II MHC expression due to a disrupted Aβ gene revealed only a modest reduction ($\simeq$ 40 to 50%) of the frequency of TCR $\alpha\beta^+$ DN thymocytes, an effect not restricted to $V_{\beta 8}^+$ cells. Interestingly, the description of a human DN$\alpha\beta$T cell line specific for the class I-like CD1 molecules prompts the question as to whether this T cell subset undergoes positive selection by nonpolymorphic class I molecules (Porcelli *et al.* 1989). Therefore, similar to their thymic counterparts, the biased $V_{\beta 8}$ repertoire expressed by splenic DN$\alpha\beta$T cells may suggest prior influence by positive selection events. However, employing H-2^q mice, known to selectively increase the expression of $V_{\beta 17a}^+$ CD4$^+$ T cells in the absence of I-E, Huang and Crispé (1992) were unable to demonstrate the influence of positive selection on the usage of the alternate V_β genes in peripheral DN$\alpha\beta$T cells. Although mature SP T cells expressing either $V_{\beta 8.1}$ or $V_{\beta 6}$ are reactive towards Mls^a determinants and therefore deleted as a consequence of negative selection in Mls^{a+} strains, frequencies of TCR $\alpha\beta^+$ DN thymocytes expressing either $V_{\beta 8.1}$ or $V_{\beta 6}$ have not been shown to differ significantly in Mls^{a+} versus Mls^{a-} mice (Egerton and Scollay, 1990; Takahama *et al.* 1991). Therefore it was suggested that $V_{\beta 8}$ overexpression was not a consequence of self tolerance. Furthermore, the majority of thymocytes as well as peripheral T cells expressing the transgenic anti-male TCR in male transgenic mice were of the DN phenotype (Kisielow *et al.* 1988; Teh *et al.* 1989). This apparent escape from clonal deletion is not thought to be attributed to the absence of CD8 expression since the majority of thymocytes and peripheral T cells expressing both anti-male TCR and CD8.1 transgenes in double transgenic mice lacked expression of both CD4 and the endogenous CD8.2 and were therefore considered to be DN$\alpha\beta$T cells (Robey *et al.* 1992). It has therefore been suggested that TCR $\alpha\beta^+$ DN cells may be intrinsically indifferent negative selection. However introduction of the bacterial superantigen SEB into the neonatal differentiation environment of both

CBA/J and B6 strains, resulted in the markedly reduced frequency of $V_{\beta 8.1}$, $V_{\beta 8.2}$ and $V_{\beta 8.3}$ usage amongst DN thymocytes. Furthermore, TCR $\alpha\beta^+$ DN HSA^- thymocytes expressing either $V_{\beta 3}$, $V_{\beta 11}$ or $V_{\beta 17a}$, are thought to be influenced by negative selection as suggested by the significant reduction of DN thymocytes bearing either of these V_β TCRs in I-E^+ mice relative to I-E^- strains (Takahama *et al.* 1991; Suda and Zlotnik, 1993). Interestingly, the precursors of TCR $\alpha\beta^+$ DN HSA^- thymocytes, namely TCR $\alpha\beta^+$ DN HSA^+ thymocytes, of I-E^+ mice have been shown to have similar frequencies of $V_{\beta 3}$ and $V_{\beta 11}$ usage as do SP T cells of I-E^- strains suggesting that the negative selection of $V_{\beta 3}^+$ and $V_{\beta 11}^+$ DN thymocytes may have occurred during the transition from the HSA^+ to HSA^- phenotype. With respect to TCR V_β expression in peripheral DN$\alpha\beta$T cells, Prud'homme *et al.* (1991) have observed low numbers of $V_{\beta 5}^+$, $V_{\beta 6}^+$ and $V_{\beta 11}^+$ cells of the DN phenotype in the spleens of I-E^+ Mls-1^{a+} mice which were comparable to those obtained for untreated splenic T cells. Bone marrow DN$\alpha\beta$T cells, in contrast resist clonal deletion of their $V_{\beta 3}^+$, $V_{\beta 5}^+$, $V_{\beta 8.1}^+$ and $V_{\beta 11}^+$ cells in murine strains where SP T cell expressing any of these V_β genes are deleted (Martinez-A. *et al.* 1993). Although similar to TCR $\alpha\beta^+$ DN thymocytes in their resistance to the negative selective influences of Mls-1^a superantigen on $V_{\beta 6}$ and $V_{\beta 8.1}$ usage, Huang and Crispe (1992) have shown that the percentage of lymph node DN$\alpha\beta$T cells expressing either $V_{\beta 5}$, $V_{\beta 11}$ or $V_{\beta 17a}$ was not reduced in I-E^+ strains. Furthermore, $V_{\beta 17a}^+$ cell of the DN$\alpha\beta$T phenotype were actually found to be enriched in the lymph nodes of I-E^+ mice. However, significant individual variation in V_β expression amongst lymph node DN$\alpha\beta$T cells must be taken into consideration before conclusions may be drawn from this study. Analysis of DN$\alpha\beta$T cells isolated from human peripheral blood has also shown preferential V_β expression, namely $V_{\beta 2}$, $V_{\beta 8}$, $V_{\beta 11}$, $V_{\beta 13}$ or $V_{\beta 19}$, from multiple unrelated donors unlike SP T cells (Porcelli *et al.* 1993; Brooks *et al.* 1993; Niehues *et al.* 1994). Therefore both the lack of CD4 and CD8 expression as well as the characteristic skewing of the V_β repertoire including potentially autoreactive V_β genes not observed for SP T lymphocytes suggests that cells of the TCR $\alpha\beta^+$ DN phenotype may be governed by selection mechanisms distinct from those influencing the development of conventional T cells.

