PHYSIOLOGY OF ANERGIC CD8 T CELLS IN
A TRANSGENIC MOUSE MODEL

Submitted by

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ABSTRACT

Within the T cell system, self-tolerance is mainly maintained by thymic clonal deletion of self-reactive T cells through the mechanism of negative selection. However some self-reactive T cells escape negative selection and reach the lymphoid periphery where other mechanisms maintain tolerance. To study the peripheral tolerance mechanisms we are using a mouse experimental model of peripheral non-deletional tolerance. In this double transgenic mouse model the antigen, an influenza nucleoprotein peptide is widely expressed under the MHC I H-2K\textsuperscript{b} promoter and all CD8 T cells carry a transgenic TCR specific for the influenza peptide presented by MHC H-2D\textsuperscript{b}. Thymic selection of these T cells proceeds normally without deletion, but peripheral tolerance is maintained. The NP-specific T cells are functionally compromised in vitro to antigenic stimulation and are characterised by a CD44\textsubscript{high} activated phenotype. However these cells, upon transfer to antigen free lymphopenic host can be rescued from the anergic state, undergo several homeostatic divisions and show a long life span. Further more investigation on the functional capacities of the NP47F5 CD8 T cells showed that they could have cytotoxic capacity. Therefore, although they appear to be functionally compromised in vitro, they can react in vivo. These functional characteristics indicate that these cells are not totally anergic, as they can have effector functions. These functional characteristics also indicate that these cells could be auto reactive in vivo, and it could be that the site of Ag expression (Bone Marrow derived cells) that accounts for the lack of measurable tissue damage. The long life span and the functional characteristics may also indicate that they could play a functional role in the maintenance of peripheral tolerance.
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<table>
<thead>
<tr>
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<th>Definition</th>
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<tbody>
<tr>
<td>mAb(s)</td>
<td>Monoclonal Antibody(ies)</td>
</tr>
<tr>
<td>AIDC</td>
<td>Antigen induced cell death</td>
</tr>
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<td>AINR</td>
<td>Antigen induced nonresponsiveness</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell(s)</td>
</tr>
<tr>
<td>BIO</td>
<td>Biotinylated</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxy-Fluorescein-diacetate-Succinimidyl-Ester</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptors</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cells</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DN</td>
<td>Double Negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fas-L</td>
<td>Fas Ligand</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HEL</td>
<td>Hen egg lysozyme</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial vessels</td>
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<tr>
<td>HSA</td>
<td>Heat Stable Antigen</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFNs</td>
<td>Interferons</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Gamma Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
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<td>ABBREVIATIONS</td>
<td>Definition</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motive</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition motive</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cells Ig like receptors</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LCR</td>
<td>Locus Control Region</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic antibody cell sorter</td>
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<tr>
<td>MBP</td>
<td>Myelin-based-protein</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophages</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic mice</td>
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<tr>
<td>NP</td>
<td>Nucleoprotein peptide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pCTL</td>
<td>CTL precursors</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PDBU</td>
<td>Phorbol12.13-dibutyrate</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrine</td>
</tr>
<tr>
<td>PEV</td>
<td>Position effect variegation</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming units</td>
</tr>
<tr>
<td>PTKs</td>
<td>Protein tyrosine kinases</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombinase activating genes</td>
</tr>
<tr>
<td>RIP</td>
<td>Rat insulin promoter</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidine</td>
</tr>
<tr>
<td>SEA</td>
<td>Staphylococcal enterotoxin A</td>
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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>SPL</td>
<td>Spleen</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Tumour growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cells</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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INTRODUCTION

1. INTRODUCTION

1.1 Function of the immune system.

The immune system has evolved to protect the integrity of the organism against infections. In addition to antigen non-specific innate immune mechanisms, specific recognition events by receptors on T and B cells lead to the elimination of pathogens. The antigen specific receptors are generated by random rearrangements of non-continuous DNA fragments, creating a high diversity of antigenic specificities for non-self as well for self-antigens. One of the principal challenges of the immune system is to maintain tolerance to self-antigens. Physical elimination of self-reactive T cells during their maturation in the thymus is the main and best-characterised mechanism for the induction of self-tolerance. However not all self-antigens are expressed in the thymus and there is a variety of deletional and non-deletional tolerance mechanisms in the periphery. A detailed knowledge of tolerance induction in mature peripheral T cells is essential for the design of therapeutic approaches of autoimmune diseases and for tolerance induction in organ transplantation or in order to break tolerance to tumour antigens.

1.1.1 Components of the immune system.

Host defence depends on a concerted action of both innate and adaptive immunity. Innate immunity offers a rapid first line of defence against pathogens, based on a system of cell populations that express Ag-non-specific receptors that are relatively invariant. Key features of the mammalian innate immune system include a) the ability to recognise pathogen and/or tissue injury and b) the ability to signal the presence of danger to cells of the adaptive immune system. The innate immune system includes phagocytic cells, natural killer cells, complement and interferons (IFNs). Cells of the innate immune system use a variety of pattern recognition receptors to recognise pathogens, for instance bacterial lipopolysaccharide (LPS), carbohydrates and double-stranded viral DNA. Haematopoiesis occurs initially in foetal blood islands, liver and spleen, and is gradually taken over by the bone marrow of the flat bones. All the blood cells and cells of the immune system, originate from a common stem cell that becomes committed to differentiate along particular lineages. The myeloid pathway of development gives rise to erythrocytes, macrophages, dendritic
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cells and granulocytes such as neutrophils, basophils and eosinophils. The lymphoid pathway of development gives rise to B and T lymphocytes and to natural killer cells.

B and T cells of the adaptive immune system have the unique ability to rearrange genes of immunoglobulin family, permitting the generation of large diversity of Ag-specific clones. However this highly sophisticated system needs to be instructed and regulated by cells of the innate immune system. Cells of the innate immune system are particularly important in the initiation of an immune response of the adaptive immune system, acting as antigen presenting cells and influencing the adaptive immune response by the secretion of cytokines.

Macrophages differentiate from bone marrow derived monocytes, and are present in lymphoid and non-lymphoid organs. Their main function is to phagocytose opsonised particles such as microbes, macromolecules, and apoptotic cells. Macrophage recognition of foreign substances and injured tissue involve receptors for phospholipids, and sugars such as mannose and fucose (Sallusto et al., 1995). They also express Fc receptors and CR1, CR3 complement receptors and therefore can phagocytose opsonised particles. Apart from their role in humoral immunity macrophages can play a role in the recognition, activation and effector phase of specific immunity, as they can function as antigen presenting cells (APC) and produce cytokines like IL-12, IL-1, IL-6 and TNF that can stimulate T cell activation. Unstimulated macrophages cannot act as APC and activation is required for macrophages to express sufficient levels of MHC II and B7 molecules, which are required for naïve T cell activation.

Dendritic cells (DCs) are professional APC that have a unique capacity to stimulate primary immune responses, thus permitting establishment of immunological memory. Development of DC is initiated by bone marrow progenitors. At least two distinct pathways of DC development have been identified in mice, myeloid and lymphoid. Myeloid progenitors under the influence of Granulocyte-Macrophage-Colony-Stimulating Factor (GM-CSF) give rise to granulocytes and DCs. However purified lymphoid progenitors after transfer into irradiated hosts can also give rise to DCs. Both lymphoid and myeloid DCs express high levels of CD11-c, MHC II and co-stimulatory molecules (CD86, CD40) and up to date the most reliable marker to distinguish the two subsets is CD8α which is expressed as a homodimer on the lymphoid DCs. DC precursors are patrolling through blood and lymphatics as well as lymphoid tissues and upon pathogen recognition, release
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large amounts of cytokines, e.g. IFN-α, thereby limiting the spread of the infection. Immature resident DCs are present in peripheral tissues and in the apparent absence of maturation stimuli constitutively migrate at low rate to draining lymph nodes. These spontaneously migrating DCs do not induce effector responses possibly due to low levels of expression of co-stimulatory molecules or due to the production of inhibitory cytokines like IL-10 (Banchereau et al., 2000; Lanzavecchia and Sallusto, 2001).

DC maturation is recognised as a key event in the induction of the immune response. Work on human monocyte-derived DCs revealed that DCs mature in response to LPS, TNF-α or CD40-L, which represent pathogens, endogenous inflammatory signals or T cell feedback signals respectively. Recently more stimuli and receptors that trigger and modulate DC maturation process have been described. Toll-like receptors (TLRs) represent a family of receptors specific for pathogen-associated molecular patterns that are phylogenetically conserved conferring responses to LPS (TLR 4), lipoproteins and peptidoglycans (TLR 2), or to non-methylated DNA (TLR 9). Besides receptors for inflammatory cytokines such as TNF-α, type-I IFNs and IL-1 DC also express CD91, which bind to heat shock proteins that are released by necrotic cells (Lanzavecchia and Sallusto, 2001). Although the way that DC can integrate different stimuli to induce different T cell responses is not clear yet, it is thought to be controlled by the regulation of IL-12 production (Liu et al., 2001).

Different lineages of lymphocytes capable of antigen specific recognition, engage in different effector functions like secretion of antigen specific antibodies by B cells, destruction of virus infected cells by cytotoxic T cells (CTL), or secretion of lymphokines that support antibody production by B cells and expansion of cytotoxic T cells by helper T cells. T and B cells recognise antigens in different ways. The B cell receptor, recognises a three dimensional conformation-dependent shape, whether in solution or on a cell surface, resulting in humoral immunity by the secretion of antibodies. The T cell receptor (TCR) molecules provide each T cell with the specificity for an antigenic peptide fragment non-covalently complexed with major histocompatibility complex (MHC) molecules. CD4 and CD8 co-receptors in the surface of T helper and cytotoxic T cells respectively, assist the binding of the TCR to MHC-peptide ligand. CD8 T cells recognise primarily peptides processed from an endogenous source bound to MHC class I molecules whereas CD4 T cells recognise peptides processed mainly from an exogenous source bound to MHC class
INTRODUCTION

II molecules. Activation of T cells results in cell mediated immunity. Another type of cells of the lymphoid lineage are natural killer cells (NK) that although they do not express any of the T or B cell markers, are capable of lysing a variety of virus infected and tumour cells without overt antigenic stimulation.

The important functions of NK cells place them at the interphase between the innate and adaptive immune systems. NK cells respond rapidly to interferon IFN-α/β and to IL-12 during infections by viruses and other intracellular pathogens. Activated NK cells produce lymphokines such as IFN-γ and TNF-α that regulate immune responses. In addition NK cells kill certain tumour and virally infected cells. The cytolytic activity and lymphokine production by NK cells are under tight regulation by inhibitory receptors that are specific for MHC I antigens. NK cells kill haematopoietic target cells unless they receive an inhibitory signal from an MHC-I specific receptor. NK cells express two families of MHC I reactive inhibitory receptors: a) immunoglobulin-like killer cells Ig like receptors (KIR) that directly interact with various MHC class I molecules and b) C-type lectin-like receptors (Ly49, CD94/NKG2) which interact with peptides derived from MHC-I molecules (Long et al., 1997). These receptors inhibit NK activation in trans through an immunoreceptor tyrosine based inhibition motif. Most of these receptors contain one or more tyrosine residues in their cytoplasmic tail that upon phosphorylation they bind to SH2 domains of tyrosine phosphatases and recruit them to counteract the kinase activity of enzymes required for NK cell activation. Expression of inhibitory receptors has been reported on human or mouse memory phenotype CD8 T cells and has been suggested that inhibitory receptors may regulate CTL responses and serve as a mechanism to control the activity of self-cross-reactive T cells (Huard and Karlsson, 2000; McMahon and Raulet, 2001; Ugolini et al., 2001).

1.2 T cell development and selection.

1.2.1 Early T cell development in the thymus.

T cells are generated in the thymus from haematopoietic –multipotent progenitor stem cells that migrate there from the bone marrow. In the thymus microenvironment progenitor cells undergo the processes of TCR rearrangement, maturation, differentiation and
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selection. Developing T cells migrate from cortex to medulla and their maturation stage can be identified on the basis of the expression of CD4 and CD8 accessory molecules.

The most immature thymocytes are the Double Negative (DN) (2-5% of all the thymocytes) population lacking expression of CD4 CD8 and TCR-CD3 molecules. This population passes through a series of differentiation stages defined by the expression of CD44 (phagocyte glycoprotein-1), CD25 (IL-2 receptor γ chain) and c-kit (Stem cell factor receptor) cell surface markers. The most immature thymocytes are c-kit⁺, CD44⁺, CD25⁺. Surface expression of CD25 molecule defines the next developmental stage c-kit⁺, CD44⁺, CD25⁺. This stage is followed by downregulation of the CD44 surface marker, giving rise to a c-kit low, CD44⁺, CD25⁺ population. At the DN stage T cells undergo rearrangement of the TCR β chain locus and express TCR-β chain as a heterodimer with an invariant pre-Tα chain, in association with CD3 molecules. The pre-T cell stage is an important signalling checkpoint during thymus development as only the functionally TCR-β expressing cells are selected for further expansion and maturation.

This stage ultimately leads to the co-expression of CD4 and CD8 molecules that define the double positive (DP) maturation stage of T cells (80-90% of thymocytes) which then undergo TCR-α chain gene rearrangements. Rearrangements in the V and J segments of the TCR-α locus allow DP thymocytes to express αβ TCR on their surfaces. The specificity of the expressed αβ TCR then determines whether the cells will undergo apoptosis (negative selection) or commit to differentiate to either CD4 or CD8 mature single positive (SP) T cell lineages (positive selection) (Fehling and von Boehmer, 1997; Killeen et al., 1998).
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Figure 1: Schematic illustration of T cell development

This figure shows discrete developmental stages in thymus development. At the double negative stage there is a developmental checkpoint, which allows only those, cells that have a productive TCR β rearrangement and can express functional pre-TCR complex to progress, a process known as pre-TCR selection. At the double positive stage another important developmental checkpoint of positive and negative selection operates on TCR expressing thymocytes. At single positive stage cells have committed to CD8 or CD4 T cell lineages.
Figure 1: T cell development in the thymus
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1.2.2 Positive selection in the thymus.

Because of the random nature of the rearrangement process that creates a vast TCR diversity, there is a need for selection events to operate in thymocytes to allow survival of only those thymocytes that express self-MHC restricted TCRs. This process, which is called positive selection (von Boehmer, 1994), operates already from the DN stage (pre-T cell selection) and mainly at the DP stage of development. The result of this process is the selection for survival of thymocytes that express TCRs able to bind to the polymorphic part of a set of MHC molecules that are present in a given individual (Bevan, 1997; Jameson and Bevan, 1998). DP cells have a very limited life span (3.5 days). Cells that express TCRs that do not recognise MHC ligands are eliminated by programmed cell death, (a process that is also called “death by neglect”).

Although the maturation step at which positive selection occurs is the “point” at which T cells express αβ TCR (CD4+, CD8+, TCRlow), positive selection is a multistep phenomenon. The first step involves maturation of TCR low subset to a DP TCR high subpopulation following interaction with the thymus environment. DP TCR high cells are not found in TCR transgenic animals of non-selecting haplotypes whereas DP TCR low precursors are (Ohashi et al., 1990).

The major consequence of positive selection is the alteration of the life span of a cell. Binding of the αβ-TCR to MHC is essential for rescuing relatively short-lived DP thymocytes from cell death. Also the specificity of TCR determines further differentiation of thymocytes to CD4 or CD8 lineage (Kisielow et al., 1988b; Scott et al., 1989; Teh et al., 1988; von Boehmer, 1994).

