Naive T Cell Survival and Homeostasis

Analysis of Monoclonal and Polyclonal T Cell Populations

Submitted by

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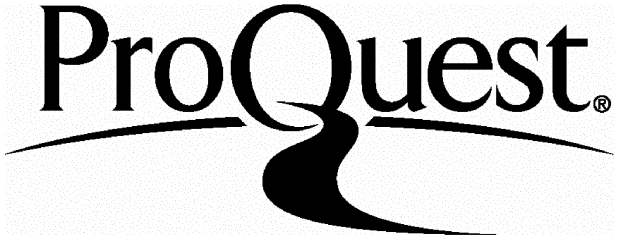

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Abstract

Understanding the mechanisms that regulate life span and survival of the naive T cell clones that compose the peripheral pool is essential to understand the functioning of the immune system. In this study the survival characteristics of several individual monoclonal T cell populations, obtained from TCR transgenic mice, as well as polyclonal controls were analysed using different survival criteria. Initially, T cell life span was established for each population by monitoring T cell persistence after thymectomy. Subsequently susceptibility to apoptosis *in vitro* and homeostatic proliferation were also determined. It was observed that TCR transgenic CD4 T cells, when compared with CD8 T cells, have an intrinsically lower capacity for survival, which is reflected in their gradual disappearance in thymectomised hosts, their increased sensitivity to apoptosis in vitro and fewer divisions upon transfer into syngeneic lymphopenic hosts. Also in the case of polyclonal T cells, naive CD8 T cells appear to survive better than their CD4 counterparts, which is reflected in a drop of the CD4/CD8 ratio observed in old animals and in thymectomised animals, when compared with young controls. Homeostatic proliferation was not universally accompanied by phenotypic conversion of activation markers. Some T cells, such as A18 T cells, maintained a naive phenotype while dividing, whereas others, like AND T cells acquired a memory-like phenotype. It is currently unknown whether phenotypic conversion is a reflection of high avidity or indicates the presence of cross-reactive antigens in the host.

In the second part of the project two other parameters were studied for their influence on T cell homeostasis. These were overexpression of an adhesion molecule on thymic epithelium (EVA molecule) or of CD4 co-receptor on A18 T cells. Although further work will still have to be performed on the subject, the data obtained suggests that both factors might affect the peripheral pool size. The larger peripheral pools found in both situations might be a consequence of increased thymic export, better cell survival resulting from more efficient imprinting of survival signals during thymic development or increased proliferative capacity associated with high avidity peripheral interactions.

" I never could make out what those damned dots meant" Lord Randolph Churchill (1849-94). W.S. Churchill. *Lord Randolph Churchill.* 1906

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Abbreviations

7-AAD	7-aminoactinomycin D
AICD	Activation induced cell death
APC	Antigen presenting cell
ATx	Thymectomised
BCR	B cell receptor
BM	Bone marrow
BrdU	5-Bromo 2'-deoxyuridine
C5	Fifth component of complement
CD	Cluster of differentiation
CD40L	CD40 ligand
cDNA	Complementary DNA
CDR3	Complementary-determining region 3
CFSE	Carboxy-fluorescein-diacetate succinimidyl ester
CLA	Cutaneous lymphocyte antigen
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
DC	Dendritic cell
DN cell	Double negative cell
DNA	Deoxyribonucleic acid
DP cell	Double positive cell
ELC	Epstein-Barr virus-induced molecule 1 ligand Chemokine
ER	Endoplasmic reticulum
EVA	Epithelial V-like antigen
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H chain	Heavy chain
HEV	High endothelium venule
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin

Ii	Invariant chain
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
INF	Interferon
L chain	Light chain
LCMV-D	Lymphocytic choriomeningitis virus-docile
LFA	Leukocyte functional antigen
LKLF	Lung Kruppel-like factor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger RNA
NK cells	Natural Killer Cells
NTx	Non-thymectomised
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
pTα	Pre-Tα
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
SL	Surrogate light chain
SLC	Secondary lymphoid chemokine
SP cell	Single positive cell
TAP	Transporter associated with antigen processing
TARC	Thymus and activation-regulated chemokine
TCR	T cell receptor
Tet	Tetracycline
TECK	Thymus expressed chemokine
Th	T helper cell
TLR	Toll like receptor
TNF	Tumour necrosis factor

1. Introduction

1.1. Immune system

The immune system is responsible for the elimination of pathogens such as bacteria, fungi, viruses and parasites and protects the host against infection. Two types of immune responses can be triggered by an antigen – innate and adaptive – and each of these responses is mainly carried out by different cells.

1.2. Innate immunity

Cells from the myeloid lineage, e.g. macrophages and neutrophils, together with some enzyme systems form the innate immune system. They provide the early phases of host defence until the adaptive immune response becomes effective. Their action does not depend upon prior exposure to the pathogen and it does not generate immunological memory. That is, these cells will react the same way even when a second encounter with the same antigen takes place.

One of the first lines of defence that an invading pathogen faces is the activation of the alternative pathway of complement. This consists of a humoral reaction cascade occurring spontaneously in the plasma and from which the host cell is protected by regulatory proteins. Lacking these protective proteins the microbial cells are lysed or opsonized. In addition to engulfing opsonized particles, macrophages can also recognise many pathogens directly. Their surface receptors recognise constituents common to many pathogens, known as pathogen-associated molecular patterns (PAMPs). These represent conserved molecular structures essential for the survival of the microbial agents, but not expressed by the host organisms. They are often shared by a pathogen class. For instance, lipopolysaccharide (LPS) and lipoteichoic acid (LTA) are "molecular signatures" of gram-negative and gram-positive bacteria, respectively (Medzhitov and Janeway Jr, 2000). The receptors that recognise the PAMPs are called pattern recognition receptors and can be found in organisms as far apart in

the evolutionary scale as *Drosophila* and humans. The Toll-like receptor (TLR) family is a good example of this point. A mutation in the *Drosophila* Toll protein affects the fly's ability to recognise and eliminate fungal pathogens, but not bacteria (Lemaitre et al., 1996). On the other hand, mutations in another *Drosophila* TLR, the *18-wheeler* (18*w*) caused a decreased viability of the flies in response to bacterial challenge (Williams et al., 1997). These observations clearly suggest that different Toll receptors specialise in the recognition of different classes of pathogen. In the case of mammals the same mechanism appears to be present. The mouse strains C3H/HeJ and C57BL/10ScCr have a mutated *lps* gene which renders them hyporesponsive to LPS and highly susceptible to Gram-negative infection. Recent work has identified *lps* as TLR4, yet another member of the TLR family (Poltorak et al., 1998; Qureshi et al., 1999). Finally, mammalian TLR2 was shown to mediate recognition of Gram-positive bacteria via the bacterial cell wall components LTA and peptidoglycan (Schwandner et al., 1999; Yoshimura et al., 1999). Thus, also in mammals a single TLR protein seems to be responsible for the detection of a given pathogen class.

Another function of phagocytes is the production of cytokines. Interleukin- (IL –) 1, IL-6 and tumour necrosis factor- (TNF-) α can elevate body temperature, which in general inhibits microbial expansion and enhances the immune response. Furthermore, they activate hepatocytes to synthesise acute-phase proteins, molecules that can bind to the bacteria surface and activate complement or phagocytes. Cytokines can also induce a series of changes in the surface properties and permeability of blood vessels increasing the recruitment of leukocytes to the site of infection. Finally, IL-12 in synergy with interferon- (IFN-) α and IFN-β activates natural killer (NK) cells, which can distinguish between infected and uninfected host cells eliminating the former.

1.3. Adaptive immunity

Adaptive immunity is triggered when a pathogen eludes the innate mechanisms of defence. Several days are required for this adaptive response to become

effective; time necessary for the proliferation and differentiation of antigen-specific lymphocytes – the cells that form the adaptive immune system.

Conversely to the innate system, the adaptive system is able to change and adapt so that in a subsequent encounter with a particular antigen, the lymphocytes can mount a swifter and more efficient response. Specificity and memory are thus two essential characteristics of the adaptive immune response.

However the division between innate and adaptive immunity is only a very simplified way of looking at the immune system. In reality there is extensive co-operation between lymphocytes and phagocytes both in the form of direct cell-cell contact and through the production of soluble molecules such as cytokines and antibodies that act as messengers between cells.

1.4. Lymphocytes

The effectors of the adaptive immune response are the lymphocytes and can be divided into two categories: T and B lymphocytes. Both T and B cells are derived from hematopoietic stem cells. B cells differentiate in the liver before birth and in the bone marrow afterward. They then progress to a mature functional stage, likely in peripheral tissues, such as the spleen. T cell precursors have also origin in bone marrow stem cells. Once they leave this organ they colonise the thymus where the T differentiation process occurs.

An essential characteristic of an efficient immune system is its ability to recognise a wide variety of pathogens to which the organism is exposed during its lifetime. Each lymphocyte expresses on its surface a receptor responsible for the specific recognition of antigen. The mechanism that ensures the generation of diverse receptors is the rearrangement of the genes that encode for these molecules and it is similar for both cell types. The lymphocyte receptors are composed of two (α and β or γ and δ) polypeptide chains in the case of T cells or four polypeptide chains in the case of B cells. The four-chain unit consists of two identical light (L) chains associated with two identical heavy (H) chains.

The genes encoding for the T cell receptor (TCR) α, TCR γ and for the L chain are organised in multiple variable (V), joining (J) and constant (C) segments; the TCR β, TCR δ and H chain genes have diversity (D) segments in addition. During T and B cell development any V gene segment can be joined to any J or DJ gene segment by somatic recombination, resulting in an enormous diversity of receptor specificities.

1.4.1. B cell differentiation and activation

The B cell antigen receptor is an immunoglobulin (Ig) and the different forms in which it is expressed have been used to identify different stages of B cell development within the bone marrow (ten Boekel et al., 1995; Ghia et al., 1996). Thus, three major groups of developing B cells can be defined by this method: pro-B cells do not express either L or H chains, pre-B cells express H, but not L chains and B cells express both. During the pre-B cell stage, the product of the rearranged H chain allele, the μH chain, is tested for fitness by its capacity to associate to a molecule expressed on the cell surface and known as surrogate light chain (SL). Only the μH chains capable of paring with SL chains are selected and they form what is known as the pre-B cell receptor (pre-BCR). If the gene rearrangement was non-productive or if the μH chain generated is non-paring, the second allele gets rearranged. Selection is achieved through the proliferation of the cells expressing a pre-BCR over the ones that have not produced a fitting μH chain (Rolink et al., 1993). After the production of the pre-BCR both the SL chain and the gene rearrangement machinery are switched off. However, as soon as the cell runs out of SL chain and for this reason ceases to produce pre-BCR, gene rearrangement is reactivated this time at the L chain loci (κL and λL). Productive rearrangement at one of the L loci followed by paring with the pre-existing μH chain results in transition to immature B cells expressing surface IgM. Those IgM^+ immature B cells that express autoreactive receptors have a chance to survive by undergoing secondary L chain gene rearrangements (Gay et al., 1993; Tiegs et al., 1993). As soon as a non-autoreactive receptor is expressed the recombination mechanisms are turned off

ensuring that each B cell produces only one type of receptor. This mechanism is known as allelic exclusion. Remaining autoreactive cells undergo apoptosis.

The last stage of B cell differentiation takes place in the periphery, most probably in the spleen (Loder et al., 1999) and is characterised by the expression of IgD on the cell surface, so that mature B cells are both IgM and IgD positive cells. When this stage is reached B cells become competent to respond to antigen provided they receive the necessary stimuli.

Antigen is bound by B cells, internalised, processed and returned to the cell surface in association with a molecule known as major histocompatibility complex (MHC) class II. The MHC/peptide complex is then recognised by activated T cells carrying a specific TCR (Lanzavecchia, 1985). These cells express additional cell surface molecules that interact with receptors on B cells providing important activation signals. The immediate effect of activation by T cells is to trigger B cell proliferation. However another important effect can be observed when CD40 ligand (CD40L), a molecule expressed by activated T cells (Armitage et al., 1992; Lane et al., 1992) binds CD40 (constitutively expressed on B cells). CD40L/CD40 interactions in synergy with the effects of cytokines, such as IL-4, IL-5 and IL-6 which are secreted by the T cell, induce B cell differentiation into plasma cells and isotype switching (Jabara et al., 1990; Snapper et al., 1995). Plasma cells produce and secrete large amounts of the soluble form of the immunoglobulin (antibodies). These circulating antibodies can bind to the pathogen protecting the host in three major ways. They facilitate antigen recognition and uptake by phagocytes, they activate the complement system and they neutralise pathogenic effects by coating the microbial surface.

1.4.2. T cells

In contrast to what happens with B cells, T cells cannot recognise antigen free in solution or on the surface of other cells. It must be presented to them in the context of MHC. MHC molecules are glycoproteins that fall into two categories: MHC class I and MHC class II. MHC class I and II bind largely non-

overlapping sets of peptide that are generated in distinct intracellular locations. MHC/ TCR interactions in conjunction with other co-stimulatory molecules provide the necessary signals to activate T cells.

The TCR is a glycoprotein formed by two polypeptide chains (usually αβ chains, although the γδ form is found in some minor T cell populations). Each chain consists of two external Ig-like domains, a transmembrane peptide and a short cytoplasmic tail. The domains that interact with the MHC molecule are extremely diverse and result from the recombination process that takes place in the thymus during T cell differentiation. They are known as variable (V) regions. Conversely, the two domains closer to the T cell membrane are encoded by a limited number of alleles and are thus known as constant (C) regions.

Two main populations of T cells, CD4 T cells and CD8 T cells, can be found in the organism and are named according to the co-receptor they express.

1.4.2.1. CD4 T cells

CD4 T cells also known as helper T cells (Th) recognise the antigen in the context of MHC class II. These molecules are expressed constitutively by dendritic cells (DCs) and B cells and can be induced in other cells by cytokines such as IFN-γ. They generally bind peptides derived from an antigenic exogenous source such as pathogens or bacterial toxins, which were internalised by macrophages, dendritic cells or B cells. Once internalised the antigen becomes enclosed in a series of vesicles known as endosomes, where progressively decreasing pH activates proteases that reside within the vesicles leading to protein degradation. Simultaneously class II molecules are synthesised in the endoplasmic reticulum (ER) where they assemble with an invariant chain (Ii). The function of the invariant chain is to prevent the binding of ER intracellular peptides by the MHC class II molecule (Roche and Cresswell, 1990) and to direct its export through the Golgi apparatus to the acidified endosomes containing the antigen. Here Ii undergoes sequential proteolysis and the MHC class II molecule is loaded with antigenic peptide and

subsequently transported to the cell surface where it is presented to the CD4 T cell.

Although usually MHC class II proteins present exogenous antigen it should be mentioned that class II presentation of peptides derived from intracellular proteins is also possible (reviewed by Lechler et al., 1996).

Two types of CD4 T cells can be distinguished by their cytokine secretion profile: Th1 and Th2 cells. Th1 cells synthesise cytokines like IL-2, IFN-γ and TNF-β, which are not produced by Th2 cells. On the other hand, IL-4, IL-5, IL-6 and IL-10 are examples of cytokines exclusively made by Th2 cells. Finally, cytokines such as IL-3, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) were found to be secreted by both Th1 and Th2 cells. The different cytokine secretion profile is reflected on the different functions exerted by each of the subsets. Hence, while Th1 cells interact mainly with macrophages stimulating their microbicidal activity, Th2 cells help B cells to differentiate and produce antibodies. The fact that each of these subsets regulates a different group of cells means that they also target different pathogens. Thus, Th1 cells act mainly against antigens which have gained access to the intracellular environment, such as viruses while Th2 cells protect against extracellular agents, e.g. bacterial toxins.

1.4.2.2. CD8 T cells

CD8 T cells are lymphocytes that recognise antigen when it is presented to them in association with MHC class I (Townsend et al., 1986). This molecule, in contrast with MHC class II is normally expressed in every nucleated cell of the organism. When a cell is infected, the pathogen's DNA is transcribed and the protein synthesised is transported to the cell surface bound to MHC molecules. MHC class I proteins are synthesised in the ER and generally bind peptides derived from intracellular antigens (Rammensee et al., 1993). The antigens are degraded in the cytoplasm by proteasomes generating peptide fragments. These are then translocated to the ER lumen by a heterodimeric transporter consisting

of two proteins, transporter associated with antigen processing-1 and –2 (TAP-1 and TAP-2) and bind to MHC class I (Lehner and Cresswell, 1996). Finally, loaded MHC class I molecules are released from the TAP complex and exported to the cell surface (Neefjes and Momburg, 1993). CD8 T cells (also known as cytotoxic T cells) are then able to mount a specific response against the cells that present the peptide. Infected cells are destroyed by apoptosis and the resulting apoptotic bodies are internalised by phagocytes.

Although MHC class I mainly presents peptides derived from intracellular proteins, as for class II molecules, a minority pathway of antigen presentation has been described. Evidence emerging from different studies indicates that processing of exogenous antigen can lead to MHC class I-restricted presentation (reviewed by Reimann and Schirmbeck, 1999). This mechanism is known as cross-presentation and dendritic cells are thought to be the major antigen presenting cell (APC) capable of cross-presenting (Carbone et al., 1998).

Two major mechanisms are used by cytotoxic cells to kill the target cell: release of granule cytotoxins and receptor-mediated signalling (reviewed by Shresta et al., 1998). Perforin is one of the cytotoxins present in the cell. It is a protein that can polymerise to form pores in the target cell membrane (Masson and Tschopp, 1985). Other granule proteins include granzymes. Granzymes are serine proteases that are thought to act in collaboration with perforin and have been shown to induce DNA fragmentation and subsequent apoptosis (Nakajima and Henkart, 1994). The second mechanism by which cytotoxic cells kill their targets involves direct cell-cell contact. The activation of a cell surface molecule (Fas) present in mature lymphocytes was shown to cause rapid cell death (Rouvier et al., 1993). Its ligand is called Fas-ligand (Suda et al., 1993) and can be induced in the cytotoxic cell membrane (Hanabuchi et al., 1994).

1.5. Professional antigen presenting cells

There are three main types of cells that can express both MHC class II and co-stimulatory molecules and can thus initiate an immune response: macrophages, B cells and dendritic cells.

Each of these cell types vary in the mechanisms of antigen uptake, expression of MHC class II and co-stimulatory molecules and location in the body. Therefore, each group is optimally equipped for the presentation of a specific type of antigen.

1.5.1. Macrophages

Macrophages are derived from a myeloid precursor and distribute widely in the body tissues. They circulate in the blood as immature monocytes and differentiate into macrophages upon migration into the tissues.

Phagocytosis is the main process utilised by macrophages for antigen uptake and is mediated through specific receptors expressed on the phagocyte surface (Griffin and Silverstein, 1974; Griffin et al., 1975). Once bound, the pathogen is engulfed and processed and can be presented by MHC class II molecules. Resting macrophages, however, do not express MHC class II or co-stimulatory molecules constitutively. The expression of these molecules is induced by the presence of IFN-γ or by the ingestion of microorganisms (Liu and Janeway, 1991). Thus, activated macrophages display increased cytotoxicity to parasites and tumour cells and enhanced expression of both MHC and co-stimulatory molecules, which enables them to act as APCs towards T cells.

1.5.2. B cells

In contrast to macrophages, B cells are non-phagocytic and are therefore restricted in their uptake of extracellular antigen. On the other hand, they are uniquely adapted to bind, internalise and present specific soluble antigen. Uptake is mediated by the surface immunoglobulin receptor (BCR) and

subsequently antigen is processed in intracellular vesicles where it binds to MHC class II molecules (Lanzavecchia, 1985). MHC /peptide complexes are then transported to the surface and, because internalisation is highly efficient and B cells express MHC class II constitutively, can be displayed in high levels on the B cell surface. In fact, B cells are able to present their specific antigen at concentrations up to 1000-fold lower than required for presentation by macrophages and dendritic cells.

When the antigen is not specifically recognised by the B cell, its internalisation is inefficient and presentation on the cell surface is only done at low density.

Expression of co-stimulatory molecules is also initiated by engagement of the receptor (Lenschow et al., 1994) and can be induced by various microbial constituents (Hathcock et al., 1994).

1.5.3. Dendritic cells

DCs are bone marrow derived cells that can be found in their immature stage in the peripheral tissues and as mature cells in the lymphoid organs. The immature phenotype is associated with low levels of MHC proteins and co-stimulatory activity and with efficient antigen uptake, both by phagocytosis and by macropinocytosis (Sallusto et al., 1995), a process by which large volumes of extracellular fluid are internalised. Infection stimulates these cells to migrate to the lymphoid tissues acquiring, during the process, a completely different phenotype. It has been shown that systemic administration of TNF-α, IL-1 or LPS induces DC migration into lymphoid organs (Cumberbatch and Kimber, 1992; Roake et al., 1995), suggesting that these might be some of the factors responsible for DC maturation *in vivo*. Mature DCs loose their phagocytic ability and increase expression of B7, MHC class I and class II molecules and adhesion molecules (Sallusto et al., 1995). These dramatic alterations favour antigen presentation transforming the cells into highly efficient inducers of T cell activation. Due to the unique characteristics which make them highly competent at capturing processing and presenting antigen as well as to their migratory

capacity, DCs play a critical role in immune surveillance. They are, in fact, the specialised cell type that can activate naive T cells (Steinman, 1991) and therefore initiate an immune response.

1.6. T cell differentiation

The thymus is the organ where the majority of T cells are produced, although the gut mucosa is also a site of T cell differentiation. The lymphoid progenitors that colonise the thymus are produced, in an early stage in the fetal liver, and later in the adult bone marrow.

According to the expression of the surface markers CD4 and CD8, thymocytes can be classified into four major subpopulations: the double negative (DN) subset constituted by $CD4^-CD8^-$ cells, the double positive (DP) subset where the cells up-regulate the expression of both markers becoming $CD4^+CD8^+$ cells, and the two single positive (SP) subsets resulting from the downregulation of either the CD4 or the CD8 molecule.

1.6.1. DN cells

Further subdivisions based on other surface markers and functional competence can be made within each subset. Thus, the earliest double negatives are cycling cells with a $CD44^+CD25^-$ phenotype which can give rise to B cells, NK cells and DCs in addition to T cells and that have unrearranged TCR genes. On a second stage of differentiation these cells up-regulate the expression of the CD25 molecule becoming $CD44^+CD25^+$ cells referred to as pro-T cells. Pro-T cells form a rapidly dividing population now fully committed to the T lineage, but still containing the TCR genes in the germline configuration. Rearrangements are first detected in the early pre-T cells ($CD44^-CD25^+$ cells) which follow the pro-T cell stage. Within this subset the TCR β, γ and δ genes are rearranged but not the α genes. At this stage a lineage split can be observed. Those cells that have managed to express functional $\gamma\delta$ TCRs at the surface will engender the $\gamma\delta$

lineage while the ones that have rearranged the β chain gene will follow the differentiation pathway conductive to the formation of αβ T cells.

Once a productive β chain rearrangement has occurred, CD25 is downregulated and the TCRβ is expressed on the cell surface in association with the CD3 molecules and an invariant α chain named pre-Tα (pTα). pTα is equivalent to the SL chain expressed during B cell development and the pTα/ TCRβCD3 complex is known as pre-TCR. Pre-TCR expression suppresses further rearrangements of the β locus, triggers rapid cell proliferation and eventually induces expression of the CD4 and CD8 co-receptors. The cells that failed to productively rearrange the TCRβ gene die by apoptosis while the ones that express a pre-TCR enter the DP stage.

1.6.2. DP cells

The DP subset represents about 80% of the total thymocytes and it is during this stage that important events like TCRα rearrangement and positive and negative selection take place. In contrast to what can be observed in the TCRβ locus, the expression of an α TCR chain does not prevent further rearrangements of the TCRα locus (Borgulya et al., 1992). Therefore, mature T cells expressing two TCRs composed of identical β chains paired with two different α chains are not infrequent (Padovan et al., 1993; Heath et al., 1995; Simpson et al., 1995).

Following completion of TCRα rearrangements and production of the TCRαβ complex, DP thymocytes are subject to positive and negative selection. Positive selection promotes the survival and differentiation of the DPs which TCR can interact with self-peptide/ self-MHC complexes. This mechanism ensures that the cells that leave the thymus will eventually be able to recognise self-MHC molecules complexed with foreign antigens. The cells that fail to recognise self-MHC molecules in the thymus never progress further than the DP stage and die within 3-4 days after the last division. This process is called "death by neglect".

The second selection mechanism is called negative selection and it eliminates thymocytes that express self-reactive TCRs. As in the case of positive selection, the signal is delivered through engagement of the TCR by self-peptide/ self-MHC complexes. A possible explanation for the two different outcomes (cell survival or cell death) that result from the same kind of TCR/ MHC interaction is based on the strength of the signal delivered by the receptor on binding. Thus, accordingly to this hypothesis thymocytes weakly signalled would undergo further maturation (positive selection) whereas thymocytes that received strong signals would die by apoptosis (negative selection). A signal can be stronger or weaker depending on the affinity of the TCR for the peptide/MHC complex or on the density of the complex on the thymic epithelial cell.

An alternative model is based not on quantitative, but on qualitative differences between the signals. Hence, negative selection would be prompted by a complete activating signal, while positive selection would be caused by a partial activation signal being transduced into the cell. Peptides that induce full activation are known as agonists while the peptides that deliver partial activation signals are called antagonists.

Whatever the mechanism by which thymocytes are selected the number of cells that reach the last differentiation stage (SP stage) is extremely reduced (about 2% of total thymocytes in normal mice). This could be attributed to low efficiency of the rearrangements occurring in the TCR genes or to a high frequency of DP cells expressing self-reactive TCRs, which would then be negatively selected. However, even in TCR transgenic mice where most DP thymocytes express a selectable TCR only 20% of the $CD4^+CD8^+$ cells become mature (Huesmann et al., 1991). A possible explanation for this phenomenon was provided by a series of experiments that demonstrated that the availability of selecting stromal microenvironments is rate limiting for positive selection and that during positive selection, immature thymocytes engage only one (or very few) stromal cells. By constructing *in vitro* chimeras between normal and MHC deficient thymi Merkenschlager et al. (1994) showed that the generation of mature SP thymocytes was proportional to the fraction of MHC expressing stroma. Thus thymocytes do not appear to migrate between microenvironments,

which reduces their chances of encountering a selecting ligand. A second experiment using a tracing system for thymocyte/ stromal cell interactions confirmed that each thymocyte engages one (rather than multiple) stromal cell partners (Merkenschlager, 1996). The tracing system used was based on the acquisition by thymocytes of stroma-derived MHC class II determinants. Placing MHC class II-deficient precursors in an environment where all stromal cells express simultaneously H-2^b and H-2^k leads to the acquisition of determinants of both haplotypes by individual thymocytes. However when placed into mosaic stromal environments where stromal cells express either H-2^b or H-2^k evenly interspersed, thymocytes acquire determinants of one or the other haplotypes, but rarely both.

At the beginning of positive selection cells express both CD4 and CD8 co-receptor molecules, but at the end of the selection process, mature thymocytes express only one of the markers. Thus, positive selection also determines the functional potential and the $CD4^+$ or $CD8^+$ phenotype of the thymocytes.

Most TCRs have a "preference" for being expressed in association with either the CD4 or the CD8 marker, but how the lineage commitment process occurs is still not clear. Two models have been proposed initially to explain the mechanism. The instructional model stipulates that the recognition of the specific MHC by the TCR induces the downregulation of either the CD4 (if the TCR is MHC class I specific) or the CD8 co-receptor (if the TCR is MHC class II specific) (von Boehmer, 1986). On the contrary, the stochastic model proposes that the downregulation of the co-receptors is independent of the MHC specificity of the TCR (Robey et al., 1990; Chan et al., 1993). Therefore, DP thymocytes commit themselves to one of the lineages, but only the ones which TCR and co-receptor recognise the same class of MHC differentiate into SPs. Cells with mismatched co-receptor/ TCR combinations die at this stage.

More recently, and taking into account new sets of findings, other models have been formulated. A hemi-instructive model (Lucas et al., 1995; Suzuki et al., 1995) postulates that the MHC recognition requirements for CD4 commitment and for CD8 commitment differ fundamentally. In this variation, CD4

commitment is stochastic but CD8 commitment is instructive. Thus, while entering the CD8 lineage requires MHC class I-dependent instructional signals, commitment to the CD4 lineage is MHC independent and it may represent a default pathway. Another model bases the lineage commitment decisions on the differential binding of the CD4 and CD8 co-receptors to $p56^{lck}$ (an important molecule in TCR signalling) (Itano et al., 1996). It proposes that the quantity of $p56^{lck}$ associated with the TCR complex might impose a bias on lineage commitment, such that MHC engagement producing weak $p56^{lck}$ activation will give rise mainly to CD8 T cells whereas strong $p56^{lck}$ activation will more likely result in CD4 T cells. Since CD8 binds $p56^{lck}$ more weakly, engagement of MHC class I would tend to produce CD8 lineage T cells while recognition of MHC class II molecules would be more likely to produce CD4 lineage cells. A modification of this model was introduced by Basson et. al. in 1998. The authors proposed that it was the relative amounts of TCR and $p56^{lck}$ signals that induced lineage commitment in DP thymocytes. Hence, CD8 differentiation would be induced by TCR engagement combined with relatively little $p56^{lck}$ activation whereas stimuli that preferentially induced $p56^{lck}$ signals relative to the TCR signal would promote CD4 differentiation.

Despite the varied experimental approaches used to identify the mechanism by which DP thymocytes become SP cells, little consensus has been achieved in this matter. Consequently, the debate continues and none of the models has yet been completely accepted or eliminated.

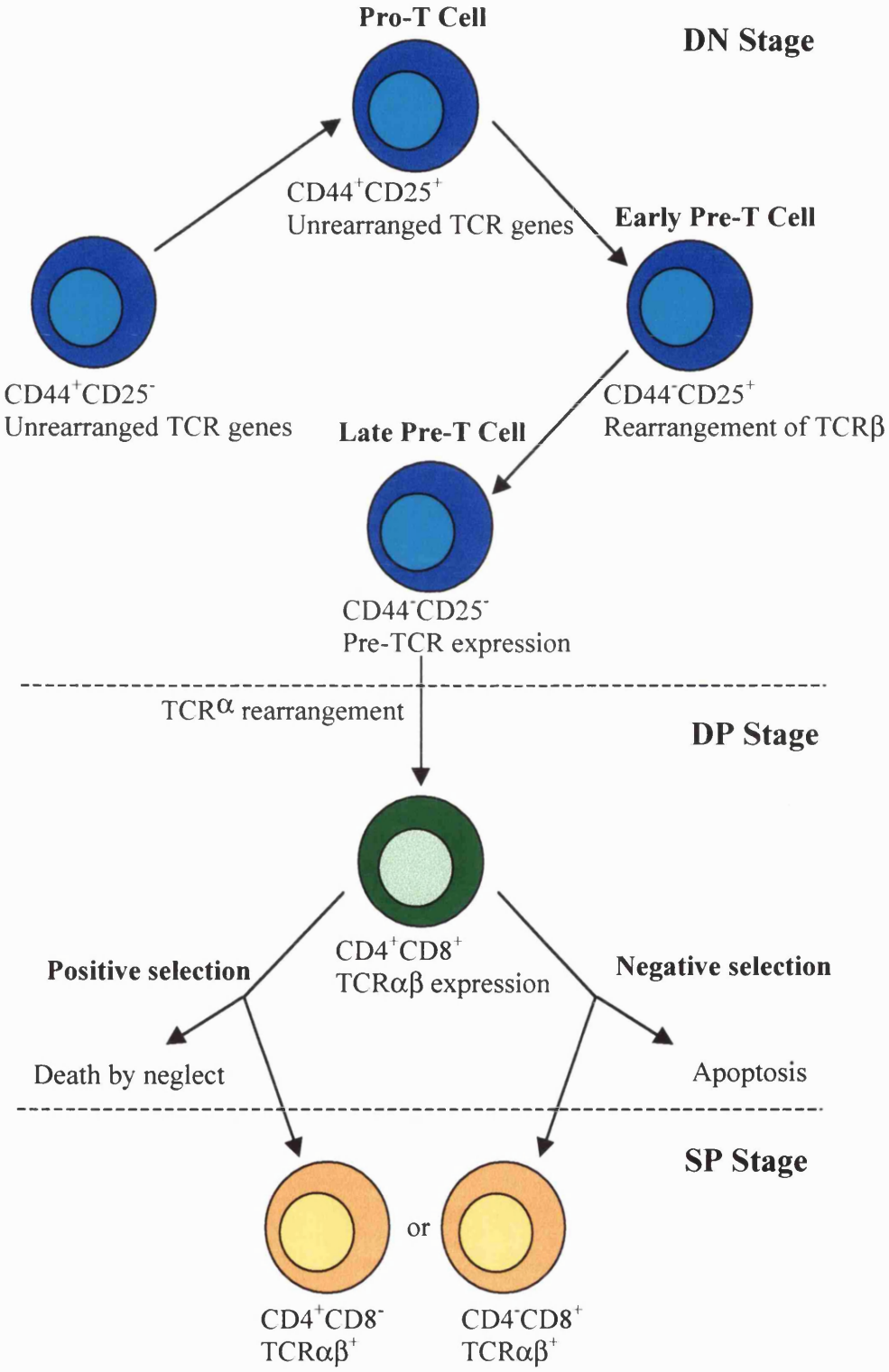
1.6.3. SP cells

As mentioned previously, usually it's the specificity of the TCR for self-MHC molecules that determines which co-receptor a mature T cell will express. It is however, possible to force an MHC class II specific TCR to be expressed on CD8 T cells in the absence of the CD4 co-receptor (Matechak et al., 1996). This situation can be observed in different TCR transgenic mouse strains including one used in this project. The TCR expressed on the A18 strain is generally selected into the CD4 lineage, but when the CD4 gene is deleted from the

genome, A18CD4negative (A18CD4neg) mice express the same TCR on the CD8 T cells.