In addition both splenic as well as thymic TCR $\alpha\beta^+$ DN cells are responsive to antigen independent stimulation by IL-7. Although initially identified as a product of stromal cells providing support for the growth of B cells, a role for IL-7 in the development of T cells has since been suggested based on the detection of high levels of IL-7 mRNA in the murine thymus (Henney, 1989) as well as the demonstration of responsiveness to this cytokine by the $CD4^-$ $CD8^-$ subset of thymocytes (Murray *et al.* 1989) . It has been subsequently shown that IL-7 plays a dual role in terms of its effect on this DN thymocyte population (Vissinga *et al.* 1992; Suda and Zlotnik, 1991). More specifically, IL-7 maintains the viability of the $CD3^-$ $CD4^-$ $CD8^-$ subset and therefore allows

this thymic population to undergo their precommitted differentiation without the induction of proliferation. In contrast, $CD3^+$ $CD4^-$ $CD8^-$ thymocytes expressing either TCR $\alpha\beta$ or $\gamma\delta$ respond to IL-7 with the induction of proliferation but not differentiation. Similarly, as shown in Table I, splenic DN$\alpha\beta$T cells also proliferate in response to IL-7 although to a lesser extent than TCR $\alpha\beta^+$ DN thymocytes. This response, however, appears to be influenced by the presence of monocytes/macrophages as suggested by the decrease in ^{3}H-thymidine incorporation levels observed when DN splenic T cells were further purified by the depletion of Mac-1^+ cells. Reconstitution of the proliferative response by the addition of IL-1, a known macrophage product, provides further support for a potential co-stimulatory role of cells of the monocyte/macrophage lineage. Furthermore, monocytes have indeed been reported to produce IL-1 as well as IL-6 and TNFα in response to IL-7 (Alderson *et al.* 1991).

The TCR of both splenic (Table II, Prud'homme *et al.* 1991) and thymic (MacDonald *et al.* 1988) TCR $\alpha\beta^+$ DN T cells is thought to be functional based on the ability of MAb which crosslink the TCR associated CD3 complex to activate these two T cells populations. Both splenic (Table III) and thymic (MacDonald *et al.* 1988) TCR $\alpha\beta^+$ DN T cell subsets can also be activated by a combination of PMA and ionomycin which bypass the TCR by their direct effects on PKC and $[Ca^{+2}]_i$ respectively.

One final similarity between splenic DN$\alpha\beta$T cells and their thymic counterparts is the potential for cytokine production. As shown in Figure 11, splenic DN$\alpha\beta$T cells were capable of both IFNγ and IL-4 mRNA expression following the crosslinking of the TCR associated CD3 complex. Under the conditions tested, splenic DN$\alpha\beta$T cells were not capable of expressing either IL-2 or IL-6 messages. A recent study has demonstrated a similar cytokine pattern for the phenotypically similar subset of thymocytes . More specifically, Zlotnik *et al.* (1992) observed both IFNγ and IL-4 production as well as TNF-α synthesis by HSA^+ TCR $\alpha\beta^+$ DN thymocytes cells following anti-CD3 crosslinking by the use of *in vitro* bioassays. Furthermore IL-2 production was not detected under this condition. It has been subsequently shown that IL-4 production by thymic DN$\alpha\beta$T cells is independent of V_β expression (Suda and Zlotnik, 1993). Whether TCR $\alpha\beta^+$ DN thymocytes constitutively express IFNγ specific mRNA, as does their splenic DN$\alpha\beta$T counterparts, remains to be demonstrated.

Therefore, based on similarities with respect to phenotype, $V_{\beta 8}$ overexpression, responsiveness to IL-7, CD3 crosslinking and a combination of phorbol ester and calcium ionophore as well as potential cytokine production, splenic DN$\alpha\beta$T cells may be developmentally related to their thymic TCR $\alpha\beta^+$ DN counterparts which have emigrated from the thymus to populate this peripheral

organ. Evidence for a thymic dependence of splenic DNαβT has been provided by the virtual absence of $NK1^+$ $TCR\alpha\beta^+$ T cells, of which a significant proportion are of the DNαβT cell phenotype, in the spleen of athymic nude mice and the reconstitution of this splenic subset following fetal thymic engraftment (Levitsky *et al.* 1991). In addition, although DN T cells expressing a transgenic TCR αβ were identified in the murine thymus as well as spleen, bone marrow, and axillary, mesenteric and inguinal lymph nodes, T cells of this phenotype could not be detected in the peripheral tissues of transgenic nude mice (Scott *et al.* 1989). Furthermore, following intrathymic reconstitution with TN thymocytes precursors, donor derived DNαβT cells have been detected in other peripheral organs including lymph nodes and blood suggesting a thymic dependence of this subpopulation (Guidos *et al.* 1989). In order to provide further support for a developmental relationship between thymic and splenic DNαβT cells, additional studies are required including the determination of whether splenic DNαβT cells are present following intrathymic reconstitution with either TN or TCR $\alpha\beta^+$ DN thymocytes in either sublethally irradiated or congenic (Thy1.1 versus Thy1.2) hosts. The use of retrovirally introduced cytokines such as IL-7 for the expansion of this minor $TCR\alpha\beta^+$ DN thymocyte population would further facilitate the reconstitution studies.

How the potential thymic precursors of splenic DNαβT cells, namely TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ thymocytes, fit into the T cell differentiation pathway has been the subject of numerous studies. TCR $\alpha\beta^+$ DN thymocytes are thought to be composed of late stage differentiated cells based on studies demonstrating the lack of HSA expression, the relatively late appearance during ontogeny and in FTOC (Fowlkes *et al.* 1987) and the lack of precursor activity following intrathymic transfer (Crispe *et al.* 1987). Several studies have suggested that TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ thymocytes and their peripheral counterparts may be derived from $CD4^+$ $CD8^+$ DP thymocytes which, due to potential self reactivity, have escaped clonal deletion by the downregulation of surface expression of their CD4 and CD8 determinants. In male transgenic mice, for example, the majority of thymocytes and T cells expressing the transgenic anti-male TCR were of the DN phenotype and expressed high levels of the transgenic TCR but were nonetheless tolerant to male self antigen (Teh *et al.* 1989). Furthermore, the demonstration of demethylation of DNA sequences 5' to the CD8α gene has implied that TCR $\alpha\beta^+$ DN thymocytes may be derived from a T cell population that had at one time expressed the CD8α gene locus (Wu *et al.* 1990; Takahama *et al.* 1991). Cytosine residues of developmentally regulated genes are often demethylated at the time of initial expression and progressive demethylation of several CCGG sites has been observed as T cells develop from immature TN through the DP stage to peripheral mature SP T cells (Carbone *et al.*1988). The demethylation pattern observed in TCR $\alpha\beta^+$ DN thymocytes was however distinct from that of either thymic or peripheral $CD8^+$ T cells and therefore provided