As a consequence of TCR-initiated signals a number of changes occur. One of these changes is termination of expression of recombinase activating genes (RAG). It is shown in TCR Tg mice that early surface expression of an αβ TCR by DP thymocytes did not interfere with TCR α rearrangement in these cells unless the αβ TCR bound to intrathymic MHC ligands, resulting in an immediate shut off of the RAG genes. (Borgulya et al., 1992). Another change that occurs following αβ TCR initiated signals is upregulation of surface expression of activation markers like CD69, indicative of the fact that positive selection is an active process that induces signalling pathways in thymocytes (Bendelac et al., 1992).
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The nature of the MHC ligand that induces positive selection is under investigation. Positive selection, unlike negative selection of immature T cells, is independent of the specific peptide required for activation of mature T cells with the same TCR. For instance positive selection of a TCR specific for a male specific peptide is observed in female mice (Kisielow et al., 1988a). Multiple peptides were found to have the ability to select single TCR (Hogquist et al., 1997; Hu et al., 1997) and multiple different TCRs can be selected in mice in which a single peptide/MHC complex predominates (Amsen and Kruisbeek, 1998; Ignatowicz et al., 1996; Surh and Sprent, 1994). Probably the requirement for specific binding of a TCR to a given peptide will be less stringent for positive selection than activation of mature T cells or negative selection (von Boehmer, 1994). This is also supported by the fact that during thymocyte maturation there is loss of TCR sensitivity in response to weak agonist as a result of changes in the biochemistry of TCR signalling (Lucas et al., 1999).

1.2.3 Lineage commitment.

At subsequent steps of positive selection, one of the co-receptors is fully downregulated and cells migrate from the thymic cortex to the medulla. In the medulla, where the cells are already SP, further functional maturation occurs. These steps can be taken without an apparent need for cell division (Huesmann et al., 1991). Thus a consequence of positive selection is the determination of whether the cell matures to CD4 SP, which is MHC II, restricted or matures to CD8 SP T cell, which is MHC I restricted. The specificity of the $\alpha \beta$ TCR for either MHC class I or MHC class II determines whether rescued cells would be of the CD8 cytotoxic or CD4 helper T cell precursor phenotype respectively.

The mechanism that regulates this process, which is called CD4-CD8 lineage commitment, is yet unclear. At the moment two major models have been described to explain lineage commitment. The instructive model (von Boehmer, 1996) postulated that interaction of DP cells with MHC II resulted in downregulation of CD8 molecule, to produce CD4 SP cells, whereas interaction with MHC I produced CD8 cells. In the stochastic/selective model, lineage commitment occurs stochastically by downregulation of either CD4 or CD8 co-receptors and a cell with “fitting” TCR and TCR co-receptor
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combination that can engage at the same time MHC molecules would be selected for survival, whereas cells that take the "wrong" decision would quickly disappear (Robey et al., 1991).

Apart from the MHC restriction of the TCR there may be other factors that could influence CD4-CD8 lineage commitment. According to this view a quantitative model has also been proposed to explain CD4-CD8 lineage commitment (Itano et al., 1996) suggesting that strong signals, through p56 lck for example (Hernandez-Hoyos et al., 2000) may favour CD4 lineage choice, whereas CD8 lineage choice may be mediated by weaker signals (Basson et al., 1998; Volkmann et al., 1998).

In addition to appropriate interactions between the TCR and MHC molecules, other instructive signals are believed to be critical for lineage commitment to CD4 or CD8 cells. One such candidate-signalling molecule is Notch-1. Notch proteins are large transmembrane receptors that play crucial roles in cell-fate decisions in many developmental systems. The cytoplasmic domain of Notch proteins harbour six ankirin repeats and is involved in intracellular signalling. Notch-1, -2, -3 and the ligands jagged-1 and -2 are expressed in thymocytes and in thymic stromal epithelium (Felli et al., 1999; Rebay et al., 1991). Several lines of evidence are consistent with the hypothesis that Notch-1 signalling is involved in CD4-CD8 lineage commitment. Transgenic expression of constitutively active cytoplasmic domain of Notch-1 in immature thymocytes leads to increased numbers of either CD8 SP cells (Robey et al., 1996), or both CD4 and CD8 SP thymocytes (Deftos et al., 1998). Expression of antisense Notch-1 retroviral vector in reaggregated foetal thymus organ cultures selectively blocked the maturation of CD8 SP cells (Yasutomo et al., 2000). However although Notch-1 signalling has been shown to be required for embryonic development, T-B and αβ-γδ T cell lineage commitment its role in CD4-CD8 lineage commitment is unclear. In a recent study tissue-specific inactivation of gene encoding Notch-1 in immature T cell precursors did not affect subsequent thymocyte development, excluding an essential role of Notch-1 in CD4-CD8 lineage commitment.

Once thymocytes have reached the fully mature, HSA low, single positive stage, they respond to anti-TCR stimulation in a manner analogous to peripheral T cells (Barthlott et al., 1997; Ramsdell et al., 1989; Volkmann et al., 1997). When fully matured SP thymocytes have completed their maturation, selection and differentiation processes, they
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migrate to the peripheral lymphocyte pool and recirculate through secondary lymphoid organs.

1.2.4 Negative selection in the thymus.

In a highly diverse T cell repertoire of approximately 25 million specificities (Arstila et al., 1999) generated by random gene rearrangements, there is high possibility to generate TCRs with specificity not only for foreign but for self-antigen as well. Therefore there is need for a control process to eradicate T cells with potentially autoreactive receptors, a process called negative selection. Negative selection is the main tolerance mechanism that ensures that T cells that express potentially autoreactive receptors die in the thymus by apoptosis (Kappler et al., 1987; Kisielow et al., 1988a).

During thymus development the majority (>95%) of the DP thymocytes die due to either death by neglect or by negative selection, and only a small fraction of cells (< 5%) are positively selected for further maturation into functionally mature cells. The processes of positive and negative selection present a paradox of how interaction of TCR with MHC peptide ligands presented by thymic stromal cells can have diametrically opposed outcomes, in positive selection or in negative selection.

The current consensus is that the fate of a developing thymocyte is determined by the overall avidity of their TCR and its ligand MHC/self-peptide. Thymocytes with TCRs that cannot bind with sufficient avidity will undergo apoptosis and the majority of the cell death in the thymus may in fact be a consequence of lack of positive selection signal. In contrast TCR ligation with low to intermediate avidity (low number of high affinity peptide/MHC complexes or high-density of low affinity interactions) result in signals sufficient to rescue from programmed cell death and positive selection occurs. However high avidity TCR ligation results in negative selection and cells die by apoptosis. It is through this combined process of positive and negative selection that the fate of each T cell is determined and the repertoire of T cells that will populate the peripheral lymphoid organs is generated.
Probability of selection

Avidity for MHC-peptide complex

Avidity | Low | Intermediate | High
--- | --- | --- | ---
Cell fate | Cell death | Survival | Cell death
Process | Neglect | Positive Selection | Negative Selection

Figure 2: **Avidity model of thymus selection.**

According to the avidity model of thymus selection, the avidity of the interactions between TCRs on developing thymocytes and self-MHC complexes on thymic stromal cell, determine the fate of the thymocytes. Thymocytes with TCRs that can not bind with sufficient avidity will undergo apoptosis. TCR ligation with intermediate avidity provide signals that rescue cells from apoptosis. High avidity TCR ligation results in negative selection and cell death by apoptosis.
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Direct demonstration of clonal deletion of autoreactive cells in the thymus came from studies of the T cell repertoire to endogenous super antigens (Kappler et al., 1987; MacDonald et al., 1988). Using antibody (Ab) specific for Vβ17α it was showed that T cells bearing Vβ segments that conferred binding to endogenous mouse mammary tumour viruses (Mtv) were absent from mice carrying these superantigens (sAg) in their genome.

Negative selection was directly verified in a T-cell receptor transgenic mouse strain (Kisielow et al., 1988a) bearing TCRs specific for an epitope on the male Ag H-Y. In female transgenic mice the majority of CD8 T cells expressed the transgenic TCR however in male mice massive deletion of DP thymocytes expressing the transgenic TCR was observed in the thymic cortex.

Since the TCR-ligand threshold determines whether deletion or selection occurs, all parameters that determine avidity are important for the outcome of selection. The parameters that determine avidity are the affinity of TCR-ligand interaction and the ligand density. Therefore the avidity of TCR-ligand interaction will reflect TCR affinity for MHC-peptide ligand, TCR density, density of co-receptors and density of MHC-peptide on the presenting thymic stromal cell. It is likely that the TCR is a particularly versatile receptor that can signal very different cellular responses when confronted with only small differences in affinity/avidity.

Various transgenic models illustrated that TCR/MHC peptide interactions that exceed a certain threshold of avidity result in deletion. In one of them over-expression of CD8 co-receptor analogous to an increase in TCR affinity was shown to change positive selection to negative selection (Lee et al., 1992; Robey et al., 1991). Also expression of different levels of Vβ11 transgene resulted in different degrees of deletion in the presence of H-2E allele (Homer et al., 1993), supporting the notion that there is a quantitative signal threshold which is critical for tolerance induction. In support of the avidity model of selection is the work of Auphan et al (Auphan et al., 1992) that showed that the density of H-2Kb alloantigen influenced the deletion pattern of thymocytes with class I alloreactive receptor. Recent studies of the TCR affinity using biosensors to study kinetics of TCR ligand interactions demonstrate that the “window” of avidity resulting in positive selection is much more narrow than that of negative selection (Alam et al., 1996).

Several other receptor-ligand interaction systems affect the signals delivered by TCR and thus will affect the outcome of selection. Such receptors are the CD4 and CD8 co-
receptors that stabilise the interaction of the TCR with MHC and bring Lck to the TCR. An important role of these co-receptors is to increase the efficacy of TCR signalling in selection (Lee et al., 1992; Robey et al., 1991).

Adhesion molecules like Lymphocyte function associated antigen-1 (LFA-1) can modulate TCR signalling during thymic selection indirectly through promoting contact with APC. Indeed, antibodies to intracellular adhesion molecule (ICAM) and (LFA-1) (Carlow et al., 1992) can block negative selection by dendritic cells. However positive selection is not dependent on ICAM (Sligh et al., 1993). Co-stimulatory molecules are very important in the activation of mature T cells, as they provide indispensable accessory signals distinct from those generated by the TCR. As these molecules can modulate mature T cell activation, it is most likely to influence thymic selection. The best-defined co-stimulatory interactions, those between T cell specific receptor CD28 and B7.1 and B7.2 ligands expressed on various APC, do not seem obligatory for thymus negative selection. Also the contribution of B7 molecules to negative selection is ambiguous (Amsen and Kruisbeek, 1998; Jones et al., 1993; Shahinian et al., 1993). A possible explanation is that there is redundancy in receptors that are involved in co-stimulation of thymocytes, and other ligand-receptor interactions like CD40-CD40L (Foy et al., 1995) are also involved in thymus selection.

Although the exact sensitivity for negative selection within the thymus is not known, it is quite clear that negative selection is a very sensitive procedure. One evidence comes from (Adelstein et al., 1991) where a large number of founder lines for antigen Hen egg lysozyme (HEL) were created, each expressing different amounts of HEL Ag. It was found that serum levels less than $10^{-10}$ M produced tolerance within the T cell population, whereas approximately $10^{-8}$ M were required for B cell tolerance. A second example for the high sensitivity of negative selection came from mice transgenic for H-2K\textsuperscript{b} gene under the control of Rat insulin promoter (RIP) (Heath et al., 1992). This promoter directed Ag expression mainly in insulin secreting cells of pancreas. However low levels of Ag expression were detected in the thymus at m-RNA level. The presence of such low levels of Ag expression in the thymus was sufficient to induce negative selection of those thymocytes with high level of anti-H-2K\textsuperscript{b} TCR expression. Also it has been shown that deletion of antigen-specific thymocytes requires a lower threshold of antigen stimulation than activation of mature T cells (Pircher et al., 1993; Vasquez et al., 1994).
1.2.5 What is the nature of thymic APC involved in positive or negative selection?

Although there is clearly an important role for the affinity/occupancy of TCR engagement in thymocyte selection, developing T cells are affected by other ligands than just peptide MHC complexes like co-stimulatory signals that “fine-tune” the TCR response and affect the overall avidity. As different types of APC express different co-stimulatory molecules the fate of developing thymocytes is also dictated by the nature of APC they interact with.

For positive selection to occur, thymocytes have to have contact with the selecting MHC on cortical epithelial cells. Expression on medullary epithelial cells or cells of haematopoietic origin fails to support significant positive selection (Brocker et al., 1997; Cosgrove et al., 1992).

With regard to cell types that induce negative selection it is clear that dendritic cells serve as APC for deletion (Brocker et al., 1997; Marrack et al., 1993) but the capacity of thymic epithelium to induce deletion has been controversial. Medullary epithelial cells have been shown to induce deletion for endogenous super-antigens in mice selectively expressing MHC II-IE molecules only on these cells (Burkly et al., 1993). It has also been shown that medullary thymic epithelial cells can utilise a MHC II endogenous pathway to induce tolerance to β-galactosidase nuclear protein, to the CD4 compartment (Oukka et al., 1996). The finding that medullary epithelial is a unique cell type that express a diverse range of tissue specific antigens enhances their role in thymus selection (Derbinski et al., 2001). Although some reports have shown cortical epithelium to play a role in deletion (Laufer et al., 1996), others report at least partial effects such as anergy induction (Ramsdell et al., 1989; Roberts et al., 1990). In one TCR transgenic system both cortical and medullary epithelium were as efficient as dendritic cells (DC) in negative selection (Volkmann et al., 1997). Therefore thymic epithelial cells from cortex or medulla can be involved in negative selection, but may not be equally efficient to thymic DC in low avidity TCR/MHC-peptide interactions.
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1.2.6 Involvement of Fas and TNF surface molecules in negative selection.

Both Fas and TNF play an important role in peripheral T cell deletion following activation, a process which is also called antigen induced cell death (AICD) and is an homeostatic mechanism necessary for the termination of the immune responses. However the role of Fas and TNF in negative selection is controversial. In most studies, negative selection occurs in the absence of Fas and Fas-ligand. Additionally no effects on deletion were observed from deficiency for CD95 (Fas). Also blocking of TNF did not interfere with negative selection either (Amsen and Kruisbeek, 1998). Therefore although some types of accessory molecules may act in negative selection, the role of death receptors is minimal.

1.2.7 Escape from negative selection.

Although negative selection is the main tolerance mechanism, there is ample evidence that self-reactive T cells can escape deletion and reach the periphery. In a study using transgenic mice for the TCR β chain of a CTL clone directed against male-specific peptide, comparison of the pre-immune repertoire between male and female mice showed that in the presence of deleting ligand only 25-40% of reactive T cells, persist in the periphery (Bouneaud et al., 2000) compared with the repertoire in female mice. In another study using H-2M mice in the presence of CLIP, peptide bound to MHC II molecules, it was shown that thymic selection directed by a very restricted set of peptides, generated a post-thymic CD4 cell repertoire highly reactive to self-peptides, but with only low affinity (Lee et al., 1999).