Once the selection process is finished SP thymocytes accumulate in the inner region of the thymus (thymic medulla). These cells express now higher levels of the TCR and interestingly can remain in the medulla for up to 14 days (Scollay and Godfrey, 1995). The reason for this prolonged retention period is yet unknown, but a final maturation step may be a possible explanation. Petrie et al. (1993) have demonstrated that cultures of $CD3^{high}$ DP thymocytes which are believed to have undergone positive selection, but have not yet downregulated either the CD4 or the CD8 marker, do not give rise to significant levels of $CD4^{+}CD8^{-}$ cells. Also supporting the idea of post-selection maturation is the work of Dyall and Nikolic-Zugic (1995) who isolated and characterised two types of SP thymocytes: $CD4^{+}CD8^{-}$ and $CD4^{+}CD8^{low}$. They observed that while $CD4^{+}CD8^{-}$ cells were fully functional, $CD4^{+}CD8^{low}$ cells were unable to mount an immune response *in vivo* and failed to expand in the peripheral lymphoid organs of lymphopenic hosts. Thus, despite their post-selection status, these cells still appear to require some kind of processing for final development.

T Cell Differentiation



1.7. Peripheral T cells

Once they leave the thymus mature T cells accumulate in the periphery recirculating continuously through the organism. Different subsets of cells at various stages of activation and differentiation contribute to this peripheral lymphoid pool. In a simplified way, peripheral T cells can be divided into three sub-populations: naive cells, activated/ effector cells and memory cells.

Naive cells are cells that have never encountered their specific antigen. After being activated these cells become effector cells and are able to neutralise the pathogen. During the course of an immune response there is a massive proliferation of specific lymphoid clones. However, when the foreign antigen is eliminated the relative proportions of T cell subpopulations returns to normal by a homeostatic mechanism. The majority of the effector cells die by apoptosis, a phenomenon termed activation-induced cell death (AICD), and are cleared by phagocytes. The third phase of the T cell response is characterised by the appearance of specific memory cells that can persist for many years and that can be rapidly summoned into action in case of a recurrent infection. The elimination of effector cells prevents the immune system from being overwhelmed with cells of a certain specificity, which would compromise the capacity of the organism to respond properly upon encountering a new pathogen.

Interestingly, although it appears that most T cell dependent immune responses culminate in memory cell generation, it has been shown that a different outcome can occur. Evidence was presented that exposure of mature T cells to superantigens leads to a marked expansion of $V\beta$ specific T cells followed by their disappearance from the T cell repertoire (Webb et al., 1990). Therefore, the end result of this mechanism of clonal deletion is not memory, but tolerance by exhaustion. Also, in the case of viral infections similar results were obtained (Moskophidis et al., 1993). When infected with a high dose of lymphocytic choriomeningitis virus-docile (LCMV-D), mice bearing virus specific transgenic cells were unable to clear the infection. After a brief but massive proliferation the specific T cells stopped dividing and disappeared from the spleen reaching

undetectable levels by 2 weeks post-transfer. However, when a low dose of virus was used the outcome of the infection was different; the virus was cleared and only a partial elimination of the specific T cells was observed. The remaining specific T cells survived by becoming memory cells.

A very important characteristic of the cells that constitute the peripheral lymphoid pool is their mobility. Lymphocytes recirculate through the whole organism carrying out a routine function of immunosurveillance, which is ultimately dependent on their capacity to move through different tissues. Chemokines play a key role in lymphocyte traffic and in the regulation of cell positioning and cell-cell interactions, which must take place in order to initiate an immune response. Two essential chemokines for T cell homing are the secondary lymphoid chemokine (SLC) and the Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC), which are expressed in the T cell areas of the secondary lymphoid organs (Gunn et al., 1998; Cyster, 1999) and, in the case of SLC, also in the high endothelium venules (HEVs) of Peyer's patches and lymph nodes (Gunn et al., 1998). Both chemokines are associated with lymphocyte homing into these tissues, with preferential activity towards naive T cells (Campbel et al., 1998; Ngo et al., 1998; Pachynski et al., 1998; Stein et al., 2000; Warnock et al., 2000). Analysis of mice deficient in SLC and ELC (Gunn et al., 1999; Luther et al., 2000) reveals that T cells and DCs fail to accumulate in the T cell areas of spleen and lymph nodes of these animals, supporting the idea that the chemokines regulate the entry of both naive T cells and DCs in the secondary lymphoid organs (Gunn et al., 1999). Since co-localisation of T cells and DCs in T cell areas is an essential step in the development of primary immune responses, it is not surprising that the authors found resistance to infection markedly decreased in this strain.

While SLC and ELC are expressed in most lymphoid organs, other chemokines play essential roles in directing particular specialised T cell subsets into non-lymphoid tissues. That is the case of the thymus and activation-regulated chemokine (TARC) present in the skin endothelium, which attracts skin-memory T cells (characterised by their expression of cutaneous lymphocyte antigen

(CLA) (Campbell et al., 1999). It is also the case of the thymus expressed chemokine (TECK) found in intestinal epithelial cells (Wurbel et al., 2000) and responsible for the homing of a subset ($\alpha 4\beta 7^{high}$) of intestinal-memory T cells to the gut (Zabel et al., 1999).

In conclusion, evidence of the importance of chemokines in tissue specific homing is accumulating and it is becoming increasingly clear that these molecules play an essential role in the allocation of certain lymphocyte subsets to particular microenvironments.

1.8. T cell activation

The activation of T cells during an immune response requires contact with antigen presenting cells (APCs). Several adhesion molecules expressed by both T cells and APCs mediate this initial contact. Thus, CD2 a glycoprotein present in the surface of T cells establishes adhesion interactions with Leukocyte Functional Antigen-3 (LFA-3), an APC cell surface molecule. Leukocyte Functional Antigen-1 (LFA-1) and Intercellular Adhesion Molecules 1 and 2 (ICAM-1 and ICAM-2) also promote adhesion between T cells and APCs. However, unlike CD2 and LFA-3 this may be a bidirectional interaction since both molecules can be expressed by T cells as well as by some APCs. Finally, the CD4 and CD8 co-receptors also play a role in the APC-T cell adhesion by interacting with MHC class II or class I respectively.

The initial intercellular contact mediated by these accessory molecules can have two different outcomes: in the absence of the correct peptide-MHC ligand the interactions dissociate allowing the cells to part. If, however enough specific complexes are formed signals are initiated leading to the activation of the T cell.

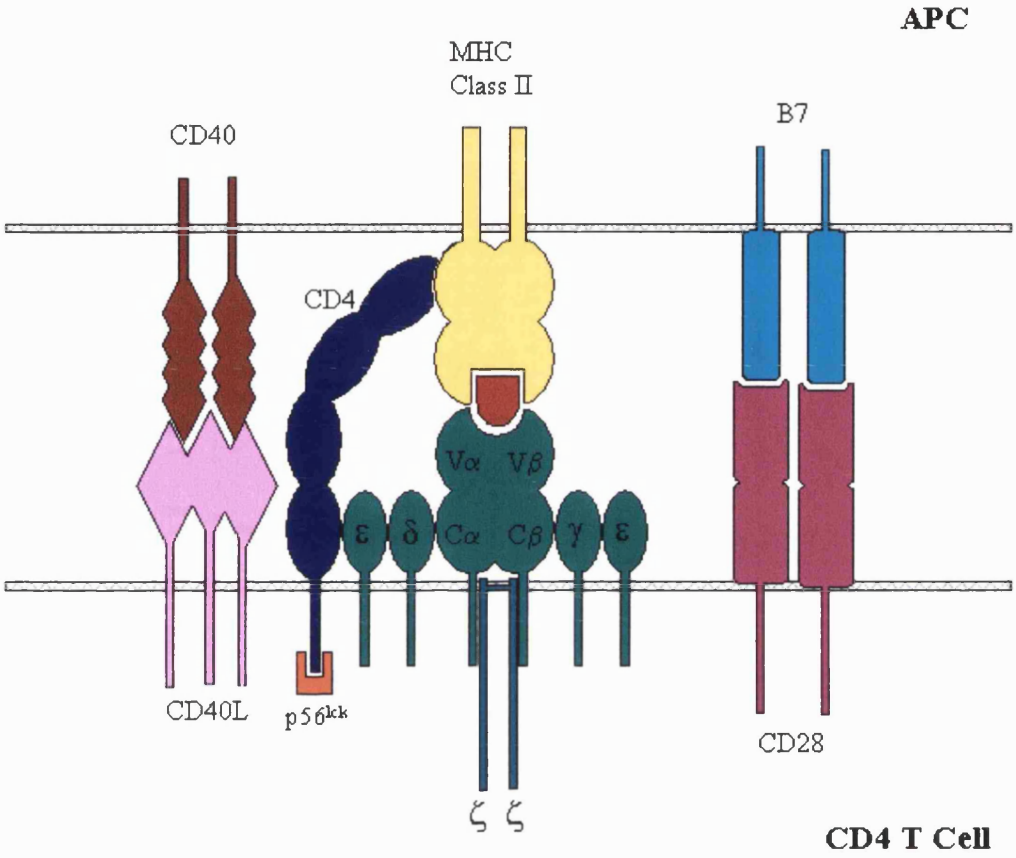
The TCR signals into the T cell through the CD3, a TCR associated complex of proteins. It consists of six molecules which form two heterodimers ($\varepsilon\gamma$ and $\varepsilon\delta$) and a homodimer ($\zeta\zeta$). Simultaneously, the co-receptors CD4 or CD8 recruit $p56^{lck}$ into the immune complex bringing this molecule into close proximity with

the CD3 complex. It is thought that $p56^{lck}$ phosphorilates the CD3 chains and together with the TCR-mediated signal initiates the signalling cascade that can lead to the activation of T cells.

In addition to the signal via the TCR/CD3 complex, naive T cells require a second signal to become activated. This is known as the co-stimulatory signal and should be delivered by the same APC that presents the antigen. Cells that express co-stimulatory molecules on their surface are denominated professional APCs. There are three types of cells that can function as professional APCs: macrophages, B cells and dendritic cells. Both macrophages and B cells express these co-stimulatory molecules only when suitably activated by infection, but dendritic cells express them constitutively.

The most well studied costimulatory pathway involves the binding of two distinct glycoproteins (B7-1 and B7-2) to two homologous ligands expressed on T cells (CD28 and CTLA-4). CD28 and cytotoxic T lymphocyte associated antigen-4 (CTLA-4) can bind both B7 molecules and appear to have opposing effects on the response of T cells to stimulation. Thus, CD28 deficient mice show a marked decrease in the mature T cell proliferative response and in the production of IL-2 after stimulation (Shahinian et al., 1993). Contrarily to CD28, CTLA-4 is found in activated T cells only (Freeman et al., 1992). CTLA-4 deficient mice (Waterhouse et al., 1995) display a profound *in vivo* lymphoproliferation and early lethality, providing evidence that CTLA-4 can function as a negative regulator of T cell activation. These data taken together suggest that the outcome of T cell activation is determined by the integration of stimulatory (CD28) and inhibitory (CTLA-4) signals delivered by these two molecules (Krummel and Allison, 1995).

T cells that receive both TCR mediated and co-stimulatory signals differentiate into effector cells that are able to initiate a specific immune response against the invading pathogen. However the lack of proper co-stimulation can lead to a state of long-lived immunological unresponsiveness called anergy. In the anergic state T cells are incapable of producing their own growth hormone, IL-2 and to proliferate when challenged with the specific antigen (Schwartz, 1990). This



T cell activation – The α β heterodimer that is responsible for the TCR specificity is present on the surface of the cell in association with the various elements of the CD3 complex (γ, δ, ε and ζ). To become activated a naive T cell requires two different signals. The first is delivered by both the TCR/CD3 complex and the co-receptor. Upon activation the TCR is engaged by the MHC-peptide complex and signals into the cell via the CD3 complex. At the same time, the co-receptor binds to the MHC molecule bringing the $p56^{lck}$ molecule into the immune complex. The second signal results from the interactions established between the co-stimulatory molecules CD28 and B7 and CD40 and CD40L. Although not sufficient by themselves for activation, together with the TCR mediated signals they rescue the T cells from anergy, inducing proliferation and differentiation into fully functional effector cells.

mechanism may allow the immune system to inactivate potentially autoreactive peripheral T cells.

1.9. T cell survival

Understanding the specific immune mechanisms depends on the understanding of the life-span of lymphocytes and the factors that influence their survival. But as it was mentioned previously, the peripheral T cell pool is not a homogeneous population and each of the subsets which constitute it seem to have different half-lives and require specific factors for survival.

1.9.1. Naive T cells

The subject of the naive T cell life-span has been a topic of debate for several years and it has not yet been completely understood. Data from experiments performed with euthymic mice points to a relatively short life-span (approximately 8 weeks) of naive T cells (von Boehmer and Hafen, 1993). However, when thymectomised animals were used naive CD8+ T cells were shown to persist without dividing for up to 5 months (Rocha et al., 1995). Recently some light was shed on the role of thymus output that can explain these seemingly contradictory results (Tanchot and Rocha, 1997b). It was shown that thymic migrants substitute peripheral T cells in an age-independent manner. That is when T cells are produced in the thymus they do not have an intrinsic pre-defined life span. Peripheral T cells are randomly replaced by thymic migrants that are competing for the same niche. But if the thymus output decreases or stops completely, naive peripheral T cells will then be able to survive indefinitely because of a lack of competition for the same niche.

For the CD4 subset however, a different situation has arisen. Both in the case of thymectomised animals (Swain et al., 1991; Barthlott et al., 1998) and in the case of transfer of transgenic T cells to euthymic mice (London et al., 1999), significant reductions in CD4 naive T cell numbers have been reported. The reason for the differences observed between the CD4 and CD8 subsets have not

yet been clarified, nevertheless these results seem to point for a reduced survival capacity of the CD4 naive T cells. This possibility will be further explored during the course of this study.

1.9.1.1. TCR/MHC interactions

As far as naive T cells are concerned, several factors have been reported to play a role in their survival. For example, it was demonstrated that MHC/TCR interactions are essential for the survival of both CD8+ (Tanchot et al., 1997a) and CD4+ (Kirberg et al., 1997) T cells in the periphery.

In the case of the naive CD8 T cells, their capacity to survive and the relation between survival and the TCR interactions was studied by comparing their fate after transfer into irradiated hosts that expressed different MHC class I alleles. Tanchot et al. (1997a) showed that monoclonal naive CD8 T cells from a TCR transgenic mouse restricted to MHC class I H-2D^d survived when transferred to mice that lacked H-2K^b but expressed normal levels of H-2D^d. However, in mice that expressed lower levels of H-2D^d, only a fraction of the cells injected survived and this fraction was proportional to the level of H-2D^d expression. In the cases where no H-2D^d was present in the host, the cells disappeared rapidly and could no longer be detected two weeks after the transfer. Confirming this observation and suggesting that these requirements apply to naive CD8 T cell populations in general are the results obtained by Nesic and Vukmanovik (1998). By grafting wild-type thymic epithelium into MHC class I deficient hosts, they demonstrated that differentiation of immature T cells takes place in the graft, but CD8 T cells do not accumulate in the periphery. In fact, significant numbers of peripheral CD8 T cells were only observed after injection of MHC class I-expressing cells in the periphery.

Naive CD4 T cells survival was also shown to be dependent on expression of selecting MHC molecules. When CD4 T cells expressing a transgenic TCR restricted by I-E^d were transferred into immunodeficient hosts of H-2^d haplotype they survived and even proliferated. However, when the transfer was made into H-2^b recipients the cells disappeared within 7 weeks (Kirberg et al., 1997). In the

case of CD4 polyclonal populations identical results were obtained. Using an adenovirus vector to deliver MHC class II into the thymus of MHC class II-deficient mice, Rooke et al. (1997) induced the generation and export of CD4 T cells into MHC class II negative peripheral pools. Alternatively, Takeda et al. (1996) grafted wild-type thymi into class II negative hosts and followed the fate of the naive CD4 T cells overtime. In either case a gradual decrease in peripheral T cell numbers was observed, but while in the first situation the cells were reported to survive less than 2 months, in the second they survived for more than 6 months. Brocker (1997) presents us with a possible explanation for the discrepancy. He demonstrates that when the thymus is depleted of all bone-marrow class II^+ derived cells prior to transplantation into MHC class II^- hosts, CD4 T cells do not accumulate in the periphery. However, when the transplanted host expresses MHC class II molecules exclusively on DCs, CD4 T cells accumulate in the periphery. Thus, it is possible that the extended survival observed by Takeda et al. is caused by the presence of class II^+ cells exported by the grafted thymus.

The issue of precisely how the signal resulting from engagement of the MHC molecule by the TCR promotes survival was addressed in a recent work by Witherden et al. (2000). In this study MHC class II expression is limited to thymic epithelial cells in a tetracycline (tet)-controllable manner. That is in the presence of tet no MHC class II molecules are expressed in both the thymus and the periphery; in it's absence MHC class II is found in the thymus but nowhere else. Interestingly when the authors examined CD4 T cells obtained with this method from a class II^- periphery they found abnormally low levels of CD3 ζ chain phosphorylation and ZAP-70 recruitment. In naive T cells from normal mice the CD3 ζ chain was observed to be partially phosphorylated (van Oers et al., 1993) and to associate with ZAP-70 (van Oers et al., 1994). Thus, it is likely that weak MHC/TCR interactions deliver a signal into the T cell which is translated in basal phosphorylation of the CD3 ζ chain, ultimately promoting survival. In the case of T cells that are released into a class II-deficient periphery though, no MHC/TCR interactions can be established, there will be no

phosphorylation of the CD3 ζ chain and consequently T cells will not be able to accumulate.

Another situation where low affinity interactions between MHC and TCR results in better survival of T cells is during thymic differentiation. The low avidity reactions involved in the positive selection of these cells increase the chance of double positive thymocytes surviving death by neglect. Therefore, it is possible that the same mechanism may be used both by immature and mature T cells, but it remains unclear if the same MHC/ peptide complexes are involved in both situations. In fact different conclusions have been drawn by different authors on this subject. Ernst et al.(1999) showed that CD4 T cells expressing a transgenic TCR that is not positively selected on an H-2M$^-$ background failed to proliferate in irradiated H-2M$^-$ hosts and gradually disappeared. By contrast, when the cells injected can undergo positive selection in an H-2M$^-$ background, they survive and proliferate well. The authors concluded thus that the peptides that mediate positive selection in the thymus play a crucial role in peripheral T cell survival and homeostatic proliferation. Contradicting this hypothesis, Bender et al. (1999) did not observe homeostatic division of CD4 T cells transferred into irradiated recipients that express class II molecules occupied solely by the peptide responsible for positive selection. However, when an identical experiment was performed using the same system, but with later time points, CD4 T cells survived and expanded (Muranski et al., 2000). Hence, the different settings used may be responsible for the distinct conclusions obtained in similar studies.

1.9.1.2. Cytokines

Although the presence of complementary MHC molecules in the periphery appears to be essential for mature T cell survival, other factors have been identified as possible survival elements. It has been shown in several *in vitro* studies that resting T cells can be kept alive for longer, when cultured in the presence of some cytokines. IL-4 and IL-7 are two of these cytokines. Resting T cells isolated from a transgenic mouse strain and cultured during 48 hours in the presence of IL-4 and IL-7 showed less than half of the death rate observed in

control cultures (Vella et al., 1997). In the same study, two other cytokines, IL-2 and IL-15 were shown to have some effect on increasing the viability of the cell cultures, although this effect was only evident at very high concentrations. Also in the case of human $CD45RA^+$ T cells, addition of IL-4 and IL-7 allowed the cells to survive for longer in culture (Soares et al., 1998). Moreover, the increase in cell recovery induced by IL-7 resulted in part from proliferation, which suggests a role for this cytokine not only in prolonging cell life, but also in the expansion of the naive repertoire.

A common factor to IL-2, IL-4, IL-7 and IL-15 is that all of them can signal through the γ chain of the IL-2 receptor (IL-2R). A possible mechanism through which this signalling can retard apoptosis is by the induction of anti-apoptotic genes.

The Bcl-2 gene family encodes for apoptosis regulatory proteins and is, for this reason, a strong candidate to the role of mediator in the cytokines' pathway. At least fifteen Bcl-2 mammalian family members have already been identified (Adams and Cory, 1998) and it is possible that other relatives are yet to be described. The most well studied family members are the bcl-2, bcl-x and bax proteins. Bcl-2 is a 26 KDa protein, which has been localised to the mitochondrial outer membrane, smooth endoplasmic reticulum and nuclear envelope (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993) and has an inhibitory effect in apoptosis. Conversely to bcl-2, bax is a pro-apoptotic molecule, promoting death rather than survival (Oltvai et al., 1993). The third gene, bcl-x, encodes two different proteins with opposing effects. The longer bcl-x_L variant has an anti-apoptotic function while the shorter form of the protein (bcl-x_S) promotes apoptosis (Boise et al., 1993). It appears that is the relative concentration of pro- and anti-apoptotic family members which influences the cell fate to survive or die (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994; Sedlak et al., 1995).

Akbar et al. (1996) presented evidence that indeed the four cytokines mentioned above influence the expression of Bcl-2 family genes in activated T cells. The expression of Bcl-2 and Bcl-x_L relative to Bax and Bcl-x_S was shown to be

increased in cells cultured in the presence of these cytokines. A similar effect was observed in resting T cells cultured with IL-4 (Vella et al., 1997). Bcl-2 and Bcl-x_L protein levels persisted in cells co-cultured with IL-4, but gradually disappeared in control samples. In the case of IL-2 however, a different situation was encountered. Pre-treatment with IL-2 was shown to enhance human T cell resistance to γ irradiation, but neither Bcl-2 nor Bcl-x_L levels were affected (Boise et al., 1995).

IL-6 is another cytokine that can rescue resting T cells from apoptosis (Teague et al., 1997). In the presence of this factor no decrease of Bcl-2 expression was observed in cultured CD4 T cells. However, when compared with the effects of both IL-4 and IL-7, the percentage of cells rescued from death was somewhat reduced (Marrack et al., 1998). The reason why some T cells are IL-6 insensitive is not yet known, but, based on preliminary observations, the authors suggested that prior exposure to antigen was a possible contributing factor.

All the cytokine studies mentioned until now were performed *in vitro*, but recently, using IL-7 depletion and IL-4 deficient mice, it was possible to assess the effect of each of these cytokines on naive T cells *in vivo*. Although the absence of only one of the cytokines did not appear to have an effect on the naive CD4 T cell populations of thymectomised animals, when both IL-4 and IL-7 were absent CD4 T cell numbers decreased significantly (Boursalian and Bottomly, 1999).

Finally, two other observations support the hypothesis that IL-4, IL-6 and IL-7 may play an important role in maintaining resting T cells *in vivo*: first, IL-7 and IL-6 are constitutively made in animals and second resting T cells bear high affinity receptors for IL-4, IL-6 and IL-7.

1.9.2. Memory T cells

Memory T cells arise from naive cells that have been challenged with specific antigen and constitute the last stage of differentiation in an immune response.

However, the precise lineage by which naive T cells differentiate into memory cells is still not well understood. It is not clear if memory and effector cells differentiate along separate pathways through parallel lineages or if memory T cells are the progeny of effector cells that escaped AICD. A recent report by Opferman et al. (1999) appears to supports the later model. Performing adoptive transfer experiments with TCR transgenic cells, the authors measured the ability of post- and pre-effector CD8 T cells to give rise to memory cells. They demonstrated that generation of memory cells required al least five cell divisions in response to antigenic challenge. This observation sustains the hypothesis that naive T cells have to go through an effector phase before they can reach the memory stage.

But whether effector cells give rise to memory cells or not, it is clear that the great majority of effectors are short-lived cells. On the other hand, since the challenge with viruses during infancy leads usually to life-long immunity it is generally assumed that memory is long-lived. Comparison of the expression levels of Bcl-2 family members in both subsets confirms this idea. The ratio between anti- and pro-apoptotic mRNA is observed to decrease in effector cells compared with naive cells, but in memory cells the anti-apoptotic message is increased again (Garcia et al., 1999).

Peripheral T cells undergo homeostatic proliferation to reconstitute the immune system, when transferred into lymphopenic hosts. In the case of memory cells, this division continues even after lymphoid homeostasis is reached. In normal mice a fraction of memory cells have been shown to be cycling and dividing continuously (Tough and Sprent, 1994). The factors responsible for the maintenance of this population, however, have been the subject of a long-standing debate. Several authors have made the case that long-term memory is dependent on persistent antigenic stimulation (Gray and Matzinger, 1991; Oehen et al., 1992). Later experiments, though have demonstrated that at least some CD8 T cells can survive in the absence of specific antigen (Hou et al., 1994; Lau et al., 1994; Mullbacher, 1994). In the case of CD4 T cells, opinions are still divided. Some authors maintain that the presence of antigen is essential for CD4 memory T cell survival (Gray and Matzinger, 1991; Gray et al., 1996) while

others show long-term persistence of transferred cells into antigen free environments (Swain, 1994; Garcia et al., 1999).

Also the role of MHC molecules in T cell memory has been discussed extensively with different conclusions arising from different studies. Tanchot et al. (1997a) observed that memory CD8 T cells expressing a transgenic TCR specific for the H-Y male antigen survived and expanded when transferred into irradiated hosts that differed in MHC class I expression, but disappeared in mice lacking both β2-microglobulin and the MHC restricting element. They concluded that maintenance of CD8 T cell memory did not depend on the restricting MHC allele, but still required some type of TCR/MHC interaction. Using the same transgenic strain, Markiewicz et al. (1998) arrived to similar conclusions. Memory cells survived for at least 70 days in mice expressing TAP-1, while most transgenic cells transferred into TAP-1 deficient hosts (expressing less than 10% of normal levels of MHC class I) disappeared in two weeks. In a different study however, the authors observed persistence and proliferation of both monoclonal and polyclonal memory CD8 T cells after transference into irradiated mice completely lacking the classical MHC class I molecules (K^b and D^b) and β2-microglobulin. The reason for the differences observed has not been clarified yet, but they may reflect different requirements for different TCR specificities. Further work in the subject will have to be done before any definite conclusions can be drawn.

The MHC class II requirements for CD4 memory T cells were evaluated by transferring TCR transgenic memory cells into class II knockout mice (Swain et al., 1999). Donor cells were shown to persist for more than 60 days, when the experiment was terminated. The authors concluded that the transferred memory cells did not require further class II recognition to survive. It remains to see if the results of this experiment can be extrapolated to other TCR specificities and to the polyclonal population in general.

Another possibility is the stimulation of memory T cells in a non-antigen-specific manner through contact with cytokines. For instance, injection of mice

with purified IFN-I induces marked proliferation of memory-phenotype CD8 T cells ($CD8^+CD44^{high}$) (Tough et al., 1996). IL-12 can also stimulate CD8 memory expansion and, to a smaller extent CD4 T cell memory turnover (Belardelli et al., 1998). IL-15, on the other hand seems to induce a potent and selective proliferation of $CD8^+CD44^{high}$ T cells, but it has only a minimal effect on CD4 T cells (Zhang et al., 1998). In fact, in IL-15 receptor-deficient mice, memory phenotype CD8 T cells are selectively reduced in number when compared with their CD4 counterparts (Lodolce et al., 1998). A recent report by Ku et al. (2000) not only confirms that the division of CD8 memory T cells is driven in large part by IL-15, but also confers to IL-2 an inhibitory role. Thus, the authors suggest that IL-15 and IL-2 have opposing effects on the CD8 memory subset and are kept in check by each other, allowing the immune system to respond vigorously, but not uncontrollably to infections.

1.10. Peripheral homeostasis

Despite its complexity and the variety of challenges that it is subject to, the immune system maintains a dynamic equilibrium in terms of size and subset composition throughout much of normal adult life.

It has been shown by studying different lines of mutant mice that B and T cells are independently regulated. Similar numbers of B cells are found in normal, thymectomised, athymic and TCR knockout mice (Mombaerts et al., 1992). The same was observed in the opposite situation, mice that lack B cells (μMT) (Kitamura et al., 1991) have identical T cell numbers to normal mice. These observations led to the conclusion that B and T cells require different factors for the maintenance of population numbers, that is they do not compete for resources. Resources are considered to be present in limited amounts in the immune system and to be the combination of elements that promote cell survival and growth. Many molecules can function as resources: MHC molecules, hormones, cytokines, ligands for co-receptors or adhesion molecules, mitogens, antigen, chemokines, etc. In a situation where two populations compete for survival signals, the removal of one will lead to the expansion of the other.

Populations that share common resources are said to occupy the same "niche". Niche differentiation ensures the coexistence of different cell types (Freitas and Rocha, 2000).

1.10.1. B cells

Within the B cell population three different compartments can be found (pre-B cells, mature resting B cells and activated B cells). Results presented by Agenes et al. (1997) suggest that the size of each compartment is autonomously regulated. In chimeras reconstituted with different ratios of bone marrow (BM) cells from normal and B-cell deficient hosts, the size of the pre-B cell pool has been shown to depend exclusively on the number of immature stem-cells present. Moreover, B cell production is not influenced by the number of mature peripheral B cells. The rates of pre-B and B cell division are constant and independent of the peripheral pool size, *i.e.* there is no feed-back regulation of the central pre-B cell compartment by the number of mature B cells. This mechanism ensures the maintenance of a diverse repertoire by preventing the accumulation of specific B cell clones that may have expanded during the course of ongoing immune responses. On the other hand, although a minimal continuos input of new cells is required for the maintenance of physiological peripheral B cell numbers, the size of the peripheral pool is not determined by the number of B cell precursors. In fact, using parabiosis between normal and B cell deficient mice the authors were able to show that the B cell production of one single animal is enough to replenish the B cell pool of three mice. Thus, the survival of newly produced B cells is not only limited by the avidity of the interactions between each B cell and its ligands, but also by the presence of other cells that compete for the same resources. Finally, in the case of the activated B cell compartment it was observed that both the number of IgM-secreting cells and the levels of serum IgM were constant and independent of the size of the pre-B and mature B cell pools. So the autonomous homeostatic regulation of each of the three compartments allows the efficient functioning of the immune system. On one hand it prevents the restriction of the peripheral B cell repertoire and on

the other ensures the presence of normal IgM levels that act as a first natural barrier of protection against invading organisms.