further evidence that TCR $\alpha\beta^+$ DN thymocytes may be the progeny of DP thymocytes rather than SP thymocytes or peripheral T cells that have returned to thymus and downregulated CD8 expression. Cyclosporin A treatment of radiation bone marrow chimeras, previously known to arrest development of conventional T cells at the DP thymocyte stage, has also been shown to significantly block the appearance of TCR $\alpha\beta^+$ DN thymocytes (Takahama *et al.* 1991). Tiefenthaler *et al.(*1992) have recently provided direct evidence for the generation of TCR $\alpha\beta^+$ DN thymocytes and their peripheral counterparts from DP thymocytes. Following a 2 day coculture with the stimulatory pair of anti-rat CD2 MAb, OX-54/55, a significant proportion of TCR $\alpha\beta^+$ $CD4^+$ $CD8^+$ thymocytes were shown to have downregulated both CD4 and CD8 while upregulating TCR $\alpha\beta$ expression. The absence of detectable DNA synthesis ruled out a selective expansion of preexisting $CD4^{lo/-}$ $CD8^{lo/-}$ cells. Confirmation of this conversion *in vivo* was provided by the 3 to 4 fold increase in TCR $\alpha\beta^+$ DN thymocytes observed in 4 week old Lewis rats following repeated injections with OX-54/55 since birth. Furthermore, while virtually absent in controls, DN$\alpha\beta$T cells constituted approximately one fourth of all T cells isolated from the lymph nodes of CD2 treated rats. A concomitant depletion of $CD4^+$ T cells was considered to be unrelated to the peripheral accumulation of DN$\alpha\beta$T cells the proportion of $CD4^+$ T cells, unlike DN$\alpha\beta$T cells, was also influenced by the nonstimulatory anti-CD2 MAb, OX-34.

Recent evidence, however, has suggested that TCR $\alpha\beta^+$ DN thymocytes may not be the progeny of DP thymocytes but may instead represent a distinct T cell lineage which has branched off from the classical T cell differentiation pathway prior to the DP stage. First of all, in conflict with the suggestion that TCR $\alpha\beta^+$ DN thymocytes are derived from potentially self reactive DP thymocytes following the downregulation of the CD4 and CD8 accessory molecules, was the demonstration that the frequency of TCR $\alpha\beta^+$ DN thymocytes expressing various anti-self TCR including either I-E reactive $V_{\beta 11}$ and $V_{\beta 17a}$ or Mls-1^a reactive $V_{\beta 6}$ or $V_{\beta 8.1}$ was not increased in those strains expressing their putative self antigen but was either unaffected or significantly reduced (Egerton and Scollay, 1990; Takahama *et al.* 1991; Suda and Zlotnik, 1993). In addition, unlike DP thymocytes which are not capable of cytokine production upon stimulation with calcium ionophore and phorbol ester (Fischer *et al.* 1991), TCR $\alpha\beta^+$ DN thymocytes have been shown to produce IFNγ, IL-4 and TNFα in response to CD3 crosslinking (Zlotnik *et al.* 1992). Furthermore, although peripheral SP progeny were observed, Guidos *et al.* (1989b) were unable to obtain detectable levels of $CD3^+$ DN cells following the intrathymic transfer of DP thymocytes. With respect to the association of CD8α demethylation with a potential DP thymic origin for TCR $\alpha\beta^+$ DN thymocytes, Nikolic-Zugic *et al.* (1989) have shown that an early thymic subpopulation referred to as TN based on FACS analysis actually express low levels of CD8α. Furthermore, CD8α methylation has indeed been detected in a portion of the TN thymocytes and may correspond

to the $CD8^{lo}$ subset (Wu *et al.* 1990). Although the majority of $CD4^{-/lo}$ $CD8^{lo}$ thymocytes, isolated by anti-CD8α MAb coated plates yet identified as $CD4^-$ $CD8^-$ by FACS, spontaneously differentiated into thymic DP cells following short term culture, a significant proportion of these cells maintained their DN phenotype and acquired CD3 expression (Nikolic-Zugic and Moore, 1989). Therefore the demethylated CD8α genes identified in TCR $\alpha\beta^+$ DN thymocytes may actually be reminiscent of a TN phenotype expressed during a previous step in development. Several recent studies have provided support for the TN origin of TCR $\alpha\beta^+$ DN thymocytes (Vissinga *et al.*1991; Suda and Zlotnik, 1991; Suda and Zlotnik, 1993). Those TN thymocytes which express CD25 have been shown to consist of precursor cells for all TCR^+ subsets found within the thymus including $CD3^+$ DN thymocytes as well as DP and SP thymocytes. Following coculture with IL-7, a proportion of the $CD25^+$ TN thymocytes acquired the expression of CD3 associated with either of the αβ or γδ TCR. Furthermore the majority of those TCR $\alpha\beta^+$ $CD3^+$ DN thymocytes derived from the $CD25^+$ HSA^+ $CD44^-$ thymocytes were shown to be HSA^- $CD44^+$. It was therefore proposed that $CD25^+$ TN thymocytes represent a stage in T cell development when those cells destined to become $CD3^+$ DN branch off from the classical differentiation pathway to acquire either TCR αβ or γδ and differentiate from HSA^+ $CD44^-$ via HSA^- $CD44^-$ to the HSA^- $CD44^+$ phenotype.

On the other hand, splenic DNαβT cells may represent a heterogeneous population of T lymphocytes. Although the majority of splenic DNαβT cells are most likely derived from TCR $\alpha\beta^+$ DN thymocyte having emigrated to this peripheral organ, as based on the numerous similarities discussed in this thesis between these two T cell subsets, a small percentage of these splenic T lymphocytes may have alternate origins. It is possible that a minor proportion of splenic DNαβT cells may represent a population of spent single positive T cells whose CD4 and CD8 coreceptors have been downregulated. Indeed there are similarities between splenic DNαβT cells and mature SP T cells which might support this relationship. In addition to the expression of the TCR αβ, for example, both splenic DNαβT and mature SP T cells lack the heat stable antigen, a marker typically found on immature thymocytes and lost as a consequence of maturation (Crispe and Bevan, 1987). Furthermore, the response of splenic DNαβT cells to crosslinking of the TCR associated CD3 complex as assessed by ^{3}H-thymidine incorporation, is comparable to that of mature SP T cells isolated by the nylon wool column method (Table II). Evidence for the previous activation of splenic DNαβT cells including Pgp-1, transferrin receptor and ICAM-1 expression as well as IFNγ mRNA expression may provide further support for the possible origin of this splenic subset from spent SP T cells following the down regulation of the CD4 or CD8 coreceptors. Interestingly, Erard *et al.* (1993) have recently demonstrated the generation of DNαβT cells from small $CD8^+$ SP lymph node cells obtained from C57BL/6 mice following 6 day coculture with