One constraint on central tolerance is the requirement for the relevant self-antigen to be present in the thymus. Tissue specific antigens or antigens expressed in an age dependent manner are examples of antigens not expressed in the thymus, a situation that could allow self-reactive T cells to escape deletion. Although recent data indicate that some tissue specific antigens are expressed at m-RNA levels in medullary epithelial cells, it is not clear whether expression at protein levels is sufficient enough to induce negative selection (Derbinski et al., 2001; Klein and Kyewski, 2000). In an experimental autoimmune
encephalomyelitis (EAE) animal model for multiple sclerosis, induced by activation of a subset of myelin-based-protein (MBP) specific T cells that have escaped deletion, it was shown that the efficacy of central-tolerance is age dependent, reflecting the developmentally regulated expression of MBP (Huseby et al., 2001). Also, although antigens injected into the periphery can migrate into the thymus where they can be processed and presented by thymic MHC II molecules, this mechanism cannot be operative for MHC class I-restricted antigens, because these predominantly present peptides derived from endogenously synthesized antigens (Hammerling et al., 1991).

Because of the window in the avidity of TCR-MHC peptide ligand interaction that operates in selection, self-reactive T cells can escape negative selection even if self-antigen is present in the thymus. Self-reactive T cells with reduced TCR-ligand avidity might receive signals that are insufficient to induce apoptosis and therefore escape deletion. TCR or co-receptor down regulation has been shown to lower the avidity of TCR-ligand interaction and allow T cells to be selected, but to remain unresponsive in the periphery. In male H-Y specific TCR transgenic mice it is shown that deletion of auto reactive T cells spares cells with normal TCR expression levels but low CD8 levels, that are accumulated in the periphery where they are tolerant to H-Y antigen (Teh et al., 1989).

In some double transgenic mice, expressing transgenic TCR and the specific antigen, self-reactive T cells that exist in the periphery have lower levels of TCR expression (Lanoue et al., 1997; Mamalaki et al., 1996; Schonrich et al., 1991). Partial deletion due to TCR down-regulation can be the outcome of expression of a second endogenously rearranged TCR α chain. Incomplete allelic exclusion in the TCR α chain locus can lead to the expression of endogenous TCR α chains in TCR transgenic mouse strains which are not rearrangement gene deficient Rag-/-, and generate TCR αβ molecules with reduced TCR ligand avidity, that can escape deletion. Expression of endogenous TCR α chain has been shown to allow escape of T cells from thymic deletion despite the presence of self-antigen (Girgis et al., 1999; Lanoue et al., 1997; Legrand and Freitas, 2001; Zal et al., 1996). However partial deletion of self-reactive CD8 T cells in double Rag-/- transgenic mice expressing NP specific TCR and endogenous NP peptide spare self-reactive T cells in the periphery that have reduced TCR and co-receptor levels (Mamalaki et al., 1996).

In all cases of partial deletion in the thymus, despite the presence of self-antigen in the thymus, self-reactive T cells that have escaped deletion and migrated in the periphery
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were unresponsive and tolerant to self-antigen (Lanoue et al., 1997; Zal et al., 1996). Some evidence suggests that induction of unresponsiveness to self-reactive T cells that escape deletion already takes effect in the thymus. In MHC K\(^b\) specific transgenic mice expressing K\(^b\) on thymic medullary epithelial cells there was no deletion of clonotypic CD8 T cells, however thymocytes were unresponsive (Hammerling et al., 1991). Also in the case of HA specific T cells (Lanoue et al., 1997) abnormal expression of activation markers on single positive thymocytes indicates that unresponsiveness might have been induced in the thymus (Stockinger, 1999).

In another study on double transgenic mice specific for HA, it was shown that incomplete deletion of self reactive T cells allowed selection of CD4\(^+\)CD25\(^+\) thymocytes bearing high affinity TCR. The CD4\(^+\)CD25\(^+\) peripheral T cells, although unresponsive to the specific antigenic stimulation, could function as regulatory T cells. This finding indicates that the development into CD4\(^+\)CD25\(^+\) regulatory T cells may be an alternative mechanism to deletion of thymocytes bearing a TCR with high intrinsic affinity for self-peptide.

All these data clearly show that negative selection is incomplete, and that there are self-reactive T cells that can escape deletion in the thymus. Given the promiscuity of TCR recognition, the more stringent negative selection becomes, the greater the risk becomes of narrowing dangerously the peripheral repertoire available to respond to foreign antigens. In that sense incomplete central tolerance with low cost to the peripheral repertoire could allow a degree of self reactive T cells to escape and be regulated in the periphery. Although self-reactive T cells can escape negative selection in the thymus, incidents of autoimmune disease are rare, which indicates that there are peripheral tolerance mechanisms to maintain tolerance induction. Given the importance of maintaining tolerance it is not surprising that there are several potential peripheral tolerance mechanisms described. (See 1.4)

1.3 Dynamics of immune responses.

T cell activation is a dynamic process, the outcome of which depends on many factors, including the TCR-ligand binding strength, co-receptor binding, the presence of cytokines and the differentiation stage of T cells. Triggering of TCRs on peripheral T cells can initiate immune responses that lead to prominent clonal expansion and further
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differentiation into a variety of functional subsets of T cells that determine the fate of the immune response.

1.3.1 Naïve T cells and the preimmune phase.

Prior to contact with Ag, naïve T cells congregate in secondary lymphoid tissues (spleen, lymph nodes and Payer's patches) and migrate continuously from one lymphoid organ to another, via blood and lymph. In contrast to the spleen, entry to the lymph nodes and Payer's patches from blood is highly specific and occurs by entry through high endothelial vessels (HEV).

Naïve T cells express L-selectin (CD62-L) (Arbones et al., 1994), the chemokine receptor CCR7 (Forster et al., 1999) and the β2 integrin LFA-1, which interact with peripheral node addressin (PNAd), CCL21 chemokine and intracellular adhesion molecule (ICAM-1/-2) (Lanzavecchia and Sallusto, 2000; Sprent and Tough, 1994; Weninger et al., 2001). This combination of traffic molecules is uniquely found on HEV and is essential for efficient homing of naïve T cells in lymphoid tissues. Continuous migration of naïve T cells through secondary lymphoid tissues is highly important for allowing T cells to make rapid contact with antigens released from pathogens.

During their normal pattern of blood to lymph recirculation naïve T cells are quiescent and have a prolonged life span (Ernst et al., 1999). The long life span of naïve T cells is not innate but requires continuous contact with self-peptide MHC complexes on dendritic cells, and signals through the common cytokine receptor γ-chain, (Lantz et al., 2000), and particularly IL-7 (Tan et al., 2001). Recognition of these signals presumably delivers low-level signals, which keep naïve T cells metabolically active to avoid passive death (Sprent and Tough, 1994).

1.3.2 Initiation of immune responses.

T cell responses are initiated in the T cell areas of secondary lymphoid organs where naïve T cells encounter Ag presented on the surface of specialised “professional” antigen presenting cells (APC). Therefore, initiation of immune responses depends upon antigen capture, transport from the site of infection to secondary lymph nodes and efficient
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presentation to T cells. Initiation of immune responses is the primary function of dendritic cells (DC). However other cell types, including activated T cells or macrophages can present Ag to T cells, and may contribute to the expansion of already primed T cells and possibly modulation of T cell responses.

Dendritic cells are resident throughout all non-lymphoid tissues where they reside in a resting-immature state. MHC-bound peptides displayed on resting DC are poorly immunogenic. For optimal immune responses DC first need to be activated. Immature DC efficiently capture antigens using several pathways such as macropinocytosis, receptor mediated endocytosis and phagocytosis (Lanzavecchia and Sallusto, 2000). In response to “danger” signals such as pathogens, inflammatory cytokines or necrotic cells, DCs migrate to the T cell areas of secondary lymphoid organs and switch from antigen capturing mode to antigen presentation and stimulation mode. During this maturation phase DC assemble peptide-MHC complexes, up-regulate co-stimulatory molecules and elaborate cytokines.

1.3.3 Antigen presentation to T cells

Antigens captured by DC are subsequently degraded in endosomes and the generated polypeptides are transported into MHC II rich compartments, for loading into MCH II molecules. A fraction of peptides are loaded onto empty MHC II molecules recycled from the cell surface. Mature DCs shut off antigen capture and MHC II synthesis, however increase the half-life of MHC II complexes, thereby maximising Ag presentation to CD4 T cells.

DCs also have the unique ability to present antigen peptides on MHC class I molecules, which can be loaded through both an endogenous and an exogenous pathway. The endogenous pathway operates through the degradation of cytosolic proteins and the loading of peptides onto newly synthesised MHC I molecules within the endoplasmic reticulum (ER). Degradation of cytosolic proteins starts by ubiquitin conjugation and continues with enzymatic cleavage by the proteasome and proceeds to translocation of peptides to the ER via ATP dependent TAP transmembrane transporters (Banchereau et al., 2000). In the exogenous pathway (cross-presentation) MHC I molecules present peptides derived from endocytosed proteins rather than newly synthesised cytosolic ones. DCs have been shown to efficiently load MHC I for the exogenous pathway, with peptides derived
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from immune complexes, bacteria and apoptotic cells dying because of viral or bacterial infection. Two routes for the exogenous MHC I pathway have been described, a TAP-independent pathway in which Ag is most likely hydrolysed in endosomes and a TAP dependent phagosome pathway (Banchereau et al., 2000; den Haan et al., 2000; Heath et al., 1998; Kurts et al., 2001).

1.3.4 Activation requirements for naïve T cells.

Initial interaction of T cells with APC leads to the formation of tight synapses at the T cell/APC contact site. Synapse formation precedes T cell activation and is associated with rapid clustering of TCR molecules binding to peptide-MHC complexes on APC, plus local accumulation of intracellular signalling molecules such as lck and LAT (Tomlinson et al., 2000). The latter associate with TCR/CD3 multi-subunit transmembrane complex in lipid rafts, and together with various other intracellular molecules initiate downstream signalling events that cause T cells to have cellular responses of activation, proliferation, differentiation and effector function.

Naïve T cells must interact with APC for about 2 hours to become committed to activation (Bongrand and Malissen, 1998), and studies on human T cells report that they need Ag stimulation for at least 20 hours in order to be committed to proliferate. This high activation threshold needed for naïve T cell activation is due to an inefficiently coupled signalling machinery (Croft et al., 1994; Sagerstrom et al., 1993; Valitutti et al., 1996; Viola and Lanzavecchia, 1996). CD4/CD8 co-receptors, co-stimulatory and adhesion molecules aid TCR-CD3 triggering. Some co-stimulatory and adhesion molecules may provide essential second signals for T cell activation, but others may act largely by enhancing TCR triggering either by stabilising synapse formation, or/and by recruiting intracellular signalling molecules (Lanzavecchia and Sallusto, 2001). There are several adhesion molecules that mediate DC-T cell clustering like intergrins $\beta_1$ and $\beta_2$, immunoglobulin molecules CD2, CD50 and LFA-1. However the crucial factor that is required to sustain T cell activation is the interaction between co-stimulatory molecules expressed on DC and their receptors expressed on T cells.
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1.3.5 Co-stimulatory molecules.

B7.1 and B7.2 are rapidly up regulated upon DC maturation and so far are the most critical molecules for amplification of T cell responses. B7 molecules are important in the initial phase of T cell responses by engagement of CD28 molecules, but also at later stages they can down-regulate T cell responses by engaging CTLA-4. Expression of other members of B7 family molecules on mature DCs like PDL-1 and PDL-2 oppose T cell activation and attenuate cytokine production by binding to PD-1 molecules on T cells which contain immunoreceptor inhibitory motifs on their cytoplasmic tails (Coyle and Gutierrez-Ramos, 2001).

CD28 is constitutively expressed on T cells and upon binding to B7 molecules delivers signals that enhance T cell responses to the antigen resulting in cell cycle progression, proliferation and differentiation. CD28 ligation also promotes the survival of T cells by turning on optimal IL-2 production and expression of IL-2 receptor and anti-apoptotic molecules, such as Bcl-2 and Bcl-xL. Alternatives to CD28 co-stimulatory molecules like ICOS or CTLA-4 appear to act at different stages of T cell activation (Coyle and Gutierrez-Ramos, 2001; Watts and DeBenedette, 1999).

CTLA-4 molecules, which belong to the CD28 family, are not constitutively expressed, but induced upon T cell activation. These receptors bind with higher affinity to B7 molecules and deliver inhibitory signals to activated T cells. ICOS is another member of the same family that was recently identified. ICOS is expressed on activated T helper cells and on memory T cells and upon binding to its counterpart B7RP-1 (member of the B7 family) delivers a CD28 independent signal for IFN-γ, IL-4 and IL-10 but not IL-2 production (Coyle and Gutierrez-Ramos, 2001; Schwartz, 2001; Sperling and Bluestone, 2001).

Other molecules expressed on DCs that may have co-stimulatory function are OX-40 ligand and 4-1BB ligand. T helper cells can activate DCs via upregulation of CD40 ligand. CD40-CD40L signalling to DCs leads to increased B7 molecules expression, IL-1 cytokine release and TNF expression. In that way DCs are “licensed” to prime CD8 T cells without the simultaneous presence of CD4 T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). Triggering CD40 on DCs results in upregulation of OX-40L which signals T cells to produce cytokines and chemokine receptors. Also OX-40 was
shown to be essential for long-term survival of activated CD4 T cells by promoting Bcl-xl and Bcl-2 expression on T cells (Rogers et al., 2001). OX-40 L does not appear to be capable to provide signal 2 alone for naïve T cell activation, rather it enhance proliferation and cytokine production by effector T cells (Watts and DeBenedette, 1999). Mature DCs also express 4-1BB ligand, which binds to 4-1BB co-stimulator on activated T cells. 4-1BB has been shown to co-stimulate T cell responses independently of signals through CD28 in the cases where there are strong TCR signals. Also 4-1BB molecules can stimulate both primary and secondary responses and preferentially induce CD8 T cell proliferation and effector function (Banchereau et al., 2000; Watts and DeBenedette, 1999).

1.3.6 T cell differentiation to effector cells.

Activation of T cells results in proliferation and clonal expansion. As they divide under continuous TCR and cytokine stimulation, T cells differentiate to different effector subsets, with distinct effector functions and capacity to produce effector cytokines. CD4 T cells can polarise towards T helper-1 (Th-1) or Th-2 subsets and this differentiation process is promoted by IL-12 and IL-4 cytokines respectively. Activation of DCs by polysaccharide (LPS) poly (I:C) bacteria and viruses, induce IL-12 release, for Th-1 polarisation. Th-1 cells typically produce IFN-γ but they also secrete IL-2, TNF-β and LT cytokines and protect against intracellular pathogens. The signature cytokine of Th-2 cells is IL-4, but they also secrete IL-5, IL-9, IL-10 and IL-13 cytokines that provide help to B cells for the production of antibodies against antigens originating from extracellular pathogens (Glimcher and Murphy, 2000; Mosmann and Coffman, 1989).

CD8 T cells differentiate into cytotoxic T cells capable of killing virus infected cells (Lanzavecchia and Sallusto, 2000; Sprent and Tough, 1994). In contrast to naïve T cells, primed, effector T cells have a low activation threshold, because their signalling machinery is fully coupled. Short TCR stimulation in the absence of CD28 engagement is sufficient to trigger proliferation and IFN-γ production in Th-1 cells whereas even shorter stimulation triggers cytotoxicity by CD8 T cells (Croft et al., 1994; Pihlgren et al., 1996; Sagerstrom et al., 1993; Viola and Lanzavecchia, 1996).