1.10.2. T cells

Different observations suggest that the size of the peripheral T cell pool is relatively independent of the thymic output. For instance, the fact that thymectomised mice do not show a drastic reduction in T cell numbers has been known for long (Miller, 1962). More recently other experiments have also supported this notion. Mice transplanted with neonatal thymi show a constant thymic export rate irrespective of whether the peripheral pool is oversupplied (thymic graphs) or undersupplied (neonatal thymectomy), and have constant peripheral numbers (Berzins et al., 1998). Quantification of the expansion potential of CD4 T cells by serial passages *in vivo*, revealed that each cell is able to divide up to 56 times. However, recipient nude mice injected with different numbers of mature T cells reconstituted their peripheral compartment to similar levels (Rocha et al., 1989). These observations suggest an important role for peripheral mechanisms in the regulation of the immune system.

Within the peripheral T cell pool the CD4 and CD8 subsets may partially share common resources. In the absence of one of the populations the other expands, so that the total T cell number is identical to that of normal mice. This can be seen in CD4 knockout mice (Rahemtulla et al., 1991), β2-mycroglobulin knockout mice (Zijlstra et al., 1990), MHC class II knockout mice (Cosgrove et al., 1991) and in reconstituted nude mice treated with either anti-CD4 or anti-CD8 antibodies (Rocha et al., 1989).

1.10.2.1. Naive and memory T cell niches

Within the CD8 T cell subset, naive and memory T cells appear to occupy different "niches". Experiments realised using euthymic transgenic or non-transgenic mice as well as euthymic irradiated chimeras reconstituted with transgenic or non-trangenic BM and mature CD8 T cells, showed that all the

animals had similar numbers of peripheral CD8 T cells. About half of these were memory cells. However when the mice were manipulated to contain only either naive or memory cells, the peripheral pool was reduced by half (Tanchot and Rocha, 1995). To reconstitute a normal-sized compartment, both naive and memory cells are required. The independent homeostatic regulation of each subset ensures the maintenance of repertoire diversity while optimising the immune response. Thus, the preferential incorporation of thymus migrants (naive T cells) in the periphery whereas increasing the capacity of the immune system to react to new antigen would reduce the presence of resident memory cells and consequently the capacity of response of the immune system to recurrent infections. Alternatively accumulation of memory cells would lead to the restriction of the T cell repertoire jeopardising the capacity of response to new antigen.

The notion that naive and memory T cells belong to different niches is also supported by studies of substitution of peripheral T cells by thymus migrants. It was shown that naive T cells decayed, while memory cells persisted in the presence of thymic output (Tanchot and Rocha, 1997b). These results suggest the existence of competition within the naive subset, but not between the naive and memory pools. Interestingly, tolerant T cells were also dislodged by thymic migrants in an exponential way. The majority of the cells disappeared, but a few tolerant T cell clones remained for prolonged periods of time (Tanchot and Rocha, 1997b). Therefore, continuous thymic output ensures that self-reactive clones are maintained at very low frequencies in the periphery. A possible role suggested for the residual tolerant cells is the regulation of immune responses and maintenance of tolerance through the production of cytokines like IL-10 and IFN-γ. These experiments were performed using CD8 T cells. It is not yet known if CD4 T cells follow the same strategy.

1.11. Aims

This project is concerned with the investigation of the mechanisms involved in the maintenance of homeostasis within the peripheral naive T cell pool. The use

of several different TCR transgenic mouse strains crossed onto a Rag knockout background made possible to study individual T cell clones determining specific survival and proliferative characteristics for each one of them. Models to study competition between monoclonal T cell populations were also generated by making cell transfers between TCR transgenic mouse strains bred into identical genetic backgrounds.

The second part of the project, seeks to assess the influence of level of co-receptor expression or expression of an adhesion molecule (EVA molecule) in thymic selection, *in vitro* survival and homeostatic proliferation. In this case, the animals analysed were A18 TCR transgenics expressing a second transgene. CD4VA2B.A18 and A18EVA mice were used respectively.

2. Materials and Methods

2.1. Animals

The strains used for these analysis were A18 TCR transgenic ($Rag1^{-/-}$ $C5^{-/-}$) (Zal et al., 1994), A1 TCR transgenic ($Rag1^{-/-}$ $C5^{-/-}$) (Zelenika et al., 1998), A18 $CD4^{-/-}$ ($Rag1^{-/-}$ $C5^{-/-}$), CD4VA2B.A18 TCR transgenic mice ($Rag1^{-/-}$ $C5^{-/-}$) (generated by Rose Zamoyska), AND $Rag1^{-/-}$ (Kaye et al., 1989), F5 $Rag^{-/-}$ (Mamalaki et al., 1993), BM3 $Rag^{-/-}$ (Sponaas et al., 1994) as well as syngeneic $Rag1^{-/-}$ $C5^{-/-}$ $H\text{-}2^a$, $Rag1^{-/-}$ $H\text{-}2^k$ and $Rag1^{-/-}$ $H\text{-}2^b$ mice. Polyclonal controls were B10 ($H\text{-}2^b$), CBA ($H\text{-}2^k$) or A/J ($H\text{-}2^a$). All mice were kept in conventional, but pathogen-free, animal facilities at the National Institute for Medical Research (London, U.K.)

Table 1. TCR specificities, restriction elements and MHC haplotypes of the different mouse strains used in this project

STRAIN	TCR SPECIFICITY	RESTRICTION ELEMENT	MHC HAPLOTYPE
A18	C5 – (106-121)	$H2\text{-}E^k$	$H\text{-}2^a$
A1	Dby peptide 8	$H2\text{-}E^k$	$H\text{-}2^k$

CD4VA2B.A18	C5 – (106-121)	H2-E^k	H-2^a
A18EVA	C5 – (106-121)	H2-E^k	H-2^a
AND	Pigeon Cytochrome c (88-104)	H2-E^k	H-2^{bxk}
A18CD4neg	C5 – (106-121)	H2-E^k	H-2^a
F5	NP-(366-379)	H2-D^b	H-2^b
BM3	H2-K^b	H-2^k	H-2^k
CBA	-----	-----	H-2^k
B10	-----	-----	H-2^b
A/J	-----	-----	H-2^a

2.2. Monoclonal antibodies

Table 2. Specificity, name and reference of the monoclonal antibodies used in this study

Specificity	Name	Reference
CD4 (PE)	H129.19	Pharmingen (San Diego, CA)
CD4 (APC)	RM4-5	Pharmingen (San Diego, CA)
CD8 (PE)	53-6.7	Pharmingen (San Diego, CA)
CD8 (APC)	53-6.7	Pharmingen (San Diego, CA)
CD8α (FITC or biotin)	YTS 169.4	(Cobbold et al., 1984)
CD45RB (biotin)	16A	Pharmingen (San Diego, CA)
CD44 (biotin)	IM7	Pharmingen (San Diego, CA)
TCR (FITC)	H57-597	Pharmingen (San Diego, CA)
TCR (PE)	H57-597	Cambridge Bioscience (Cambridge, UK)
TCR-Vβ8.3 (FITC or biotin)	7G8.2	(Forster et al., 1995)
TCR Vβ8.2 (FITC or biotin)	F23.2	(Kappler et al., 1988)
CD5 (FITC)	53-7.3	Pharmingen (San Diego, CA)
Bcl-2	3F11	Pharmingen (San Diego, CA)
Bcl-2 isotype control	---------	Pharmingen (San Diego, CA)

2.3. Media

The culture medium used was Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% heat inactivated fetal calf serum (FCS), $2x10^{-3}$ M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and $5x10^{-5}$ M β-mercaptoethanol (all Sigma). Medium for washing cells was air buffered IMDM containing 25mM HEPES and L-glutamine and supplemented with 0.21% NaCl, 100 U/ml penicillin,100 μg/ml streptomycin and 12.5mM NaOH (AB medium).

2.4. FACS analysis

Expression of cell surface antigens was determined by analytical flow cytometry using a FACScan or a FACScalibur (Becton Dickinson) followed by data processing in CellQuest software (Becton Dickinson).

Stainings were performed with fluorescein isothiocyanate- (FITC-), phycoerythrine- (PE-), allophycocyanin- (APC-) or biotin-conjugated monoclonal antibodies (mAb). Cells were preincubated with unlabeled mAb to FcγRII/III (2.4G2) to minimise unspecific staining. All stainings were performed on ice and washed with washing buffer (PBS, 2% FCS, 0.1% azide). Staining with biotinylated mAb was followed by a second staining step with streptavidin-RED670 or streptavidin -RED613 (Life Technologies, Paisley, UK).

For analysis of Bcl-2 expression, cells were first stained with PE-anti-CD4 or -anti-CD8 mAbs, fixed in PBS and 1% paraformaldehyde, and then incubated with FITC-conjugated anti-Bcl-2 mAb or FITC-conjugated isotype control in saponin buffer for 30 minutes on ice.

2.5.Cell death analysis

To obtain single cell suspensions, spleens were digested using collagenase type 4 (Worthington Biochemical Corporation, UK) and Deoxyribonuclease I (Sigma, St. Louis, MO) at a concentration of 1mg/ml and 0.02 mg/ml respectively, in AB medium. Spleens were kept in the solution, shaking gently, for 30 minutes at room temperature, until fully digested. Lympho-Sep Mouse Lymphocyte Separation Medium (Harlan, UK) was used to eliminate red blood cells from the preparation. The cells were then plated in round bottom 96-well plates (Falcon, Becton Dickinson) at a density of $2x10^5$ cells/well in a final volume of 200 μl/well. The percentage of dead cells was determined in the beginning of the experiment and then, following incubation at 37°C, after 1, 2 and 3 days of culture.

The method used to assess cell death was previously described by Schmid et al. (1992) and modified later by Lecoeur and Gougeon (1996). Briefly, after pre-incubation with unlabelled mAb against FcγRII/ III (2.4G2) to minimise unspecific staining, cells were incubated with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 for 30 minutes at 4°C. Cells were then washed with washing buffer (PBS, 2% FCS, 0.1% azide) and incubated with 20 μg/ ml of 7-AAD (Sigma, St. Louis, MO) for 20 minutes at 4°C, for detection of early apoptosis. Subsequently cells were acquired by FACS.

2.6.Adult thymectomy and blood analysis

Adult thymectomy was performed under anaesthesia with Hypnorm/Hypnoval and Temgesic (Hoffmann La Roche). The thymus was removed by inserting a tube connected to a suction pump through an incision made above the sternum area. Once the extremity of the tube reached the area of the thymus localisation, the pump was connected and the organ was removed by suction. The successful extraction of the thymus was carefully checked in all animals once they were killed at the end of the test period.

The animals were bled weekly during 6-9 weeks and the blood samples were stained with anti-CD4 or anti-CD8 monoclonal antibodies. Erythrocytes were lysed using FACS Lysing Solution (Becton Dickinson, San Jose, CA).

2.7.Carboxy-fluorescein-diacetate succinimidyl ester (CFSE) cell labelling and adoptive transfer

For CFSE labelling, mouse spleen and lymph nodes were removed and gently pressed through a fine nylon mesh to obtain single cell suspensions. The cells were resuspended in AB medium and pelleted by centrifugation. The pellet was resuspended in PBS at a concentration of 10^7cells/ ml and incubated with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 2.5 μM for 10 minutes at 37°C. Cells were then washed twice with AB medium and injected into the tail vein of the adoptive host. At different time points after injection spleen and lymph node single cell suspensions were stained with anti-CD4 or anti-CD8, anti-CD44, anti-CD45RB and anti-TCR. Cells were analysed by FACS as described previously. Due to the high fluorescence intensity displayed by the CFSE dye, compensation was increased at the FL2-FL1 level in order to avoid detection of the signal in the FL2 channel. A homogeneous decrease of CFSE intensity was observed 24-48 hours after labelling and can probably be attributed to membrane turnover, which would eliminate any CFSE molecules that had remained attached to it.

2.8.CDR3 length analysis

CDR3 length analysis was performed using a modified protocol similar to the one described by Pannetier et al. (1993). The following steps were followed during the process:

- **2.8.1.Cell sorting**

Pooled spleen and lymph node cells from thymectomised and non-thymectomised CBA mice were stained with anti-CD4 and anti-CD44 mAbs, and $CD4^+CD44^-$ cells were sorted on a MoFlo machine (Cytomation).

- **2.8.2.RNA and cDNA preparation**

Total RNA was extracted from $5x10^5$ to $2x10^6$ $CD4^+CD44^-$ T cells using the TRIZOL reagent according to the recommended procedure (Gibco-BRL, Life Technologies, Paisley, UK). Single-strand cDNA synthesis was performed by using the RNA PCR Core Kit (Perkin Elmer, Roche Molecular Systems).

- **2.8.3.PCR amplification**

From each cDNA PCR reactions were performed using a Vβ8.3 primer (TGCTGGCAACCTTCGAATAGGA) and a Cβ primer (GCCAGAAGGTAGCAGAGACCC). The amplification started with a denaturation step of 30 seconds at 94°C, followed by 40 cycles consisting of 30 seconds at 94°C, 45 seconds at 60°C and 45 seconds at 72°C, and finally a 5 minute step at 72°C.

- **2.8.4.Run-Off reactions**

Using 2 µl of the PCR products as a template, run-off reactions were performed with an internal fluorescent FAM-labelled Cβ primer (CTTGGGTGGAGTCACATTTCTC) at a final concentration of 0.2 µM. A denaturation step of 2 minutes at 94°C, was followed by 3 cycles consisting of 45 seconds at 94°C, 1 minute at 60°C and 45 seconds at 72°C, and finally a 15 minute incubation at 72°C.

- **2.8.5.Data Analysis**

Run-off products were denatured in 10 µl formamide and run on a 6% acrylamide/ 8M urea gel using a 373A DNA sequencer (Applied Biosystems). Data analysis was performed with the immunoscope software (Pannetier et al., 1993).

3. Results

3.1. Determination of absolute T cell numbers in TCR transgenic and non-transgenic mouse strains

To investigate the survival characteristics of CD4 and CD8 T cells, several TCR transgenic and non-transgenic mouse strains were analysed during the course of this study.

The A18 TCR transgenic strain expresses a TCR restricted by H-2E^k and specific for the 106-121 peptide derived from the circulating self-protein C5 (fifth component of complement). A natural mutation occurring in about 40% of the mouse strains results in the absence of C5 protein allowing the selection and export of mature C5 specific T cells into the periphery (Zal et al., 1994).

Previous data obtained from the analysis of the A18 TCR transgenic strain crossed onto a Rag knockout background have shown that although the thymic selection processes occurring in these mice are normal and that the generation of CD4 SP thymocytes is comparable with normal mice, in the periphery very few CD4 T cells can be detected (Barthlott et al., 1998).

The first question to address was whether similar situations could be observed in other TCR transgenic mouse strains. Four additional TCR transgenic mouse strains were therefore analysed and a comparison between spleen cellularity was performed. All TCR transgenic strains used were bred onto a Rag knockout background to avoid the expression of additional unrelated TCR specificities.

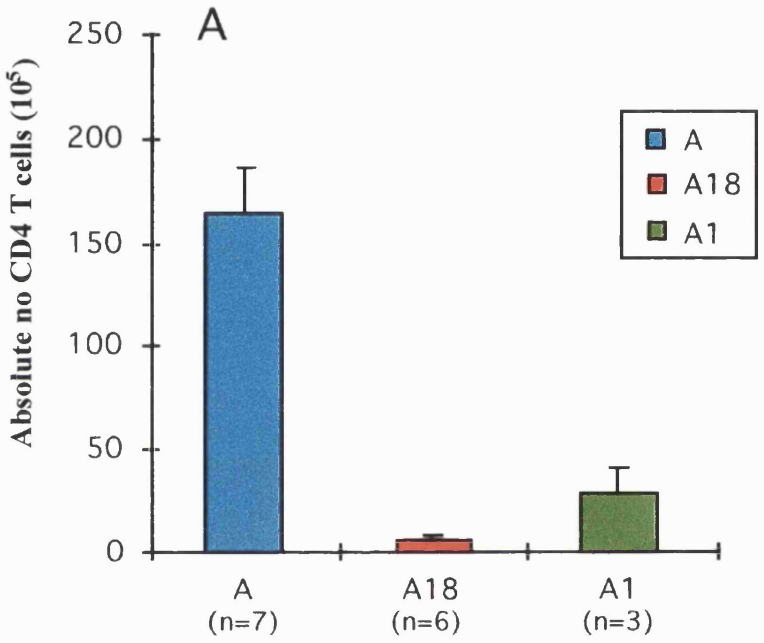
The A1 TCR transgenic strain selects CD4 T cells recognising the Dby peptide 8 derived from H-Y presented by H-2E^k (Scott et al., 2000), the F5 TCR transgenic strain selects CD8 T cells reactive to influenza nucleoprotein epitope 366-374 in the context of H-2D^b and the BM3 TCR transgenic strain selects CD8 T cells restricted by H-2^k and specific for the alloantigen H-2K^b. A18 mice when bred with mice that lack CD4 (A18CD4neg) select the A18 TCR

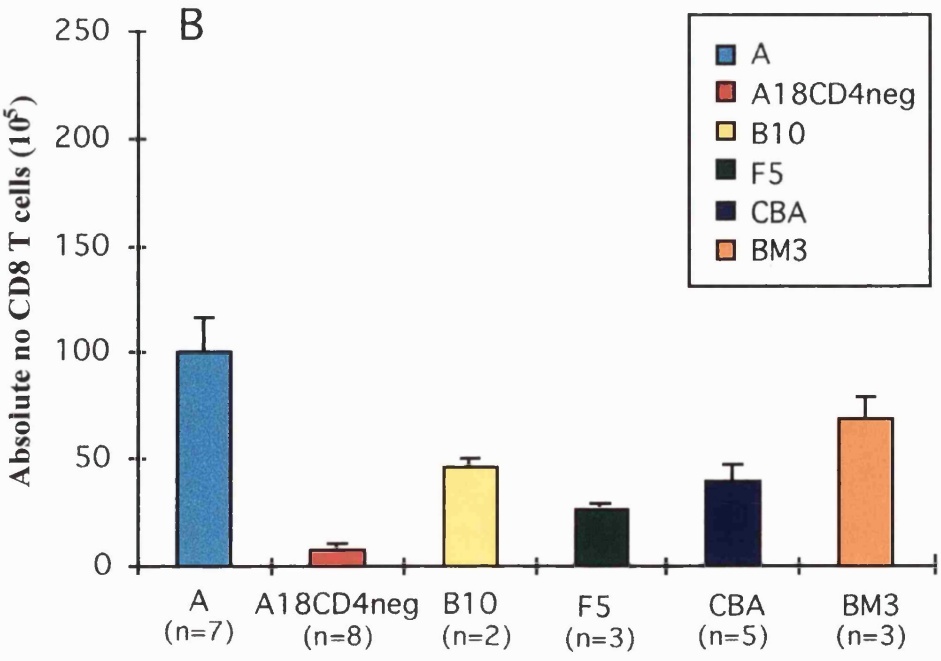
Figure 1 A-B

Spleen T cell numbers from five transgenic mouse strains, all on a Rag knockout background, as well as from three wild type mouse strains were calculated from frequency determination of CD4 and CD8 T cells, as evaluated by immunofluorescence, and the total number of cells recovered. The number of animals analysed per group is indicated in the figure. A) CD4 T cell numbers ($x10^5$) $\pm$ SEM in spleens from A, A18 and A1 strains. B) CD8 T cell numbers ($x10^5$) $\pm$ SEM in spleens from A, A18CD4neg, B10, F5, CBA and BM3 strains.

Fig. 1 A and B

Absolute T Cell Numbers in TCR Transgenic and Non-transgenic Mouse Strains





exclusively into the CD8 lineage as observed previously in other CD4 negative TCR transgenic strains (Matechak et al., 1996).

A comparison between the cellularity of spleens from A1 and A18 TCR transgenic mice and a nontransgenic control with identical MHC background (A strain) confirmed the observation that A18 mice have a much reduced number of peripheral T cells. In fact T cell numbers in A18 spleens are more than 25-fold lower than the ones determined for the wild type strain. Also in the case of the A1 strain a significant reduction in CD4 T cell numbers was observed. This reduction however, being in the order of 5-6 fold, was not as pronounced as the one found in the A18 strain (Fig. 1A).

In the situations where TCR transgenic strains that select into the CD8 lineage were used though, a different picture was observed (Fig.1B). Peripheral T cell numbers in the F5 mice are only minimally reduced (less than 2-fold when compared with its wild type syngeneic control B10) and the BM3 strain actually shows higher numbers of CD8 T cells than its control (CBA). For the A18CD4neg strain, T cell numbers are still very decreased when compared with wild type animals (approximately 13-fold), but this reduction is clearly less drastic than the one seen when the A18 TCR is selected into the CD4 lineage.

3.2. Differential survival of CD4 and CD8 T cells after thymectomy

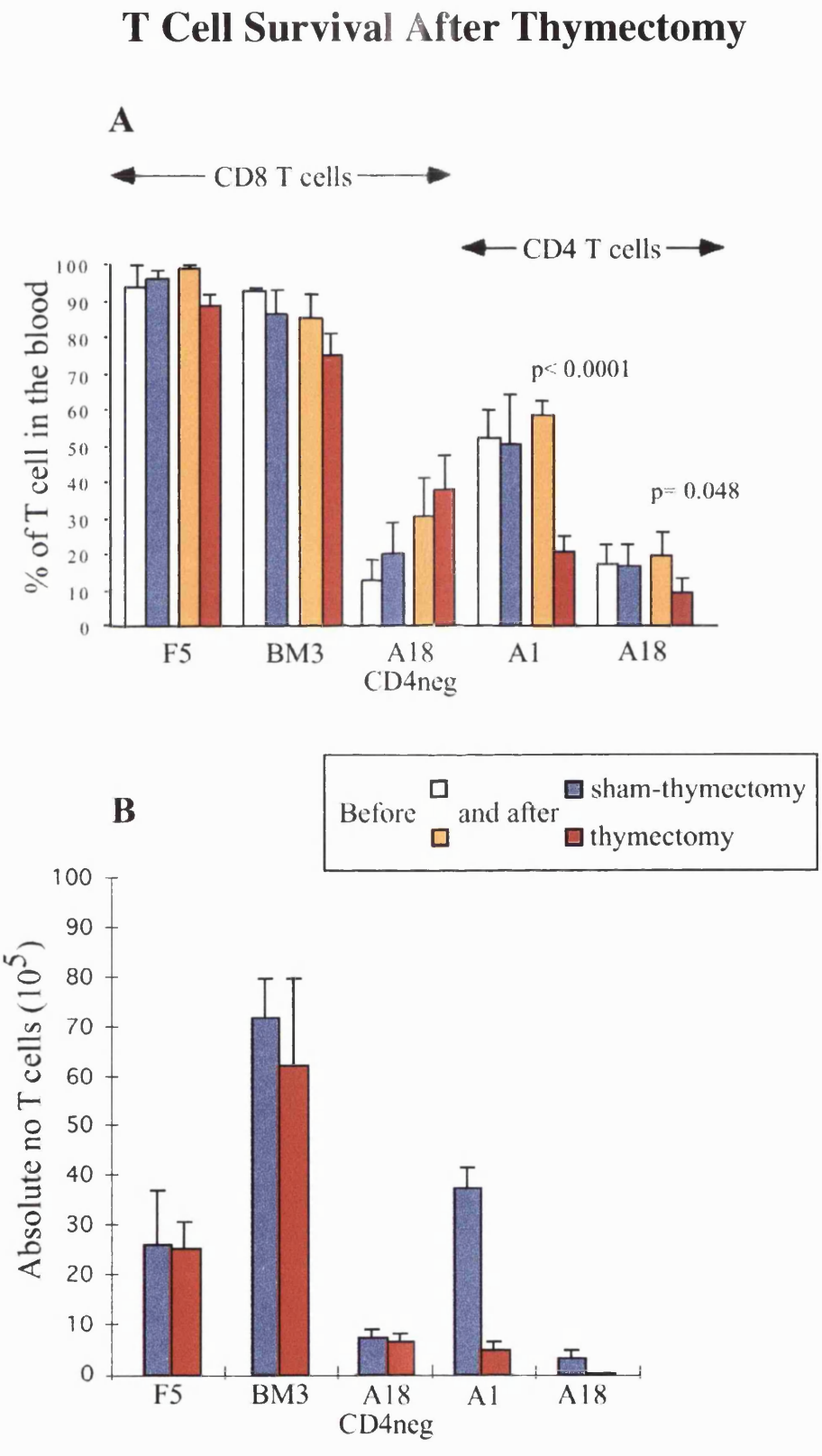
A18 mice were shown to gradually loose their peripheral T cells over a period of 6 weeks following thymectomy, indicating a high turnover of peripheral CD4 T cells and providing an explanation for the reduced peripheral T cell numbers found in these animals (Barthlott et al., 1998).

To investigate whether this was a general phenomenon in TCR transgenic mice and if a similar correlation between peripheral T cell numbers and T cell life-span could be observed in the other TCR transgenic strains used in this study, F5, BM3, A18CD4neg and A1 animals were thymectomised. After an interval of

Figure 2 A-B

Four to eight mice per group from five transgenic mouse strains (all on a Rag knockout background) were thymectomised or sham-thymectomised as indicated in the legend. A) The figure shows mean ± SEM percentage of CD4 (A1 and A18 strains) or CD8 T cells (F5, A18CD4neg, BM3 strains) in blood, 1 day before thymectomy and 60 days after thymectomy. The *p* values, determined by paired *t* test, show significant differences for A1 and A18 before and after thymectomy, whereas all other groups are not significantly different. B) Spleen T cell numbers were calculated from frequency determination of CD4 and CD8 T cells as evaluated by immunofluorescence, and the total number of cells recovered.

Fig. 2 A and B


T Cell Survival After Thymectomy
A
CD8 T cells
CD4 T cells
% of T cell in the blood
100
90
80
70
60
50
40
30
20
10
0
p< 0.0001
p= 0.048
F5
BM3
A18
A1
A18
CD4neg
B
Before and after
sham-thymectomy
thymectomy
Absolute no T cells (10^5)
100
90
80
70
60
50
40
30
20
10
0
F5
BM3
A18
A1
A18
CD4neg

two weeks to allow the animals to recover from the operation, the percentage of T cells in the blood was determined by flow cytometry at regular intervals over a period of 6 weeks.

During the course of the experiment CD4 T cells showed a similar trend for decline in A1 and A18 mice, whereas the percentage of CD8 T cells in F5, BM3 and A18CD4neg mice remained largely unaffected by thymectomy. Data is summarised in Fig.2A showing a comparison of the percentage of CD4 or CD8 T cells in the blood before thymectomy and 60 days after thymectomy.

Also in terms of absolute T cell numbers, a decrease in peripheral CD4 T cells, but not CD8 T cells could be observed (Fig.2B). At the end of the experimental period a comparison between the numbers of T cells found in the spleens of thymectomised versus sham-thymectomised animals revealed that the percentage of T cells found in the blood correlated with spleen T cell numbers. Thus, while in the CD8 T cell populations studied no difference between control and experimental animals could be observed, in both A1 and A18 mice spleen T cell numbers were significantly reduced by thymectomy.

3.3. Susceptibility to apoptosis *in vitro*

Resting T cells have drastically reduced life spans when removed from their stromal environment and cultured *in vitro*, and survival under these conditions has been shown to be promoted by cytokines such as IL-4 or IL-7 (Vella et al., 1997).

To evaluate whether peripheral T cells from different strains show differences in their susceptibility to apoptosis when removed from their stromal environment, spleen cells from the five TCR transgenic strains as well as from nontransgenic controls were cultured during 3-4 days in the absence of antigen or cytokines. Apoptosis was determined by staining with 7-Aminoactinomycin D (7-AAD).

Since the Rag knockout transgenic mice do not have B cells whereas the polyclonal control mice do, we first tested whether the presence of B cells influences survival under these conditions. As shown in figure 3A, B cell depletion from spleens of wild type mice did not alter their susceptibility to apoptosis. Therefore we performed the following experiments with total spleen cells for wild type mice. It should also be mentioned that the assay was used with the intention of assessing the inherent susceptibility of the cells to undergo apoptosis once removed from their natural environment, rather than as a reflection of the real apoptosis occurring *in vivo*.

Background levels of apoptosis were between 5 and 10% in T cells for all strains at the start of culture and no proliferation was observed during the experiment. However, there was a striking difference in the apoptosis rate of A18 CD4 T cells compared with polyclonal CD4 T cells. About 50% of the A18 CD4 T cells showed signs of apoptosis already on day 1 of culture (Fig. 3B). The percentage of apoptotic cells in A1 CD4 T cells was similar to that of polyclonal cells for the first two days of culture, but then their rate of apoptosis exceeded that of polyclonal CD4 T cells, similar to the situation found for A18 T cells (Fig.3C). In contrast, CD8 T cells from F5 or BM3 TCR transgenic mice did not differ in their rate of apoptosis from polyclonal CD8 T cells (Fig.3D and E). Interestingly, CD8 T cells expressing the A18 TCR showed a similar slow increase in the rate of apoptosis over the 4-day culture period (Fig. 3F). Analysis of the percentage of apoptotic cells found in polyclonal CD4 versus polyclonal CD8 T cell cultures revealed that although the death rate was kept at low levels for both subsets, the CD4 population showed an increased susceptibility to apoptosis (Fig.3G). Thus, given that even polyclonal CD4 T cells undergo apoptosis more readily than polyclonal CD8 T cells, it seems possible that the differential susceptibility to apoptosis is an intrinsic property of each lineage.

An obvious molecule that could be playing a role in the differential survival of CD4 and CD8 T cells is Bcl-2 (Veis et al., 1993). To analyse Bcl-2 expression intracellular staining was performed using anti-Bcl-2 mAb or the respective isotype control. However no significant difference in the levels of Bcl-2

Figure 3 A-I

Spleen and lymph node cells from five transgenic mouse strains, all on a Rag knockout background (green bars), as well as polyclonal T cells (dark blue bars) were cultured during 3-4 days in medium without antigen (A-G). The percentage of apoptotic cells $\pm$ SEM in the gated CD4 or CD8 population was determined by staining with 7-AAD before culture and after each day of culture. A) The figure shows percentage of apoptotic CD4 or CD8 polyclonal T cells from the A.J strain when B cell depletion has or has not been performed prior to the set up of the cultures, as indicated in the legend. B-F) Polyclonal controls are from the A.J strain in B, C, F and G; from CBA mice in E; and from B10 mice in D. H) Expression of Bcl-2 on fresh A18 CD4 T cells (yellow line) compared with A18 CD8 T cells (brown line). The stippled line represents the isotype control curve. I) Mean fluorescence values for Bcl-2 expression in fresh polyclonal CD4 or CD8 T cells (from CBA mice). The *p* values, determined by student's *t* test, were <0.005 for days 1-3 in B and <0.03 for days 3 and 4 in C. The *p* values in D-F indicated no statistical differences between cells from transgenic mice and control cells. The *p* values in G were <0.01 for days 1-3.

Amendment to figure 3

Figure 3 A

Spleen and lymph node cells were cultured during 3 days in medium without antigen. The percentage of apoptotic cells $\pm$ SEM in the gated CD4 or CD8 population was determined by staining with 7-AAD before culture and after each day of culture. The figure shows percentage of apoptotic CD4 or CD8 polyclonal T cells from the A.J strain when B cell depletion has or has not been performed prior to the set up of the cultures, as indicated in the legend.