PMA, ionomycin, IL-2 and IL-4. $CD4^+$ SP T cells under the same culture conditions, however, maintained expression of the CD4 accessory molecule. Following the gradual loss of surface expression of both CD8α and CD8β chains and concomitant decrease of CD8α encoded mRNA but not CD8β message, the $CD8^-$ phenotype appeared to be stable as suggested by the lack of CD8 reexpression during an additional 6 days of culture in the absence of IL-4. Unlike their $CD8^+$ SP progenitor cells, this $TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ T cell population possessed no lytic activity attributed not to the lack of CD8 but to the absence of perforin mRNA induction. Interestingly, this DNαβT cell population was capable of a T_H2-like pattern of cytokine production including IL-4, IL-5 and IL-10 in addition to a lower level of IFNγ secretion and were able to induce the production of immunoglobulin G1 (IgG1) from B cells. The $CD8^+$ SP progenitor cells, on the other hand, were only capable of IFNγ production and failed to induce detectable antibody production. This switch to non-cytotoxic T_H2-like DNαβT cells has since been extended to viral peptide specific $CD8^+$ SP T cells. Kyburz *et al.* (1993) demonstrated that in transgenic mice, approximately 30% of $CD8^+$ SP T cells specific for a lymphocytic choriomeningitis viral peptide restricted to class I MHC, switched to the $CD8^-$ phenotype upon activation in the presence of IL-4 and were capable of IL-4, IL-5 and IL-10 production following restimulation with the viral peptide. Although splenic DNαβT cells are also capable of IL-4 mRNA production in response to activation of the TCR-CD3 complex as shown in this thesis, additional studies including the demonstration of CD8β chain mRNA expression are required before a developmental relationship can be drawn between $CD8^+$ SP T cells and splenic DNαβT cells. If splenic DNαβT cells are indeed derived from spent SP T cells following the downregulation of the CD4 and CD8 molecules, this T cell subset may be capable of coreceptor reacquisition under the appropriate culture conditions. Prud'homme *et al.* (1991) demonstrated the acquisition of CD4 by splenic DNαβT cells following 4 day coculture with either ConA or anti-TCR αβ MAb accompanied by the development of responsiveness to syngeneic and allogeneic MLC. There was a gradual reversion to the DN phenotype in culture following activation. Thymocytes of the TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ $CD38^+$ have been shown to transiently express CD8α following activation with solid-phase anti-CD3 MAb for 18 hours (Zlotnik and Bean, 1994).

Although it has been suggested that DNαβT cells may be derived from SP T cells following the downregulation of the CD4 and CD8 co-receptors, evidence has been provided to challenge this hypothesis. First of all, the demethylation pattern of the CD8α gene in TCR $\alpha\beta^+$ DN thymocytes has been shown to be distinct from that of thymic and peripheral $CD8^+$ SP T cells. Analysis of $\beta_2m^{+/-}$ chimeras has demonstrated an inverse correlation in the frequency of $V_{\beta8}{}^+$ DN thymocytes versus $CD8^+$ SP T cells which further argues against a precursor-product relationship (Bix *et al.* 1993). Furthermore this thesis provides significant evidence to suggest that splenic

DNαβT cells represent a unique class of T cells distinct from the what is considered to be the classical type of mature T lymphocyte, namely SP T cells. In contrast to nylon wool nonadherent SP T cells, an unusually high proportion of splenic DNαβT cells express a particular member of the V_β family of TCR genes, namely $V_{\beta 8}$. Splenic DNαβT cells also differ from the nylon wool nonadherent SP T cells with respect to their response to antigen independent activation by IL-7. Whereas splenic DNαβT cells proliferate in the presence of IL-7 and this response is enhanced by the addition of IL-1, the proliferation of nylon wool purified SP splenic T cells in the presence of IL-7 is minimal and is not influenced by the addition of IL-1. Furthermore the characteristic pattern of cytokine expression differs between the two types of mature T cells. Whereas both splenic DNαβT cells as well as nylon wool purified SP T cells expressed IL-4 mRNA following the crosslinking of TCR associated CD3 complex, IL-2 message was only detected for the latter splenic subset under similar conditions. A more striking difference between splenic DNαβT and SP T cells is the pattern of IFNγ mRNA expression. Only in response to crosslinking of the TCR associated CD3 complex was significant levels of IFNγ message detected for mature SP T cells. With respect to splenic DNαβT cells, the expression of IFNγ specific message was constitutive and dependent only on the viability of this splenic population. Therefore these results suggest that the majority of splenic DNαβT cells are a unique class of mature T cells distinct from the classical type of mature T cell which expresses the SP phenotype.

Alternatively, a proportion of splenic DNαβT cells may not be of a thymic origin at all but may be the products of an extrathymic differentiation pathway. Interestingly, a significantly higher percentage of bone marrow T cells express the TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ phenotype than in either the thymus or spleen suggesting that the bone marrow is a potential site for extrathymic T cell development (Sykes 1990, Palathumpat *et al.* 1992b; Martinez-A. *et al.* 1993). Palathumpat *et. al.* (1992b) provided support for the potential extrathymic origin of bone marrow DNαβT cells with the observation of an apparent *in vitro* conversion of TCR $\alpha\beta^-$ $CD4^-$ $CD8^-$ bone marrow cells to the TCR $\alpha\beta^+$ DN phenotype. Although TCR rearrangement, as detected by PCR amplification of genomic DNA employing $V_{\beta 7}$ and $J_{\beta 2}$-$C_{\beta 2}$ primers, was not observed in freshly isolated TCR $\alpha\beta^-$ $CD4^-$ $CD8^-$ marrow cells, β chain rearrangement associated with the differentiation of this marrow subset to the TCR $\alpha\beta^+$ $CD4^-$ $CD8^-$ phenotype was demonstrated following 48 hr incubation in tissue culture medium suggesting an apparent maturation of bone marrow T cells in the absence of a thymic microenvironment. In a second study, IL-3 was shown to induce the generation of DNαβT cells with predominant $V_{\beta 2}$ gene usage from $CD4^+$ /$CD8^+$ depleted bone marrow of normal BALB/c mice (Kubota *et al.* 1992). Also consistent with the putative lack of dependence of this bone marrow T cell subset on the presence of a mature thymus was the demonstration of the absence of negative selection of normal bone marrow DNαβT cells