In typical infections, prominent clonal expansion of specific T cells is followed by differentiation into effector cells generally causing rapid elimination of pathogens. After
elimination of the Ag, the enormous numbers of effector cells decline and only some cells survive to form a subset of memory cells. The capacity of activated T cells to migrate to non-lymphoid tissues, such as lung, liver, gut, kidney, bone marrow and thymic medulla, reflect the upregulation of several classes of homing receptors, including selectins, chemokine receptors and β1 and β2 integrins, notably LFA-1. In general homing to non-lymphoid tissues is skewed towards effector and memory cells that have downregulated lymph-node homing receptors CD62-L and CCR-7. This proposes that primed T cells preferentially migrate to non-lymphoid tissues and subsequently travel to lymph nodes via afferent lymph vessels. However in rodents substantial homing of memory T cells occurs via HEV (Westermann and Pabst, 1996).

The mechanisms responsible for eliminating effector cells are poorly understood, but are clearly complex. For CD8 cells the inhibitory influence of certain cytokines, mainly IFN-γ, may be sufficient to eliminate effector T cells. For CD4 T cells effector cell elimination reflects a tightly regulated instructive process involving multiple mechanisms, including negative signalling by CTLA-4 and PD-1 receptors, activation of Fas pathway (Siegel et al., 2000) and onset of sensitivity to several cytokines such as IL-2, IFN-γ and TNF (Chan et al., 2000). In addition to these active mechanisms for cell death, effector cells may undergo passive death through loss of contact with protective cytokines.

1.3.7 Memory cells.

At the end of primary immune responses, the wide-scale elimination of effector T cells is incomplete, and a small proportion of T cells survive, to become memory cells. The precursor frequency of antigen-specific memory cells is far higher than that for naïve T cells, also memory cells have the ability to mount a qualitatively and quantitatively enhanced response upon re-encounter with the same pathogen (Ahmed and Gray, 1996; Dutton et al., 1998; Goldrath and Bevan, 1999; Sprent and Surh, 2002; Zinkernagel, 1996). Memory cells show distinct phenotypic differences from naïve T cells. CD4 and CD8 memory cells are characterised by expression of CD44 surface marker. In addition CD8 cells are characterised by high expression of Ly6C and CD122 (IL-2 R β chain) (Cho et al., 1999; Pihlgren et al., 1996). Other markers that are used to define memory cells are low
levels of CD62-L and CD45RA/B/C, which can be partly reversible on late memory cells (Sprent and Surh, 2002).

Memory cells do show a shorter lag time for entering cell cycle, synthesising cytokines, differentiating to CTL and migrating to non-lymphoid tissues, (Cho et al., 1999; Garcia et al., 1999; Kedl and Mescher, 1998; Veiga-Fernandes et al., 2000; Zimmermann et al., 1999) and in some cases show increase sensitivity to limiting concentrations of antigen relative to naïve T cells (Rogers et al., 2000).

Although memory cells are more metabolically active than naïve T cells, and can survive for long time, maintenance of CD8 and CD4 T cells is independent of the presence of the specific antigen and signalling through the TCR (Garcia et al., 1999), (Tanchot et al., 1997). Direct evidence that memory cell survival is Ag independent came after following the survival of these cells in MHC -/- host, where both CD4 and CD8 memory T cells were shown to survive for prolonged periods (Hu et al., 2001; Murali-Krishna et al., 1999; Swain et al., 1999). However in the absence of MHC despite survival memory cells show distinct functional defects (Kassiotis et al., 2002).

The survival of memory T cells is also dependent on cytokines. CD8 CD44h CD122+ memory cells are strongly dependent on IL-15 both for their survival and turnover. However IL-2 inhibits memory CD8 T cell survival (Ku et al., 2000; Zhang et al., 1998).

1.3.8 Homeostasis in peripheral T cell pools.

Although naïve T cells have a prolonged life span at a population level, the total size of the T cell pool is highly regulated (Freitas and Rocha, 1993; Tanchot et al., 1997). Also, expansion of the T cell pool during an immune response is followed by a deletion phase in which most of the newly generated effector cells are eliminated at the end of the response, thereby restoring total cell numbers to normal levels (van Parijs et al., 1998; Webb et al., 1990). On the other hand the lymphoid system has great resilience, and it is well established that when the T cell pool is reduced both CD4 and CD8 cells have the capacity to undergo extensive proliferation. Such “homeostatic” or cell density dependent proliferation of T cells occurs when small numbers of T cells are adoptively transferred into T cell depleted syngeneic nude, SCID, recombination deficient RAG-/- or irradiated hosts (Bell et al., 1987; Rocha et al., 1989).
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The factors regulating homeostatic expansion of T cells are not fully understood yet. A body of evidence suggests that homeostatic proliferation is driven by low affinity interactions with self-MHC molecules loaded with self peptides, that may be related to those that induced positive selection (Ernst et al., 1999; Goldrath and Bevan, 1999; Muranski et al., 2000; Viret et al., 1999).

Unlike antigen-induced T cell proliferation, homeostatic proliferation occurs without effector cell formation and without requiring IL-2 or CD28 co-stimulation (Cho et al., 1999). Homeostatic proliferation of naïve T cells to self-MHC peptide complexes is relatively slow and is not associated with upregulation of acute activation markers such as CD25 and CD69 (Ernst et al., 1999; Goldrath and Bevan, 1999; Muranski et al., 2000; Oehen and Brduscha-Riem, 1999). Nevertheless, the dividing cells can acquire cell surface markers typically expressed on memory cells, and become CD44 hi, Ly6C high (Cho et al., 1999; Goldrath and Bevan, 1999; Murali-Krishna et al., 1999; Oehen and Brduscha-Riem, 1999). Homeostatic proliferation however does not always result in phenotypic conversion of activation markers (Ferreira et al., 2000).

CD44 upregulation is slower than antigen activated T cells, and cells become CD44 high only after multiple rounds of cell division (Cho et al., 1999; Murali-Krishna et al., 1999). Other cell surface markers expressed on homeostatically proliferating cells are CD122 (IL-2, IL-15 receptor) and CD132 (common cytokine γc receptor). Also these cells show decreased expression of CD45RB but not CD62-L (Surh and Sprent, 2000). In some cases it has been shown that after homeostatic proliferation naïve T cells do acquire characteristics of true memory cells, including the capacity to mount accelerated functional responses to cognate antigen. However the intensity of the effector responses displayed by homeostasis-activated cells were clearly less marked than responses of overtly activated effector T cells (Surh and Sprent, 2000).

Homeostatic proliferation is not limited to naïve T cells but also apply to memory cells (Murali-Krishna et al., 1999). In contrast to naïve T cells homeostatic division of memory cells in T deficient hosts is MHC independent, (Murali-Krishna et al., 1999) indicating that the mechanisms controlling homeostatic proliferation of naïve and memory cells seem to be fundamentally different.

Homeostatic expansion of T cells can be also regulated by cytokines. In particular, IL-7 cytokine is required for homeostatic expansion of naïve CD4 and CD8 T cells in
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lymphopenic hosts and for CD8 in normal hosts, and to some extent is required for memory cells (Schluns et al., 2000).

1.4 Peripheral tolerance mechanisms.

As described in (1.2.7) thymic deletion of autoreactive T cells is incomplete, either because some self antigens are presented only in peripheral tissues and do not get access in the thymus, or because some self-reactive T cells although they contact self antigens in the thymus the avidity of the interaction is too low and permits them to escape tolerance. Despite that, autoimmune incidents are rare. That means that, fortunately there are mechanisms that maintain tolerance in the periphery and provide the necessary safety net against autoimmunity. The mechanisms responsible for peripheral tolerance can either act directly on the responding T cell, or can evoke other cell subsets. T cell intrinsic mechanisms of peripheral tolerance include ignorance, deletion, anergy and immune deviation. T cell extrinsic mechanisms can induce tolerance indirectly via additional cell types including “tolerogenic” DCs or regulatory T cells.

1.4.1 Ignorance.

The simplest mechanism contributing to the maintenance of peripheral tolerance involves T cell ignorance of self-antigens. In that case potential auto reactive T cells remain naïve despite the presence of the antigen in the periphery, either because self antigens are expressed and presented in sites not easily accessible to blood-lymph borne immune system, or because the amount of antigen or TCR signalling does not reach the threshold required to trigger a T cell response.

Ignorance due to lack of contact between naïve T cells and self-antigens expressed in peripheral tissues other than secondary lymphoid tissues mainly depend on the migration properties of naïve T cells. As naïve T cells mainly recirculate through secondary lymphoid tissue, but do not access extra lymphoid tissue, antigens that are not present on migrating professional APC and stay outside of lymphoid organs will be ignored by naïve T cells.

This was demonstrated by Ohashi et.al (Ohashi et al., 1991) using double transgenic mice expressing lymphocytic choriomeningitis virus glycoprotein (LCMV GP) on
pancreatic β cells and TCR specific for LCMV GP. The transgenic T cells remained unresponsive (tolerant) and phenotypically naïve unless the mice were infected with LCMV virus. Infection abolished unresponsiveness and resulted in CD8 T cell mediated diabetes (Ohashi et al., 1991). In another set of studies it was shown that ignorance and central deletion interplay can occur and that is dependent of differential avidity of T cells. In these studies mice which express H-2K\(^b\) transgene driven by a rat insulin promoter (RIP) in pancreatic islet cells, crossed with H-2K\(^b\) specific TCR transgenic mice, specifically deleted in the thymus CTL precursors (pCTL) bearing high affinity K\(^b\) –directed TCRs (Heath et al., 1995). The remaining low-affinity pCTL ignored K\(^b\) bearing islet cells, even after specific immunization with K\(^b\). The mice were not tolerant of K\(^b\) as they rejected K\(^b\) expressing skin grafts. When bone marrow radiation chimeric mice were made to prevent deletion of high-affinity T cells, pCTLs still generally ignored K\(^b\) expressing islet cells, but became autoreactive after priming, resulting in islet infiltration, destruction and diabetes.

The view for ignorance as a result of lack of contact between naïve T cells and antigen not present on migrating professional APC on secondary lymphoid issues is challenged by some observations that show that there is some accessibility of naïve T cells to extra lymphoid antigens. In one of them large scale trafficking of naïve T cells through extra-lymphoid sites early in ontogeny was reported, indicating that early in development there is contact between naïve T cells and tissue-specific antigens (Kimpton et al., 1995). Also in a transgenic model it was shown that neonatal T cells had access to alloantigen expressed exclusively on keratinocytes (Alferink et al., 1995).

As mentioned earlier not all cells have the ability to activate and prime T cells, as apart from TCR signals naïve T cells require co-stimulation to be fully activated. Recognition of self-antigens on non-professional APC such as tissue cells, due to lack of co-stimulatory molecule expression, is not able to induce an immune response, and such a recognition may have several outcomes from ignorance, to peripheral activation or unresponsiveness. Professional APC like DCs express co-stimulatory molecules upon activation and up-regulate MHC II molecules. However resting DCs do not constitutively express co-stimulatory molecules. According to the “Danger theory” danger signals like bacterial or viral products, necrotic death or inflammatory cytokines generated by an infection, that activate APC are required for T cell activation. Such “danger” signals could
activate resting tissue APC that in their term could activate self-reactive T cells and break ignorance to self-antigens (Matzinger, 1994).

Also sufficient priming of some naïve CD8 T cells to become cytotoxic effector T cells requires recognition of specific antigen by TCR and other "help" factors like IL-2 by CD4 T cells. For that reason CD8 ignorance had been proposed to be an adequate means of CD8 T cell peripheral tolerance. However this cannot be the case as DC are efficient to prime CD8 T cells with tissue antigens (Bevan, 1987; Kurts et al., 1997) without the simultaneous presence of CD4 T cells.

Another way of retaining T cell ignorance could be poorly processed and presented epitopes of self-proteins to self-reactive T cells. Each protein contains a number of dominant epitopes that will be preferentially processed by APCs. These dominant epitopes presented in the thymus will induce negative selection to those T cells expressing high affinity TCR for these peptides. However the majority of epitopes which are poorly presented will be unavailable for tolerance, and T cells specific for these epitopes are not tolerant and ignored because they have never encountered their antigen. However priming with an environmental factor that mimics self-epitopes could activate these T cells that cross-react with self-antigen, initiating autoimmunity (Lanzavecchia, 1995; Mamula and Craft, 1994).

1.4.2 Peripheral deletion.

Exposure of T cells to their cognate antigen in the periphery, can lead to their elimination. In order to prevent autoimmune destruction by a given T cell clone, the most efficient way may possibly be to delete that T cell clone from the repertoire by activation induced cell death (AICD). Evidence that excessive contact of mature T cells with their specific antigen or that persistence of antigen, can lead to peripheral deletion come from studies of excessive T cell responses to either, superantigens, or conventional antigens (Jones et al., 1990; Kawabe and Ochi, 1991; Kearney et al., 1994; MacDonald et al., 1988; Renno et al., 1995; Rocha and von Boehmer, 1991; Webb et al., 1990). In contrast to negative selection of immature thymocytes, mature T cells undergo a period of activation and expansion before they are deleted (Kurts et al., 1997; Renno et al., 1995).
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Variables that determine whether peripheral deletion proceeds efficiently include, the extent of TCR occupancy, the affinity of the antigenic peptide for MHC, and the affinity of the TCR for its MHC-peptide ligand. Therefore as for negative selection in the thymus, deletion in the periphery seems to follow an avidity model, in which high avidity T-cell-APC interactions in the periphery can promote deletion. Clearly high antigen dose and chronic stimulation favour elimination both in CD4 and CD8 mature T cells (Fink et al., 1994; Huang and Crispe, 1993; Kearney et al., 1994; Kyburz et al., 1993; Moskophidis et al., 1993; Salmon et al., 1994; Sprent and Webb, 1995; Zal et al., 1994).

The dose of antigen is generally correlated with the degree of tolerance induced. The extent of deletion of T cells following an in vivo proliferate response to lymphocytic choriomeningitis virus (LCMV, was strongly dependent on Ag-dose. Only at high antigen doses of LCMV the effector cells disappeared after a brief proliferative response and the virus persisted (Moskophidis et al., 1993).

Using lines of mice which express variable amounts of influenza virus haemagglutinin transgene driven by RIP promoter it appears that antigen concentration and precursor frequency determine the rate of CD8 T cell tolerance (Morgan et al., 1999). Similarly thymectomised female H-Y antigen specific TCR transgenic mice receiving 3, 30, or 100 x10^6 male cells showed no deletion, partial deletion or massive deletion of transgenic CD8 cells respectively (Sheng et al., 1998). Also when myelin basic protein (MBP)-reactive TCR transgenic T cells were adoptively transferred to naïve recipients, multiple injections of MBP or peptide were required for efficient deletion. (Critchfield et al., 1994). This requirement for chronic stimulation for deletion may reflect lower avidity interactions, which only lead to deletion following repeated contact with antigen (Sprent and Webb, 1995).

1.4.2.1 Mechanisms of deletion.

Self-reactive T cells might be deleted following contact with self-antigen by AICD involving Fas and TNF-mediated cell death. Other molecules such as cytokines and signalling by CTLA-4 may also play role in AICD.