Fig. 3 A

Susceptibility to Apoptosis *in vitro*

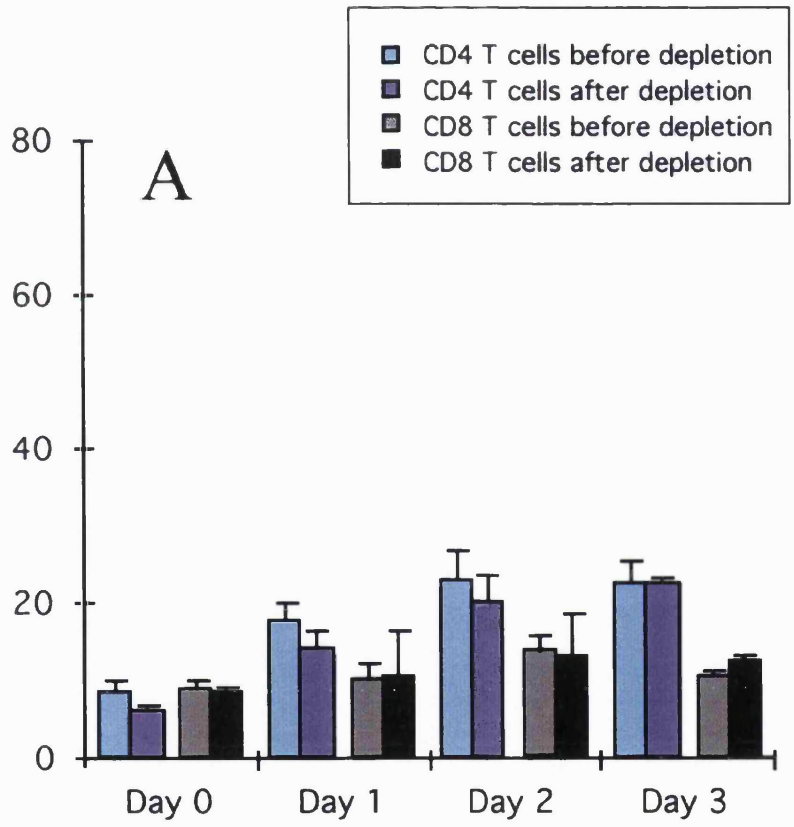


Figure 3 B-G

Spleen and lymph node cells from five transgenic mouse strains, all on a Rag knockout background, as well as polyclonal T cells were cultured during 3-4 days in medium without antigen. The percentage of apoptotic cells $\pm$ SEM in the gated CD4 or CD8 population was determined by staining with 7-AAD before culture and after each day of culture. B-F) Polyclonal controls are from the A.J strain in B, C, and F; from CBA mice in E; and from B10 mice in D. G) Comparison between the CD4 and the CD8 T cell populations of A.J mice. The *p* values, determined by student's *t* test, were <0.005 for days 1-3 in B and <0.03 for days 3 and 4 in C. The *p* values in D-F indicated no statistical differences between cells from transgenic mice and control cells. The *p* values in G were <0.01 for days 1-3.

Fig. 3 B, C, D, E, F and G

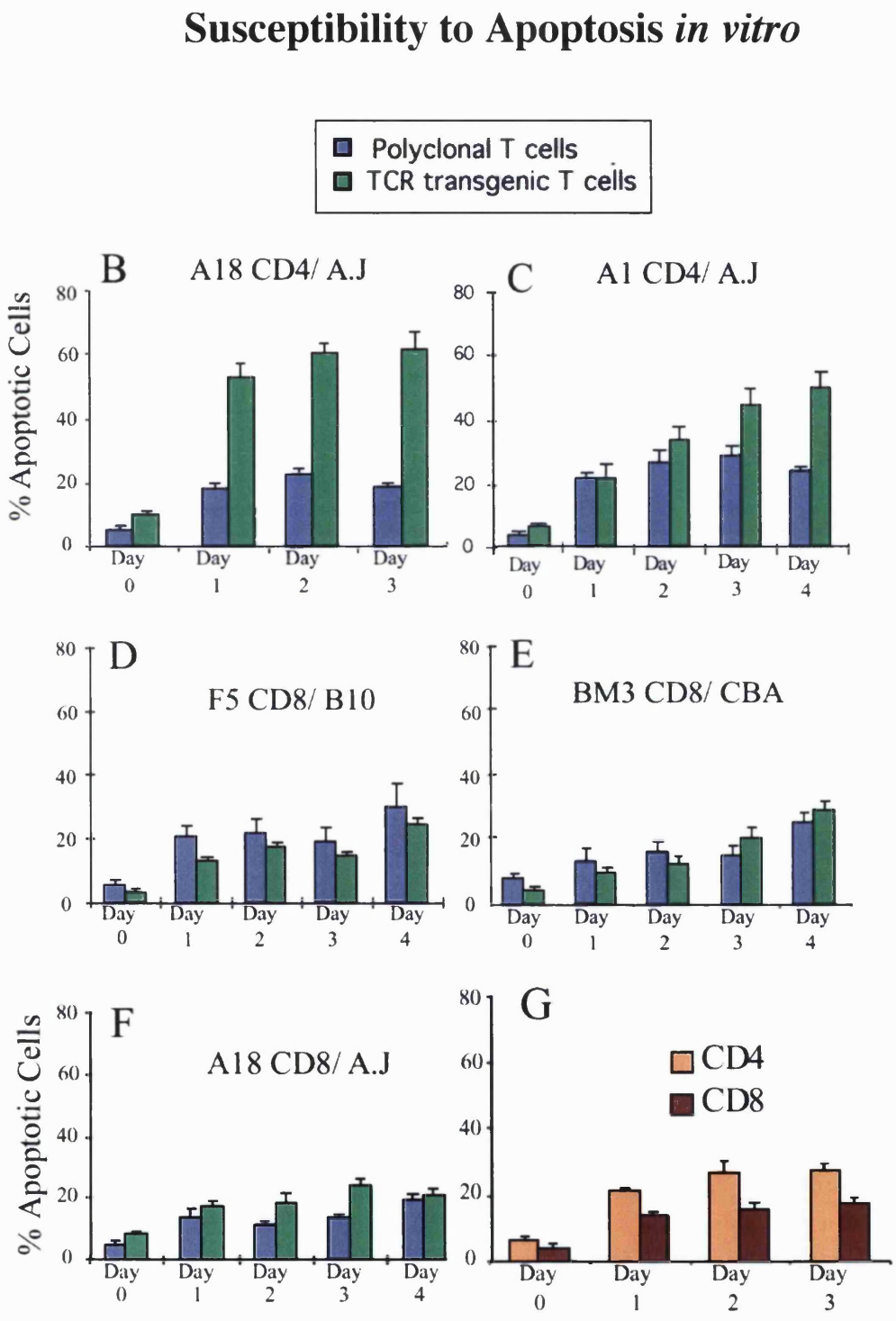
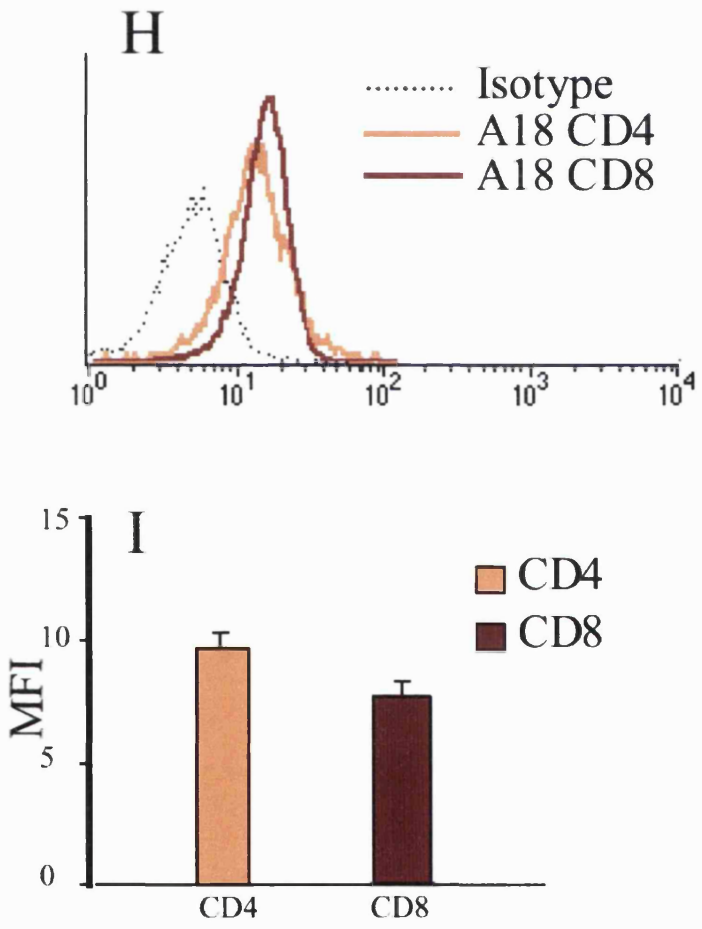


Figure 3 H and I

H) Expression of Bcl-2 on fresh A18 CD4 T cells (yellow line) compared with A18 CD8 T cells (brown line). The stippled line represents the isotype control curve. I) Mean fluorescence values for Bcl-2 expression in fresh polyclonal CD4 or CD8 T cells (from CBA mice).

Fig. 3 H and I

Susceptibility to Apoptosis *in vitro*



expression between CD4 and CD8 subsets in either transgenic A18 T cells or $CD44^{low}$ polyclonal T cells could be detected (Fig.3H and I).

3.4. Homeostatic proliferation of naive CD4 and CD8 T cells

A number of recent reports described cell division (homeostatic proliferation) of naive peripheral T cells when these are transferred into syngeneic, supposedly antigen-free lymphopenic hosts, where they do not encounter competition for survival niches with other lymphocyte populations (Bruno et al., 1996; Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999a; Oehen and Brduscha-Riem, 1999; Cho et al., 2000; Goldrath et al., 2000). These observations led to the establishment of the concept of maintenance as the sum of survival and proliferation (Goldrath and Bevan, 1999b). Homeostatic proliferation depends on MHC/peptide recognition, but the nature of the peptides involved and their relation to peptides that effect positive selection in the thymus are still unresolved (Bender et al., 1999; Ernst et al., 1999; Viret et al., 1999).

To compare homeostatic proliferation of different cell populations, spleen cells from the five TCR transgenic strains as well as from nontransgenic controls were labelled with the tracker dye carboxy-fluorescein-diacetate succinimidyl ester (CFSE) (Lyons and Parish, 1994) and injected into syngeneic Rag knockout mice. Due to the very limited number of T cells present in A18 mice, the pooled spleens of 15-20 animals were used per injection.

Figure 4 shows the CFSE patterns 9 or 15 days after transfer. While CD8 T cells (Fig.4B) have undergone several divisions by day 9 after transfer, CD4 T cells divide much slower (Fig.4A). A18 and A1 CD4 T cells show only one peek of division 9 days after transfer and two or three peaks after 15 days. In contrast, homeostatic proliferation of CD8 T cells expressing the A18 TCR is comparable with that of other CD8 T cells.

Figure 4 A-B

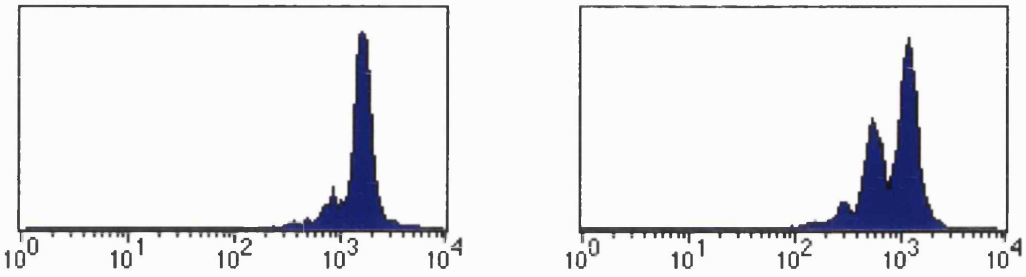
Homeostatic proliferation of CFSE-labelled lymph node cells from five transgenic mouse strains and polyclonal T cells, 9 and 15 days after transfer into untreated syngeneic Rag knockout recipients. 2-5x10^6 T cells were injected per mouse and 3-6 animals per group were studied. The histograms show CFSE levels on gated T cell subpopulations. A) CD4 T cell proliferation; B) CD8 T cell proliferation.

Fig 4 A

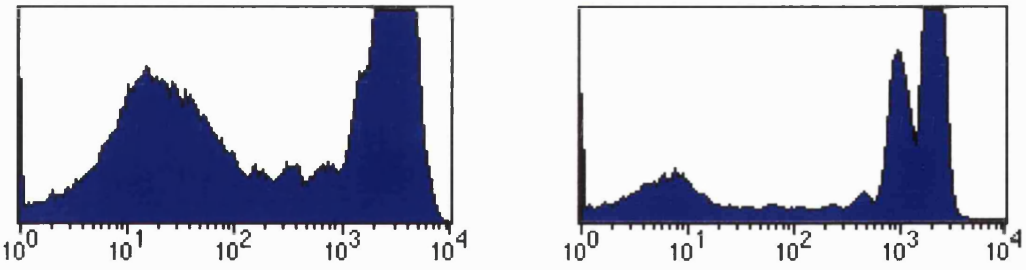
Homeostatic Proliferation - CD4 T Cells

Day 9 Day 15

A1 CD4



CBA CD4



A18 CD4

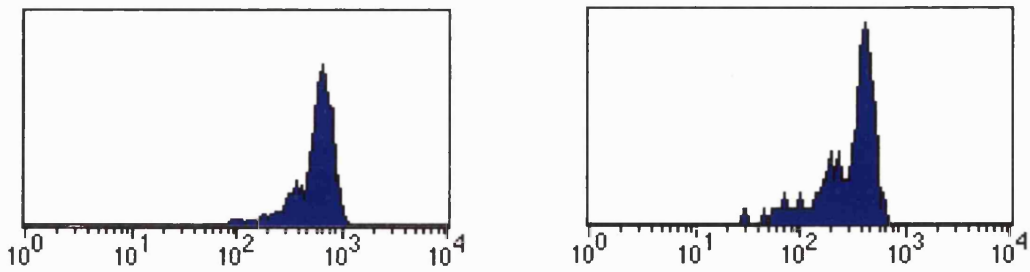
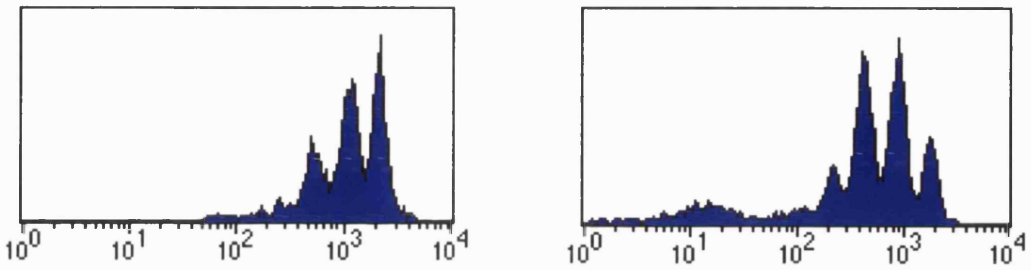


Fig 4 B

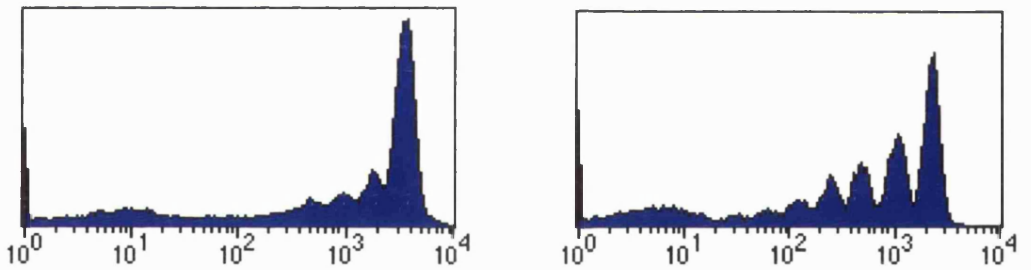
Homeostatic Proliferation – CD8 T Cells

Day 9 Day 15

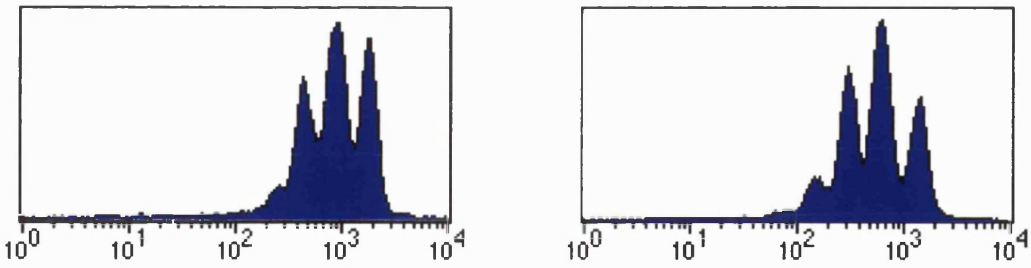
F5 CD8



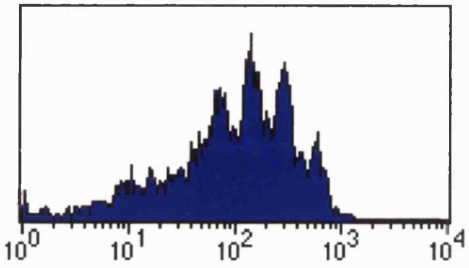
CBA CD8



BM3 CD8



A18 CD8



In polyclonal T cells a fraction of both CD4 and CD8 T cells divided rapidly and lost CFSE label. In all likelihood these were preactivated T cells, as indicated by their high levels of the CD44 activation marker (Fig.5). The naive cells, in contrast, showed the same pattern of proliferation for CD4 and CD8 as that seen in transgenic T cells, emphasising that there must be intrinsic differences in the way homeostatic survival signals are transmitted in CD4 and CD8 T cells.

Hence, it is possible that the shorter or longer T cell life-spans observed in the thymectomised strains studied, reflects at least in part, the different expansion capacities of the respective TCR transgenic populations.

3.5. Homeostatic proliferation does not generally affect the expression of activation markers

Since naive T cells can be distinguished from activated T cells by the expression of activation markers such as CD44 or CD45RB, it was possible to address the question of whether the homeostatic proliferation observed after transfer resulted in phenotypic conversion.

T cells from the five TCR transgenic strains used in this analysis did not show any changes in their expression levels of CD44 or CD45RB during homeostatic proliferation. It should be noted that the CFSE negative, $CD44^{high}$, $CD45RB^{low}$ population visible in recipients of transgenic T cells is not a T cell population since it does not express TCR. Also the majority of naive polyclonal CD4 and CD8 T cells did not change the expression of activation markers, although a small fraction of polyclonal CD8 T cells slightly increased CD44 expression after the fourth or fifth division (Fig.5). In contrast phenotypic conversion was clearly evident after transfer of CFSE-labelled HY-specific A1 T cells into syngeneic male Rag knockout mice, where they encounter their cognate antigen.

Figure 5

The dot plots show staining for CFSE (y-axis) and either CD44 or CD45RB (x-axis) of gated CD4 and CD8 T cell populations 15 days after transfer (9 days after transfer for A18 CD8) into untreated syngeneic Rag knockout recipients. The CFSE-negative populations (with the exception of those seen in Rag knockout recipients of polyclonal T cells) are not T cells; they are $CD44^{high}$ and negative for TCR expression.

Fig. 5

Expression of Activation Markers in A1, F5, CBA, A18CD4 and A18CD8 T Cells, Undergoing Homeostatic Proliferation

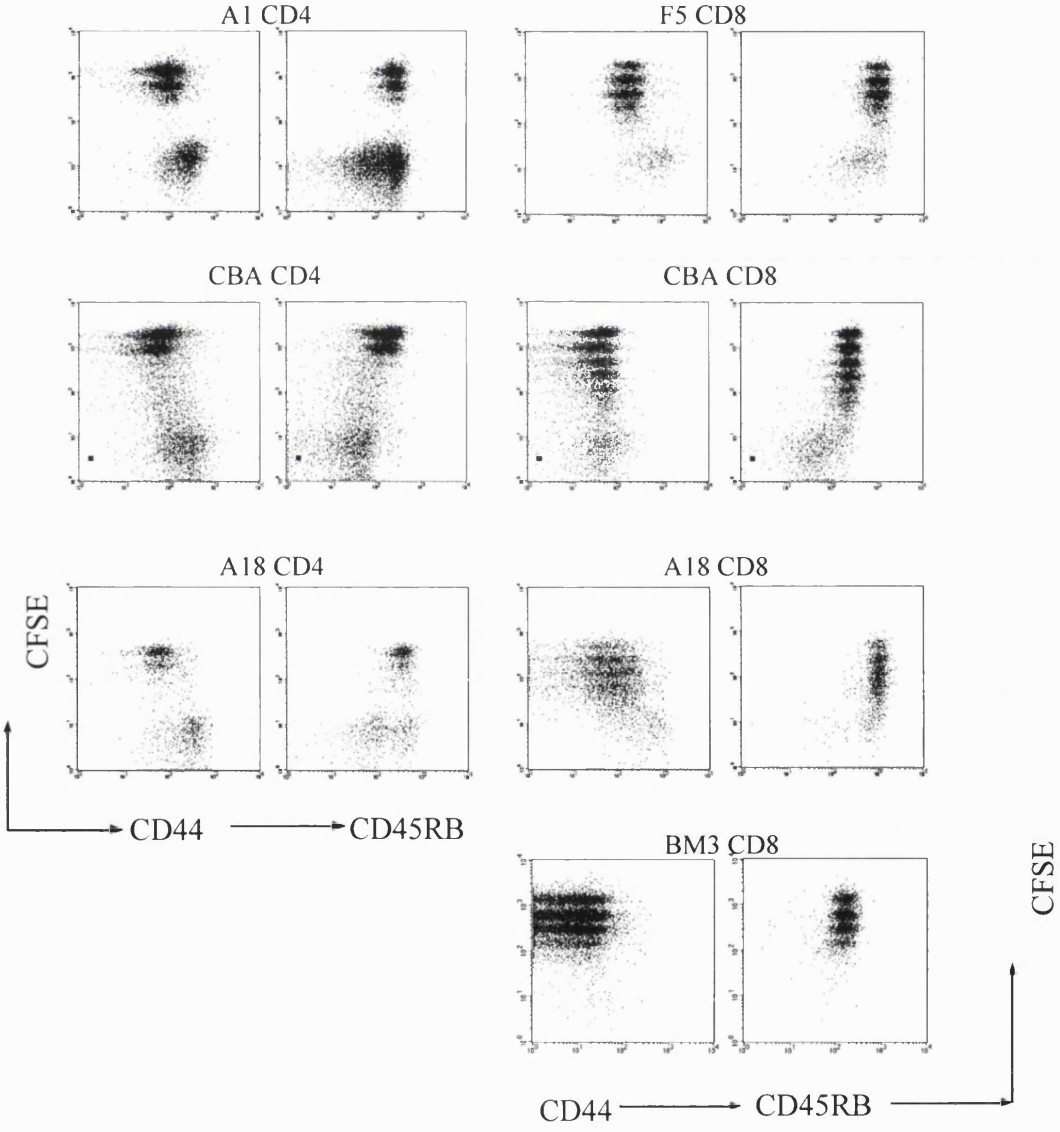
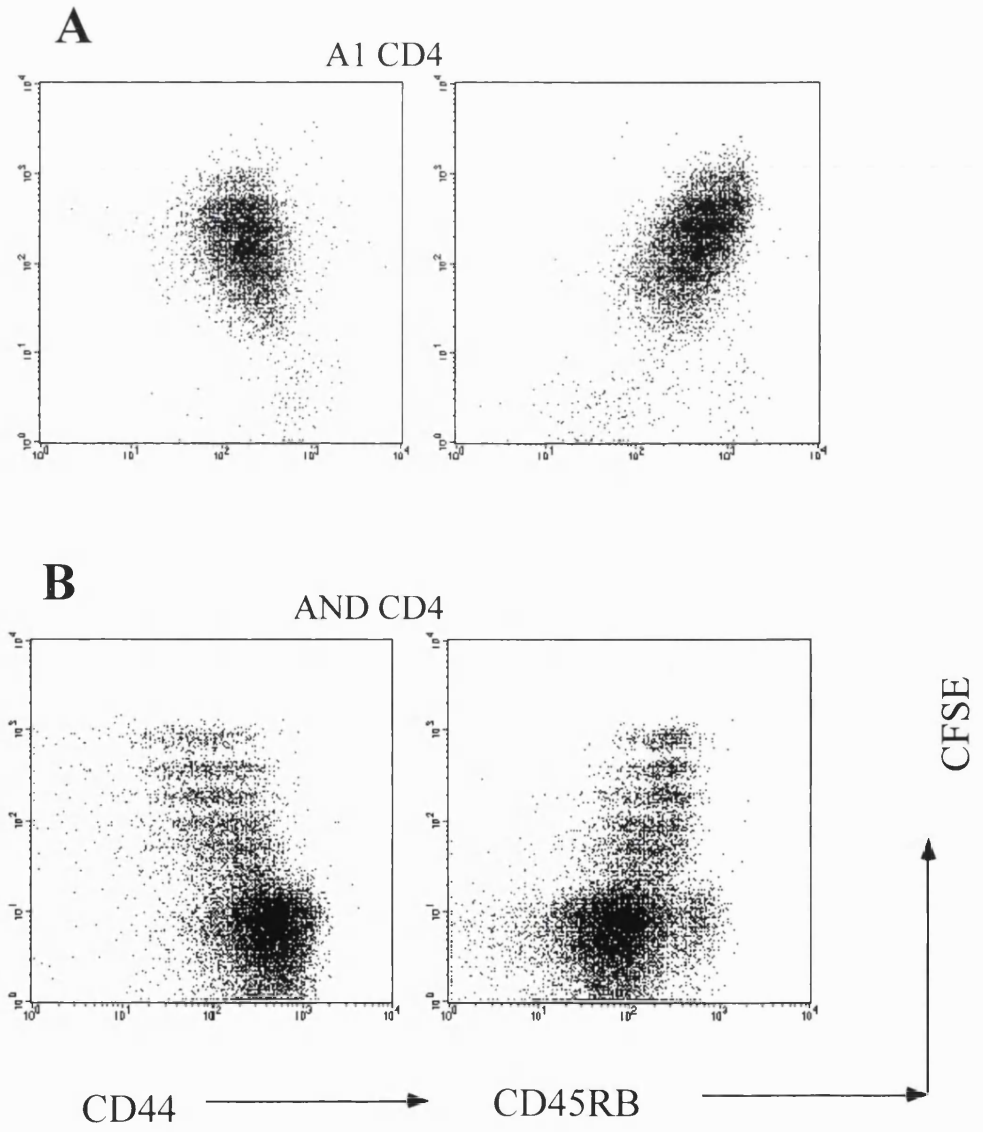


Figure 6

A) CD44 and CD45RB expression on A1 CFSE-labelled CD4 T cells 3 days after transfer into syngeneic male Rag knockout mice expressing cognate antigen. B) CD44 and CD45RB expression of AND (kxb) CFSE-labelled CD4 T cells 9 days after transfer into untreated Rag knockout (H-2^b) recipients.

Fig. 6

Expression of Activation Markers in Activated A1 T Cells and in AND T Cells Undergoing Homeostatic Proliferation



This resulted in an increase in CD44 expression and a reduction of CD45RB expression already evident on day 3 after transfer (Fig.6A). A different situation was observed for the AND TCR transgenic strain. CD4 T cells expressing this TCR proliferated rapidly, upregulated CD44 progressively with each division and downregulated CD45RB despite the absence of a defined antigenic ligand in the adoptive host (Fig.6B).

It is possible that upregulation of CD44 during proliferation in empty hosts signifies T cell activation rather than mere homeostatic proliferation. This could be a consequence of recognition of bona fide antigen due to expression of additional TCRs in Rag positive donors or because some T cells are activated under these conditions by cross-reactive antigens present in the adoptive host (Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999; Cho et al., 2000; Goldrath et al., 2000). This might more easily happen for TCRs with high avidity, which can interact with a wide range of ligands such as the AND TCR (Kaye et al., 1989; Matechak et al., 1996).

3.6. Homeostatic proliferation of naive polyclonal T cells

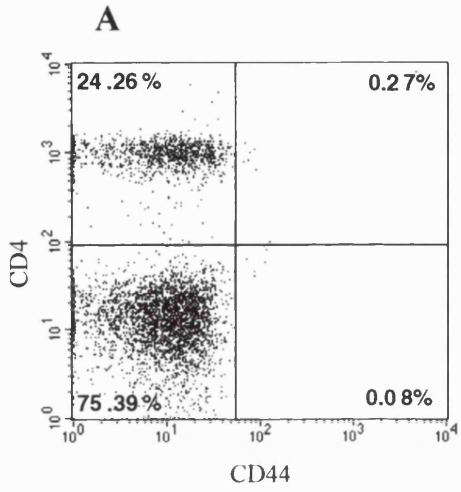
Since in the previous experiment total polyclonal T cells were injected into the Rag knockout hosts, it was reasonable to assume that the majority of the CFSE negative, $CD44^{high}$ T cells that developed in these mice, were derived from the pre-existent memory subset. However it was also possible that some of these cells had its origin in the naive T cell population. To distinguish between these two possibilities, pooled spleen and lymph node cell populations from CBA mice were depleted of $CD44^{high}$ cells before injection into syngeneic lymphopenic hosts. In order to determine CD44 levels within the T cell compartment but avoid direct manipulation of the TCR, which might result in signalling, the cells were also stained for CD4. The CD8 compartment in this strain expresses identical or slightly lower levels of the CD44 molecule than the CD4 subset and is therefore included in the $CD4^{-}CD44^{low}$ quadrant of the dot plot (Fig.7A).