expressing the self reactive $V_{\beta 5}$ receptor in BALB/c mice (Palathumpat *et al.* 1992b), $V_{\beta 8.1}$ and $V_{\beta 3}$ in DBA/2 and CBA/J mice (Martinez-A. *et al.* 1993). If the hypothesis suggesting an extrathymic origin for bone marrow DN$\alpha\beta$T cells was indeed valid then one would expect that the proportion of DN$\alpha\beta$T cells in the bone marrow of athymic nude mice to be similar to that in euthymic strains. Two studies have shown that the proportion of NK1.1$^+$ TCR $\alpha\beta^+$ T cells actually decrease in the bone marrow of nude mice as compared to normal mice (Sykes 1990; Levitsky *et al.* 1991). The majority of bone marrow NK1.1$^+$ TCR $\alpha\beta^+$ cells, however, have been shown to express CD4 or CD8 and therefore the reduction of this population in nude mice can not necessarily be extrapolated to the DN subset. Subsequent studies, however, have demonstrated that unlike the percentage of typical TCR $\alpha\beta^+$ CD4$^+$ and CD8$^+$ SP T cells which was markedly reduced in the spleens of nude mice, the proportion of DN$\alpha\beta$T cells in the bone marrow of this athymic murine strain was indeed similar to that of normal euthymic mice (Palathumpat *et al.* 1992b; Kubota *et al.* 1992). It is possible that a proportion of splenic DN$\alpha\beta$T cells may be derived from the phenotypically similar cells identified in murine bone marrow. Similar to their splenic counterparts, bone marrow DN$\alpha\beta$T cells are Ly-1$^+$ Thy-1$^+$ and lack HSA and B220 (Figure 6; Martinez-A. *et al.*1993). Kubota *et al.* (1992) have also demonstrated the IL-3 induced generation of DN$\alpha\beta$T cells from both CD4$^+$/CD8$^+$ depleted bone marrow as well as spleen of normal and nude mice. Furthermore, Palathumpat *et al.* (1992b) demonstrated that the percentage of DN$\alpha\beta$T cells in both the bone marrow and spleen of nude mice were comparable to that of normal mice. In addition, the neonatal spleen contained a similar percentage of DN$\alpha\beta$T cells but a significantly reduced number of typical T cells when compared to adult mice suggesting the presence of DN$\alpha\beta$T cells in the murine spleen prior to the emigration of SP T cells from the thymus. However, unlike splenic DN$\alpha\beta$T cells where almost one half of the population expresses $V_{\beta 8}$ genes, the frequency of $V_{\beta 8}$ usage for bone marrow cells of this phenotype has been shown to be only 14-35% (Martinez-A. *et al.* 1993). In addition, whereas splenic DN$\alpha\beta$T cells expressing either $V_{\beta 5}$, $V_{\beta 6}$ or $V_{\beta 11}$ are reduced in I-E$^+$ Mls-1^{a+} strains (Prud'homme *et al.* 1991), phenotypically similar bone marrow cells expressing these V_β genes appear to be resistant to clonal deletion (Palathumpat *et al.* 1992b; Martinez-A. *et al.* 1993). Although both are responsive to stimulation with anti-CD3 MAb (Figure 11; Marinez *et al.* 1993) suggesting functional TCR, the patterns of cytokine production are somewhat different. Whereas bone marrow DN$\alpha\beta$T cells secrete IL-2, IL-3, IL-4 and IL-5 in response to anti-$V_{\beta 5}$ and anti-$V_{\beta 11}$, (Martinez-A. *et. al.* 1993) the corresponding splenic subset produce IFNγ, IL-4 and TNFα following activation of the TCR associated CD3 complex (Figure 11; Zlotnik *et al.* 1992). Additional studies to further elucidate the developmental relationship between bone marrow and splenic DN$\alpha\beta$T cell could include the determination of the presence DN$\alpha\beta$T spleen cells following reconstitution of sublethally irradiated or congenic hosts with bone marrow TCR$\alpha\beta^+$ DN T cells. Furthermore it

would be of interest to determine whether DNαβT cells exist in the bone marrow and spleen of mice deficient in IL-3 production such as an IL-3 gene knockout murine strain.

The liver has been proposed as an alternate site for development of DNαβT cells. Seki *et al.* (1991) have shown that while a single peak pattern of bright TCR αβ and V_β expression was observed in intestinal epithelia as well as mononuclear cells of lymph nodes, spleen and blood of normal mice, a two peak pattern, specifically intermediate and high expression of both TCR αβ V_β was detected in the liver. Further characterization of such intermediate hepatic cells revealed that although a large proportion of V_β^+ T cells with intermediate TCR also expressed either CD4 or CD8, a significant percentage of TCR $\alpha\beta^{intermediate}$ (TCR $\alpha\beta^{int}$) cells were of the DN phenotype. Although TCR $\alpha\beta^{int}$ cells constitute a small proportion of cells in the liver of young mice (6-8 weeks of age), Ohteki *et al.* (1992) demonstrated a significant expansion of this population as a consequence of aging with DNαβT cells assuming the predominant phenotype in the older mice (60 weeks of age). At older ages when the thymus is characteristically involuted, this extrathymic pathway was in its most activated state and the apparent TCR $\alpha\beta^{int}$ progeny of this pathway began to appear in the periphery, first in the peripheral blood and then eventually in the spleen and lymph nodes. Confirmation that the liver is a site of extrathymic differentiation was provided by the observation of only TCR $\alpha\beta^{int}$ cells including DNαβT cells in the liver of congenitally athymic nude mice. Furthermore, an age dependent increase in the proportion of this hepatic population was also observed in the athymic mice. The expansion of DNαβT cells, including those bearing self reactive TCRs, has also been demonstrated in the liver of normal mice following bacterial stimulation (Abo *et al.* 1991). This proposed extrathymic pathway has also been shown to be activated following estrogen administration at the expense of intrathymic T cell development (Okuyama *et al.* 1992). Subcutaneous injection of 1 mg of estrogen resulted in the increase of hepatic mononuclear cells enriched for TCR $\alpha\beta^{int}$ cells including V_β^+ cells and forbidden clones while the thymus underwent estrogen induces atrophy with depletion of TCR $\alpha\beta^{dull}$ $CD4^+$ $CD8^+$ thymocytes. Interestingly, the liver has been shown to be a major site for the proliferation of DNαβT cells in numerous autoimmune mice including MRL-*lpr/lpr,* C3H/HeJ-*gld/gld*, BXSB, NOD and NBZB/W F_1 strains (Ohteki *et al.* 1990, Masuda *et al.* 1991). Following the onset of the autoimmune disease, DNαβT cells expressing both Pgp-1 and B220 have been shown to accumulate in the liver of *lpr* mice where they reside in hepatic sinusoids in close association with Kupffer and endothelial cells. While DNαβT cells accounted for greater than 50% of total hepatic TCR $\alpha\beta^+$ cells, the vast majority of TCR αβ expressing T cells in the lymph nodes and spleen of *lpr* mice were of the DNαβT phenotype. Assessment of CD3 depleted hepatic MNC cultured for three days revealed a prominent increase in expression of TCR αβ and CD3 but not CD4 or CD8 and suggested the possible extrathymic development of DNαβT cells