T cell homeostasis in the periphery is regulated by interactions between Fas and Fas-Ligand (Fas-L), as Fas engagement leads to the apoptosis of activated T cells. In that way T cells can regulate their own responses to protect tissues from being damaged by excess
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effector cells. T cell receptor stimulation increases Fas expression and re-stimulated cells become sensitive to apoptosis triggered by TCR through autocrine production of Fas-L and signalling through Fas (Siegel et al., 2000; Van Parijs et al., 1996). The prominent role of the interactions between Fas and its ligand in the periphery is demonstrated by the lymphoproliferative disorders in Fas or Fas-L defects in mice and humans. In mice naturally occurring defects in Fas or Fas-L are associated with a lymphoproliferative lupus-like syndrome (Watanabe-Fukunaga et al., 1992). Also defects in Fas pathway in humans are associated with lymphoproliferative disorder (ALPS) (Fisher et al., 1995). Other evidence supporting the role of Fas in T cell deletion and self tolerance come from TCR transgenic mice, where peptide deletion requires expression of Fas receptor (Singer and Abbas, 1994), and also in models where self antigen is expressed as a transgene in which transferred antigen-specific T cells which lack Fas fail to be deleted and induce autoantibody production from B cells (Van Parijs et al., 1998).

Apart from Fas-FasL interactions, members of the tumour necrosis factor (TNF) receptor superfamily play critical role in lymphocyte apoptosis during immune regulation. TNF receptor performs similar role to Fas, and in vitro evidence shows that whereas Fas interactions mostly induce AICD in CD4 T cells, the TNF receptor is responsible for induction of apoptosis to CD8 T cells (Chan et al., 2000; Kruisbeek and Amsen, 1996).

Another peripheral mechanism of deletion that is reported to protect the organism of the effects of over-reaction to self or non-self antigens is the lack of lymphokines. Activated T cells undergo apoptosis when cytokines are withdrawn, at the end of an immune response. This particular mechanism could be prominent for CD8 T cells, as the absence of CD4 “help” and especially lack of IL-2 leads to CD8 T cell deletion, and this mechanism appears to be independent of Fas or TNF signalling (Van Parijs et al., 1998). However it has also been shown that IL-2 apart from a role as growth factor has a crucial role in AICD. T cells from mice that lack IL-2 or IL-2 high affinity receptor, are still able to proliferate, with the help of other growth factors than IL-2, but notably fail to undergo Fas-mediated AICD (Lenardo, 1991; Van Parijs et al., 1998). Also mice deficient in IL-2 production or signalling show autoimmunity (Sadlack et al., 1995; Willerford et al., 1995). However it is unclear whether lack of IL-2 in these mice has any abnormalities in thymic selection or production of regulatory T cells (Walker and Abbas, 2002).
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It has been suggested that CTLA-4 membrane receptor that has an inhibitory role in T cells expansion, functions by induction of apoptosis (Gribben et al., 1995). In contrast to Fas, ligation of which on activated cells is sufficient for induction of cell death, CTLA-4 induced cell death is dependent on simultaneous TCR triggering (Gribben et al., 1995). CTLA-4 induced cell death can be reversed by IL-2 or anti CD28 cross-linking. It is thus possible that the apoptosis associated with CTLA-4 engagement observed under some conditions reflects cell death as a consequence of IL-2 withdrawal.

1.4.2.2 Cross presentation-cross tolerance.

Deletion of self-reactive T cells in the periphery requires antigen recognition and activation. Since mature naïve T cells exclusively re-circulate through secondary lymphoid organs the question is how do naïve T cells meet peripheral tissue antigens for activation and deletion? CD8 or CD4 priming associated with capture and indirect presentation of extra-lymphoid (peripheral) tissue derived cellular antigens by professional APC, permit recognition of peripheral antigens by lymph node naïve T cells (Bevan, 1976; Bevan, 1987; Kurts et al., 1996; Rock, 1996).

Experiments by Kurts et al. (Kurts et al., 1997) showed that CD8 T cells were activated and proliferated in lymph nodes draining the site of peripheral antigen expression, and long-term examination of these CD8 T cells revealed their gradual deletion from the peripheral pool. Such deletion was mediated by AICD since it was preceded by a proliferative response and was dependent on Fas signalling. These studies revealed that cross presentation of self-antigens by bone marrow derived APC provided a mechanism for induction of peripheral tolerance at least for CD8 T cells. This mechanism is termed cross-tolerance and refers to tolerance induced by cellular antigens indirectly presented by bone marrow derived APCs.

Like CD8 T cells, CD4 T cells specific for a model autoantigen, SV40 T antigen have been reported to be activated in the draining lymph nodes of those organs expressing this protein. (Forster et al., 1995). In this case also, proliferation was followed by deletion, although some remaining cells exhibited properties of anergy. These findings were extended by another work where mice expressing haemagglutinin in various parenchymal cells, showed that CD4 T cell tolerance was mediated by cross-presentation of this antigen on bone marrow derived APC (Adler et al., 1998). In this case tolerance induction was
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more consistent with anergy although contribution by a deletional mechanism was difficult to rule out.

In a recent study, analysis of CD8 T cells undergoing peripheral deletion in response to cross-presentation of self antigen showed that the phenotype of these cells was CD44, CD69 high, yet only partially CD62-L low. A functional analysis of these cells also showed that they lack the ability to produce IFN-γ in response to antigen or cytolytic ability, and the cells disappeared after a few rounds of division. Therefore it was suggested that, antigen recognition by naïve CD8 T cells results in localised proliferation and deletion, without the production of effector cells (Hernandez et al., 2001).

In studies of transgenic mice expressing different levels of OVA peptide in the pancreas, it was shown that antigen concentration is critical in determining whether such antigens are cross-presented in the lymph nodes. High antigen dose can be cross-presented constitutively resulting in the activation of autoreactive CD8 T cells. This does not lead to autoimmunity, but to Fas-mediated deletion of the T cells.

Low doses of self-antigen are not cross-presented and were consequently ignored by the immune system (Heath et al., 1998; Miller et al., 1998). In another system (RIP-m-OVA) where the auto-antigen was cross presented, low numbers of autoreactive CD8 T cells were activated, proliferated, but underwent AICD before any damage resulted. High numbers, on the other hand, did induce diabetes, and low numbers in the presence of CD4 help were just as harmful. Therefore in that system it was shown that another important factor that determines whether the T cells response to cross-presented auto antigen is indifference or tolerance or autoagression is the precursor frequency of circulating autoreactive T cells (Miller et al., 1998).

1.4.2.3 Escape from peripheral deletion.

It should be noted that as well as negative selection in the thymus, peripheral deletion of self-reactive T cells is rarely complete and in many models the remaining cells that have escaped deletion are unresponsive to cognate antigen (Kearney et al., 1994; Lanoue et al., 1997). This indicates that deletion of a T cell clone from the peripheral repertoire is probably the end point of a continuous process ranging from activation, to unresponsiveness, to deletion, with signal strength and exposure time together determining
the outcome (Arnold et al., 1993; Dillon et al., 1995; Forster and Lieberam, 1996; Rocha and von Boehmer, 1991; Schonrich et al., 1992). Otherwise deletion might serve to decrease the precursor frequency to a level at which unresponsiveness or other tolerance mechanisms can be efficiently induced.

1.4.3 Anergy.

Not all APCs have the ability to prime and activate T cells, as only some "professional" APC like DC can trigger both the TCR and the T cell co-stimulatory receptors. Although "dual" signalling induces T cell responsiveness and differentiation to effector function, the antigenic TCR signal alone has been shown to induce a state of unresponsiveness called anergy (Boussiotis et al., 1994b; Jenkins and Schwartz, 1987; Lamb et al., 1983; Mueller et al., 1989; Quill and Schwartz, 1987; Schwartz, 1990). Anergy or T cell unresponsiveness is not only mediated by lack of co-stimulatory signal, yet there are other mechanisms that can also drive a T cell unresponsive. Apart from the fact that the degree of unresponsiveness can vary in different models of anergy, and can range from total to partial unresponsiveness which affect only some effector functions, unresponsiveness can be either permanent or is in some cases reversible. Therefore, there is not a general definition of anergy, and the term is used vaguely in the majority of cases to describe a cellular state of partial or total unresponsiveness to optimal antigenic stimulation (TCR and co-stimulation), in which antigen experienced cells are alive but fail to display certain functional properties. This state is different from antigen induced unresponsiveness (AINR) resulting from temporary refractory state when T cells are exposed to activating stimuli too soon after the last antigen encounter, when they are still at the effector state.

1.4.3.3 Mechanisms of anergy induced in vitro.

Among the different in vitro mechanisms that have been observed to induce T cell anergy, TCR ligation in the absence of co-stimulation is the best established (Boussiotis et al., 1994b; Mueller et al., 1989). This mechanism has been defined in vitro using artificial lipid bi-layers, impregnated with H-2E molecules together with antigen a pigeon cytochrome c peptide. Th-1 clones specific for this peptide-MHC complex when exposed to this planar membranes failed to proliferate and were refractory to further stimulation in
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response to fully competent APC (Quill and Schwartz, 1987). Anergy induction in the absence of full co-stimulation was mainly shown in mouse or human T cell clones (Lamb et al., 1983; Schwartz, 1996), however naïve T cells have also been reported not to be susceptible to anergy induction under these conditions (Davis and Lipsky, 1993; Hayashi et al., 1998; Sagerstrom et al., 1993).

The molecular basis of T cell anergy has been defined in vitro based on Th-1 (CD4) T cell clones that have been rendered anergic by activation with APC that lack co-stimulatory molecules expression. Anergic T cells showed a block in IL-2 production that occurs at the level of transcription of IL-2 gene. The production of other cytokines like IL-3 and IFN-γ was also affected but to varying degrees, whereas IL-4 production in Th0 clones was unaffected (Schwartz, 1997). The current model for the effect on IL-2 gene transcription on T cells anergized in vitro has been shown to involve defects in the signal transduction pathways downstream TCR, and other multiple components acting on the IL-2 promoter.

Early signalling defects occurring before protein kinase C activation and [Ca²⁺] release were reported in human T cell clones anergized in vitro by T cell presentation of auto-antigen (Lasalle et al., 1992). Later it was shown that T cells anergized in vitro have a block in p21 ras activation (Fields et al., 1996) which prevented activation of ERK kinases. MAPK kinase signalling blockage on anergized T cells leads to failure to induce and activate c-fos and Jun-B, thus a failure to form and phosphorylate AP-1 transcription factor, required for IL-2 gene transcription (Becker et al., 1995; Schwartz, 1997). In addition, some evidence emerged showing the presence of negative regulatory elements that also function in anergic cells to repress IL-2 gene transcription like NIl-2 and NRE-A (Schwartz, 1997). In vivo studying of CD8 T cells specific for H-2K^b MHC class I antigen when transferred into Alb-K^b transgenic mice expressing the H-2K^b antigen on hepatocytes, showed that unresponsive CD8 T cells expressed no AP-1 and had only weak NF-kB transcriptional activity. The differences in NF-kB transcriptional activity co-related with the generation of discrete NF-kB complexes, suggesting that fine regulation of NF-kB complex formation may determine T cell responses (Guerder et al., 2001).

The scenario of anergy induction due to lack of co-stimulation is not applicable to all models of T cell unresponsiveness, and there are other mechanisms of anergy induction in vitro distinct from lack of co-stimulation. Small structural variations in a peptide component, that do not decrease its ability to bind to the MHC molecules, can result in
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differences in TCR: (peptide/MHC) bond lifetime and can translate into markedly distinct signalling outcomes, thus determining the extent of T cell responses. Such altered peptide ligands can initiate partial T cell activation due to an assembly of partial signalling transduction complexes (partial agonist) and result in desensitisation of the cell to subsequent full stimulation (antagonist) (Bongrand and Malissen, 1998). Amino acid substituted peptide variants have been described not only to just fail to induce T cell proliferation, but to induce T cell anergy (Sloan-Lancaster et al., 1993). Anergy induced by such APC was due to partial T cell signalling with induction of altered TCR phospho-ζ species and lack of association with Zap-70 kinase (Sloan-Lancaster et al., 1993). Thus, even in the presence of co-stimulation, presentation of suboptimal ligand can induce T cell unresponsiveness, via altered signalling pathways.

When TCR engages an optimal ligand both IL-2 and IL-2 receptor (IL-2 R) synthesis take place, promoting the subsequent expansion and terminal differentiation of the corresponding T cell clone. IL-2 dependent proliferation seems to be a final common pathway for T cell activation and itself a critical event. Prevention of IL-2 proliferation by anti-IL-2 or anti-IL-2 receptor antibodies induces anergy in otherwise fully stimulated CD4 T cells in vitro, and can be reversed by inducing proliferation by exogenous IL-2 (DeSilva et al., 1991). T cell clones that have received an energizing stimulus can be rescued by antibody-mediated cross-linking of common γ chain (γc) shared by receptors for IL-2, IL-4 and IL-7 (Boussiotis et al., 1994a). Therefore, in vitro provision of TCR excitation signals in the presence of co-stimulatory signals, but not accompanied by growth factor signals also result in unresponsiveness.

IL-10 is an important cytokine in the regulation of the immune responses. The role of IL-10 in anergy induction was first shown in human CD4 T cells. In vitro activation of human CD4 T cells in the presence of IL-10 induced a state of unresponsiveness that could not be reversed by IL-2 (Groux et al., 1996). Later it was shown that chronic in vitro stimulation of mouse CD4 T cells in the presence of IL-10 results in the emergence of T cells which, although unresponsive themselves, they produce high amounts of IL-10 and some TGF-β and are able to suppress the immune responses of naive T cells in vitro via a mechanism dependent on IL-10 and TGF-β (Groux et al., 1997). Therefore, activation in the presence of IL-10 regulatory cytokine can induce unresponsiveness in vitro (Roncarolo and Levings, 2000).
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Anergy induction in human T cell clones when cultured in vitro with antigen in the absence of APC, was attributed to T-to-T cells antigen presentation in the absence of costimulation. However activated human T cells as used in these experiments express both high levels of MHC II and B7 molecules (Azuma et al., 1993; Wyss-Coray et al., 1993). The molecular basis of this type of anergy induction is not known, although signalling through CTLA-4 has been proposed to play a role (Lechler et al., 2001).

1.4.3.4 Anergy in vivo.

Although anergy is well defined in vitro, very little is known about the in vivo mechanisms of peripheral tolerance, especially regarding the induction of unresponsiveness. Evidence that anergy can occur during normal development in vivo was provided by a study of mice transgenic for an H-2E alloantigen under the rat insulin promoter. The T cells from these mice were hyporesponsive in vitro when challenged with transgene encoded H-2E (Burkly et al., 1989). Induction of anergy in vivo was also shown in transgenic mice, in which the Kb alloantigen expression was targeted to a variety of peripheral sites, using tissue specific promoters (Arnold et al., 1993). Other studies using injection of super-antigens, showed that after a wave of cell death of T cells expressing particular TCR Vβ families, some T cells persisted in an unresponsive state (Kawabe and Ochi, 1991; Rammensee et al., 1989). In other studies using transgenic mice and more conventional antigen, induction of anergy was displayed in vivo for CD4 and CD8 T cells (Kyburz et al., 1993; Lanoue et al., 1997; Rocha et al., 1995).