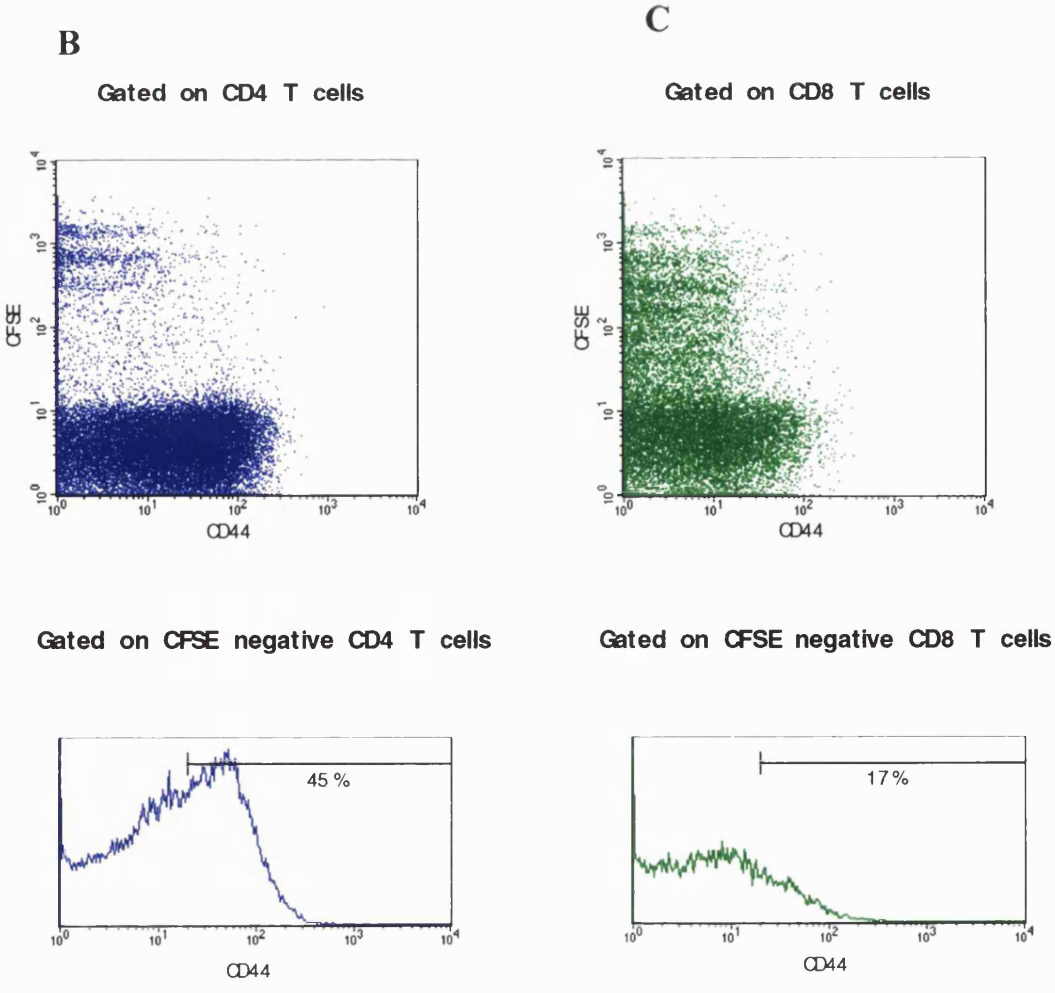
Figure 7 A-C

Spleen and lymph node pooled cells from CBA mice were stained with anti-CD4 and anti-CD44 mAbs and depleted of $CD44^{high}$ cells by sorting. A) Dot plot shows CD44 levels on the sorted population. The cells were then labelled with CFSE and injected into Rag knockout syngeneic hosts. Homeostatic proliferation was assessed 3 weeks after cell transfer in spleen and lymph nodes. Identical results were obtained in both organs. B) The dot plot is gated on $CD4^{+}TCR^{+}$ lymph node cells and it shows CD44 expression and CFSE contents on transferred T cells; the histogram is further gated on CFSE negative CD4 T cells. C) The dot plot is gated on $CD8^{+}TCR^{+}$ lymph node cells and it shows CD44 expression and CFSE contents on transferred T cells; the histogram is further gated on CFSE negative CD8 T cells. The experiment was repeated two other times and similar results were observed in both occasions.

Fig.7 A, B and C

Homeostatic Proliferation of Naive Polyclonal T Cells





Within three weeks of transfer, it was observed that a proportion of both CD4 and CD8 donor T cells had upregulated the CD44 marker (Fig.7B and C). These cells were CFSE negative indicating that they had undergone several rounds of division and could only have derived from the transferred population since the adoptive host did not contain endogenous T cells.

A possible explanation for this phenomenon is that the $CD44^{high}$ population could have resulted from a preferential expansion of a few contaminant T cells (Tanchot and Rocha, 1995). Another possibility is that some minor histocompatibility differences between the donor population and the adoptive host may be playing a role in this situation. Since we are using a polyclonal population and the host has not been extensively backcrossed into the CBA ($H\text{-}2^k$) background it is possible that some naive TCR specificities have been activated by minor histocompatibility antigen differences still present in the recipients. Finally, it is also possible that this group of T cells that has divided rapidly and acquired an activated phenotype is constituted by T cell clones expressing high affinity TCRs which can cross-react with host antigens. Each of these possibilities is currently being investigated.

3.7. Peripheral T cell populations in the absence of thymic output

Experiments described so far suggest that naive CD4 T cells have an intrinsically lower capacity for survival compared with CD8 T cells. This characteristic is reflected in their gradual disappearance in thymectomised hosts, their increased sensitivity to apoptosis *in vitro*, and their fewer divisions upon transfer into syngeneic lymphopenic hosts. If this is the case, naive CD4 T cells should be eliminated more rapidly than their CD8 counterparts *in vivo*.
However thymic export ensures a constant supply of newly generated naive T cells into the periphery which substitute resident cells and take the place of those that have died, maintaining T cell numbers. Thus, even if the CD4 subset has a higher turnover than the CD8 population, this will not be immediately visible.

In a situation where thymic output is absent and can not influence the peripheral T cell pool though, it should be possible to observe a decrease in the CD4/CD8 ratio with time. To test this hypothesis, spleen and lymph node cells from CBA mice were transferred into lymphopenic syngeneic hosts and the naive CD4/CD8 ratio determined prior to injection and 3 months after transfer. No decrease on CD4/CD8 ratio could be detected at this time point (Fig.8A). This could signify that either, despite our earlier observations, CD4 polyclonal T cell numbers did not decrease *in vivo* compared with CD8 T cells or that a longer time span was needed to detect these alterations.

To verify if a decrease in CD4 T cell numbers could be detected at a later time point, CBA mice were thymectomised and the CD4/CD8 ratio was determined 12 months after the operation. A comparison between ratios in thymectomised mice, non-thymectomised age-matched animals and a group of young (4-5 weeks old) controls revealed that naive CD4/CD8 ratios were significantly reduced in the thymectomised animals in comparison with both age-matched controls and young mice. Also in the case of the older non-thymectomised animals the CD4/CD8 ratio was significantly lower than the one present in the younger animals (Fig.8B).

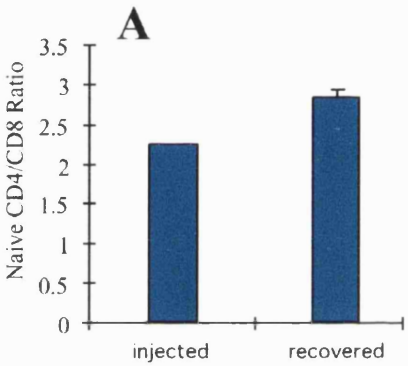
In order to exclude the possibility that the alterations observed were due to the expansion of the CD8 population rather than to the accelerated decline of the CD4 T cells, absolute naive T cell numbers were determined. Results showed a reduction of the CD8 T cell population in the thymectomised animals compared with both control groups (approximately 2-3 fold) and a much more drastic decrease in CD4 T cell numbers (Fig.8C). Thus, while old non-thymectomised mice showed only a small reduction in CD8 T cell numbers when compared with young animals, their CD4 T cell numbers were reduced in almost 40%. On the other hand, in the thymectomised group both CD4 and CD8 T cells numbers decayed although the decline of the CD4 population appeared to occur at a slightly more accelerated pace. Finally, further analysis of the experimental groups demonstrated that naive T cell decay is reflected by a drop in the naive/memory ratio in a manner that correlates remarkably well with the differential rates of decline observed for naive CD4 and CD8 T cells (Fig.8D).

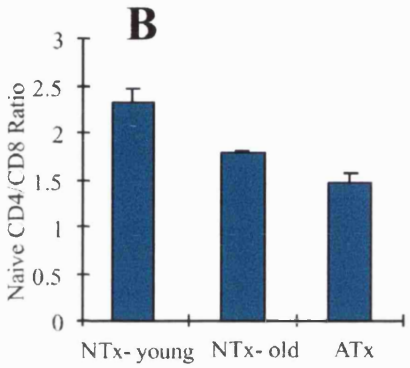
Figure 8 A-D

Absolute T cell numbers were calculated from frequency determination of $CD44^{low}$ TCR^+ $CD4^+$ or $CD8^+$ (naive T cells) and from $CD44^{high}$ TCR^+ $CD4^+$ or $CD8^+$ cells (memory T cells) as evaluated by immunofluorescence analysis, and the total number of cells recovered. The total number of peripheral T cells recovered was considered to be equal to that obtained in the spleen plus twice that obtained in the pool of lymph nodes studied (axillar, brachial, inguinal and mesenteric) (Zatz and Lance, 1970). CD4/CD8 ratio was subsequently calculated from these values. Three to six mice per group were used. A) Pooled spleen and LN cells from CBA mice were transferred into Rag knockout syngeneic hosts and naive CD4/CD8 ratios $\pm$ SEM determined before injection and 3 months after injection. B) Naive CD4/CD8 ratios $\pm$ SEM were determined for non-thymectomised young (NTx-young), non-thymectomised old (NTx-old) and thymectomised (ATx) mice. The *p* values, determined by Student's *t* test, were 0.027 for NTx-young and NTx-old and 0.0191 for NTx-old and ATx. C) The table shows absolute naive CD4 and CD8 T cell numbers ($x10^6$) $\pm$ SEM recovered from NTx-young, NTx-old and ATx animals. NTx-young were 4-5 weeks old and both NTx-old and ATx were 13-14 months old. D) Naive/memory T cell ratios ($CD44^{low}/CD44^{high}$) $\pm$ SEM were determined for both the CD4 and the CD8 populations in NTx-young, NTx-old and ATx animals.

Fig. 8 A, B, C and D

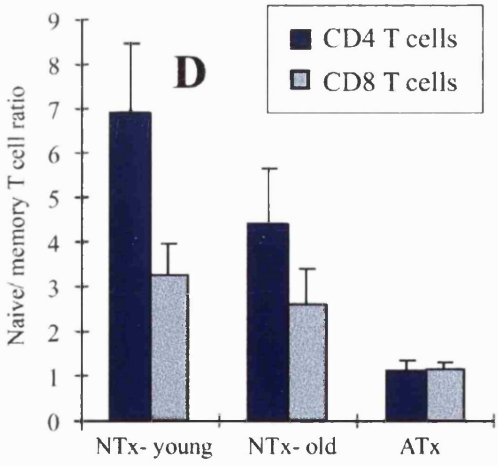
T Cell Populations in the Absence of Thymic Output





C

	CD4	CD8 (x10^6)
NTx—young	35 ± 4	15 ± 2
NTx-old	22 ± 3	12 ± 2
Atx	7 ± 2	5 ± 1



The results suggest thus that naive CD4 T cells are shorter lived than CD8 T cells and that the effects of these different survival characteristics become visible in situations where the thymus output has been reduced or is completely absent.

3.8. CDR3 length of naive CD4 T cells in the absence of thymic output

The complementary-determining region 3 (CDR3) is thought to play an important role in the specificity and outcome (Jorgensen et al., 1992; Kalergis and Nathenson, 2000) of antigen recognition by T cells. In fact, selection for homogeneity in the length of CDR3 restriction has been observed to occur during the immune response, indicating the importance of this parameter in T cell receptor recognition (McHeyzer-Williams and Davis, 1995).

Since T cell survival has also been shown to be dependent on TCR/ MHC-peptide interactions, it was possible that a similar selection process had taken place during the contraction of the T cell population observed in the previous experiment. In this case some T cells might have a survival advantage over others, and CDR3 size could be a readout for reduced heterogeneity or oligoclonality.

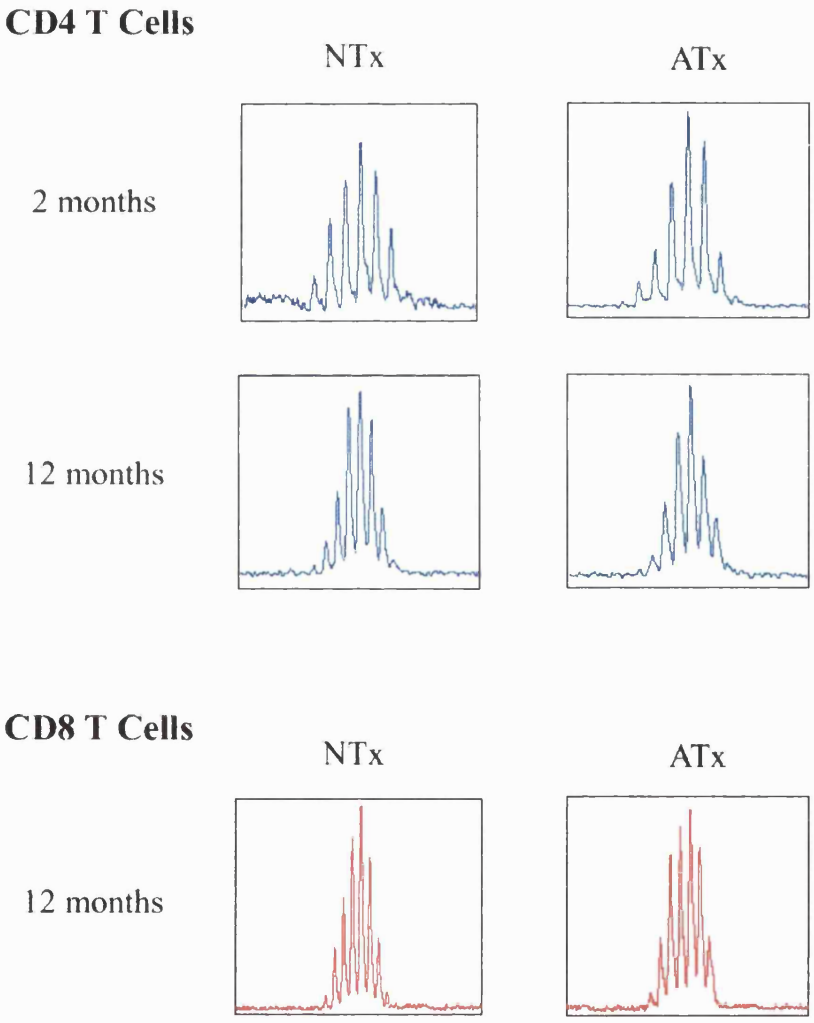
In order to test this possibility, RNA was obtained from naive CD4 and CD8 T cells of thymectomised and non-thymectomised animals 2 and 12 months after thymectomy, and CDR3 size was determined by the immunoscope method. Since skewed CDR3 profiles in old mice have been previously reported within the Vβ8.3 family (Mosley et al., 1998) and since this family is well represented in the Vβ repertoire (approximately 10%) (Forster et al., 1995), we used a Vβ8.3-specific primer together with a Cβ-specific primer to determine CDR3 sizes in this experiment.

Figure 9

TCRβ chain CDR3 sizes in thymectomised (ATx) and non-thymectomised (NTx) mice, 2 and 12 months after thymectomy. Total RNA was extracted from sorted $CD4^{+}CD44^{low}$ and $CD8^{+}CD44^{low}$ T cells obtained from spleen and lymph nodes. cDNA was synthesised and amplified with primers specific for the Cβ and Vβ8.3 gene segments. Aliquots of the amplifications were subject to run-off reactions using a Cβ-specific fluorescent primer and analysed on a 373A Applied Biosystems sequencer. The experiment was repeated a second time with identical results being obtained.

Fig. 9

CDR3 Length of Naive T Cells in the Absence of Thymic Output



In all situations analysed the profiles yielded a typical bell-shaped distribution of the CDR3 lengths and did not suffer any alteration with age (Fig.9). The results suggest therefore that the decrease in T cell numbers observed in the thymectomised animals is not accompanied by a narrowing or skewing of the T cell repertoire, within the Vβ8.3 family. To determine if this is a feature common to the whole Vβ repertoire though, further analysis will have to be conducted using other Vβ families.

3.9. Competition between CD4 T cells expressing different transgenic TCRs

A mechanism closely linked with homeostasis, which plays an important role in the shaping of the immune system, is competition. T cell repertoire diversity and T cell survival rely not only on the interactions of each T cell with their respective ligands, but also on the nature and number of other competing cells.

Competition within the peripheral T cell pool is especially relevant when thymic output is reduced or absent. Thymus migrants have been shown to substitute peripheral naive resident cells in an age-independent manner, which has the potential to alter relative clone sizes and thus to influence the T cell repertoire (Tanchot et al., 1997b). Ultimately, non-age-dependent substitution is associated with a rapid contraction of large clones and with a relatively longer survival of small clones, which ensures the maintenance of a diverse peripheral T cell repertoire. When thymic export decreases however, clones that show an advantage in terms of survival or homeostatic proliferation and which were kept under control by the constant input of newly produced T cells might expand at the expense of less competitive clones.

In order to verify if the type of TCR expressed could be an important factor in competition and if it could indeed provide a T cell population with a competitive advantage over other TCR specificities, mice from the A1 and A18 TCR transgenic strains were used. CFSE-labelled A1 cells were transferred into

syngeneic Rag knockout hosts and also into A1 and A18 animals, which contained their respective monoclonal T cell populations. Competition was then assessed by determining to which extent homeostatic proliferation was inhibited by the presence of another TCR specificity, when compared with the situation observed in a lymphopenic host.

The data obtained from this first assay reveals that, although A1 T cells can no longer expand in a fully populated A1 mouse (possibly due to the fact that all niches are already occupied by native cells), the proliferative extent observed in the A18 and in the Rag knockout host is comparable (Fig.10). Hence, T cells from the A1 strain do not appear to compete with A18 T cells. However, two different explanations can account for the results obtained. In a first scenario, competition would not be present due to the fact that A1 and A18 T cells would use different survival resources and thus occupy different niches. In a second situation, the absence of competition would be explained not by the usage of different resources, but by the fact that A18 mice have very few peripheral T cells and consequently many of their niches would be unoccupied and available for A1 T cells.

To discriminate between the two possibilities, a second complementary experiment was performed in which the same adoptive hosts were used, but the cells injected were now CFSE-labelled A18 T cells. Because A18 T cells proliferate very slowly, an extra time point (30 days after transfer) was used in this second case. Analysis of the results obtained revealed that although A18 T cells can not proliferate in A1 mice, their expansion does not appear to be impaired in A18 hosts (Fig.11). Therefore the most likely explanation is that the small number of endogenous T cells present in A18 TCR transgenic mice is not sufficient to fill all niches available, leaving them free to be occupied by other cells that have identical survival requirements.

The data suggests that A18 and A1 T cells occupy overlapping ecological niches, however we can not rule out the possibility that A1 T cells occupy a wider niche than A18 T cells in the peripheral pool, and thus would still show

Figure 10

Homeostatic proliferation of CFSE-labelled lymph node cells from the A1 strain on day 15 after transfer. Adoptive hosts used were Rag knockout, A18 TCR transgenic and A1 TCR transgenic mice (both in a Rag knockout background) as indicated in the figure. 3-5x10^6 CD4 T cells were injected. The histograms show CFSE levels on transferred CD4 T cell populations.

Fig. 10

Competition between CD4 T Cells Expressing Different Transgenic TCRs – A1 Cells Transferred into A18 Host

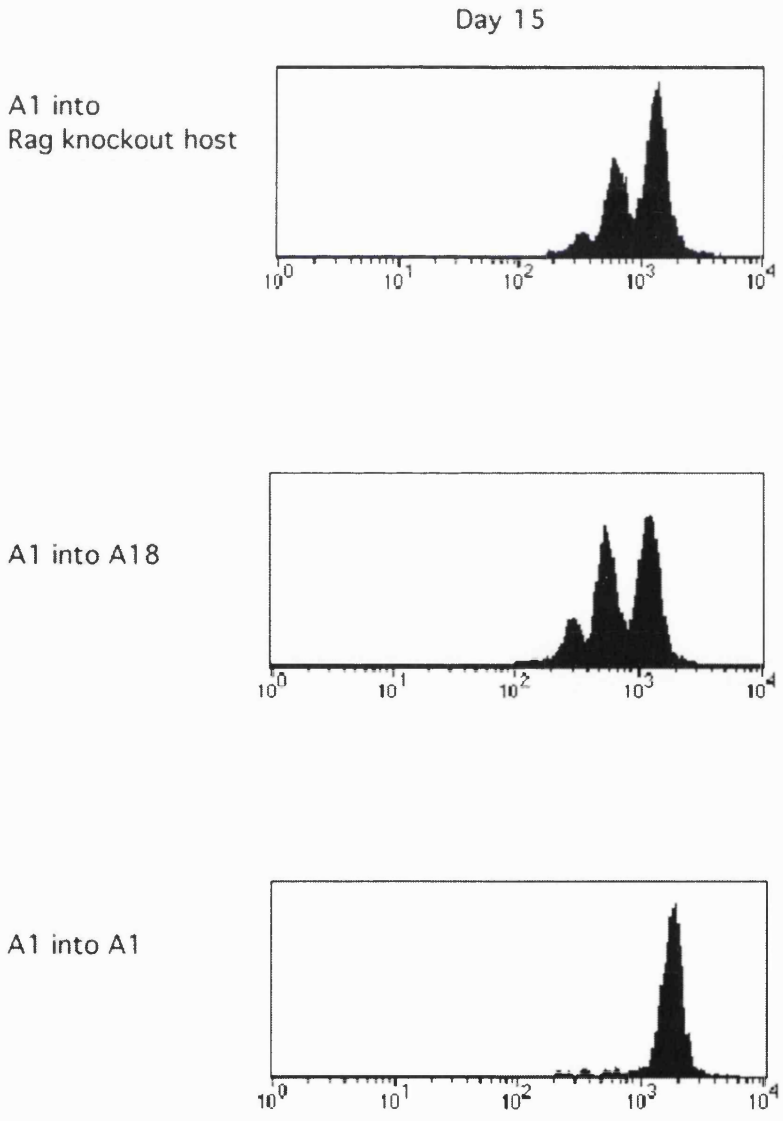
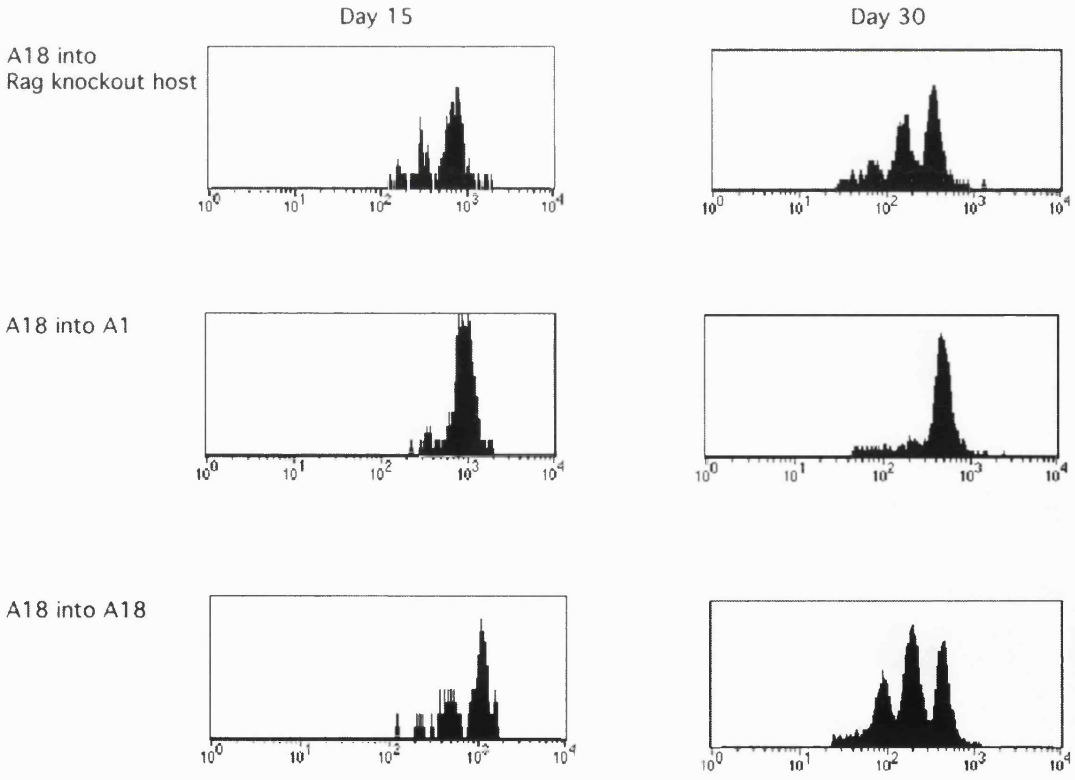


Figure 11

Homeostatic proliferation of CFSE-labelled cells from the A18 strain on days 15 and 30 after transfer. Adoptive hosts used were Rag knockout, A18 TCR transgenic and A1 TCR transgenic mice (both in a Rag knockout background) as indicated in the figure. 2-3x10^6 CD4 T cells were injected. The histograms show CFSE levels on transferred CD4 T cell populations.

Fig. 11

Competition between CD4 T Cells Expressing Different Transgenic TCRs – A18 Cells Transferred into A1 Host



some degree of homeostatic proliferation even in a fully populated A18 host. To determine if one of the factors defining a niche for T cells is the TCR specificity further experiments will have to be performed, this time using a second TCR transgenic mouse strain in which peripheral T cell numbers do not represent a limiting factor. Future experiments are planned using AND TCR transgenic mice crossed onto an H-2^k background so that cells can be co-transferred with A1 and A18 T cells.

3.10. Influence of overexpression of CD4 in T cell survival and homeostatic proliferation

Previous work by Maroto et al. (1999) indicated that interactions between CD4 and MHC class II molecules may be playing an important role in survival of resting CD4 T cells in peripheral lymphoid organs.

To verify if overexpression of the CD4 co-receptor would have an effect in survival and proliferation of naive T cells, we analysed A18 Rag knockout TCR transgenic mice that expressed a transgenic CD4 molecule in addition to the endogenous CD4 (CD4VA2B.A18 mice). Total CD4 expression in these animals is increased approximately 50% in comparison with single transgenic A18 mice.

Determination of absolute T cell numbers revealed an increase in the number of CD4 single positive thymocytes as well as in the numbers of peripheral T cells both in the spleen and in the lymph nodes when compared with age-matched A18 controls (Fig.12). A possible explanation for this phenotype is that overexpression of the CD4 molecule might in the A18 case rescue thymocytes from apoptosis, possibly by increasing TCR affinity for selecting MHC/peptide complexes during positive selection.

As a next step, we determined if the increment observed in the CD4VA2B.A18 peripheral T cell population was being caused solely by a higher thymic output or if the intrinsic survival and proliferative characteristics of the mature T cells had been themselves affected by the expression of the transgene. Spleen cells from CDVA2B.A18, A18 and AJ animals were therefore cultured in vitro during 3 days and percentage of apoptotic cells assessed after each day of culture, as described before.

A comparison between the three groups studied showed that the double transgenic cells did not differ in their rate of apoptosis from single transgenic A18 controls (Fig.13). It appears then, that in the case of A18, overexpression of the CD4 co-receptor does not alter the *in vitro* T cell survival profile.

The proliferative capacity of the CD4VA2B.A18 T cells was evaluated as in previous experiments that is, pooled spleen cells were CFSE labelled and transferred into Rag knockout syngeneic hosts and CFSE patterns were determined by immunoflourescence analysis 9 and 15 days after transfer. Contrary to what was observed for single transgenic A18 mice, CD4VA2B.A18 T cells were seen to divide very rapidly. In fact the majority of cells had undergone at least one division by day 9 and some of them had already lost their CFSE label (Fig.14) indicating that they had divided more than 6 times.

It seems thus, that although overexpression of the CD4 molecule does not have any visible effect on the *in vitro* survival of A18 naive T cells, it can improve their selection in the thymus and their proliferative capacity in a lymphopenic host. To distinguish between increased production and increased survival however, and to determine exactly how much each of these mechanisms influences the size of the peripheral T cell pool, further experiments will have to be conducted, this time using thymectomised animals.

Figure 12

Absolute T cell numbers on CD4VA2B.A18 mice. T cell numbers were calculated from frequency determination of $CD4^+$ $CD8^-$ TCR^+ cells as evaluated by immunofluorescence analysis, and the total number of cells recovered. In the thymus, single positives were further distinguished from double negatives by their $CD5^{high}$ phenotype. The total number of peripheral T cells recovered was considered to be equal to that obtained in the spleen plus twice that obtained in the pool of lymph nodes studied (axillar, brachial, inguinal and mesenteric). The figure shows mean $\pm$ SEM of data obtained from four animals.

Fig. 12

Absolute T Cell Numbers on CD4VA2B.A18 Mice

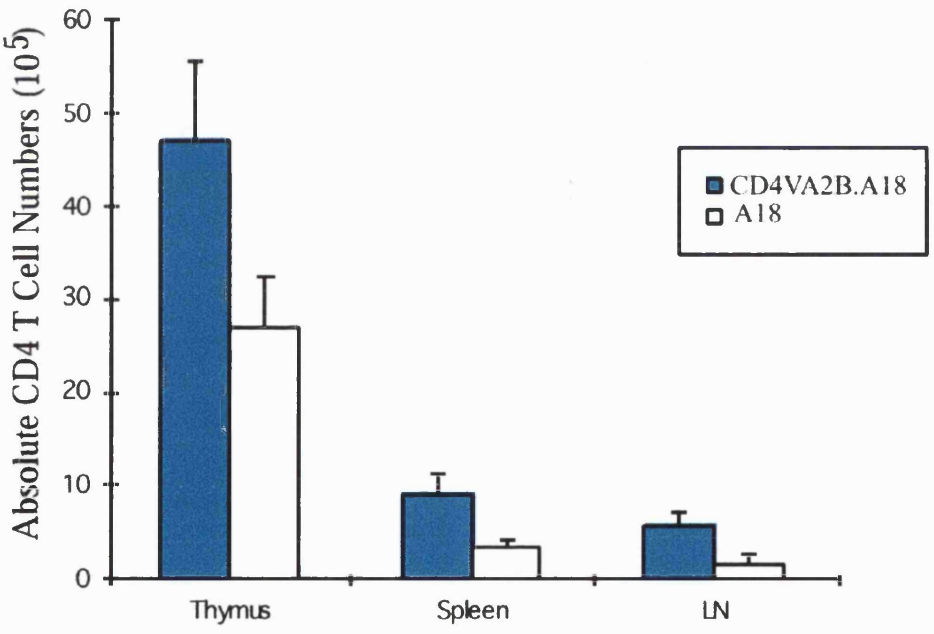


Figure 13

Susceptibility to apoptosis *in vitro* of CD4VA2B.A18 T cells. Spleen cells from four CD4VA2B.A18 double transgenic mice, as well as A18 single transgenic and AJ polyclonal controls were cultured for 3 days in medium without antigen. The percentage of apoptotic cells ± SEM in the gated CD4 population was determined by staining with 7-AAD before and after each day of culture.

Fig. 13

Susceptibility to Apoptosis *in vitro* of CD4VA2B.A18 T cells

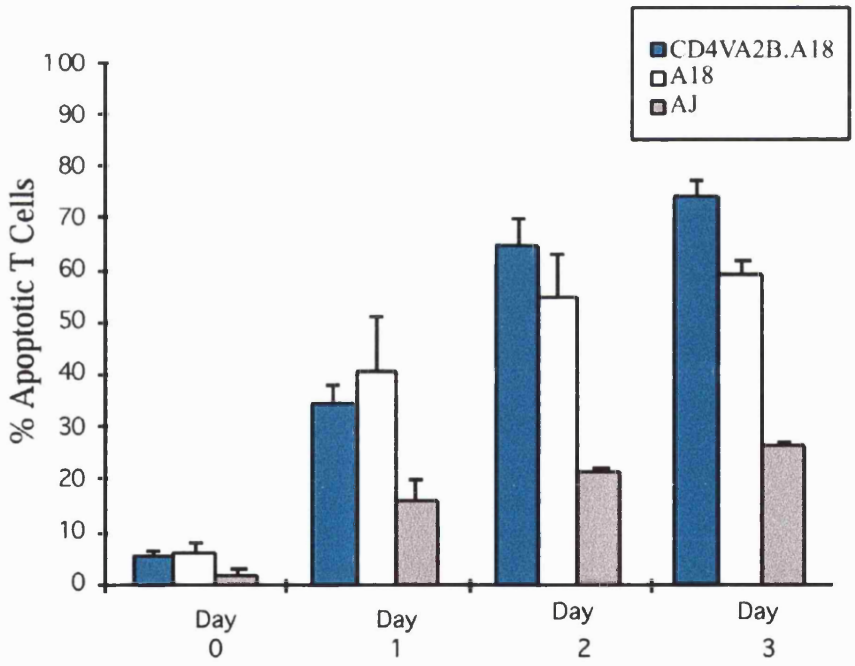
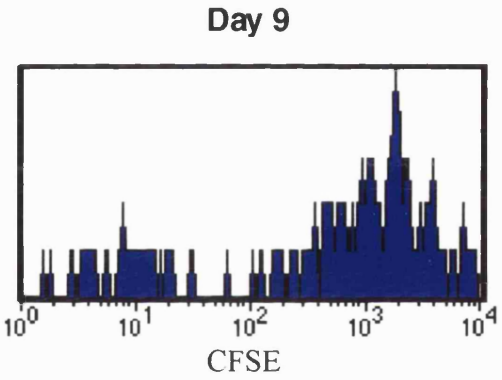


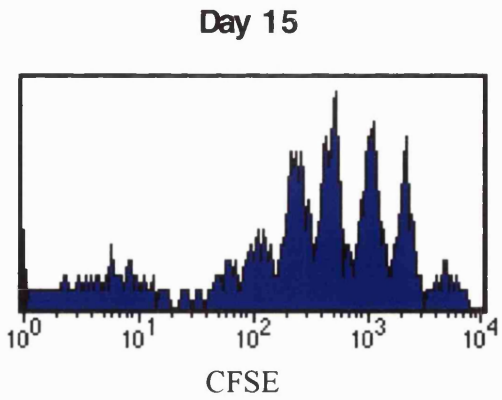
Figure 14

Homeostatic proliferation of CFSE-labelled lymph node cells from the CD4VA2B.A18 double transgenic mouse strain 9 and 15 days after transfer into untreated syngeneic Rag knockout recipients. The histograms show CFSE levels on gated CD4 T cells.