null cell precursors. Furthermore, *in vivo* intravenous injection of ^{3}H-thymidine demonstrated peak ^{3}H-TdR incorporation by hepatic MNC on day 1 at levels almost comparable to that of bone marrow. A peak of radioactivity on day 5 in lymph node MNC provided support for the possible migration of hepatic derived DNαβT cells to peripheral tissues and the resultant lymphadenopathy in MRL-*lpr/lpr* mice. Although these studies have implicated the hyperactivity of this extrathymic T cell development pathway in the pathogenesis of the marked T lymphocyte accumulation observed in *lpr* mice, a recent study has proposed an alternate role for the liver in this autoimmune disorder. Employing a modified separation procedure which combined mild enzymatic digestion, FCS gradient centrifugation and panning, Huang *et al.* (1994) were able to increase the liver T cell yield over Ficoll density gradient centrifugation and were therefore able to demonstrate that the predominant intrahepatic lymphocyte in the normal adult mouse were of the TCR $\alpha\beta^{int}$ CD4$^-$ CD8$^-$ B220$^+$ HSA$^+$ phenotype. Furthermore, this hepatic TCR$\alpha\beta^{int}$ DN T cell subset, whose phenotype is similar to those T cells massively expanded in *lpr* mice, were shown to be actively proliferating and undergoing apoptosis. Together with the absence of significant *RAG-1* and *RAG-2* expression required for TCR α and β chain rearrangement (Crispe and Huang, 1994), it was therefore suggested that the liver is a site for T cell destruction as opposed to T cell development and that failure of T cells to undergo apoptosis possibly due to the insertional mutation in the *Fas* gene (Watanabe-Fukunaga *et al.* 1992) may result in the accumulation of TCR $\alpha\beta^{int}$ CD4$^-$ CD8$^-$ B220$^+$ T cells in the liver and subsequent spill over into the periphery of *lpr* mice. Therefore based on the demonstration of a B220$^+$ HSA$^+$ phenotype as well as significant apoptotic activity, it is unlikely that hepatic DNαβT cells represent precursors of splenic DNαβT cells which are HSA$^-$ B220$^-$ (Figure 7; Prud'homme *et al.* 1991). In order to confirm that hepatic and splenic DNαβT cells are indeed developmentally unrelated, sublethally irradiated or congenic hosts could be assessed for the absence of splenic DNαβT cells following reconstitution by hepatic TCRαβ$^+$ DN T cells. Furthermore the failure of bacterial stimulation or subcutaneous estrogen injection to induce the expansion of splenic DNαβT cells would provide further evidence for a developmental pathway distinct from that of phenotypically similar hepatic cells.

Although both splenic DNαβT cells and TCR γδ$^+$ DN T cells share the HSA$^-$ CD3$^+$ DN phenotype as well as the ability to respond to antigen independent activation of IL-7 with or without the addition of IL-1, one significant difference has been observed between these two T lymphocyte subsets. DNαβT cells proliferated vigorously in the presence of immobilized anti-CD3 MAb, a response comparable to that of nylon wool purified SP T cells. TCR γδ$^+$ DN spleen cells, on the other hand, were nonresponsive to anti-CD3 crosslinking despite the expression of the TCR/CD3 complex. It has recently been shown that TCR/CD3 signaling by crosslinking with immobilized MAb specific for either TCR γδ or CD3 complex in the presence of IL-2 induces

death by apoptosis in nontransformed IL-2-dependent TCR $\gamma\delta^+$ DN T cells isolated from normal human peripheral blood (Janssen *et al.* 1991). It would therefore be interesting to determine whether TCR $\gamma\delta^+$ DN spleen cells, which are nonresponsive when cultured with immobilized anti-CD3, actually undergo apoptosis under these conditions. Interestingly, the addition of IL-1 to TCR $\gamma\delta^+$ DN cells cultured with immobilized anti-CD3 appears to restore the responsiveness of this splenic subset towards the activation of the TCR/CD3 complex. Therefore whereas anti-CD3 crosslinking is sufficient to stimulate splenic DN$\alpha\beta$T cells to proliferate, the activation of TCR $\gamma\delta^+$ DN spleen cells appears to require a comitogenic stimulus.

Splenic DN$\alpha\beta$T cells are considered to be functionally mature as suggested by the absence of expression of the heat stable antigen, a responsiveness to crosslinking of the TCR associated CD3 complex which is comparable to that of nylon wool nonadherent mature SP T cells and an ability to express cytokine specific messages. Although the functional significance of this mature T cell population in the immune response remains to be established, several possible functions have been proposed.