In vitro experiments indicate that T cells can be tolerized by non-professional APCs that are unable to deliver the co-stimulatory CD28/B7 signal. The in vivo equivalent is that auto-reactive T cells may be tolerized upon encounter with antigen expressed in non-haematopoietic tissues in the periphery. However the two-signal model is only part of a chain of events that determines T cell activation and there could be additional mechanisms to lead to anergy induction. CD8 T cells were shown to become anergic following activation in vivo with allogeneic tumour cells expressing B7.1 co-stimulatory molecules (Deeths et al., 1999). Also transgenic mice expressing B7.1 molecule in pancreatic islet cells rarely develop autoimmune diabetes (Guerder et al., 1994), suggesting that additional mechanisms operate besides lack of co-stimulatory molecules to maintain tolerance.
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Signalling through alternative receptors rather than just lack of co-stimulation is involved in the induction of anergy. The finding that blocking of co-stimulatory ligands B7.1 (CD80) and B7.2 (CD86) inhibited tolerance rather than promoting it supported this view (Lane et al., 1996). CTLA-4 inhibitory receptor has been reported to play a role in anergy induction \textit{in vivo}, and consequently in peripheral tolerance. The significance of CTLA-4 function is indicated by the lymphoproliferative disorder resulting from its absence shown in CTLA-4 deficient mice, and provide direct evidence for a crucial role of CTLA-4 in down-regulating T cell activation (Tivol et al., 1995; Waterhouse et al., 1995). It has been shown that induction of anergy is prevented when specific antibodies block CTLA-4 (Perez et al., 1997). Blocking of CTLA-4 was also shown to reverse CD8 T cell (OT-1) tolerance to EG 7 tumour by a CD4 and IL-2 dependent mechanism (Shrikant et al., 1999). CTLA-4 up-regulation was also shown to play a role in allograft tolerance mediated by antibody against CD45 RB isoforms (Fecteau et al., 2001). Using naïve TCR transgenic T cells lacking CTLA-4 it was demonstrated that CTLA-4 regulates anergy induction \textit{in vivo} by controlling cell cycle progression (Greenwald et al., 2001). Thus CTLA-4 has an essential role in determining the outcome of T cell encounter with a tolerogenic stimulus (Coyle and Gutierrez-Ramos, 2001; Walunas and Bluestone, 1998), and may regulate anergy by block of IL-2 production and progression into cell cycle.

Another molecule, which could play a role in anergy induction, is programmed cell death (PD-1) molecule, which is expressed at high levels on anergic T cells (Lechner et al., 2001). Some mice strains lacking these molecules were shown to have autoimmune disorders, and it has been reported that PD-1 might function by inhibiting cytokine secretion or by causing cell-cycle arrest (Walker and Abbas, 2002). Therefore, unresponsiveness could be controlled by other cell surface receptors as well as by B7 co-stimulatory molecules, and together with TCR generating tolerizing stimuli by generating a complex of signalling pathways.

CD8 T cells can become unresponsive by down-regulating their co-receptor or TCR. Down-regulation of TCR or CD8 molecules has already been mentioned as a mechanism by which self-reactive T cells can escape negative selection in the thymus. TCR and CD8 down-regulation as a mechanism of peripheral tolerance was shown in transgenic mouse models. In one of these models, where transgenic mice express MHC I K\textsuperscript{b} gene under the control of liver specific promoter, increasing levels of transgene expression induced an
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increasingly anergic state in transgenic-specific CTLs. It was shown that the mechanism of anergy induction in that system was down-regulation of the specific TCR (Hammerling et al., 1991; Schonrich et al., 1991). Very low levels of transgene expression induced tolerance by partial down-regulation of TCR on CD8 T cells. Further exposure to higher concentrations of antigen transgene led to complete down-regulation of TCR on already tolerant cells. TCR down-regulation was reversible, as removal of promoter inducing agent resulted in TCR up-regulation. In further experiments where the expression of K\(^{b}\) transgene was targeted to peripheral tissues (hepatocytes, or keratinocytes, or cells of neuroectodermal origin) using tissue specific promoters, it was shown that distinct levels of progressively more profound TCR and CD8 down-regulation occurred, depending on the level of antigen expression (Schonrich et al., 1992). Also in another study where antigen is expressed exclusively on hepatocytes, partial down regulation of TCR on self-reactive CD8 T cells induced tolerance, with further down regulation of TCR to increase the degree of tolerance depending on antigen dose (Ferber et al., 1994). Reversible anergy followed by down-regulation of TCR and CD8 molecules was also reported in mice transgenic for a TCR specific for H-Y antigen (Rocha et al., 1993).

Down-regulation of TCR complex is believed to be intimately tied to T cell activation, allowing serial triggering receptors and desensitization of stimulated cells. Such TCR down-regulation appears to be regulated by two mechanisms, one that requires direct TCR engagement, and is independent of signalling, and a second signal transduction dependent mechanism that down-regulates non-triggered receptors (San Jose et al., 2000).

CD8 down-regulation has also been reported in some cases to characterise peripheral self-reactive T cells that have escaped deletion in the thymus. In male mice expressing transgenic TCR specific for H-Y H-2D\(^{b}\), despite deletion in the thymus, the majority of T cells that had escaped were shown to express high density of transgenic TCR but low density of CD8 molecules (Teh et al., 1989). Also in V\(\beta\) 5.2 TCR\(\beta\) transgenic mice both CD4 and CD8 T cells that express V\(\beta\) 5.2 TCR are deleted intrathymically in I-E mice that express SAg by endogenous retrovirus Mtv-9. CD4 T cells that escaped deletion were found anergic in the periphery. In the peripheral CD8 cellular compartment CD8 down-regulation was reported and identified a population targeted for deletion (Dillon et al., 1995). Therefore TCR or CD8 down-regulation has been proposed as one of multiple levels of peripheral tolerance multistep process, that precedes deletion (Arnold et al., 1993).
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together these data suggest that the level of antigen expression determines the degree of anergy induction in antigen specific T cells and that some tissues are more likely to be affected by autoimmunity than others. However, as most of these studies were done on Rag positive transgenic mice, the complication exist that TCR down regulation in the periphery may be apparent due to the selection of cells expressing endogenous TCR α chains.

Therefore induction of unresponsiveness \textit{in vivo} can be induced by different mechanisms including antigen presentation by non-professional APC and lack of co-stimulation, signalling through inhibitory receptors like CTLA-4 or PD-1, TCR and/or CD8 downregulation. Regardless of the mechanism that operates to induce unresponsiveness, in many cases the degree of unresponsiveness can vary from partial to total. In these cases of partial unresponsiveness, the term anergy might relate to only one or a few functions whereas other functions are still intact.

Partial inactivation of TCR transgenic T cells specific for hen egg lysozyme (HEL) was observed in mice expressing HEL on the thyroid epithelium. CD4 T cells in TCR/thyroid-HEL escape deletion but peripheral T cells retained sufficient function to initiate non-destructive, sub-clinical autoimmune inflammation (Akkaraju et al., 1997). Also persistence of antigen in viral immune evasions has also been reported to sustain activated cells in an unresponsive state (Ehl et al., 2000; Zajac et al., 1998).

Different levels of tolerance were described in a transgenic mouse model where cytosolic ovalbumin was expressed in intestinal epithelium after transfer of OVA specific CD8 T cells. After primary T cell activation, subsequent deletion occurred in the draining lymphoid tissue. Following initial activation, OT-1 cells encountering antigen destroyed tissue and subsequently remained partially anergic, as they exhibited high levels of lytic activity but were unable to produce cytokines (Vezys et al., 2000).

In another study it was also shown that anergy is not global loss of function, but rather characterises partial unresponsiveness to some functions. DO11.10 TCR transgenic cells specific for OVA peptide were transferred intraperitoneally into lightly irradiated tolerant nu/nu BALB mice. Fifteen days after transfer, cells were rendered tolerant by intraperitoneally injections of antigen (OVA) three times at five days intervals. 3x OVA treated cells had undergone cell divisions \textit{in vivo} displayed an activated phenotype and were defective for IL-2 production and proliferation upon Ag-stimulation \textit{in vivo}. These tolerized T cells were however equally capable of providing “help” for antibody production.
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after antigenic immunisation. This showed that otherwise anergic T cells could still participate in antibody response, and therefore retained some of their functions (Malvey et al., 1998).

Until recently anergy was believed to be a last step before deletion. This is the correct interpretation for the B cell system, where anergy in vivo is well studied. B cells in vivo were shown to have a biochemical block in the tyrosine kinase-signalling cascade initiated by IgM and IgD receptors. This inhibits the accumulation of phosphotyrosine on the receptor-associated CD79α and β chains and on the collaborating tyrosine kinase (Cooke et al., 1994; Goodnow et al., 1995). Also anergic B cells were shown to have a very short half-life as they fail to compete with normal B cells for entry into follicles (Cyster et al., 1994). In contrast to B cells, the life span of anergic T cell has not been studied systematically. Some studies have described the persistence of anergic cells in vivo for long time after the injection of the antigen that was responsible for inducing anergy (Pape et al., 1998). Other data have shown that anergy is a distinct mechanism from exhaustion and that anergy is not a functional stage before deletion (Rocha et al., 1995). It is in question though whether anergic T cells can compete with polyclonal cells for survival.

Also anergy was reported to be reversible and that continuous contact with tolerogenic antigen is required for the maintenance of the anergic state (Rocha et al., 1993). However it is not known whether reversal of the anergic state is dependent on cell divisions and progression through the cell cycle.

1.4.4 Immune deviation- phenotype skewing.

T cell tolerance is usually characterised by absence of T cell responsiveness at a cellular level. However functional tolerance at the level of the whole organism has also been described in the case of persistently active T cell responses. Even when T cells become fully activated, effective tolerance might still be maintained if the nature of the response is such that pathogenic effects are avoided. In certain autoimmune models, animals that do not develop disease still have extensive tissue infiltration, and autoreactive T cells from such mice have been reported to display activated phenotypes (Katz et al., 1995; Nicholson et al., 1995). In these cases, the T cell response has been skewed into a lineage that does not mediate disease, and prevents the development of other cell types,
which cause harmful T cell responses. This phenomenon is called immune deviation (Finkelman, 1995; Liblau et al., 1995). Given the antagonistic effects of the Th2 cytokines IL-4 and IL-10 on Th1 responses it has been hypothesised that Th2 might function as regulatory cells to suppress the harmful activity of Th1 mediated immunity, while not causing pathogenesis themselves. Th2 cytokines have been linked with downregulation of autoimmunity in experimental encephalomyelitis (Young et al., 2000), and diabetes (Bradley et al., 1999), although this is not always the case (Pakala et al., 1997).

During an immune response induction of development of different Th lineages occurs through the action of cytokines (Paul and Seder, 1994). The type of APCs involved in an immune response can also determine Th lineage skewing. In addition genetic differences could be involved in determining lineage skewing. Transgenic mice expressing HA on islet β cells, as well as TCR against this epitope presented on MHC II, develop diabetes when crossed onto the B10. D2 background but not when crossed onto Balb/c background, although in both cases T cells were activated (Scott et al., 1994).

Recent data indicate an association between cytokine differentiation and chemokine expression (Charles et al., 1999; Chensue et al., 2001). Altering lymphocyte trafficking can modulate immune responses by determining which APC T cells encounter. In response to tolerogenic antigen, T cell migration in B-cell areas in lymph nodes was shown to be defective (Kearney et al., 1994). Expression of CCR5 chemokine receptor that mediates this migration of T cells into B cell areas was shown to be dependent on CD28 ligation (Walker et al., 1999). Also absence of this chemokine has been linked with autoimmunity in murine lupus (Ishikawa et al., 2001). Regulation of migration patterns of self-reactive T cells by differential expression of chemokine receptors may be another way to prevent autoimmune destruction in the periphery (Walker and Abbas, 2002).

1.4.5. Immune regulation.

Apart from “passive” peripheral tolerance mechanisms that act directly on self-reactive T cells, like deletion, ignorance, anergy and immune deviation, there appear to be active immune regulatory control mechanisms and certain T cells were shown to actively down regulate the activation, effector function and proliferation of self-reactive T cells (Maloy and Powrie, 2001; Sakaguchi, 2000; Shevach, 2000).
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A number of experimental systems have provided evidence for the existence of a regulatory mechanism of peripheral tolerance, based on T regulatory cells capable of controlling autoimmunity. For example, removal of a regulatory T cell population by thymectomy in neonatal mice leads to the development of a wide spectrum of autoimmunity including gastritis, oophoritis and thyroiditis, all of which can be inhibited by transfer of a small number or CD4 T cells, from normal mice (Maloy and Powrie, 2001; Shevach, 2000). Similarly autoimmune disease can be induced by transferring CD4 T cells from normal adult mice that have been depleted of CD4CD25⁺ cell subset, into immunodeficient nude mice. CD4CD25⁺ cells prevent the development of autoimmunity induced by CD4CD25⁺ counterparts (Sakaguchi et al., 1995). Also transfer of CD4 T cells to non-obese diabetic (NOD) mice, who spontaneously develop insulin dependent diabetes mellitus (IDDM), and autoimmune thyroiditis, prevents IDDM (Shevach, 2000). Studies on transplantation tolerance also show that T cells can actively inhibit effector T cells from rejecting grafts (Qin et al., 1993).

All these data support the existence of regulatory T cells capable of inhibiting autoimmune disease, and a lot of studies have been concentrated to identify surface markers- specific for these cells. The surface markers that have been employed to date are CD25 and CD45RB (Powrie et al., 1994; Stephens and Mason, 2000). CD25 is the IL-2 receptor α chain, which is up regulated on activated T cells. CD45RB/RC belongs to a family of protein phosphatases and plays a critical role in lymphocyte activation by regulating Src protein tyrosine kinases (PTKs) and the TCR ζ chain (Trowbridge and Thomas, 1994). The expression of different CD45 isoforms is associated with the activation status of T cells and with memory formation.

Peripheral murine CD4 CD25⁺ T cells have been shown to have regulatory activity in vitro (Takahashi et al., 1998; Thornton and Shevach, 1998) and in vivo (Sakaguchi et al., 1995). These CD4CD25⁺ cells are partially anergic as they show little proliferation in vitro in-response to TCR stimulation and their growth is dependent on exogenous IL-2. CD4CD25⁺ T cells can have regulatory role by inhibiting diabetes in mice (Salomon et al., 2000), induce tolerance to alloantigens (Taylor et al., 2001), and regulate the expansion of other peripheral CD4 T cells (Annacker et al., 2001) even those responding to an unrelated antigen (Thornton and Shevach, 2000). The CD4 CD25⁺ population has been reported to constitutively express CTLA-4, and inhibiting this pathway using antibodies or CTLA-4
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deficient regulatory cells, suppressor function is reduced in some cases. This indicates that their mode of regulatory activity may require cell-to-cell contact. However in other studies it has been reported that CD25 regulatory cells function independently of CTLA-4. CD4 CD25^+ T cells are reported to be produced and selected in the thymus, as shown in the study of Jordan et al. (Jordan et al., 2001) where self-reactive high affinity H-A specific CD4 CD25 T cells are selected in double transgenic mice expressing H-A peptide, and function as regulatory cells. However, in the peripheral CD4 T cell pool not all regulatory cells are found to be CD25^+, and there are cells with regulatory activity which are CD4 CD25^- (Stephens and Mason, 2000).

Another mechanism utilised by other types of regulatory cells to regulate indirectly immune responses is the release of immunoregulatory cytokines, like IL-10, TGF-β and IFN-γ. IL-10 was identified as a critical cytokine, which suppresses multiple activities of the immune response. IL-10 is produced by different kinds of cells, including activated T cells, monocytes and B cells. In vitro studies have demonstrated that IL-10 inhibits the accessory function of macrophages by reducing the expression of MHC II molecules and co-stimulatory B7 molecules. Also it inhibits macrophages from producing TNF, IL-1 and IL-12. IL-10 inhibits the IFN-γ, IL-2 and IL-2 receptor α chain up-regulation on T cells after stimulation with dendritic cells. Finally IL-10 can down-regulate its own production, and mice lacking IL-10 develop colitis and were susceptible to a condition resembling rheumatoid arthritis (Rennick et al., 1997). Overall these properties of IL-10 make it a potent negative regulator of the immune responses. The action of TGF-β on T cells is critical for prevention of autoimmunity as demonstrated in TGF-β deficient or TGF-β receptor inactivated mice, which develop autoimmunity (Gorelik and Flavell, 2000).