Fig. 14

Homeostatic Proliferation of CD4VA2B.A18 T Cells





3.11. Influence of EVA expression on thymic and peripheral T cell numbers of A18 mice

Adhesion molecules are key factors in lymphocyte homing and recirculation and consequently play an important role in immune homeostasis (Butcher and Picker, 1996). Epithelial V-like antigen (EVA) is an adhesion molecule expressed in the thymic epithelium which is strongly downregulated by thymocyte development progression (Guttinger et al., 1998). The gene is expressed in thymus-derived epithelial cell lines and in various epithelia both during development and adulthood, but not in T cells, B cells, fibroblasts, macrophages or dendritic cells. Although highly expressed in the thymic epithelium of Rag-2 knockout mice, it is almost completely downregulated following *in vivo* treatment with anti-CD3ε mAbs. Guttinger et al. (1998) suggest that EVA downregulation by double positive transition could reflect the need of thymocytes to move to a more appropriate stage specific niche in the organ.

Since TCR/MHC interactions and consequently cell-cell interactions seem to be key factors in both thymic development and T cell survival, it is possible that increasing the avidity of these interactions, for instance by constitutively expressing an adhesion molecule, might improve selection and survival of low affinity TCRs. To test this hypothesis, A18 TCR transgenic mice were bred with mice expressing a transgene for the EVA molecule under the K14 promoter, which directs expression into stratified squamous epithelia (Vassar et al., 1989), such as the skin and the thymic epithelia. All animals were kept on a Rag knockout background.

Preliminary data revealed that the number of CD4 single positive thymocytes appears to be reduced when compared with an A18 control (Fig.15A). This might be explained by a high increase in avidity caused by the expression of the transgenic adhesion molecule, which could lead to an increment in negative selection.

Surprisingly though, the total numbers of peripheral T cells are not decreased. On the contrary they even appear to be slightly augmented (approximately two fold) and TCR levels, as determined by mean fluorescent intensity, show identical increase (Fig.15B). This might argue in favour of an improved survival and/or homeostatic proliferation for these cells.

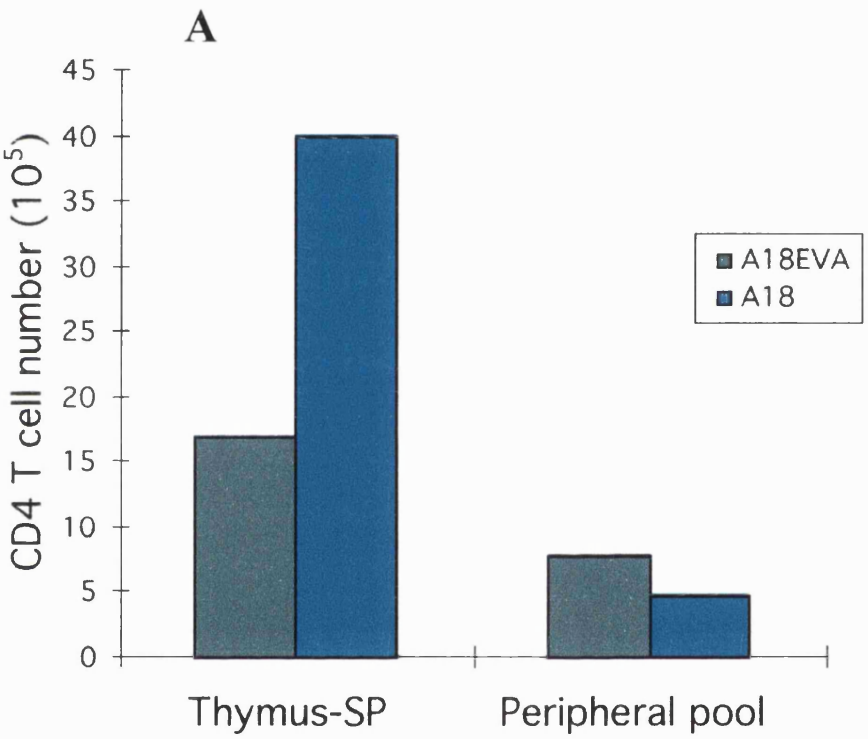
Since the A18 EVA line is still being established, only one double transgenic and one littermate control were available for experimental use. Therefore, to verify if the results obtained in this preliminary study will remain consistent, more animals will have to be analysed in the future. It will also be necessary to conduct tests similar to the ones done for the CD4VA2B.A18 mice, which will allow us to determine if the higher number of peripheral T cells detected in these animals is due to alterations in the survival and/or proliferation characteristics of the CD4 T cells.

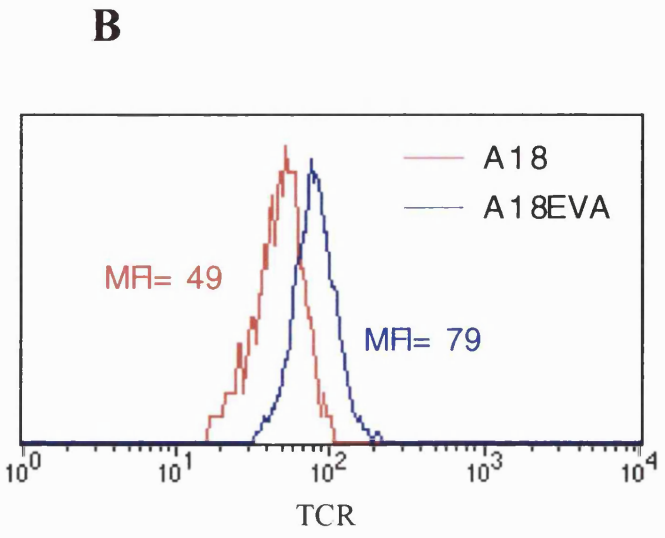
Figure 15 A-B

A) Absolute T cell numbers in A18EVA mice. T cell numbers were calculated from frequency determination of $CD4^+$ $CD8^-$ TCR^+ cells as evaluated by immunofluorescence analysis, and the total number of cells recovered. Thymus-SP (CD4 single positive thymocytes); Peripheral pool (total number of peripheral T cells). The total number of peripheral T cells recovered was considered to be equal to that obtained in the spleen plus twice that obtained in the pool of lymph nodes studied (axillar, brachial, inguinal and mesenteric). B) Histogram shows mean fluorescence intensity (MFI) of TCR in A18 EVA and A18 control.

Fig. 15 A and B

Thymus and Peripheral Pool of A18EVA Mice





4. Discussion

Following positive selection and maturation in the thymus, CD4 and CD8 T cells colonise the periphery and recirculate through lymphoid organs until they encounter antigen. Activation by antigen results in transient expansion of antigen-specific clones followed by contraction, so that the total number of peripheral T cells remains in homeostatic equilibrium (Tanchot et al., 1997c).

In adult mice new lymphocytes are constantly being produced in the primary lymphoid organs or by peripheral cell division at a much higher rate then the one required to replenish the periphery. In fact, the thymic export rate appears to be constant and independent of the peripheral T cell pool (Berzins et al., 1998). In this situation newly generated cells have to compete between themselves or with resident cells already established in the periphery to survive.

The mechanisms that determine the life span and survival of naive T cells in the periphery have been a subject of debate for many years and are still not clearly defined at present. Initial experiments attributed T cells an average life span in the order of 4 to 6 months (Sprent and Basten, 1973). However at this time no distinction was made between naive and memory cells, which have now been shown to possess different survival and turnover rates (Sprent and Tough, 1994; Tanchot et al., 1997a). In later studies, transferred T cells have been observed to persist in severe combined immunodeficiency (SCID) hosts up to 10 months and even to expand gradually. Since many of them maintained a virgin phenotype the authors concluded that naive T cells can survive indefinitely (Sprent et al., 1991). A point that should be taken in consideration though is that the experiment was realised using lymphopenic mice. Thus, in a situation like this, the transferred cells are not subject to the effects of competition from newly produced naive T cells or other resident cells and can survive and proliferate taking full advantage of the available resources. Therefore the data reflects the survival potential rather than the real life span of naive T cells in normal physiological circumstances.

Another important distinction that should be made is between CD4 and CD8 T cells. Experiments during this project have exposed the differential survival and proliferative capacities of these two subsets both on the level of individual T cell clones and on the polyclonal populational level. Hence, the analysis of the naive T cell population without further subdivisions may result in loss of information, which might be fundamental for the complete understanding of the situation being studied.

An essential factor for both naive CD4 and CD8 T cells seems to be the presence of syngeneic MHC molecules displaying a diverse repertoire of peptides (Takeda et al., 1996; Brocker, 1997; Kirberg et al., 1997; Rooke et al., 1997). Also memory T cells maintain constant TCR/MHC interactions but conversely to naive cells, they do not require the restricting MHC allele for survival. Hence, the data suggests an important role for TCR-mediated signals in T cell survival. Another observation that corroborates this point is the existence of a hierarchy of thymic and peripheral selection in which lymphocytes with certain TCR specificities are more easily replaced by competitors than others (Freitas et al., 1996). This is most likely a reflection of the avidity of TCR interactions with a varying choice of selecting and/or survival ligands.

Similar conclusions can be drawn from the study of TCR transgenic mice. While in polyclonal animals T cell numbers decline only very slowly in the absence of thymic output (for instance, after adult thymectomy), this does not appear to be the case for all TCR transgenic mice (Barthlott et al., 1998; Boursalian and Bottomly, 1999). Once more a likely explanation for the discrepancies observed is that individual T cell clones may have different requirements for their maintenance depending on TCR avidity and the availability of selecting ligands. The influence of TCR specificity and affinity in survival and competition will be analysed later in this chapter.

In addition to the intrinsic capacity for survival of each T cell clone, another mechanism that may be playing an important role in the maintenance of a T cell population is homeostatic proliferation. Although naive T cells have been shown

to occasionally undergo division in their physiological environment (Tough and Sprent, 1994; Bruno et al., 1996; Bell and Sparshott, 1997), both CD4 and CD8 T cells proliferate much more efficiently in lymphopenic hosts (Bruno et al., 1996; Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999a; Oehen and Brduscha-Riem, 1999; Cho et al., 2000; Goldrath et al., 2000). This is presumably due to the absence of cells competing for survival signals, since co-transfer of large numbers of T cells can suppress this expansion (Rocha et al., 1989; Ernst et al., 1999). Indeed homeostatic proliferation, as opposed to antigen-induced proliferation, tends to maintain stability in a manner similar to the one seen in other ecological systems. Thus, homeostatic expansion takes place in situations where there is a surplus of resources, such as in lymphopenic mice, and is reduced or absent in polyclonal animals where the presence of large numbers of cells increases competition and makes resources scarce. An example of an important resource that mediates homeostasis is the cytokine IL-7, which has been shown to play a crucial role in homeostasis-driven proliferation and survival of naive CD8 T cells (Schluns et al., 2000).

Potentially all T cells should be able to proliferate equally in a competition-free environment, however there seem to be substantial differences in the expansion capacity of cells with different TCRs. Despite the absence of bona fide cognate antigen in the adoptive hosts, some T cells appear to "fill the space" and even up-regulate certain activation markers, whereas others show restrained proliferation without changes in such markers (Rocha et al., 1989; Rocha and von Boehmer, 1990; Sprent et al., 1991; Tough and Sprent, 1994; Bruno et al., 1996; Ernst et al., 1999; Kieper and Jameson, 1999). This could have important consequences for peripheral homeostasis after a loss of peripheral T cells in adults, since the homeostatic proliferative capacity of each clone could determine to which extent it would contribute to reconstitution of the depleted pool.

In this project several survival criteria for T cells from TCR transgenic mice and nontransgenic controls have been analysed and different T cell populations were compared. The manner in which these data can be integrated in the context of T

cell physiology and the resulting implications will be further explored in the following sections.

4.1. Different T cell populations have different survival capacities

Previous work by Barthlott et al. (1998) has shown that the A18 TCR transgenic strain on a Rag knockout background develops few peripheral CD4 T cells, which are relatively short lived compared with normal polyclonal CD4 T cells. The thymectomy experiments described at the beginning of the study demonstrated that another TCR transgenic strain (the A1 strain) exhibits similar behaviour. Also in terms of homeostatic proliferation these two strains appear to fall in the same category showing only a small number of divisions when transferred into lymphopenic hosts. The phenotype that both A18 and A1 T cells display is that of T cells with a narrow specificity and/or low affinity TCR recognition.

As mentioned previously, the T cell expansion potential is very limited for both strains, however it is possible to make a distinction between A18 and A1 T cells. Transfer of T cells of each strain into a Rag knockout host shows that within a very limited range of divisions, A1 T cells still perform better than A18 T cells. This increased homeostatic proliferation may be a possible explanation for the fact that A1 mice have larger peripheral T cell pools than A18 animals. Another factor that has to be taken into account though, is the thymic export rate. Although, the percentage of single positives in A1 and A18 appears to be similar (Barthlott et al., 1998; Zelenika et al., 1998), it is still possible that A1 CD4 T cells are being produced and exported at a higher rate than A18 T cells. Further experiments would have to be performed to test this hypothesis. Using bromodeoxyuridine (BrdU) incorporation to determine the proportion of recent thymic migrants in the periphery would be a possible method to clarify the subject.

Although it is still a matter of debate whether ligands involved in thymic selection are the same as those that procure peripheral survival signals (Bender et al., 1999; Ernst et al., 1999; Viret et al., 1999), selection in the thymus appears to play a critical role in the survival of T cells in the periphery (Janeway et al., 1998). For instance, T cells from the highly selecting AND strain show extensive expansion upon transfer into lymphopenic hosts. The fact that proliferation of AND T cells *in vivo* is accompanied by acquisition of activation markers similar to those seen in antigen driven expansion indicates that the AND TCR is more promiscuous, with a lower threshold for survival signals, some of which might represent cross-reactive antigens. A similar phenotype in CD4 T cells expressing the hemagglutinin-specific ABII TCR, which incorporated BrdU and upregulated CD44 following thymectomy or transfer into lymphopenic hosts was attributed to recognition of an unidentified ligand (Bruno et al., 1996).

The high proliferative potential inherent to certain T cell clones, such as AND and ABII appears to give them an advantage over other T cells. So why is the T cell repertoire not dominated by only a few T cell specificities instead of containing, in a minimum estimate, about $2x10^6$ clones (Casrouge et al., 2000)? A factor that plays a very important role in this situation is the thymic export. The constant input of newly produced T cells ensures the maintenance of diversity and the contraction of large clones while sustaining small clones for longer periods of time (Tanchot and Rocha, 1997b). When the influence of the thymus is reduced or completely abolished though, there is a reduction of the T cell repertoire diversity and some clonal expansions become visible (Callahan et al., 1993; Posnett et al., 1994; Roux et al., 1996; Mosley et al., 1998; LeMaoult et al., 2000). Since high-affinity clones appear to acquire a partially activated phenotype during homeostatic proliferation (discussed later in this chapter), it would be interesting to determine the state of activation of these expanded clones as well as the affinity of the TCR expressed.

Another aspect that should be taken into account when transferring cells into lymphopenic animals, besides the intrinsic characteristics of the donor cells, is

the type of host into which the cells are placed. The architectural organisation of the lymphoid organs might have some influence on the outcome of the colonising population. Thus, whereas in irradiated mice donor cells colonise adult organs that have previously gone through a normal developmental process, animals with congenital deficiencies such as SCID, TCRα or β knockouts or Rag knockouts, have developed their lymphoid organs in the absence of mature T and/or B cells. The importance of cross talk between stromal cells and T cells for thymic organisation has already been demonstrated (Ritter and Boyd, 1993), but is the structure of peripheral lymphoid organs in congenitally deficient animals disrupted in such a way that normal survival and expansion patterns can not be sustained? The evidence gathered until now appears to deny this hypothesis. TCRα knockout spleens retain roughly a normal internal anatomy (Philpot et al., 1992), nude rats injected with mature T cells acquire a functional T cell population that can persist up to two years (Bell et al., 1987) and reconstituted SCID mice display a normal splenic composition and architecture (Hilbert et al., 1994). Also cells transferred into TCRβ knockout and Rag-2 knockout mice do not show any decrease in their expansion potential when compared with the ones injected into irradiated hosts (Bender et al., 1999).

4.2. CD8 T cells are more efficient survivors than CD4 T cells

Regardless of the spectrum of survival patterns seen in different TCR transgenic strains, it was obvious that naive CD8 T cells survive better than naive CD4 T cells. In thymectomised animals, TCR transgenic CD4 populations showed a decline in both percentage of T cells in the blood and of absolute T cell numbers in the spleen. *In vitro* cultures of these same T cells displayed increased cell death in comparison with polyclonal controls, correlating with the *in vivo* data, and the homeostatic proliferative capacity of CD4 T cells was shown to be reduced. On the other hand, no reduction in CD8 T cell numbers was detected either in the blood or in the spleen of thymectomised mice, the apoptosis rate *in vitro* was comparable to the one seen for polyclonal cells and proliferation was

much higher than for CD4 T cells. This dichotomy is most clearly illustrated in the different survival characteristics of T cells from the A18 vs A18CD4neg mouse strains. Despite expressing identical TCR, A18 CD8 T cells do not decay after thymectomy, are less fragile *in vitro* and undergo more extensive homeostatic proliferation than their CD4 counterparts.

Also for polyclonal populations a difference became visible between the two T cell subsets. Although both CD4s and CD8s displayed low levels of apoptosis *in vitro*, still the percentage of apoptotic cells was consistently higher within the CD4 subset and transfer into Rag knockout recipients resulted in a very clear proliferative advantage for the CD8 T cells. Thymectomy performed in adult CBA mice resulted in a decline in both naive T cell subsets, but in a more substantial loss of CD4 T cells.

As mentioned previously, *in vitro* data can not be directly extrapolated to events that take place *in vivo* since stromal environment disruption have drastic effects on T cell life span. However, although in different time scales, there was a very clear correlation between a longer half-life *in vivo* and a reduced rate of apoptosis *in vitro*, in the mouse strains analysed. A possible explanation for this phenomenon is the persistence of survival signals in the cells for some time after they are removed from their physiological environment. In fact, recent evidence suggests that T cells can store previous signals received through the TCR (Bui et al., 2000). Hence, if the effects of signalling can be felt for some period of time even when the element that initiated the signal is no longer present, it is possible that *in vitro* culture is actually reflecting survival patterns previously imprinted on the cell.

It is unclear at the moment whether the differential survival observed reflects more efficient transmission and subsequent signalling upon interaction with survival ligands or whether CD8 T cells are more responsive to the presence of distinct survival mediators such as cytokines (Nakajima et al., 1997; Lodolce et al., 1998). However, evidence for differential signalling between CD4 and CD8 T cells has been accumulating both at the level of thymic selection and T cell

activation (Wiest et al., 1993; Ravichandran and Burakoff, 1994; Bosselut et al., 1999;). A protein that plays an important role in TCR signalling and that has been shown to associate differently with the CD4 or with the CD8 co-receptor is $p56^{lck}$ (Wiest et al., 1993). Although $p56^{lck}$ expression does not appear to be required for prolonged survival of naive peripheral T cells, its presence is essential in promoting TCR-driven homeostatic proliferation (Seddon et al., 2000). Thus, it is possible that identical stimuli will be transduced into the cell in a different manner and will have different outcomes depending on whether a CD4 or a CD8 T cell is receiving it.

The transcription factor lung Kruppel-like factor (LKLF) is another protein involved in T cell survival. It is abundantly expressed in naive T cells but its expression decreases rapidly upon activation (Kuo et al., 1997) and it is once more upregulated in memory T cells (Schober et al., 1999). The few cells found in the periphery of LKLF knockout animals display an activated phenotype confirming its importance for the viability of naive T cells (Kuo et al., 1997). Thus, LKLF might be another factor worth investigating. It would be interesting to determine if it is differentially expressed in T cell populations and if so whether this expression pattern could be correlated with T cell survival.

Yet another obvious factor that has been associated with T cell survival is the anti-apoptotic molecule Bcl-2. Several authors have reported a relation between increased survival of activated (Akbar et al., 1996) or resting (Teague et al., 1997; Vella et al., 1997) T cells and expression of the Bcl-2 gene. However, analysis of this parameter in naive CD4 and CD8 T cells showed identical expression of the protein in both transgenic and normal mice. It is possible though, that other members of the Bcl-2 family with pro- or anti-apoptotic properties may be differentially expressed in the two cell types. A similar situation has been reported previously in which, although T cells revealed increased resistance to apoptosis, it was not possible to correlate the phenomenon with either of the bcl-2 family genes analysed during the study (Bcl-2 and Bcl-x) (Boise et al., 1995).

4.3. The naive CD4/CD8 ratio is altered with age and in the absence of thymic output

The ratio of CD4 to CD8 T cells in normal mice is controlled homeostatically (Rocha et al., 1989), but less is known about its conservation in the absence of thymic output. Recent thymic emigrants contain about four times more CD4 than CD8 T cells and normalisation of the ratio in secondary lymphoid organs implies that some CD4 T cells are deleted, whereas, in contrast, some CD8 T cells are proliferating (Berzins et al., 1998). In agreement with this observation it was demonstrated that a small fraction of recent thymic emigrants is dividing and that the majority of these cells are indeed CD8 T cells (Penit and Vasseur, 1997).

A second observation that supports the idea that CD8 T cells have a higher proliferative and/or survival potential is the drop of the CD4/CD8 ratio seen in mice as a function of age (Callahan et al., 1993). Although total CD4 and CD8 T cell numbers (naive, effector and memory cells) were used to calculate the ratio in the experiment mentioned, it agrees remarkably well with the results obtained from the analysis of the naive subset performed during the course of this study. Indeed, an age-related significant drop on the naive CD4/CD8 ratio became apparent by comparing young and old wild type animals of the same strain. Determination of absolute T cell numbers revealed that the cause of the decrease was a decline of the CD4 population rather than an increase on CD8 T cell numbers. An even more pronounced reduction of the CD4 subset, and consequently of the CD4/CD8 ratio, was observed in thymectomised animals. In this case though, also the CD8 population was affected. Hence, it appears that naive CD4 T cells are more dependent on thymic output and that CD8 T cell numbers can be maintained even in a situation where thymic export has decreased, such as during old age. However, the peripheral CD8 population is not self-sufficient per se. The fact that the thymectomised group displays drastic reductions in both T cell subsets implies that a minimal input from the thymus is essential for the maintenance of both CD4 and CD8 T cells. This observation is

further supported by the drop in the naive/memory ratio seen in both populations of thymectomised mice. The difference seems to rest thus, on the minimal number of thymic migrants necessary to maintain the peripheral pool and ultimately, on the intrinsic life span of each compartment. The data is in agreement with the differential survival characteristics observed earlier between CD4 and CD8 naive T cells, which under physiological conditions may only become apparent once thymic output ceases or becomes negligible.

The question of how homeostasis is achieved after profound T cell depletion in the periphery has important consequences for therapy in humans. It is known that peripheral expansion in adults gives rise to low CD4 T cell numbers and leads to the acquisition of a substantially skewed T cell repertoire, which is dominated by cells with memory/effector phenotypes at the expense of naive cells (Mackall et al., 1997b).

Blood analysis of patients subjected to chemotherapy showed that CD8 T cell numbers returned to baseline in three months whereas, in contrast, CD4 T cell recovery remained incomplete up to 12 months posttherapy (Mackall et al., 1997a). It also revealed an inverse relationship between age and CD4 regeneration after T cell depletion, which was not detected for the CD8 subset. Therefore, it appears that also in humans, reduction of thymopoiesis has a greater impact on the CD4 than on the CD8 population.

An important function that has been attributed to a subset of CD4 T cells is that of maintaining peripheral tolerance (Sakagushi, 2000). The drastically reduced numbers of CD4 T cells found in old or thymectomised animals could then offer an explanation for the development of autoimmunity, which has been frequently reported in lymphopenic situations (reviewed by Gleeson et al., 1996). Thus, the fast decay of the CD4 population may result in the loss of these regulatory T cells, and consequently of their inhibitory effect, allowing potentially autoreactive clones to become active.

On the other hand lymphopenia-driven autoimmunity may also be explained by the preferential expansion of certain high-affinity clones. T cells expressing promiscuous TCRs such as AND T cells have an accelerated rate of expansion in lymphopenic conditions. They are also more likely to recognise self-antigen then other low-affinity T cell clones. It is therefore reasonable to assume that the probability of some of these cells encountering and reacting to self-antigen will be significantly increased in situations where the thymus export is absent or considerably reduced.

4.4. Peripheral T cell repertoire of naive CD4 T cells in the absence of thymic output

Alterations occurring in the peripheral T cell repertoire have been reported for both the CD4 and the CD8 subsets in situations where thymic export is diminished or absent (Callahan et al., 1993; Posnett et al., 1994; Roux et al., 1996; Mosley et al., 1998;). These alterations are manifested in skewing and contraction of the repertoire and can be observed in both mice and human.

However our analysis of the naive CD4 and CD8 repertoire in thymectomised and non-thymectomised animals by determination of CDR3 sizes of the TCRβ chain, revealed identical normal Gaussian distribution in both compartments for both the experimental and the control group. Since CDR3 length was determined in only one Vβ family, it is possible that alterations in the T cell repertoire can be detected in other Vβ families. Another explanation though, is based on the fact that previous experiments have been performed using total T cells, that is both naive and effector/memory cells were included in the study. Thus it is probable that the mono- or oligoclonal expansions detected in those cases are in fact resulting from activation rather than from the selective proliferation of some naive clones in relation to others. It is also possible that the expanded clones are derived from high-avidity AND-like T cells, which would then acquire a $CD44^{high}$ phenotype when proliferating and would consequently be excluded from the sorted population analysed in this experiment. To determine if indeed

the naive repertoire is not altered in lymphopenic conditions further experiments will have to be performed with additional Vβ families being examined.

4.5. Homeostatic proliferation does not generally affect activation markers

There are conflicting reports in the literature as to whether the often vigorous proliferation observed after transferring naive T cells into supposedly antigen-free lymphopenic hosts affects the expression of activation markers (Tough and Sprent, 1994; Tanchot and Rocha, 1995; Bruno et al., 1996; Bender et al., 1999; Ernst et al., 1999; Goldrath and Bevan, 1999a; Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999; Cho et al., 2000; Goldrath et al., 2000;). Even in the cases where phenotypic conversion has been reported for the naive population, there is not consensus on what the ultimate fate of these cells is, once homeostasis is restored. Thus, while some authors found that the memory-like phenotype is only transient and T cells reacquire naive characteristics once the cellularity of the lymphoid compartment returns to normal levels (Goldrath et al., 2000), others observe a persistence of the memory phenotype for several months (Cho et al., 2000; Murali-Krishna and Ahmed, 2000). A probable explanation for the phenotypic reversal described in some cases might be the presence of stem cells in the transferred population. Thus, the use of total spleen populations might result in the transfer of stem cells into the host, which can then colonise the thymus and follow the normal differentiation process. Consequently, the appearance of a naive population in such animals might result from the accumulation of newly generated T cells, rather than from a reversion of memory-like T cells into a naive phenotype.

In the experiments performed during the course of this project most TCR transgenic populations did not change the expression of CD44 and CD45RB when transferred into syngeneic Rag knockout mice, although they divided several times. The CD44-positive, CFSE-negative population evident in the recipients is not a T cell population, since it does not express a TCR. It is

possible that these cells are identical to the $CD4^{+}CD3^{-}$ population found in developing lymph nodes, which was shown to differentiate into APC, NK and follicular cells, but not into T and B cells (Mebius et al., 1997).

A TCR transgenic population that did show a phenotypic conversion and that proliferated rapidly was obtained from the AND mice. These CD4 T cells, when transferred into Rag knockout hosts upregulated CD44 and downregulated CD45RB progressively, with each division. Previous studies have reported identical results with several different TCR transgenic CD8 T cell populations, such as OT-I, 2C and P14 (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000). It appears thus, that depending on the T cell population, different outcomes can be observed in a lymphopenic situation.

However, even in the cases where cells display an activated phenotype, it is becoming increasingly clear that homeostasis-driven activation is distinct from activation induced by typical high-affinity antigen. During homeostatic proliferation activation markers such as CD62-L, CD25 and CD69 do not seem to be affected (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000; Surh and Sprent, 2000), suggesting that the mechanism used in both situations is not exactly the same. A possible reason for the fact that only some activation markers appear to be engaged in this process could be related with the period of time during which these proteins are expressed on the cell surface. While CD44 is a stable marker which once upregulated persists for long periods of time, molecules such as CD69 and CD25 are very transiently expressed (Garcia et al., 1999) and thus more difficult to detect. Opposing to this hypothesis however, is the fact that CD69 and CD25 levels were shown to remain low, not only in cells which have undergone several divisions and could for this reason have already passed the stage during which early activation markers are affected, but also in cells which have divided only one or two times (Murali-Krishna and Ahmed, 2000). Furthermore CD62-L, a molecule which is down regulated upon activation and which has been shown to remain so for more than 60 days (Garcia et al., 1999), is expressed at high levels by cells undergoing homeostatic proliferation (Goldrath and Bevan, 1999a).

Another explanation for this phenomenon might be the partial activation of the cell, caused by some low-affinity cross-reactive antigens present in the host, which might not have the capacity to stimulate the cell into a fully activated state. Indeed, it has been previously reported that some peptides can partially activate T cells causing alterations in some activation markers but not in others, which suggest the existence of different thresholds for each marker (Chen et al., 1996; Auphan et al., 1999). T cells expressing high-affinity TCRs, like the AND, OT-I or P14 would therefore be more susceptible to activation since they would more easily recognise cross-reactive peptides. As such they would enjoy preferential survival at the expense of T cells with more restricted TCR recognition, for whom survival signals may be limiting. The AND and A18 TCRs may represent opposite ends of the spectrum with respect to their responses to TCR-mediated signals.

In the case of polyclonal T cells, upregulation of the CD44 marker could be observed in a proportion of both the CD4 and the CD8 T cell populations. This was seen not only when totals T cells were transferred, which was expected due to the presence of preactivated T cells, but also in a situation where $CD44^{high}$ cells had been previously eliminated by sorting. A possible explanation is the presence of a few contaminating $CD44^{high}$ T cells in the injected sample. Mature T lymphocytes have a large expansion potential (Rocha et al., 1989) and memory T cells have been shown to proliferate faster than the naive subset (Tough and Sprent, 1994), which would account for the rapid accumulation of $CD44^{high}$ CFSE-negative cells.