DN$\alpha\beta$T cells have been suggested to play a key role in the suppression of immune responses. Cloned cell lines of the DN$\alpha\beta$T phenotype derived from the spleens of neonatal or adult mice recovering from either irradiation or bone marrow transplantation have been shown to inhibit both GVHD and MLR (Hertel-Wulff *et al.* 1984, Schwadron *et al.* 1985, Strober *et al.* 1987, Sykes *et al.* 1990). Lethal GVHD suppression was also performed by DN$\alpha\beta$T cells obtained from the spleens of Mls-1^b mice following immunization with Mls-1^a cells (Bruley-Rosset *et al.* 1990). Furthermore, suppression of acute GVHD has been detected in the low density fraction of Percoll density gradient fractionated spleen cells from normal mice and was correlated with the depletion of typical SP T cells and the enrichment of DN$\alpha\beta$T cells (Palathumpat *et al.* 1992). Similarly, fractionation of normal bone marrow cells yielded low density fractions enriched for DN$\alpha\beta$T cells with natural suppressor activity and capable of inhibiting both MLR and GVHD (Benveniste *et al.* 1990, Palathumpat *et al.* 1992). In further support of a suppressor like function for DN$\alpha\beta$T cells, consistent increases in splenic $CD3^+$ $CD4^-$ $CD8^-$ cells have been demonstrated in animals receiving GVH-inducing inocula (Abraham *et al.* 1992). Further enhancement of the percentage of this splenic subpopulation occurred when recipients of allogeneic inocula were treated with IL-2. Early administration of IL-2 following bone marrow transplantation has indeed been demonstrated to provide potent prophylaxis against GVHD mortality without preventing alloengraftment or reducing graft versus leukemia effects (Sykes *et al.* 1990b,c). A recent study has demonstrated the switch of $CD8^+$ SP T cells, following activation in the presence of IL-4, to noncytolytic DN$\alpha\beta$T cells capable of T_H2 cytokine production, including IL-4, IL-5 and IL-10, and of induction of

immunoglobulin production by B cells (Erard *et al.* 1993). It has been suggested that this IL-4 induced diversion of resting $CD8^+$ SP T cells away from the IFNγ producing cytotoxic pathway may represent one mechanism for suppression of CD8 cytotoxic responses. Furthermore, the T_H2 cytokine IL-4 and IL-10, as produced by these noncytolytic DNαβT cells, are considered to be suppressor factors for T_H1 type responses (Modlin and Nutman, 1993). Interestingly, following HIV-1 infection, progression from the protective phase of the disease, characterized by a high frequency of HIV-1 specific $CD8^+$ SP T cells, to AIDS is correlated with a significant reduction of these HIV-1 specific cytotoxic T cells and a concomitant increase in T_H2 cytokine activity (Riddell *et al.* 1993, Carmichael *et al.* 1993, Clerici and Shearer 1993). Furthermore, a significant increase in the absolute number of $CD3^+$ $CD4^-$ $CD8^-$ T cells in the peripheral blood of HIV-1 infected individuals has been shown to occur in the late stages of AIDS (Margolick *et al.* 1989). Whether the redirection of $CD8^+$ SP T cells down a noncytolytic T_H2-like DNαβT cell pathway plays a role in the progression from HIV-1 infection to AIDS remains to be clarified.

Conversely, a role for DNαβT cells in cytotoxic responses and allogeneic tumour rejection has also been suggested. CTL with the $TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ phenotype have been generated from MLTC spleen cells of anti-CD8 MAb treated B6 mice (Mieno *et al.* 1991). Similarly, rejection of an IA^- variant line of FBL-3 leukemia in CD8 depleted syngeneic B6 mice was also shown to be mediated by DNαβT cells as well as $CD4^+$ CTL generated by the activation of $CD4^+$ helper T cells (Yoshimura *et al.* 1993). Furthermore, IL-2 dependent T cell lines of DNαβT phenotype with non-MHC-restricted cytolytic activity against tumour targets, syngeneic and allogeneic fibroblasts and PHA blasts from allogeneic donours have been isolated from the peripheral blood of a patient with combined immunodeficiency and tissue injury resembling GVH who had a massive expansion of this cell type (Brooks *et al.*1990). Following activation by mitogens or PMA and IL-1, these cell lines were capable of IFNγ as well as TNFα and TNFβ production. Additional studies are required in order to determine whether freshly purified splenic DNαβT cells are capable of cytotoxic function against, for example, Yac-1 target cells.

Alternatively, splenic DNαβT cells may play a potentially important role in the development of T_H2 immune responses. Although it has been previously shown that IL-4 plays a pivotal role in the differentiation of T_H0 cells to the T_H2 phenotype (Swain *et al.* 1990), the source of this cytokine is unknown. Based on the demonstration of IL-4 production by splenic DNαβT cells as shown in Figure 11 and by Prud'homme *et al.* (1991) and Zlotnik *et al.* (1992), it is possible that this splenic T cell subset may be the source of IL-4 during the initial stage of development of the T_H2 response *in vivo*.

Several studies have suggested that DNαβT cells are autoreactive and have implicated this phenotypically unique T cell population in the development of autoimmune diseases. Following activation with either anti-TCR αβ MAb or Con A, splenic DNαβT cells acquired high reactivity in both syngeneic and allogeneic MLC (Prud'homme *et al.* 1991). Interestingly, this autoreactivity corresponded to the acquisition of CD4 expression with gradual reversion to the DN phenotype following the arrest of activation. A marked expansion of DNαβT cells has been observed in the peripheral blood of patients with autommune disorders including systemic sclerosis (Sakamoto *et al.* 1992) and systemic lupus erythematosus (SLE) (Shivakumar *et al.* 1989). Along with $CD4^+$ SP T cells, these TCR expressing double negative T cells were responsible for the induction of oligoclonal production of highly cationic anti-DNA autoantibodies. Studies of the murine model for SLE have also implicated peripheral DNαβT cells in the development of autoimmune disorders. DNαβT cells have been shown to accumulate in the massively enlarged lymph nodes and spleen of the autoimmune lymphoproliferative murine strain MRL-*lpr/lpr* (Wolfsy *et al.* 1984; Nemanzee *et al.* 1985). Strains which carry the mutant autosomal gene, *lpr*, spontaneously develop a severe and rapidly progressive autoimmune disease characterized by the production of a variety of autoantibodies, immune complex-mediated glomerulonephritis and vasculitis in addition to the massive lymphadenopathy (Andrews *et al.* 1978; Theofilopoulos and Dixon, 1981). It has been suggested that the massive accumulation of abnormal *lpr* T cells may be due to a failure to undergo apoptosis secondary to an insertional mutation in the *Fas* gene (Watanabe-Fukunaga *et al.* 1992). Both the lymphoproliferation and autoimmunity observed in *lpr* mice are thought to be thymic dependent based on the abrogation of these abnormalities in neonatally thymectomized mice (Hang *et al.* 1984). In addition, cells of the atypical $B220^+$ $Pgp\text{-}1^+$ phenotype have also been identified within both of the DN and DP thymocyte subpopulations of this autoimmune murine strain (Tutt Landolfi *et al.* 1993). Furthermore, *lpr* DNαβT cells have demethylated CD8α sequences and are thought to be influenced by thymic negative selection as suggested by the absence of $V_{\beta 8.1}{}^+$ and $V_{\beta 6}{}^+$ cells amongst DNαβT lymph node cells of $Mls\text{-}1^{a+}$ AKR *lpr* mice (Singer *et al.* 1989). A number of similarities exist between the DNαβT cells which reside in the spleen of normal mice and the abnormally expanded T cell population in *lpr* mice. Similar to DNαβT cells in the spleens of normal mice, the abnormally expanded cells of *lpr* mice are also of the TCR $\alpha\beta^+$ DN phenotype and express Lyt-1 and Pgp-1 but not HSA (Nemazee *et al.* 1985; Davidson *et al.* 1986). Furthermore, TCR gene expression in DNαβT cells of both normal and *lpr* mice is skewed in favour of the $V_{\beta 8}$ family (Singer *et al.* 1986). This preferential usage of $V_{\beta 8}$ genes is also characteristic of other murine models of autoimmune disorders including collagen-induced arthritis (Osman *et al.* 1993) and diabetes (Fukuda *et al.* 1989). However, there are differences between splenic DNαβT cells of normal mice and the abnormal *lpr* T cells, particularly with respect to surface phenotype. Unlike splenic DNαβT cells of normal mice, the