Chronic stimulation of human or murine T cell clones with antigens in the presence of IL-10 has been shown to give rise to T cells, termed Tr1, that suppress proliferation of CD4 T cells in-response to antigen, and prevent colitis induced in SCID mice by pathogenic CD4 CD45RB high T cells. These regulatory T cells rely on production of IL-10 and TGF-β for their function.

Production of IL-10 and TGF-β linked with regulatory function has been described in a system of oral tolerance. In that system orally administrated antigens have been shown to induce T regulatory cells termed Th3, that suppress pathology in animal models of autoimmunity such as EAE, diabetes and collagen-induced arthritis (Roncarolo and...
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Levings, 2000). Oral tolerance induced by Tr cells is mediated by bystander suppression also through the production of cytokines like IL-10, TGF-β that suppress pathogenic T cells which may be specific for a variety of antigens present in the microenvironment.

Immune regulation induced by the production of TGF-β was also shown in an experimental mouse model of inflammatory bowel disease (IBD). Reconstitution of severe combined immunodeficient (SCID) recipients with CD4 CD45RB high T cells from normal mice leads to the development of colitis. However co-transfer of CD4 CD45RB low T cells from normal mice, together with the CD4 CD45RB high population inhibits the disease (Maloy and Powrie, 2001; Powrie et al., 1993). Immune regulation against damaging Th1 type of response to enteric foreign antigen, by the CD4 CD45RB low T cell subset, was induced by the production of TGF-β in vivo. The fact that neutralization of this cytokine by monoclonal Abs resulted in the development of IBD suggested that the mode of action of CD4 CD45RB high population involves this cytokine.

1.4.5.1 Anergic T cells and their role in immune regulation.

Accumulating data suggest that anergic T cells, instead of being functionally inert, may develop a phenotype with functional importance in negative regulation of naïve T cells. This regulation could be either passive, by competing with normal naïve T cells for survival factors, or local cytokines or ligand binding. On the other hand, regulation could be the outcome of an active mechanism.

Anergy and suppression as a way to regulate responses to self has been suggested in many studies, including a study where CD4 transgenic T cells specific for influenza virus PR8-hemagglutinin, were rendered anergic in vivo, in mice expressing also PR8 antigen. Peripheral CD4 T cells that escaped deletion, although anergic both in vitro and in vivo, contained two distinct populations. The CD25hi/CD45RBint population was shown to be anergized, but the remaining CD4 T cells if isolated from the anergized population were shown to be fully reactive. Anergy and suppression were suggested to regulate CD4 T cell responses to this self-peptide (Jordan et al., 2000).

Different studies support the possibility that anergic T cells may have a regulatory role on other T cells through the release of cytokines like IL-10 and IFN-γ. In one of these studies influenza haemagglutinin (HA) specific TCR transgenic T cells anergized in vivo, in
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mice expressing HA have been shown to contain higher levels of IL-4 and IL-10 mRNA than naïve or recently activated T cells with the same specificity (Buer et al., 1998). Recently, using a gene transfer system where HA is expressed in skeletal muscle by two different DNA viral vectors, it was shown that these cells not only remained anergic, but they could also suppress the immune response of naïve T cells against immunogenic adenoviral proteins (Jooss et al., 2001). These findings support the possibility that anergic T cells can become regulatory and influence neighbouring immune responses through the effect of IL-10. This was also supported in another study that CD4 T cell clones were rendered anergic by autologous melanoma cells lacking co-stimulation, but were able to produce IL-10, leading to the maintenance of clonal anergy (Becker et al., 1994).

In a model of SAg-induced anergy it was suggested that anergy could be the result of an active process of suppression depending on IFN-γ release. Peripheral unresponsiveness of Vβ3/Vα11 transgenic CD4 T cells was induced in vivo by Vβ3 specific superantigen staphylococcal enterotoxin A (SEA), as measured by subsequent restimulation in vitro with antigen or mitogen. SEA-treated CD4 T cells purified of other contaminating cells, regained their ability to proliferate and secrete cytokines. In that model CD4/CD8 cells from SEA-treated mice suppressed the responses of fresh control CD4 T cells in-mixed cultures, indicating that this population had regulatory function (Cauley et al., 1997). Similarly, the potential of anergic cells retaining their capacity to produce IFN-γ and perform a regulatory role was suggested in a study where CD8 T cells were anergised in-vivo by viral SAg. In that study CD8 T cells were shown to transit through a CD8 low TCR low deletional intermediate stage, during their tolerance induction. These cells displayed an activated /memory phenotype CD44 high, CD45RB low, CD62-L low but were poorly responsive upon TCR cross-linking and non-cytotoxic in vitro. However these anergized cells were able to produce IFN-γ and accumulate at sites of inflammation, therefore indicating that they could retain some of their functions and potentially perform a regulatory role (Blish et al., 1999).

Some anergic T cell clones are suggested to mediate immune regulation by inhibiting the antigen presenting function of APC. CD4 T cell clones rendered anergic by T-T antigen presentation were shown to have profound TCR and CD3 downregulation. These activated T cells failed to proliferate and produce IL-2 or IFN-γ cytokines upon antigenic restimulation in vitro. However they were shown to regulate T cell responses by modulating T-cell activating capacity of the APC (Taams et al., 1999; Taams et al., 1998).
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Also specific mouse or human T cell clones, rendered anergic in vitro using immobilising anti-CD3 mAb, inhibited proliferation of responsive T cells specific for the same alloantigens. Anergic cells in this model were suggested to inhibit the ability of DC to stimulate T cells with the same or different specificity by downregulation of MHC II and co-stimulatory molecules expression on DC (Vendetti et al., 2000). In the same model suppression of immune responses in vivo, including the prevention of allograft rejection via a mechanism that involves antigen-presenting cells also indicated that anergic T cells could function as suppressor cells both in vitro and in vivo (Chai et al., 1999).

1.4.6 Role of DCs in peripheral tolerance.

Among antigen presenting cells, DCs play an important role in immune responses particularly in the initiation of the response. There is growing evidence that DCs apart from acting as professional APCs, they can also maintain and regulate T-cell tolerance in the periphery. DCs have a unique ability to process and present various types of antigens to T cells. Immature DCs in the periphery are specialised for antigen capture whereas maturating DCs lose this capacity but gain stimulatory properties for naïve T cells. Efforts to unravel the defining characteristics of the “tolerogenic” versus a “stimulatory” DC have recently shown that certain types of DCs play an essential role in inducing and modulating regulatory T cells (Roncarolo et al., 2001).

In contrast to the immunostimulatory properties of DCs, it has been shown that certain types of DCs like liver-derived DCs, IL-10 modulated DCs (Steinbrink et al., 1997), or CD8α lymphoid derived DCs, are able to down-regulate immune responses (Jonuleit et al., 2001). These DC subsets that are mainly characterised by an immature phenotype might play an important role in peripheral tolerance and prevent autoimmune reactions.

The production of IL-10 and other immuno-suppressive cytokines such as TGF-β, which modulate the function of DCs, has been proposed to be a way of inhibiting autoreactive T-cell responses by converting immature DCs to “tolerizing” APCs. The IL-10 cytokine has immunosuppressive properties on immature DCs resulting in reduction of the upregulation of MHC II molecules and several co-stimulatory and adhesive molecules, thereby modulating the function of immature DCs whereas mature DCs are more resistant to its action. It has been shown that human IL-10 modulated DCs from peripheral blood
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induce alloAg-specific anergy in hemagglutinin or melanoma Ag-specific CD4 and CD8 T cells. This state of anergy was characterised by inhibition of T cell proliferation, and reduced production of IL-2 and IFN-γ (Steinbrink et al., 1999). Therefore it has been suggested that induction of tolerance versus immunity could be determined by the ration between mature/ immature DC populations with the mode of action being either direct downregulation of T cell responses or through IL-10 modulated DCs by inducing T regulatory T cells.

1.4.7 Homeostasis as a regulatory mechanism of peripheral tolerance.

Although regulatory cells can inhibit harmful pathological responses directed against self- or foreign antigens, many aspects of immune regulation remain uncovered and controversial, especially with regard to the antigenic specificities of regulatory cells, and the mechanism of action. The profile (CD25+/CD45RBlow) of regulatory T cells either being anergic or Tr1 or Th3, is similar and more associated to an activated/memory phenotype, than directly associated to the regulatory function itself.

In addition to regulation their responses to cognate antigen, T cells also function in maintaining the size of the peripheral pool, by using tightly regulated homeostatic mechanism. Regulatory cells have also been shown to regulate the expansion of activated memory CD4 T cells compartment (Annacker et al., 2000). Furthermore the characteristics of the regulatory cells and their mode of action have mainly been defined in experimental systems that depend on lymphopenia.

Therefore immune regulation has been proposed (Bonomo et al., 1995; Stockinger et al., 2001) to be the result of a homeostatic peripheral mechanism between activated/memory T cells and self-reactive T cells. In that way any action of T cells in their effector phase irrespective of their specificity may be sufficient to prevent uncontrolled expansion of autoreactive T cells via activation for IL-2 and access to APC (Stockinger et al., 2001).
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1.5 NP47F5 transgenic mouse model.

As the repertoire of TCR specificities in polyclonal mice is enormous, TCR transgenic mice that allow the investigation of the development of T cells with a defined receptor specificity, are used to study the mechanisms which prevent autoimmunity, against those cells of the re-circulating T cell repertoire which are potentially autoreactive.

A common very powerful approach is to examine the immunological outcome in progeny of mice transgenic for a given antigen crossed with mice transgenic for the TCR recognising that antigen. In these (Ag x TCR) F1 systems the T cell repertoire is composed of T cells bearing TCRs which recognise the antigen which is also present, and subsequent activation or tolerisation is amenable to study.

In this study we use an (Ag x TCR) F1 mouse model, the double transgenic NP47RagNegF5 (NP47F5) (Mamalaki et al., 1996) as a system to study the physiology of CD8 T cells rendered unresponsive by a non-deletional tolerance mechanism. In the NP47F5 system the TCR specificity is provided by the F5 TCR transgenic mice (Mamalaki et al., 1993a). These F5 mice carry a transgenic TCR (F5) in all CD8 T cells, which utilises the Vα4,β11 members of the α and β chain variable gene segments, and is specific for an influenza nucleoprotein peptide αα 366-374 (NP) when presented in the context of MHC-I D\(^b\) (Townsend et al., 1986). F5 mice were crossed with mice expressing influenza nucleoprotein, of the A/NT/60/68 influenza virus, under the MHC-I H2-K\(^b\) promoter (H2NP) (Mamalaki et al., 1996). The gene for the nucleoprotein is a deletion mutant (IMP1295) containing the epitope (αα 366-374) recognised by the F5 TCR. Among the different lines of H2NP mice the H2NP47 line used in this study was reported to express the lowest levels of NP, as assessed by the degree of in vitro activation using APC from NP expressing strains. (Mamalaki et al., 1996). Characteristically, in this specific line and therefore in the NP47F5 system, integration of the NP transgene into the Y chromosome, allows expression of the transgene only on male cells.

Deficiency in the recombination activating gene-1 (Rag-1) (Corbella et al., 1994; Spanopoulou et al., 1994) in this strain and in all single transgenic mice used in this study (F5, NP47) excludes the possibility of rearrangements of the endogenous TCR genes that could generate endogenous TCR chains with specificities other than the transgenic ones.
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Therefore all CD8 T cells in the NP47F5 system are exclusively F5 TCR transgenic and NP specific. Thus this system allows the study of tolerance, without any complications from expression of endogenous TCR α chains.

NP47F5 mice are tolerant as they are healthy and show no signs of autoimmunity. Also previous studies have shown that there are peripheral CD8 T cells in the NP47F5 system which display an activated phenotype, evident of contact with antigen in the periphery, but were unresponsive to further stimulation in vitro (Mamalaki et al., 1996). For the particular interest of this thesis, which is to study anergy as a peripheral tolerance mechanism, the NP47F5 is an ideal model that allows to study the physiology of CD8 T cells rendered anergic in vivo.

1.6 Aims of this study.

Usually in systems where antigen is expressed broadly tolerance is initiated in the thymus and subsequently self-reactive T cells that escape central tolerance migrate to the periphery where they are exposed to peripheral tolerance mechanisms like the induction of unresponsiveness. Therefore it was of great importance firstly to investigate whether tolerance in the NP47F5 system is established in the thymus or in the periphery.

Different mechanisms have been described to induce unresponsiveness in peripheral self-reactive T cells that have escaped deletion in the thymus. Of specific interest in anergy induction is the role of different APCs. Classical in vitro anergy has been described to be the outcome of antigen presentation by non-professional APC that lack the expression of co-stimulatory molecules. However this is not always the case of in vitro or in vivo induced unresponsiveness. Also different APCs can affect negative selection in the thymus in different ways, and antigen presentation by non-BM derived APC like thymic cortical epithelium may allow self-reactive T cells to escape deletion especially in cases where low avidity TCR/MHC-peptide interactions occur. Bone marrow chimeras were generated to investigate whether NP47F5 CD8 T cells, escape from negative selection and whether peripheral unresponsiveness in the NP47F5 system is the result of antigen presentation by non-BM derived APC or not.

Unresponsiveness in vivo is vaguely defined and can affect different functions. Apart from the degree of the unresponsive state, anergy has been reported to be a dynamic
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process and in some cases can be reversed. In this aspect F5 CD8 T cells anergized *in vivo* were characterised, for their phenotype, and their functional capacities were assessed both *in vivo* and *in vitro*.

The life span of anergic T cells is not clear. Although in the past anergy was believed to be a step before deletion, recent studies indicate that long-term persistence of anergic cells. This study was therefore focused on the survival characteristics of anergic T cells, determining their life span and investigating whether or not they are capable to compete with other cells for survival both in lymphopenic and in a polyclonal environment. Apart from the fate, the functional status of anergic cells is also a matter in question, and anergic cells may become irreversibly committed to unresponsiveness or alternatively unresponsiveness may recover under appropriate conditions. Therefore the dynamic nature of the anergic state was assessed with particular focus on the requirement of the presence of the antigen for the maintenance of the anergic state.

An important question on anergy as a tolerance mechanism is what is the significance in the maintenance of self-reactive T cells that although in an unresponsive state are potentially autoreactive. Accumulating data support that anergic cells can retain some of their functions and can have a regulatory role on the responses of other cells. Such a function could be a reason why they are maintained. The possibility of NP47F5 anergic CD8 T cells to have a regulatory role on other T cells of the same or of different specificity was investigated.
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2. MATERIALS AND METHODS

2.1 Mice

The mice strains used in this study were F5Rag1\(^{-}\) (Mamalaki et al., 1993a), NP47Rag1\(^{-}\) (Mamalaki et al., 1993b), NP47F5 (Mamalaki et al., 1993b), GFPRag1\(^{-}\) F5 (unpublished), β2m\(^{-}\)/Rag1\(^{-}\) (Zijlstra et al., 1990), as well as syngeneic Rag1\(^{-}\)/H-2\(^{b}\) (Spanopoulou et al., 1994), gcNegRag-/-qq (Di Santo et al., 1995), B10 Nude H-2\(^{b}\) and polyclonal control mice B10 H-2\(^{b}\). All mice were kept in conventional pathogen free, animal facilities at the National Institute of Medical Research.