Another reason for the phenomenon might be the existence of minor histocompatibility differences between the donor population and the adoptive host since the Rag knockout mice have not been extensively backcrossed onto the CBA strain background. In this case the $CD44^{high}$ cells would be derived not from pre-existing memory cells, but rather from naive cells which had become activated after the transfer.

It is also possible that the affinity of the TCR is playing a role in this situation. As it was demonstrated previously, phenotypic conversion appears to be observed in some cases where T cells express high-affinity receptors, such as the AND and the OT-I strains, but not in other populations which carry low-affinity TCRs, like A18. Since a polyclonal population is composed of many different clones expressing TCRs with variable affinity for antigen, it is probable that T cells expressing high-affinity TCRs can more easily cross-react with host antigens and become activated.

The use of irradiated hosts is likely to exacerbate T cell activation and could be a reason why some authors describe a phenotypic conversion of the majority of the polyclonal cells as early as 10 days after transfer (Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999), while in our case less than 50% of the donor cells have upregulated the CD44 marker by day 20. Irradiation results in gastrointestinal damage, leading to the release of LPS (Hill et al., 1997). This could liberate cross-reactive antigens and result in mobilisation of antigen-bearing dendritic cells to lymphoid tissues, thus promoting T cell activation (Reis e Sousa and Germain, 1999).

Taken together, each of the factors mentioned or even a combination of several of them can be responsible for the phenotypic conversion observed after the transfer of T cells into lymphopenic hosts.

4.6. T cell competition between CD4 populations expressing different transgenic TCRs

A mechanism that plays an important role in the maintenance of homeostasis is T cell competition. T cells compete for limiting factors such as cytokines, MHC molecules, antigen, co-stimulatory molecules, etc. An essential factor for T cell survival is the maintenance of constant TCR/MHC-peptide interactions (Kirberg et al., 1997; Tanchot et al., 1997a). Therefore the specificity and affinity of the TCR expressed by the cell is likely to have some influence in the decision of

how represented a T cell clone will be in the T cell repertoire. In fact, Kedl et al. (2000) have shown that T cells compete for antigen and antigen-bearing cells and they suggest that high affinity cells might have an advantage over others. Also previous work by Freitas et al. (1996) points to a relation between the level of promiscuity of the TCR and the selection of a T cell population both in the thymus and in the periphery. Thus, high affinity for antigen may allow a given cell to gain and maintain TCR/MHC interactions to the exclusion of other lower affinity T cells.

By transferring a monoclonal T cell population into a host containing a resident population of a different specificity and by monitoring the proliferative capacity of the donor cells we intended to evaluate the role of the TCR in T cell competition. This set of experiments revealed that A18 expansion was inhibited in an A1 host, which suggested that both populations shared, at least in part, common resources. The fact that A1 cells could still proliferate in the inverse experiment was possibly due to the very reduced number of peripheral T cells present in the A18 hosts. Thus, while A1 T cells can no longer proliferate in an A1 mouse possibly because all niches are occupied by the native population, A18 cells show identical expansion in a Rag knockout and in an A18 recipient.

A possible way to surpass these difficulties is by making co-transfers of identical numbers of cells from different populations into the same host. This method will allow us to evaluate the influence of the transferred populations on each other, not only by evaluating their immediate effect through alterations in the expansion capacity, but also in a long-term manner, by determining the proportions in which each population is established in the host and if these proportions reflect their different proliferative characteristics.

CD4 and CD8 T cells are thought to occupy overlapping niches in the periphery, because co-transfer of large numbers of either subset can suppress the expansion of both (Rocha et al., 1989). Thus, another important follow-up on the subject will be the analysis of CD8 T cell populations and their interactions both within the CD8 subset and with CD4 T cells.

4.7. Overexpression of CD4 improves thymic selection and homeostatic proliferation but not *in vitro* survival of A18 T cells

Of all the T cell populations studied during this project, A18 T cells are the ones that display the poorest proliferative and survival capacities. Since the CD4 co-receptor has been previously associated with survival of resting CD4 T cells in the peripheral lymphoid organs (Maroto et al., 1999), we analysed double transgenic mice expressing both the A18 TCR and a CD4 transgene. A comparison between single and double transgenics revealed that a difference became visible already during the developmental stage of the T cells. Indeed positive selection appeared to be improved in the CD4VA2B.A18 animals since their thymus contained approximately the double of the number of single positive CD4 T cells than the one found in A18 controls.

Variation of CD4 levels in the thymus have been previously observed to cause different effects, depending on the TCR transgenic strain used. Thus, while both increasing and decreasing the expression of CD4 seems to cause significant disruption in thymocyte development in AND animals (Davis and Littman, 1995; Frank and Parnes, 1998), in 5C.C7 TCR transgenics bred onto a $CD4^{low}$ background, selection to the CD4 lineage is only slightly reduced (Frank and Parnes, 1998) and in $\lambda 2^{315}$-specific TCR transgenic mice, CD4 overexpression actually improves maturation towards the CD4 lineage (Dembic et al., 1998). It is possible then that the CD4 co-receptor increases the avidity of the TCR/MHC interactions which occur during thymocyte development, and leads to positive or negative selection, depending on the affinity of the TCR expressed by the cell.
In the case of A18, TCR/MHC interactions appear to be of relatively low avidity (Barthlott et al., 1998), which may explain the reason why overexpression of the CD4 molecule results in a higher accumulation of cells in the CD4 single positive compartment.

Another effect of the expression of the CD4 transgene might be the extension of the time that T cells have to encounter a positively selecting ligand. According to Merkenschlager (1994), positive selection niches are a limiting factor during T cell differentiation. The expression of the CD4 molecule as early as during the double negative stage may extend the time window during which thymocytes can encounter and interact with a stromal cell niche. Thus, TCR transgenic cells which do not need to go through the rearrangement process may express the TCR molecule relatively early. In fact, it has been shown that A18 mice develop mature CD4 T cells much faster than nontrangenic animals (Volkmann et al., 1997). The presence of high levels of the CD4 co-receptor would then allow these thymocytes to go through positive selection at an earlier time-point increasing the interval of time normally allocated to this mechanism.

Having observed an improvement in positive selection in the CD4VA2B.A18 animals, it was not surprising that also the number of peripheral T cells was augmented. Nevertheless, further analysis revealed that the proliferative capacity of these cells was another factor that should be taken in consideration. In fact, conversely to A18, CD4VA2B.A18 T cells divided extensively when transferred into Rag knockout recipients in a manner even comparable to the homeostatic proliferation seen for the CD8 T cell populations. The reason why increased levels of the CD4 molecule can enhance proliferation is not clear, but it is probable that also in the periphery, higher avidity TCR/MHC interactions are the critical factor. Thus, strong interactions would result in a more prolonged contact with the MHC bearing cell and in a higher intensity of the signal being transduced into the T cell and consequently in a more intense proliferative response. On the other hand, increased avidity can also allow the TCR complex to interact with a wider range of ligands that would not be available to low affinity TCR expressing cells. This would increase the probability of a given cell receiving a proliferation signal.

It is therefore probable that not only increased production, but also enhanced T cell expansion are playing a role in the maintenance of larger numbers of CD4 T cells in the peripheral pool of the CD4VA2B.A18 mice. To determine which is

the relative contribution of each factor, thymectomised animals can be used in future experiments and the half-life of the double transgenic T cell population compared with the one determined for A18 T cells.

Although introduction of the CD4 transgene seem to have improved thymic selection and homeostatic proliferation in the A18 T cells, no alteration was detected in the *in vitro* susceptibility to apoptosis. If, as discussed earlier in this chapter, susceptibility to apoptosis *in vitro* reflects survival signals previously imprinted on the cell, then the data suggests that overexpression of the CD4 molecule does not affect survival patterns. In fact, recent work by Seddon et al. (2000) supports the hypothesis that survival and homeostatic expansion are two different mechanisms, which are independently regulated. The authors demonstrate that $p56^{lck}$, a molecule that binds the CD4 and CD8 co-receptors and is involved in TCR signalling, is essential for homeostatic proliferation. However, long-term survival of naive T cells is not affected in the absence of $p56^{lck}$ expression.

4.8. Reduced numbers of CD4 single positive thymocytes, but increased peripheral pool in A18EVA mice

Preliminary results obtained from the analysis of double transgenic animals expressing the A18 TCR and the EVA adhesion molecule revealed that although thymocytes were reduced when compared with A18 animals, the peripheral pool size appeared to be slightly increased. A possible explanation for the data is based on the opposite effects that high-avidity TCR/MHC interactions have when they take place in the thymus or in the periphery. Thus, while T cells expressing high-affinity TCRs are eliminated by negative selection during development, in the periphery they appear to be favoured by this characteristic. Previous reports demonstrated that upon activation there is a selective expansion of T cells which bind MHC/peptide complexes with greater affinity (Busch and Pamer, 1999) and experiments performed during this project support the

hypothesis that high-affinity naive clones have higher rates of homeostatic proliferation.

Under the K14 promoter EVA is expressed in the thymic epithelia and its adhesion properties may cause an increase of avidity in T cell-epithelial cell interactions and consequently in TCR/MHC interactions. Even in the A18 case, which appears to express a low-affinity TCR, it is possible that an excessive increase in avidity will lead to negative selection resulting in the reduced number of CD4 single positives found in the thymus.Why, is then the peripheral pool increased when thymic production is lower? A reason for the phenomenon could be, as observed for the CD4VA2B.A18 strain, higher homeostatic proliferation rates due to increased TCR/MHC avidity. Although in A18EVA mice the EVA molecule is expressed only in certain epithelia such as skin, thymus, tongue and oesophagus it is possible that some T cells interact with these epithelial cells and expand at a faster rate, resulting in the modest increase in T cell numbers observed.

Another potential explanation is based on the survival capacity of the A18EVA cells. Single positive cells can remain in the thymic medulla for a period of time up to 14 days (Scollay and Godfrey, 1995), which supports the idea of a final stage of maturation for thymocytes. If survival signals are imprinted in the thymus and delivered by the thymic stroma during this period, then it is possible that the constitutive expression of an adhesion molecule increases the frequency or length of the interactions, resulting in a better survival of peripheral T cells. Thymic migrants would therefore be able to accumulate in the periphery providing an explanation for the increased T cell numbers observed in this strain.

More A18EVA animals will have to be analysed in the future in order to determine if the phenotype observed in this experiment remains consistent. If that is the case then survival and proliferation tests should be applied, since they might supply a clue on the cause of the augmented peripheral T cell pool.

4.9. Concluding remarks

The maintenance of the naive T cell pool depends on many different factors and understanding these factors is extremely important in order to be able to evaluate the consequences of natural or induced T cell depletion. During this study it became clear that TCR specificity, as well as the type and density of co-stimulatory and adhesion molecules expressed on the cell surface, can play an important role in the final outcome of a given naive T cell population. However, due to the fact that some TCR transgenic mouse lines were only very recently generated, in a few cases only a small number of animals was available for experimental use. For this reason some experiments will have to be repeated before final conclusions can be drawn.

Further investigation on the different issues approached during this project might offer new insights into the complex physiological processes that regulate T cell populations.

5. References

Adams, J. M. and Cory, S. (1998). The Bcl-2 Protein Family: Arbiters of Cell Survival. Science *281*, 1322-1326.

Agenes, F., Rosado, M. M. and Freitas, A. A. (1997). Independent Homeostatic Regulation of B Cell Compartments. Eur.J.Immunol. *27*, 1801-1807.

Akbar, A. N., Borthwick, N. J., Wickremasinghe, R. G., Panayiotidis, P., Pilling, D., Bofill, M., Krajewski, S., Reed, J. C. and Salmon, M. (1996). Interleukin-2 Receptor Common γ-chain Signaling Cytokines Regulate Activated T Cell Apoptosis in Response to Growth Factor Withdrawal: Selective Induction of Anti-apoptotic (bcl-2, bcl-X_L) but not Pro-apoptotic (bax, bcl-X_S) Gene Expression. Eur.J.Immunol. *26*, 294-299.

Armitage, R. J., Fanslow, W. C., Strokbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D. and Spriggss, M. K. (1992). Molecular and Biological Characterization of a Murine Ligand for CD40. Nature *357*, 80-82.

Auphan, N., Ghosh, S., Flavell, R. A. and Schmitt-Verhulst, A.M. (1999). Differential Requirements for NF-kB and AP-1 *trans*-Activation in Response to Minimal TCR Engagement by a Partial Agonist in Naive CD8 T Cells. J.Immunol. *163*, 5219-5227.

Barthlott, T., Wright, R. J. and Stockinger, B. (1998). Normal Thymic Selection on TCR Transgenic CD4 T Cells, but Impaired Survival in the Periphery Despite the Presence of Selecting MHC Molecules. J.Immunol. *161*, 3992-3999.

Basson, M. A., Bommhardt, U., Mee, P. J., Tybulewicz, V. L. J. and Zamoyska, R. (1998). Molecular Requirements for Lineage Commitment in the Thymus - Antibody-mediated Receptor Engagements Reveal a Central Role for LCK in Lineage Decisions. Immunol.Rev. *165*, 181-194.

Belardelli, F., Ferrantini, M., Santini, S. M., Baccarini, S., Proietti, E., Colombo, M. P., Sprent, J. and Tough, D. F. (1998). The Induction of In Vivo Proliferation of Long-lived $CD44^{hi}$ CD8+ T Cells After the Injection of Tumor Cells Expressing IFN-alpha 1 into Syngeneic Mice. Cancer Res. *58*, 5795-5802.

Bell, E. B. and Sparshott, S. M. (1997). The Peripheral T-Cell Pool: Regulation by Non-Antigen Induced Proliferation? Semin.Immunol. *9*, 347-353.

Bell, E. B., Sparshott, S. M., Drayson, M. T. and Ford, W. L. (1987). The Stable and Permanent Expansion of Functional T Lymphocytes in Athymic Nude Rats After a Single Injection of Mature T Cells. J.Immunol. *139*, 1379-1384.

Bender, J., Mitchell, T., Kappler, J. and Marrack, P. (1999). CD4+ T Cell Division in Irradiated Mice Requires Peptides Distinct from Those Responsible for Thymic Selection. J.Exp.Med. *190*, 367-373.

Berzins, S. P., Boyd, R. L. and Miller, J. F. A. P. (1998). The Role of the Thymus and Recent Thymic Migrants in the Maintenance of the Adult Peripheral Lymphocyte Pool. J.Exp.Med. *187*, 1839-1848.

Boise, L. H., Gonzales-Garcia, M., Postema, C. E., Ding, L., Lindsten, T., Turka, L. A., Mao, X., Nunez, G. and Thompson, C. B. (1993). Bcl-x, a Bcl-2-related Gene that Functions as a Dominant Regulator of Apoptotic Cell Death. Cell *74*, 597-608.

Boise, L. H., Minn, A. J., Junes, C. H., Lindsten, T. and Thompson, C. B. (1995). Growth factors can Enhance Lymphocyte Survival Without Commiting the Cell to Undergo Cell Division. Proc.Natl.Acad.Sci.USA *92*, 5491-5495.

Borgulya, P., Kishi, H., Uematsu, Y., and von Boehmer, H. (1992). Exclusion and Inclusion of α and β T Cell Receptor Alleles. Cell *69*, 529-537.

Bosselut, R., Zhang, W., Ashe, J. M., Kopacz, J. L., Samelson, L. E. and Singer, A. (1999). Association of the Adaptor Molecule LAT with CD4 and CD8 Coreceptors Identifies a New Coreceptor Function in T Cell Receptor Signal Transduction. J.Exp.Med. *190*, 1517-1525.

Boursalian, T. E. and Bottomly, K. (1999). Survival of Naive CD4 T Cells: Roles of Restricting Versus Selecting MHC Class II and Cytokine Milieu. J.Immunol. *162*, 3795-3801.

Brocker, T. (1997). Survival of Mature CD4 T Lymphocytes is Dependent on Major Histocompatibility Complex Class II-expressing Dendritic Cells. J.Exp.Med. *186*, 1223-1232.

Bruno, L., von Boehmer, H. and Kirberg, J. (1996). Cell Division in the Compartment of Naive and Memory T Lymphocytes. Eur.J.Immunol. *26*, 3179-3184.

Bui, J. D., Calbo, S., Hayden-Martinez, K., Kane, L. P., Gardner, P. and Hedrick, S. M. (2000). A Role for CaMKII in T Cell Memory. Cell *100*, 457-467.

Busch, D. H. and Pamer, E. G. (1999). T Cell Affinity Maturation by Selective Expansion During Infection. J.Exp.Med. *189*, 701-709.

Butcher, E. C. and Picker, L. J. (1996). Lymphocyte Homing and Homeostasis. Science *272*, 60-66.

Callahan, J. E., Kappler, J. W. and Marrack, P. (1993). Unexpected Expansions of CD8-bearing Cells in Old Mice. J.Immunol. *151*, 6657-6669.

Campbel, J. J., Hedrick, J., Zlotnik, A., Siani, M. A., Thompson, D. A. and Butcher, E. C. (1998). Chemokines and the Arrest of Lymphocytes Rolling Under Flow Conditions. Science *279*, 381-384.

Campbell, J. J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D. P., Warnke, R., Ruffing, N., Kassam, N., Wu, L. and Butcher, E. C. (1999). The Chemokine Receptor CCR4 in Vascular Recognition by Cutaneous but not Intestinal Memory T Cells. Nature *400*, 776-780.

Carbone, F. R., Kurts, C., Bennett, S. R. M., Miller, J. F. A. P. and Heath, W. R. (1998). Cross-presentation: a General Mechanism for CTL Immunity and Tolerance. Immunol.Today *19*, 368-373.

Casrouge, A., Beaudoing, E., Dalle, S., Pannetier, C., Kanellopoulos, J. and Kourilsky, P. (2000). Size Estimate of the αβ TCR Repertoire of Naive Mouse Splenocytes. J.Immunol. *164*, 5782-5787.

Chan, S. H., Cosgrove, D., Waltzinger, C., Benoist, C. and Mathis, D. (1993). Another View of the Selective Model of Thymocyte selection. Cell *73*, 225-236.

Chen, Y.Z., Matsushita, S. and Nishimura, Y. (1996). Response of a Human T Cell Clone to a Large Panel of Altered Peptide Ligands Carrying Single Residue Substitutions in an Antigenic Peptide. J.Immunol. *157*, 3783-3790.

Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N. and Chen, J. (2000). Homeostasis-stimulated Proliferation Drives Naive T Cells to Differentiate Directly into Memory T Cells. J.Exp.Med *192*, 549-556.

Cobbold, S. P., Jayasuriya, A., Nash, A., Prospero, T. D. and Waldmann, H. (1984). Therapy with Monoclonal Antibodies by Elimination of T-cell Subsets in Vivo. Nature *312*, 548-551.

Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C. and Mathis, D. (1991). Mice Lacking MHC Class II Molecules. Cell *66*, 1051-1066.

Cumberbatch, M. and Kimber, I. (1992). Dermal Tumour Necrosis Factor-alpha Induces Dendritic Cell Migration to Draining Lymph Nodes, and Possibly Provides One Stimulus for Langerhans' Cell Migration. Immunology *75*, 257-263.

Cyster, J. G. (1999). Chemokines and Cell Migration in Secondary Lymphoid Organs. Science *286*, 2098-2112.

Davis, C. B. and Littman, D. R. (1995). Disrupted Development of Thymocytes Expressing a Transgenic TCR Upon CD4 Overexpression. Int.Immunol. *7*, 1977-1986.

Dembic, Z., Munthe, L. A., Schenck, K., Mueller, C. and Bogen, B. (1998). Transient Overexpression of CD4 Enhances Allelic Exclusion of T-Cell Receptor (TCR) α Chains and Promotes Positive Selection of Class II-restricted TCR-transgenic Thymocytes. Mol.Immunol. *35*, 23-38.

Dyall, R. and Nikolic-Zugic, J. (1995). The Majority of Post-selection CD4+ Single-positive Thymocytes Requires the Thymus to Produce Long-lived, Functional T Cells. J.Exp.Med. *181*, 235-245.

Ernst, B., Lee, D.-S., Chang, J. M., Sprent, J. and Surh, C. D. (1999). The Peptide Ligands Mediating Positive Selection in The Thymus Control T Cell Survival and Homeostatic Proliferation in the Periphery. Immunity *11*, 173-181.

Forster, I., Hirose, R., Arbeit, J. M., Clausen, B. E. and Hanahan, D. (1995). Limited Capacity for Tolerization of CD4+ T Cells Specific for a Pancreatic β Cell Neo-antigen. Immunity *2*, 573-585.

Frank, G. D. and Parnes, J. R. (1998). The Level of CD4 Surface Protein Influences T Cell Selection in the Thymus. J.Immunol. *160*, 634-642.

Freeman, G. J., Lombard, D. B., Gimmi, C. D., Brod, S. A., Lee, K., Laning, J. C., Hafler, D. A., Dorf, M. E., Gray, G. S., Reiser, H., June, C. H., Thompson, C. B. and Nadler, L. M. (1992). CTLA-4 and CD28 Are Coexpressed in Most T Cells After Activation. J.Immunol. *149*, 3795-3801.

Freitas, A. A., Agenes, F. and Coutinho, G. C. (1996). Cellular Competition Modulates Survival and Selection of CD8+ T Cells. Eur.J.Immunol. *26*, 2640-2649.

Freitas, A. A. and Rocha, B. (2000). Population Biology of Lymphocytes: The Flight for Survival. Annu.Rev.Immunol. *18*, 83-111.

Garcia, S., DiSanto, J. and Stockinger, B. (1999). Following the Development of a CD4 T Cell Response In Vivo: From Activation to Memory Formation. Immunity *11*, 163-171.

Gay, D., Saunders, T., Camper, S. and Weigert, M. (1993). Receptor Editing: an Approach by Autoreactive B Cells to Escape Tolerance. J.Exp.Med. *177*, 999-1008.

Ghia, P., ten Boekel, E., Sanz, E., de la Hera, A., Rolink, A. and Melchers, F. (1996). Ordering of Human Bone Marrow B Lymphocyte Precursors by Single-cell Polymerase Chain Reaction Analysis of the Rearrangement Status of the Immunoglobulin H and L Chain Gene Loci. J.Exp.Med. *184*, 2217-2229.

Gleeson, P. A., Toh, B.H. and van Driel, I. R. (1996). Organ-specific Autoimmunity Induced by Lymphopenia. Immunol.Rev. *149*, 97-125.

Goldrath, A. and Bevan, M. J. (1999a). Low-Affinity Ligands for the TCR Drive Proliferation of Mature CD8+ T Cells in Lymphopenic Hosts. Immunity *11*, 183-190.

Goldrath, A. W. and Bevan, M. J. (1999b). Selecting and Maintaining a Diverse T-cell Repertoire. Nature *402*, 255-262.

Goldrath, A. W., Bogatzki, L. Y. and Beva, M. J. (2000). Naive T Cells Transiently Acquire a Memory-like Phenotype during Homeostatic-driven Proliferation. J.Exp.Med. *192*, 557-564.

Gray, D. and Matzinger, P. (1991). T Cell Memory Is Short-lived in the Absence of Antigen. J.Exp.Med *174*, 969-974.

Gray, D., Siepmann, K., van Essen, D., Poudrier, J., Wykes, M., Jainandunsing, S., Bergthorsdottir, S. and Dullforce, P. (1996). B-T Lymphocyte Interactions in the Generation and Survival of Memory Cells. Immunol.Rev. *150*, 45-61.

Griffin, F. M. J., Griffin, J. A., Leider, J. E. and Silverstein, S. C. (1975). Studies on the Mechanism of Phagocytosis. I. Requirements for Circumferential Attachment of Particle-bound Ligands to Specific Receptors on the Macrophage Plasma Membrane. J.Exp.Med. *142*, 1263-1282.

Griffin, F. M. J. and Silverstein, S. C. (1974). Segmental Response of the Macrophage Plasma Membrane to a Phagocytic Stimulus. J.Exp.Med. *139*, 323-336.

Gunn, M. D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L. T. and Nakano, H. (1999). Mice Lacking Expression of Secondary Lymphoid Organ Chemokine Have Defects in Lymphocyte Homing and Dendritic Cell Localization. J.Exp.Med. *189*, 451-460.

Gunn, M. D., Tangemann, K., Tam, C., Cyster, J. G., Rosen, S. D. and Williams, L. T. (1998). A Chemokine Expressed in Lymphoid High Endothelial Venules Promotes the Adhesion and Chemotaxis of Naive T Lymphocytes. Proc.Natl.Acad.Sci.USA *95*, 258-263.

Guttinger, M., Sutti, F., Panigada, M., Porcellini, S., Merati, B., Mariani, M., Teesalu, T., Consalez, G. G. and Grassi, F. (1998). Epithelial V-like Antigen (EVA), a Novel Member of the Immunoglobulin Superfamily, Expressed in Embryonic Epithelia with a Potential Role as Homotypic Adhesion Molecule in Thymus Histogenesis. J.Cell.Biol. *141*, 1061-1071.

Hanabuchi, S., Koyanagi, M., Kawasaki, A., Shinohara, N., Matsuzawa, A., Nishimura, Y., Kobayashi, Y., Yonehara, S., Yagita, H. and Okumura, K. (1994). Fas and Its Ligand in a General Mechanism of T-cell-mediated Cytotoxicity. Proc.Natl.Acad.Sci.USA *91*, 4930-4934.

Hathcock, K. S., Laszlo, G., Pucillo, C., Linsley, P. and Hodes, R. J. (1994). Comparative Analysis of B7-1 and B7-2 Costimulatory Ligands: Expression and Function. J.Exp.Med . *180*, 631-640.

Heath, W. R., Carbone, F. R., Bertolino, P., Kelly, J., Cose, S. and Miller, J. F. (1995). Expression of Two T Cell Receptor α Chains on the Surface of Normal Murine T Cells. Eur.J.Immunol. *25*, 1617-1623.

Hilbert, D. M., Anderson, A. O., Holmes, K. L. and Rudikoff, S. (1994). Long-term Lymphoid Reconstitution of SCID Mice Suggests Self-renewing B and T Cell Populations in Peripheral and Mucosal Tissues. Transplantation *58*, 466-474.

Hill, G. R., Crawford, J. M., Cooke, K. R., Brinson, Y. S., Pan, L. and Ferrara, J. L. M. (1997). Total Body Irradiation and Acute Graft-versus-host Disease: The Role of Gastrointestinal Damage and Inflammatory Cytokines. Blood *90*, 3204-3213.

Hou, S., Hyland, L., Ryan, K. W., Portner, A. and Doherty, P. C. (1994). Virus-specific CD8+ T-cell Memory Determined by Clonal Burst Size. Nature *369*, 652-654.

Huesmann, M., scott, B., Kisielow, P., and von Boehmer, H. (1991). Kinetics and Efficacy of Positive Selection in the Thymus of Normal and T Cell Receptor Transgenic Mice. Cell *66*, 533-540.

Itano, A., Salmon, P., Kioussis, D., Tolaini, M., Corbella, P. and Robey, E. (1996). The Cytoplasmic Domain of CD4 Promotes the Development of CD4 Lineage T Cells. J.Exp.Med *183*, 731-741.

Jabara, H. H., Fu, S. M., Geha, R. S. and Vercelli, D. (1990). CD40 and IgE: Synergism Between Anti-CD40 Monoclonal Antibody and Interleukin 4 in the Induction of IgE Synthesis by Highly Purified Human B Cells. J.Exp.Med *172*, 1861-1864.

Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C. and Raff, M. C. (1993). Bcl-2 Blocks Apoptosis in Cells Lacking Mitochondrial DNA. Nature *361*, 365-369.

Janeway, C. A. J., Kupfer, A., Viret, C., Boursalian, T., Goverman, J., Bottomly, K. and Sant'Angelo, D. (1998). T Cell Development, Survival and Signaling: a New Concept of the Role of Self-peptide: Self-MHC Complexes. Immunologist *6*, 5-12.

Jorgensen, J. L., Esser, U., de St. Groth, B. F., Reay, P. A. and Davis, M. M. (1992). Mapping T-cell Receptor-peptide Contacts by Variant Peptide Immunization os Single-chain Transgenics. Nature *355*, 224-230.

Kalergis, A. M. and Nathenson, S. G. (2000). Altered Peptide Ligand-mediated TCR Antagonism Can Be Modulated by a Change in a Single Amino Acid Residue Within the CDR3β of an MHC Class I-restricted TCR. J.Immunol. *165*, 280-285.

Kappler, J. W., Staerz, U., White, J. and Marrack, P. C. (1988). Self-tolerance Eliminates T Cells Specific for MIs-modified Products of the Major Histocompatibility Complex. Nature *332*, 35-40.

Kaye, J., Hsu, M.L., Sauron, M., Jameson, S. C., Gascoigne, N. R. J. and Hedrick, S. M. (1989). Selective Development of CD4+ T Cells in Transgenic Mice Expressing a Class II MHC-Restricted Antigen Receptor. Nature *341*, 746-749.

Kedl, R. M., Ress, W. A., Hildeman, D. A., Schaefer, B., Mitchell, T., Kappler, J. and Marrack, P. (2000). T Cells Compete for Access to Antigen-bearing Antigen-presentig Cells. J.Exp.Med. *192*, 1105-1113.

Kieper, W. C. and Jameson, S. C. (1999). Homeostatic Expansion and Phenotypic Conversion of Naive T Cells in Response to Self Peptide/ MHC Ligands. Proc.Natl.Acad.Sci.USA *96*, 13306-13311.

Kirberg, J., Berns, A. and von Boehmer, H. (1997). Peripheral T Cell Survival Requires Continual Ligation of the T Cell Receptor to Major Histocompatibility Complex-encoded Molecules. J.Exp.Med. *186*, 1269-1275.

Kitamura, D., Roes, J., Kuhn, R. and Rajewsky, K. (1991). A B Cell-deficient Mouse by Targeted Disruption of the Membrane Exon of the Immunoglobulin mu Chain Gene. Nature *350*, 423-426.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W. and Reed, J. C. (1993). Investigation of the Subcellular Distribution of the Bcl-2 Oncoprotein: Residence in the Nuclear Envelope, Endoplasmic Reticulum, and Outer Mitochondrial Membranes. Cancer Res. *53*, 4701-4714.

Krummel, M. F. and Allison, J. P. (1995). CD28 and CTLA-4 Have Opposing Effects on the Response of T Cells to Stimulation. J.Exp.Med *182*, 459-465.

Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. and Marrack, P. (2000). Control of Homeostasis of CD8+ Memory T Cells by Opposing Cytokines. Science *288*, 675-678.

Kuo, C. T., Veselits, M. L. and Leiden, J. M. (1997). LKLF: A Transcriptional Regulator of Single-Positive T Cell Quiescence and Survival. Science *277*, 1986-1990.

Lane, P., Traunecker, A., Hubele, S., Inui, S., Lanzavecchia, A. and Gray, D. (1992). Activated Human T Cells Express a Ligand for the Human B Cell-associated Antigen CD40 Which Participates in T Cell-dependent Activation of B Lymphocytes. Eur.J.Immunol. *22*, 2573-2578.

Lanzavecchia, A. (1985). Antigen-specific Interaction Between T and B Cells. Nature *314*, 537-539.

Lau, L. L., Jamieson, B. D., Somasundaram, T. and Ahmed, R. (1994). Cytotoxic T-cell Memory without Antigen. Nature *369*, 648-652.

Lechler, R., Aichinger, G. and Lightstone, L. (1996). The Endogenous Pathway of MHC Class II Antigen Presentation. Immunol.Rev. *151*, 51-79.

Lecoeur, H. and Gougeon, M.L. (1996). Comparative Analysis of Flow Cytometric Methods for Apoptosis Quantitation in Mutine Thymocytes and Human Peripheral Lymphocytes from Controls and HIV-Infected Persons Evidence for Interference by Granulocytes and Erythrocytes. J.Immunol.Methods *198*, 87-99.