majority of abnormal subset of T cells in *lpr* mice express B220, an isoform of the common leukocyte antigen Ly-5 whose expression is normally restricted to cells of the B lineage (Coffman and Weissman, 1981). Furthermore abnormal *lpr* T cells and normal splenic DNαβT cells also show a reciprocal expression pattern for the YE1/19.1 and YE1/9.9 (Takei, 1984; Davidson *et al.* 1986). Whereas abnormal *lpr* T cells express YE1/19.1, an antigen similar to the plasma cell antigen PC-1 (Takahashi *et al.* 1970), splenic DNαβT cells of normal mice are YE1/19$^-$. In contrast, abnormal *lpr* T cells lack the expression of YE1/9.9 whereas normal splenic DNαβT cells are YE1/9$^+$. The MAb YE1/9.9 is specific for the transferrin receptor which is expressed on essentially all proliferating cells including activated T lymphocytes (Takei 1983; Trowbridge and Omary, 1982; Sutherland *et al.* 1981). DNαβT cells of normal and *lpr* mice also differ in their response to TCR-CD3 engagement. Although abnormal *lpr* cells undergo a rapid increase in intracellular calcium with the generation of inositol phosphates and expression of interleukin-2 receptors, unlike normal splenic DNαβT cells the atypical *lpr* cells do not proliferate in response to TCR-CD3 activation due to the absence of IL-2 production (Davignon *et al.* 1985).

Although the role of DNαβT cells in the development of autoimmune disorders has yet to be established, the constitutive expression of IFNγ message by this phenotypically unique T cell population provides support for an association with autoimmunity. IFNγ has been shown to promote B cell growth (Morikawa *et al.* 1987) and induce IgG_{2a} production (Snapper and Paul, 1987) as well as class II MHC expression on the surface of macrophages and B cells (Goedegebuure *et al.* 1989). Increased levels of class II MHC expression as well as increased amounts of Ig including the IgG_{2a} isotype have observed in *lpr* mice. ConA-stimulated Lyt-1$^+$2$^-$ T cells isolated from the spleens of *lpr* mice are capable of significant IFNγ production despite hypoproduction of IL-2 (Santoro *et al.* 1983). In addition, RNA protection assays have demonstrated that abnormal *lpr* T cells isolated either as CD4$^-$ CD8$^-$ and B220$^+$/Thy-1$^+$ subsets spontaneously transcribe IFNγ as well as TNF-α genes *in vivo* (Murray and Martens, 1989). Furthermore PCR analysis has confirmed the expression of genes encoding IFNγ as well as TNF-α, TNF-β and IL-6 by the CD4$^-$ CD8$^-$ Thy-1$^+$ B220$^+$ subset of T cells in *lpr* mice (Murray *et al.* 1990). IFNγ has also been shown to accelerate the progression of autoimmune disease in New Zealand Black mice (Heremans *et al.* 1978) and can induce immune complex glomerulonephritis in newborn mice (Gresser *et al.* 1976). Furthermore, disturbances in IFNγ activity have been observed in patients with autoimmune disorders including SLE, rheumatoid arthritis, scleroderma and Sjogren's syndrome (Hooks *et al.* 1979). Since the splenic DNαβT cell population, which constitutively expresses IFNγ mRNA, is expanded by IL-7 and IL-1, this stimulation pathway may lead to antigen-independent production of IFNγ. On the other hand, antigen-dependent activation of splenic DNαβT cells may lead to the production of IL-4. In addition to the proliferative effects

on activated B cells (Kishimoto, 1985), IL-4 has also been shown to induce the expression of class II MHC genes on the surface of resting human and murine B cells (Smith and Rennick, 1986; Rousset *et al.* 1988) as well as enhance the production of Ig including IgG_1 by activated B cells (Coffman and Carty, 1986). IL-4 as well as IFNγ may therefore be potential sources of the B cell differentiation factor (BCDF) activity that is spontaneously produced in the autoimmune *lpr* mice (Prud'homme *et al.* 1983) and leads to polyclonal B cell activation and ultimately production of autoantibodies. Uncontrolled production of IL-4 as well as IFNγ by splenic DNαβT cells may also contribute to the increased expression of class II MHC which could induce the proliferation of $CD4^+$ autoreactive T cells. Additional studies are required in order to determine whether splenic DNαβT cells are in fact involved in the development of autoimmune diseases. It would be of interest to determine whether mice in which the DNαβT cell population has been expanded by, for example, infection by an IL-7 retrovirus develop autoimmune characteristics. Based on the results of a previous study which has demonstrated the ability of IL-3 to induce the generation of DNαβT from $CD4^+/8^+$ depleted bone marrow and spleen cells (Kubota *et al.* 1992), Il-3 overexpressing mice could be assessed for the possible expansion of bone marrow and splenic DNαβT cells and concurrent development of autoimmune characteristics. Futhermore *lpr* mice could be assessed for possible IL-3 overexpression as a source for the abnormally expanded TCR $\alpha\beta^+$ DN T cell population. A subsequent study could include the assessment of potential suppression of autoimmune manifestations in *lpr* mice following IL-3 gene knockout. The role of DNαβT cells, whether it be in the development of autoimmune disorders, cytotoxic responses and tumour rejection or the suppression of immune responses, will no doubt be the subject of future studies.

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