Lehner, P. J. and Cresswell, P. (1996). Processing and Delivery of Peptides Presented by MHC class I Molecules. Curr.Opin.Immunol. *8*, 59-67.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M. and Hoffmann, J. A. (1996). The Dorsoventral Regulatory Gene Cassette *spatzle/Toll/cactus* Controls the Potent Antifungal Response in Drosophila Adults. Cell *86*, 973-983.

LeMaoult, J., Messaoudi, I., Manavalan, J. S., Potvin, H., Nikolich-Zugich, D., Dyall, R., Szabo, P., Weksler, M. E. and Nikolich-Zugich, J. (2000). Age-related Dysregulation in CD8 T Cell Homeostasis: Kinetics of a Diversity Loss. J.Immunol. *165*, 2367-2373.

Lenschow, D. J., Sperling, A. I., Cooke, M. P., Freeman, G., Rhee, L., Decker, D. C., Gray, G., Nadler, L. M., Goodnow, C. C. and Bluestone, J. A. (1994). Differential Up-regulation of the B7-1 and B7-2 Costimulatory Molecules After Ig Receptor Engagement by Antigen. J.Immunol. *153*, 1990-1997.

Liu, Y. and Janeway, C. A. (1991). Microbial Induction of Co-stimulatory Activity for CD4 T Cell Growth. Int.Immunol. *3*, 323-332.

Loder, F., Mutschler, B., Ray, R. J., Paige, C. J., Sideras, P., Torres, R., Lamers, M. C. and Carsetti, R. (1999). B Cell Development in the Spleen Takes Place in Discrete Steps and Is Determined by the Quality of B Cell Receptor-derived Signals. J.Exp.Med. *190*, 75-89.

Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S. and Ma, A. (1998). IL-15 Receptor Maintains Lymphoid Homeostasis by Supporting Lymphocyte Homing and Proliferation. Immunity *9*, 669-676.

London, C. A., Perez, V. L. and Abbas, A. K. (1999). Functional Characteristics and Survival Requirements of Memory CD4+T Lymphocytes In Vivo. J.Immunol. *162*, 766-773.

Lucas, B., Vasseur, F. and Penit, C. (1995). Stochastic Coreceptor Shut-off Is Restricted to the CD4 Lineage Maturation Pathway. J.Exp.Med *181*, 1623-1633.

Luther, S. A., Tang, H. L., Hyman, P. L., Farr, A. G. and Cyster, J. G. (2000). Coexpression of the Chemokines ELC and SLC by T Zone Stromal Cells and Deletion of the ELC Gene in *plt/plt* Mouse. Proc.Natl.Acad.Sci.USA *97*, 12694-12699.

Lyons, A. B. and Parish, C. R. (1994). Determination of Lymphocyte Division by Flow Cytometry. J.Immunol.Methods *171*, 131-137.

Mackall, C. L., Fleisher, T. A., Brown, M. R., Andrich, M. P., Chen, C. C., Feuerstein, I. M., Magrath, I. T., Wexler, L. H., Dimitrov, D. S. and Gress, R. E. (1997a). Distinctions Between CD8+ and CD4+ T-cell Regenerative Pathways Result in Prolongued T-cell Subset Imbalance After Intensive Chemotherapy. Blood *89*, 3700-3707.

Mackall, C. L., Hakim, F. T. and Gress, R. E. (1997b). Restoration of T-cell Homeostasis after T-cell Depletion. Semin.Immunol. *9*, 339-346.

Mamalaki, C., Elliot, T., Norton, T., Yannoutsos, N., Townsend, A. R., Chandler, P., Simpson, E. and Kioussis, D. (1993). Positive and Negative Selection in Transgenic Mice Expressing a T-cell Receptor Specific for Influenza Nucleoprotein and Endogenous Superantigen. Dev.Immunol. *3*, 159-174.

Markiewicz, M. A., Girao, C., Opferman, J., Sun, J., Hu, Q., Agulnik, A. A., Bishop, C. E., Thompson, C. B. and Ashton-Rickardt, P. G. (1998). Long-term T Cell Memory Requires the Surface Expression of Self-Peptide/ Major Histocompatibility Complex Molecules. Immunology *95*, 3065-3070.

Maroto, R., Shen, X. and Konig, R. (1999). Requirements for Efficient Interactions Between CD4 and MHC Class II Molecules for Survival of Resting CD4+ T Lymphocytes In Vivo and for Activation-induced Cell Death. J.Immunol. *162*, 5973-5980.

Marrack, P., Mitchell, T., Bender, J., Hildeman, D., Kedl, R., Teague, K. and Kappler, J. (1998). T-cell Survival. Immunol.Rev. *165*, 279-285.

Masson, D. and Tschopp, J. (1985). Isolation of a Lytic, Pore-forming Protein (Perforin) from Cytolytic T-lymphocytes. J.Biol.Chem. *260*, 9069-9072.

Matechak, E. O., Killen, N., Hedrick, S. M. and Fowlkes, B. J. (1996). MHC Class II-specific T Cells Can Develop in the CD8 Lineage When CD4 Is Absent. Immunity *4*, 337-347.

McHeyzer-Williams, M. G. and Davis, M. M. (1995). Antigen-specific Development of Primary and Memory T Cells in Vivo. Science *268*, 106-111.

Mebius, R. E., Rennert, P. and Weissman, I. L. (1997). Developing Lymph Nodes Collect CD4+CD3-LTβ+ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells. Immunity *7*, 493-504.

Medzhitov, R. and Janeway Jr, C. (2000). Innate Immune Recognition: Mechanisms and Pathways. Immunol.Rev. *173*, 89-97.

Merkenschlager, M. (1996). Tracing Interactions of Thymocytes with Individual Stromal Cell Partners. Eur.J.Immunol. *26*, 892-896.

Merkenschlager, M., Benoist, C. and Mathis, D. (1994). Evidence for a Single-niche Model of Positive Selection. Proc.Natl.Acad.Sci.USA *91*, 11694-11698.

Miller, J. F. A. P. (1962). Immunological Significance of the Thymus of the Adult Mouse. Nature *195*, 1318-1319.

Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M. L. et al. (1992). Mutations in T-cell Antigen Receptor Genes Alpha and Beta Block Thymocyte Development at Different Stages. Nature *360*, 225-231.

Monaghan, P., Robertson, D., Amos, T. A., Dyer, M. J., Mason, D. Y. and Greaves, M. F. (1992). Ultrstructural Localization of Bcl-2 Protein. J.Histochem.Cytochem. *40*, 1819-1825.

Moskophidis, D., Lechner, F., Pircher, H. and Zinkernagel, R. M. (1993). Virus Persistence in Acutely Infected Immunocompetent Mice by Exhaustion of Antiviral Cytotoxic Effector T Cells. Nature *362*, 758-761.

Mosley, R. L., Meryem, M. K. and Miller, R. A. (1998). Idiosyncratic Alterations of TCR Size Distributions Affecting Both CD4 and CD8 T Cell Subsets in Aging Mice. Cell.Immuno. *189*, 10-18.

Mullbacher, A. (1994). The Long-term Maintenace of Cytotoxic T Cell Memory does not Require Persistence of Antigen. J.Exp.Med *179*, 317-321.

Murali-Krishna, K. and Ahmed, R. (2000). Naive T Cells Masquerading as Memory Cells. J.Immunol. *165*, 1733-1737.

Muranski, P., Chmielowski, B. and Ignatowicz, L. (2000). Mature CD4+ T Cells Perceive a Positively Selecting Class II MHC/Peptide Complex in the Periphery. J.Immunol. *164*, 3087-3094.

Nakajima, H. and Henkart, P. A. (1994). Cytotoxic Lymphocyte Granzymes Trigger a Target Cell Internal Disintegration Pathway Leading to Cytolysis and DNA Breakdown. J.Immunol. *152*, 1057-1063.

Nakajima, H., Shores, E. W., Noguchi, M. and Leonard, W. J. (1997). The Common Cytokine Receptor γ Chain Plays an Essential Role in Regulating Lymphoid Homeostasis. J.Exp.Med. *185*, 189-195.

Neefjes, J. J. and Momburg, F. (1993). Cell Biology of Antigen Presentation. Curr. Opin. Immunol. *5*, 27-34.

Nesic, D. and Vukmanovic (1998). MHC Class I Is Required for Peripheral Accumulation of CD8+ Thymic Emigrants. J.Immunol. *160*, 3705-3712.

Ngo, V. N., Tang, H. L. and Cyster, J. G. (1998). Epstein-Barr Virus-induced Molecule 1 Ligand Chemokine Is Expressed by Dendritic Cells in Lymphoid Tissues and Strongly Attracts Naive T Cells and Activated B Cells. J.Exp.Med. *188*, 181-191.

Oehen, S. and Brduscha-Riem, K. (1999). Naive Cytotoxic T Lymphocytes Spontaneously Acquire Effector Function in Lymphocytopenic Recipients: A Pitfall for T Cell Memory Studies? Eur.J.Immunol. *29*, 608-614.

Oehen, S., Waldner, H., Kundig, T. M., Hengartner, H. and Zinkernagel, R. M. (1992). Antivirally Protective Cytotoxic T Cell Memory to Lymphocytic Choriomeningitis Virus Is Governed by Persisting Antigen. J.Exp.Med *176*, 1273-1281.

Oltvai, Z. N. and Korsmeyer, S. J. (1994). Checkpoints of Dueling Dimers Foil Death Wishes. Cell *79*, 189-192.

Oltvai, Z. N., Milliman, C. L. and Korsmeyer, S. J. (1993). Bcl-2 Heterodimerizes In Vivo with a Conserved Homolog, Bax, that Accelerates Programmed Cell Death. Cell *74*, 609-619.

Opferman, J. T., Ober, B. T. and Ashton-Rickardt, P. G. (1999). Linear Differentiation of Cytotoxic Effectors into Memory T Lymphocytes. Science *283*, 1745-1748.

Pachynski, R. K., Wu, S. W., Gunn, M. D. and Erle, D. J. (1998). Secondary Lymphoid-tissue Chemokine (SLC) Stimulates Integrin Alpha 4 Beta 7-mediated Adhesion of Lymphocytes to Mucosal Addressin Cell Adhesion Molecule-1 (MAdCAM-1) Under Flow. J.Immunol. *161*, 952-956.

Padovan, E., Casorati, G., Dellabona, P., Meyer, S., Brockhaus, M. and Lanzavecchia, A. (1993). Expression of Two T Cell Receptor α Chains: Dual Receptor T Cells. Science *262*, 422-424.

Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zoller, M. and Kourilsky, P. (1993). The Sizes of the CDR3 Hypervariable Regions of the Murine T-Cell Receptor β Chains Vary as a Function of the Recombined Germ-Line Segments. Proc.Natl.Acad.Sci.USA *90*, 4319-4323.

Penit, C. and Vasseur, F. (1997). Expansion of Mature Thymocyte Subsets Before Emigration in the Periphery. J.Immunol. *159*, 4848-4856.

Petrie, H. T., Strasser, A., Harris, A. W., Hugo, P. and Shortman, K. (1993). CD4+8- and CD4-8+ Mature Thymocytes Require Different Post-selection Processing for Final Development. J.Immunol. *151*, 1273-1279.

Philpot, K. L., Viney, J. L., Kay, G., Rastan, S., Gardiner, E. M., Chae, S., Hayday, A. C. and Owen, M. J. (1992). Lymphoid Development in Mice Congenitally Lacking T Cell Receptor αβ-expressing Cells. Science *256*, 1448-1452.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. (1998). Defective LPS Signaling in C3H/Hej and C57BL/10ScCr Mice: Mutations in *Tlr4* Gene. Science *282*, 2085-2088.

Posnett, D. N., Sinha, R., Kabak, S. and Russo, C. (1994). Clonal Populations of T Cells in Normal Elderly Humans: The T Cell Equivalent to "Benign Monoclonal Gammapathy". J.Exp.Med. *179*, 609-618.

Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. and Malo, D. (1999). Endotoxin-tolerant Mice Have Mutations in Toll-like Receptor 4 (*Tlr4*). J.Exp.Med. *189*, 615-625.

Rahemtulla, A., Fung-Leung, W. P., Schilham, M. W., Kundig, T. M., Sambhara, S. R., Narendram, A., Arabian, A., Wakeham, A., Paige, C. J., Zinkernagel, R. M., Miller, R. G. and Mak, T. W. (1991). Normal Development and Function of CD8+ Cells but Markedly Decreased Helper Cell Activity in Mice Lacking CD4. Nature *353*, 180-184.

Rammensee, H. G., Falk, K. and Rotzschke, O. (1993). Peptides Naturally Presented by MHC Class I Molecules. Annu.Rev.Immunol. *11*, 213-244.

Ravichandran, K. S. and Burakoff, S. J. (1994). Evidence for Differential Intracellular Signaling via CD4 and CD8 Molecules. J.Exp.Med. *179*, 727-732.

Reimann, J. and Schirmbeck, R. (1999). Alternative Pathways for Processing Exogenous and Endogenous Antigens that Can Generate Peptides for MHC Class I-restricted Presentation. Immunol.Rev. *172*, 131-152.

Reis e Sousa, C. and Germain, R. N. (1999). Analysis of Adjuvant Function by Direct Visualization of Antigen Presentation In Vivo: Endotoxin Promotes Accumulation of Antigen-bearing Dendritic Cells in the T Cell Areas of Lymphoid Tissue. J.Immunol. *162*, 6552-6561.

Ritter, M. A. and Boyd, R. L. (1993). Development in the Thymus: It Takes Two to Tango. Immunol.Today *14*, 462-469.

Roake, J. A., Rao, A. S., Morris, P. J., Larsen, C. P., Hankins, D. F. and Austyn, J. M. (1995). Dendritic Cell Loss from Nonlymphoid Tissues After Systemic Administration of Lipopolysaccharide, Tumor Necrosis Factor, and Interleukin 1. J.Exp.Med. *181*, 2237-2247.

Robey, E. A., Fowlkes, B. J. and Pardoll, D. M. (1990). Molecular Mechanisms for Lineage Commitment in T Cell Development. Semin.Immunol. *2*, 25-34.

Rocha, B., Dautigny, N. and Pereira, P. (1989). Peripheral T Lymphocytes: Expansion Potential and Homeostatic Regulation of Pool Sizes and CD4/CD8 ratios in vivo. Eur.J.Immunol. *19*, 905-911.

Rocha, B., Grandien, A. and Freitas, A. A. (1995). Anergy and Exhaustion Are Independent Mechanisms of Peripheral T Cell Tolerance. J. Exp. Med. *181*, 993-1003.

Rocha, B. and von Boehmer, H. (1990). Peripheral Selection of the T Cell Repertoire. Science *251*, 1225-1228.

Roche, P. A. and Cresswell, P. (1990). Invariant Chain Association with HLA-DR Molecules Inhibits Immunogenic Peptide Binding. Nature *345*, 615-618.

Rolink, A., Karasuyama, H., Grawunder, U., Haasner, D., Kudo, A. and Melchers, F. (1993). B Cell Development in Mice with a Defective λ5 Gene. Eur.J.Immunol. *23*, 1284-1288.

Rooke, R., Waltzinger, C., Benoist, C. and Mathis, D. (1997). Targeted Complementation of MHC Class II Deficiency by Intrathymic Delivery of Recombinant Adenoviruses. Immunity *7*, 123-134.

Rouvier, E., Luciani, M. F. and Golstein, P. (1993). Fas Involvement in Ca(2+)-independent T Cell-mediated Cytotoxicity. J.Exp.Med. *177*, 195-200.

Roux, E., Helg, C., Dumont-Girard, F., Chapuis, B., Jeannet, M. and Roosnek, E. (1996). Analysis of T-cell Repopulation After Allogeneic Bone Marrow Transplantation: Significant Differences Between Recipients of T-cell Depleted and Unmanipulated Grafts. Blood *87*, 3984-3992.

Sakagushi, S. (2000). Regulatory T Cells. Cell *101*, 455-458.

Sallusto, F., Cella, M., Danieli, C. and Lanzavecchia, A. (1995). Dendritic Cells Use Macropinocytosis and the Mannose Receptor to Concentrate Macromolecules in the Major Histocompatibility Complex Class II Compartment: Downregulation by Cytokines and Bacterial Products. J.Exp.Med. *182*, 389-400.

Schluns, K. S., Kieper, W. C., Jameson, S. C. and Lefrancois, L. (2000). Interleukin-7 Mediates the Homeostasis of Naive and Memory CD8 T Cells In Vivo. Nature Immunol. *1*, 426-432.

Schmid, I., Krall, W. J., Uittenbogaart, C., Braun, J. and Giorgi, J. V. (1992). Dead Cell Discrimination With 7-Amino-actinomycin D in Combination With Dual Color Immunofluorescence in Single Laser Flow Cytometry. Cytometry *13*, 204-208.

Schober, S. L., Kuo, C. T., Schluns, K. S., Lefrancois, L., Leiden, J. M. and Jameson, S. C. (1999). Expression of the Transcription Factor Lung Kruppel-like Factor Is Regulated by Cytokines and Correlates with Survival of Memory T Cells In Vitro and In Vivo. J.Immunol. *163*, 3662-3667.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M. and Kirschning, C. J. (1999). Peptidoglycan- and Lypoteichoic Acid-induced Cell Activation Is Mediated by Toll-like Receptor 2. J.Biol.Chem. *274*, 17406-17409.

Schwartz, R. H. (1990). A Cell Culture Model for T Lymphocyte Clonal Anergy. Science *248*, 1349-1356.

Scollay, R. and Godfrey, D. I. (1995). Thymic Emigration: Conveyor Belts or Lucky Dips. Immunol.Today *16*, 268-272.

Scott, D., Addey, C., Ellis, P., James, E., Mitchell, M. J., Saut, N., Jurcevic, S. and Simpson, E. (2000). Dendritic Cells Permit Identification of Genes Encoding MHC Class II-restricted Epitopes of Transplantation Antigens. Immunity *12*, 711-720.

Seddon, B., Legname, G., Tomlinson, P. and Zamoyska, R. (2000). Long-term Survival But Impaired Homeostatic Proliferation of Naive T Cells in the Absence of $p56^{lck}$. Science *290*, 127-131.

Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B. and Korsmeyer, S. J. (1995). Multiple Bcl-2 Family Members Demonstrate Selective Dimerization with Bax. Proc.Natl.Acad.Sci.USA *92*, 7834-7838.

Shahinian, A., Pfeffer, K., Lee, K. P., Kundig, T. M., Kishihara, K., Wakeman, A., Kawai, K., Ohashi, P. S., Thompson, C. B. and Mak, T. W. (1993). Differential T Cell Costimulatory Requirements in CD28-Deficient Mice. Science *261*, 609-612.

Shresta, S., Pham, C. T., Thomas, D. A., Graubert, T. A. and Ley, T. J. (1998). How Do Cytotoxic Lymphocytes Kill Their Targets? Curr.Opin.Immunol. *10*, 581-587.

Simpson, E., Chandler, P., Sponaas, A., Millrain, M. and Dyson, P. J. (1995). T Cells with Dual Antigen Specificity in T Cell Receptor Transgenic Mice Rejecting Allografts. Eur.J.Immunol. *25*, 2813-2817.

Snapper, C. M., Kehry, M. R., Castle, B. E. and Mond, J. J. (1995). Multivalent, but Not Divalent, Antigen Receptor Crosslinkers Synergize with CD40 Ligand for Induction of Ig Synthesis and Class Switching in Normal Murine B Cells. J.Immunol. *154*, 1177-1187.

Soares, M. V. D., Borthwick, N., Maini, M. K., Janossy, G., Salmon, M. and Akbar, A. N. (1998). IL-7-dependent Extrathymic Expansion of CD45RA+ T Cells Enables Preservation of a Naive Repertoire. J.Immunol. *161*, 5909-5917.

Sponaas, A.-M., Tomlinson, P. D., Antoniou, J., Auphan, N., Langlet, C., Malissen, B., Schmitt-Verhulst, A.-M. and Mellor, A. L. (1994). Induction of Tolerance to Self MHC Class I Molecules Expressed under the Control of Milk Protein or β-globin Gene Promoters. Int.Immunol. *6*, 277-287.

Sprent, J. and Basten, A. (1973). Circulating T and B Lymphocytes of the Mouse. II. Lifespan. Cell.Immunol. *7*, 40-59.

Sprent, J., Shaefer, M., Hurd, M., Surh, C. D. and Ron, Y. (1991). Mature Murine B and T Cells Transfered to SCID Mice Can Survive Indefinitely and Many Maintain a Virgin Phenotype. J.Exp.Med. *174*, 717-728.

Sprent, J. and Tough, D. F. (1994). Lymphocyte Life-span and Memory. Science *265*, 1395-1400.

Stein, J. V., Rot, A., Luo, Y., Narasimhaswamy, M., Nakano, H., Gunn, M. D., Matsuzawa, A., Quackenbush, E. J., Dorf, M. E. and von Andrian, U. H. (2000). The CC Chemokine Thymus-derived Chemotactic Agent 4 (TCA-4, Secondary Lymphoid Tissue Chemokine, 6Ckine, Exodus-2) Triggers Lymphocyte Function-associated Antigen 1-mediated Arrest of Rolling T Lymphocytes in Peripheral Lymph Node High Endothelial Venules. J.Exp.Med. *191*, 61-76.

Steinman, R. M. (1991). The Dendritic Cell System and its Role in Immunogenicity. Annu.Rev.Immunol. *9*, 271-296.

Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993). Molecular Cloning and Expression on the Fas Ligand, a Novel Member of the Tumor Necrosis Factor Family. Cell *75*, 1169-1178.

Surh, C. D. and Sprent, J. (2000). Homeostatic T Cell Proliferation: How Far Can T Cells Be Activated to Self-ligands. J.Exp.Med. *192*, 9-14.

Suzuki, H., Punt, J. A., Granger, L. G. and Singer, A. (1995). Asymmetric Signaling Requirements for Thymocyte Commitment to the CD4+ versus CD8+ T Cell Lineages: A New Perspective on Thymic Commitment and Selection. Immunity *2*, 413-425.

Swain, S. L. (1994). Generation and In Vivo Persistence of Polarized Th1 and Th2 Memory Cells. Immunity *1*, 543-552.

Swain, S. L., Bradley, L. M., Croft, M., Tonkonogy, S., Atkins, G., Weinberg, A. D., Duncan, D. D., Hedrick, S. M., Dutton, R. W. and Huston, G. (1991). Helper T-Cell Subsets: Phenotype, Function and the Role of Lymphokines in Regulating their Development. Immunol.Rev. *123*, 115-144.

Swain, S. L., Hu, H. and Huston, G. (1999). Class II-independent Generation of CD4 Memory T Cells from Effectors. Science *286*, 1381-1383.

Takeda, S., Rodewald, H.-R., Arakawa, H., Bluethmann, H. and Shimizu, T. (1996). MHC Class II Molecules are not Required for Survival of Newly Generated CD4+ T Cells, but Affect Their Long-term Life Span. Immunity *5*, 217-228.

Tanchot, C., Lemonnier, F. A., Perarnau, B., Freitas, A. A. and Rocha, B. (1997a). Differential Requirements for Survival and Proliferation of CD8 Naive or Memory T Cells. Science *276*, 2057-2062.

Tanchot, C. and Rocha, B. (1997b). Peripheral Selection of T Cell Repetoires: The Role of Continuous Thymus Output. J.Exp.Med. *186*, 1099-1106.

Tanchot, C. and Rocha, B. (1995). The Peripheral T Cell Repertoire: Independent Homeostatic regulation of Virgin and Activated CD8+ T Cell Pools. Eur. J. Immunol. *25*, 2127-2136.

Tanchot, C., Rosado, M. M., Agenes, F., Freitas, A. A. and Rocha, B. (1997c). Lymphocyte homeostasis. Semmin.Immunol. *9*, 331-337.

Teague, T. K., Marrack, P., Kappler, J. W. and Vella, A. T. (1997). IL-6 Rescues Resting Mouse T Cells From Apoptosis. J.Immunol. *158*, 5791-5796.

ten Boekel, E., Melchers, F. and Rolink, A. (1995). The Status of Ig Loci Rearrangements in Single Cells from Different Stages of B Cell Development. Int.Immunol. *7*, 1013-1019.

Tiegs, S. L., Russell, D. M. and Nemazee, D. (1993). Receptor Editing in Self-reactive Bone Marrow B Cells. J.Exp.Med. *177*, 1009-20.

Tough, D. F., Borrow, P. and Sprent, J. (1996). Induction of Bystander T Cell Proliferation by Viruses and Type I Interferon in Vivo. Science *272*, 1947-1950.

Tough, D. F. and Sprent, J. (1994). Turnover of Naive- and Memory-phenotype T Cells. J.Exp.Med. *179*, 1127-1135.

Townsend, A. R., Rothbard, J., Gotch, F. M., Bahadur, G., Wraith, D. and McMichael, A. J. (1986). The Epitopes of Influenza Nucleoprotein Recognized by Cytotoxic T Lymphocytes Can Be Defined with Short Synthetic Peptides. Cell *44*, 959-968.

van Oers, N. S. C., Killen, N. and Weiss, A. (1994). ZAP-70 Is Contitutively Associated with Tyrosine-phosphorylated TCR ζin Murine Thymocytes and Lymph Node T Cells. Immunity *1*, 575-685.

van Oers, N. S. C., Tao, W., Watts, J. D., Johnson, P., Aerbersold, R. and Teh, H.S. (1993). Constitutive Tyrosine Phosphorylation of the T-cell Receptor (TCR) ζ Subunit: Regulation of TCR-sssociated Protein Tyrosine Kinase Activity by TCR ζ. Mol.Cell.Biol. *13*, 5771-5780.

Vassar, R., Rosenberg, M., Ross, S., Tyner, A. and Fuchs, E. (1989). Tissue-specific and Differentiation-specific Expression of a Human K14 Keratin Gene in Transgenic Mice. Proc.Natl.Acad.Sci.USA *86*, 1563-1567.

Veis, D. J., Sorenson, C. M., Shutter, J. R. and Korsmeyer, S. J. (1993). Bcl-2-deficient Mice Demonstrate Fulminant Lymphoid Apoptosis, Polycystic Kidneys, and Hypopigmented Hair. Cell *75*, 229-240.

Vella, A., Teague, T. K., Ihle, J., Kappler, J. and Marrack, P. (1997). Interleukin 4 (IL-4) or IL-7 Prevents the Death of Resting T Cells: Stat6 is Probably not Required for the Effect of IL-4. J.Exp.Med. *186*, 325-330.

Viret, C., Wong, F. S. and Janeway, C. A. (1999). Designing and Maintaining the Mature TCR Repertoire: The Continuum of Self-Peptide: Self-MHC Complex Recognition. Immunity *10*, 559-568.

Volkmann, A., Zal, T. and Stockinger, B. (1997). Antigen-presenting Cells in the Thymus That Can Negatively Select MHC Class II-restricted T Cells Recognizing a Circulating Self Antigen. J.Immunol. *158*, 693-706.

von Boehmer, H. (1986). The Selection of the α,β Heterodimeric T-cell Receptor for Antigen. Immunol.Today *7*, 333-336.

von Boehmer, H and Hafen, K. (1993). The Life Span of Naive α/β T Cells in Secondary Lymphoid Organs. J.Exp.Med. *177*, 891-896.

Warnock, R. A., Campbell, J. J., Dorf, M. E., Matsuzawa, A., McEvoy, L. M. and Butcher, E. C. (2000). The Role of Chemokines in the Microenvironmental Control of T Versus B Cell Arrest in Peyer's Patch High Endothelial Venules. J.Exp.Med. *191*, 77-88.

Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H. and Mak, T. W. (1995). Lymphoproliferative Disorders with Early Lethality in Mice Deficient in CTLA-4. Science *270*, 985-988.

Webb, S., Morris, C. and Sprent, J. (1990). Extrathymic Tolerance of Mature T Cells: Clonal Elimination as a Consequence of Immunity. Cell *63*, 1249-1256.

Wiest, D. L., Yuan, L., Jefferson, J., Benveniste, P., Tsokos, M., Klausner, R. D., Glimcher, L. H., Samelson, L. E. and Singer, A. (1993). Regulation of T Cell Receptor Expression in Immature CD4+CD8+ Thymocytes by $p56^{lck}$ Tyrosine Kinase: Basis for Differential Signaling by CD4 and CD8 in Immature Thymocytes Expressing Both Coreceptor Molecules. J.Exp.Med *178*, 1701-1712.

Williams, M. J., Rodriguez, A., Kimbrell, D. A. and Eldon, E. D. (1997). The *18-wheeler* Mutation Reveals Complex Antibacterial Gene Regulation in *Drosophila* Host Defence. EMBO J. *16*, 6120-6130.

Witherden, D., van Oers, N., Waltzinger, C., Weiss, A., Benoist, A. and Mathis, D. (2000). Tetracycline-controllable Selection of CD4+ T Cells: Half-Life and Survival Signals in the Absence of Major Histocompatibility Complex Class II Molecules. J.Exp.Med *191*, 355-364.

Wurbel, M. A., Philippe, J. M., Nguyen, C., Victorero, G., Freeman, T., Wooding, P., Miazek, A., Mattei, M. G., Malissen, M., Jordan, B. R., Malissen, B., Carrier, A. and Naquet, P. (2000). The Chemokine TECK is Expressed by Thymic and Intestinal Epithelial Cells and Attracts Double- and Single-positive Thymocytes Expressing the TECK Receptor CCR9. Eur.J.Immunol. *30*, 262-271.

Yoshimura, A., Lien, E., Ingalls, R. R., Tuomanen, E., Dziarski, R. and Golenbock, D. (1999). Recognition of Gram-positive Bacterial Cell Wall Components by the Innate Immune System Occurs Via Toll-like Receptor 2. J.Immunol. *163*, 1-5.

Zabel, B. A., Agace, W. W., Campbell, J. J., Heath, H. M., Parent, D., Roberts, A. I., Ebert, E. C., Kassam, N., Qin, S., Zovko, M., LaRosa, G. J., Yang, L. L., Soler, D., Butcher, E. C., Ponath, P. D., Parker, C. M. and Andrew, D. P. (1999). Human G Protein-coupled Receptor GPR-9-6/CC Chemokine Receptor 9 Is Selectively Expressed on Intestinal Homing T Lymphocytes, Mucosal Lymphocytes, and Thymocytes and Is Required for Thymus-expressed Chemokine-mediated Chemotaxis. J.Exp.Med. *190*, 1241-1256.

Zal, T., Volkmann, A. and Stockinger, B. (1994). Mechanisms of Tolerance Induction in Major Histocompatibility Complex Class II-restricted T Cells Specific for a Blood-borne Self-Antigen. J.Exp.Med. *180*, 2089-2099.

Zatz, M. M. and Lance, E. M. (1970). The distribution of Chromium 51-labelled Lymphoid Cells in the Mouse. Cell.Immunol. *1*, 3-17.

Zelenika, D., Adams, E., Mellor, A., Simpson, E., Chandler, P., Stockinger, B., Waldmann, H. and Cobbold, S. P. (1998). Rejection of H-Y Disparate Skin Grafts by Monospecific CD4+ Th1 and Th2 Cells: No Requirement for CD8+ T Cells or B Cells. J.Immunol. *161*, 1868-1874.

Zhang, X., Sun, S., Hwang, I., Tough, D. F. and Sprent, J. (1998). Potent and Selective Stimulation of Memory-phenotype CD8+ T Cells In Vivo by IL-15. Immunity *8*, 591-599.

Zijlstra, M., Bix, M., Simister, N. E., Loring, J. M., Raulet, D. H. and Jaenisch, P. (1990). β2-microglobulin Deficient Mice Lack CD4-CD8+ Cytolytic T Cells. Nature *344*, 742-746.