2.2 Antibodies

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MATERIALS AND METHODS

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<td>RatIgG1 (Isotype control)</td>
<td>PE</td>
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</table>

2.3 Media

The culture medium was Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco BRL, Paisley, Scotland) supplemented with 5% heat inactivated fetal calf serum (FCS) (Gibco BRL), 2x10⁻³ M L-glutamine, 100U/ml penicilin, 100μg/ml streptomycin and 5x10⁻⁵M β-mercaptoethanol (all Sigma, Poole, GB).

Medium for washing cells was air buffered IMDM (Gibco BRL) supplemented with 0.21%NaCl, 100U/ml penicillin, 100μg/ml streptomycin (AB medium).

2.4 Determination of cell viability and cell number.

Trypan blue (Sigma) at a final concentration of 0.08% in phosphate buffered saline (PBS, 10.1g NaCl, 0.32g KH₂PO₄, 1.449gNa₂HPO₄ in H₂O) was used to determine the viability of cells. Cells were counted in a 1:1 mixture of Trypan blue, using a Neubauer...
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counter chamber (BDH Ltd, UK) and light microscopy. Dead cells (stained blue) were excluded from counting.

To determine the absolute number of each thymic subpopulation, the percentage of each thymic subset, as determined by Flow cytometry, was multiplied by the total number of thymic viable cells.

The absolute cells number of peripheral CD8 T cells in spleen or in Lymph Nodes (inguinal, branchial, mesenteric and axillary, pooled together) was calculated as the percentage of positive cells in these organs, as detected by Flow cytometry, multiplied by the total number of viable cells. Finally the absolute cell number of peripheral CD8 T cells was calculated as the total number of CD8 T cells in the spleen plus two times the total number of CD8 T cells in the Lymph Nodes.

2.5 FACS staining

2.5.1 Surface staining and analysis.

Antibodies used in flow cytometry are shown in Table 2.2. Prior to staining, $1 \times 10^6$ cells were preincubated with unlabelled mAb to FcyRII/III (2.4G2) to minimise unspecific staining. Cells were washed and re-suspended in 50μl ice-cold FACS buffer (PBS containing 0.1% sodium azide and 1% FCS), containing an optimal dilution of the appropriate Abs. Cells were incubated for 30 minutes at 4°C. Surface staining was performed with fluorescein-isothiocyanate-(FITC), phycoerythrine-(PE), allophycocyanine-(APC) or/and biotin (BIO)- conjugated monoclonal antibodies (mAb). Cychrome 7-PE streptavidin (Cy-7 PE, Caltag Laboratories, Burlingame, CA) or streptavidin –RED 670 (Gibco BRL) were used as secondary reagents for biotin. For surface staining of peripheral blood lymphocytes, the same procedure was followed with an additional red-blood lysis step at the end. Erythrocytes were lysed after staining upon incubation at room temperature, with 100μl 1% FACS lysing solution (Becton Dickinson). For each sample $2 \times 10^4$ live gated events were analysed. Cell surface antigens expression was determined by analytical four-colour flow cytometry using a FACS Calibur Fow cytometer (Becton Dickinson). Forward and side-scatter characteristics were used to exclude dead cells. FACS data were analysed with Cell Quest software (Becton Dickinson).
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2.5.2 Intracellular staining.

Cells, either ex-vivo or after antigenic stimulation or after PDBU/Ionomycin re-stimulation, were harvested, washed and distributed to micro well plates. Surface staining was performed as described earlier (2.5.1). Following surface staining cells were washed and then fixed with 100μl 1% paraphormaldehyde for 20 min at 4°C. Cells were the permeabilized upon incubation for 3 min at 4°C with 100μl 0.1% NP40 (IGEPAL Sigma) in PBS. After washing in FACS buffer (PBS containing 0.1% sodium azide and 1% FCS) cells were stained with anti IFN-γ mAb FITC or PE conjugated for 30 minutes at 4°C. As negative control cells were stained with IgG-1 PE or FITC conjugated Ab for 30 minutes at 4°C. After staining cells were washed and re-suspended in 100μl FACS buffer prior to flow cytometric analysis. For each sample, 10^4 live-gated events were analysed.

2.6 FACS Cell Sorting.

Pooled spleen and lymph node cells from NP47F5 or F5 or B10 mice were stained with surface mAbs, as described in section (2.5.1). Cells were sorted on a MoFlo cell sorter (Cytomation, Fort Collins). GFP positive cells were sorted directly according to green fluorescent intensity without surface staining, on a MoFlo cell sorter as well.

2.7 Carboxy-Fluorescein-diacetate-Succinimidyl-Ester (CFSE) cell labelling and adoptive transfer.

For CFSE labelling, spleen and lymph node cells were removed and gently pressed through a fine nylon mesh to make single cell suspensions. The cells were resuspended in AB medium and pelleted by centrifugation. The pellet was resuspended, at a concentration of 10^7 cells /ml, in prewarmed PBS (Dulbecco’s) at 37°C. Then the cells were incubated with CFSE (molecular probes, Eugene, OR) at a final concentration of 2.5 μM for 15 minutes at 37°C. For in vivo cytotoxicity assay cells were labelled with CFSE at 5μM or 0.5μM final concentration. Cells were then washed twice with AB medium, resuspended in PBS (Dulbecco’s) and injected into the tail vein of the adoptive host. At different time
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points after injection spleen and lymph node cells from the host mice were isolated and stained with m-Abs for different surface markers. Cells were analysed by FACS as described previously (2.5.1).

2.8 Cultured Bone marrow dendritic cells, as APC.

Bone marrow derived dendritic cells (DC) were generated as described previously (Stockinger et al., 1993) with some modifications. Briefly 2x10^6 bone marrow cells were cultured in 9 cm petri dishes (Nunclon) in 10ml culture medium containing 10% supernatant of Ag8653 myeloma cells transfected with murine granulocyte macrophage colony stimulating factor (GM-CSF) cDNA = 25U/ml. On day 6 of culture non-adherent granulocytes were removed. Loosely adherent cells were transferred onto second dish on day 6 of culture. From day 6 to day 12 these loosely adherent cells were used as a source of dendritic cells for in vitro activation assays.

2.9 In vitro T cell activation Assay

To analyse the proliferation of CD8 T cells either ex-vivo or after adoptive transfer, spleen or lymph node cells were isolated and a fraction of the cells was stained with anti-CD8 and anti TCR Abs. The percentage of CD8 T cells in each sample was determined by FACS. The same number of responder CD8 T cells per well (10^3-5x10^4) of each sample, as determined by FACS analysis, was cultured with 10^4 BM B10 (H-2^b) DCs and with increasing amounts (0-100nM) of NP68 peptide. When the percentage of CD8 T cells in a sample was higher than 2 fold, cells were diluted with RagNegbb lymph node cells, to equalise the total cell number per well between all samples. Cells were cultured in a total volume of 200μl per well of 96 round bottomed well plates. Triplicate cultures were plated of each T cell population tested. Cells were incubated at 37°C in 5% CO_2 for 48 hours. Proliferation of the responding cells was measured as incorporation of ^3H thymidine, given for the last 16 hours. Data reflect the mean of triplicate cultures of each peptide concentration.
2.10 IFN-γ Detection by ELISA.

IFN-γ was detected by standard sandwich ELISA method (Slade and Langhome, 1989). Microtiter plates were coated overnight with capture anti-IFN-γ antibody (AN18) at 10μg/ml at 4°C. Plates were blocked at room temperature with 5% FCS, 5% horse serum in PBS. After washing, 50μl of proliferation cultures supernatant were added neat, for 2 hours at room temperature. The plates were later incubated with 1.25μg/ml of biotin-conjugated R46A2 anti-IFN-γ antibody and were subsequently incubated with streptavidine-horseradish-peroxidase (Southern Technology). The enzyme substrate Azino-bis-3Ethylbenz-thiazoline-6-sulfonic acid (ABTS) was used for colour development. The level of coloured product generated, was measured at 415 nm using an ELISA plate reader (Thermo Systems U.K.). Data reflect the mean of triplicate cultures supernatant from each peptide concentration. Serial dilution in triplicates of 1.4x10⁵ U/ml standard IFN-γ protein was used to develop standard curves for each individual IFN-γ ELISA test.

2.11 CTLL assay

Proliferation assay culture supernatants were collected at 48 hours culture and IL-2 cytokine levels were measured by the growth of IL-2 dependent CTLL T cell hybridoma cell line. 50μl of culture supernatant were transferred into fresh flat bottom 96 microtiter plates, together with 5000 IL-2 dependent CTLL cells / well. Incorporation of ³H thymidine by CTLL, present for the last 9 hours of culture, was measured 24 hours later. Alternatively to ³H thymidine incorporation, an Alamar blue-based (Ahmed et al., 1994) CTLL assay was used in some experiments. In the Alamar blue-based CTLL assay proliferation of CTLL line is measured by changes of the colour of a specific REDOX fluorometric/colorimetric growth indicator alamar-blue using Luminescence spectrophotometer (Perkin Elmer).

In each independent experiment an IL-2 standard curve was used by measuring the growth of IL-2 dependent CTLL cells in 50 μl of culture medium containing 20%-0% IL-2. Data represent the mean of triplicate supernatant from each peptide concentration, or from each IL-2 concentration for the standard curve.
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2.12 Restimulation of T cells Prior to intracellular staining.

In the experiment described in section (3.3.1), total spleen cells were restimulated *in vitro* for 4 hours with PDBU (50ng/ml)(Sigma) and Ionomysin)(Sigma) (50ng/ml) in the presence of 10µg/ml Brefeldin A (Sigma). Brefeldin A prevents exit of newly synthesised proteins from the Golgi compartment. In the experiment described in section (3.3.3), total spleen cells were restimulated for 19 hours with B10 H-2b BM cultured DCs in the presence of 1µM NP68 peptide. Brefeldin A at a final concentration of 10µl/ml was added for the last 4 hours of culture.

2.13 Antigens and immunisations

NP68 9-mer peptide (NP_366-374) (ASNENMDAM) from the nucleoprotein of influenza virus A/NT/60/68, was used for in-vitro activation assays and for in-vivo immunisation. NP47F5 or F5 mice ≥ 6eeks old were injected intraperitoneally for 2 days with PBS or NP68 peptide solution in the same medium.

For virus infection mice ≥ 6eeks old were injected intraperitonealy with 10⁶ PFU of A/NT/60/68 influenza A virus.

2.14 *In vivo* cytotoxicity assay

To prepare target cells for *in vivo* evaluation of cytotoxic activity, cells from C57B/10 spleen suspensions were pulsed with 1µM NP68 peptide for 90 min at 37°C, washed and labelled with high CFSE fluorescence intensity at a final concentration 5µM as described above. Uncoated control target cells were labelled at a low CFSE fluorescence intensity at a final concentration 0.5µM. For i.v. injection 5x10⁶ cells of each population were mixed in 500 µl PBS. Specific *in vivo* cytotoxicity was determined by collecting spleen and lymph node cells from mice 19 hours after injection of the target cells. Detection of the differentially labelled fluorescent target cell population was analysed by flow cytometry. The ratio R between the percentages of uncoated versus NP68 coated (CFSE<sub>low</sub>/ CFSE<sub>high</sub>) was calculated to obtain a numerical value of cytotoxicity.
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2.15 Generation of memory T cells

Spleen and lymph node cells from Tg F5 RagNeg/- (H-2b) mice which contained ~ 5X10^6 NP68 specific Tg F5 CD8 naive T cells per recipient, were transferred together with syngeneic (C57B/10) bone marrow-derived DCs (at a ratio 1:3) into allogeneic adoptive hosts, deficient for the common cytokine γ chain and Rag (gcNegRag-/-qq) (Di Santo et al., 1995). Before transfer DCs were pulsed in vitro with 10^6 PFU of A/NT/60/68 influenza A virus. Activation of F5 D8 T cells occurs by presentation of nucleoprotein (NP) epitopes only by donor-derived APC as F5 CD8 T cells recognise antigen in the context of H-2b. Also the hosts are unable to reject the allogeneic transferred T cells, since their genetic deficiency results in the absence of B, T and NK cells. Memory T cells were isolated > 6 weeks after transfer.

2.16 Generation of BM Chimeras.

Two months old mice GFPF5 or NP47F5 were treated with 1,000 rads of ionising radiation (^60Co), and then reconstituted with 10x10^6 BM total cells from NP47F5 or GFPF5 respectively. Animals were given 2 months recovery period before analysis of the developing transferred cells. After this time period the quality of the chimerism was analysed by FACS for the presence of endogenous or transferred T cells that can be differentiated by the expression or not of GFP. After that time period most of the peripheral T cells were of donor origin (> 95%) for the NP47F5 → GFPF5 chimeras and (>80%) for the GFPF5 → NP47F5 chimeras.

2.17 Magnetic cell separation

2.17.1 Magnetic labelling of cells in suspension for auto-MACS separation.

For direct labelling cells were suspended in MACS buffer (5mM EDTA; 0.5% BSA in PBS ph 7.2-7.4) at 400 μl per 1x10^6 cells. Cells were incubated with 100μl anti-CD8α (Ly-2) or with anti-CD4 or anti-mouse CD11-c MicroBeads (Miltenyi Biotech Ltd.) per 1x10^6 cells for 15 min at 4°C. Then cells were washed in 10-20X labelling volume of buffer.
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for 10 minutes at 300g. For separation, cells were re-suspended in 500μl MACS buffer per 10^8 cells.

For indirect labelling cells were suspended in PBS 2%FCS and incubated with Biotynylated antibodies (F4/80), at 50μl per 1X10^6 cells, for 15 minutes at 4°C. The cells were then washed twice with sterile PBS containing 2% FCS. To select BIO-stained cells the streptavidin MicroBeads (Miltenyi Biotech Ltd.) were used. Cells were re-suspended in biotin-free MACS buffer (5mM EDTA in PBS ph 7.2-7.4) and incubated with 100μl streptavidin microbeads per 10^8 cells for 15 minutes at 4°C. The cells were washed with 10-20X labelling volume of buffer for 10 minutes at 300g. For separation cells were re-suspended in 500μl MACS buffer per 10^8 cells.

2.17.2 Magnetic separation with auto MACS.

Magnetic separation of labelled target cells was performed using positive selection program POSSEL. During this program, the labelled target cells were retained on the autoMACS separation column, which is attached on a magnet, while the negative fraction is released at port neg 1. Subsequently the positive fraction is eluted at port pos 1. In that way there was high enrichment of magnetically labelled target cells. The positively selected cells were unaffected by the magnetic beads and were used for in vitro cultures or for adoptive transfers.

2.17.3 Magnetic separation with Dynabeads.

Depletion of DP and CD4 SP thymocytes from F5 or NP47F5 mice was performed by magnetic cell sorting using CD4 mouse Dynabeads (Dynal, Meyerside, UK). 1X 10^7 Dynabeads per ml were incubated with total thymocytes in ice cold AB medium for 20 minutes at 4°C. The amount of beads was calculated so as the bead to cells target ratio to be below 10, as recommended. After the incubation the rosetted cells were isolated by the use of a magnetic device (Dynal MCP- Magnetic Particle Concentrator). The negatively selected cells in the supernatant were collected and labelled with anti-CD4 and anti HSA mAbs for further depletion of CD4 positive HSA high cells by FACS sorting.
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Depletion of APC from NP47 mice transferred with F5 spleen cells, was performed using biotinylated M5/114 (anti MHC I-A\textsuperscript{b,d,q} and I-E\textsuperscript{b,d,k}) antibody, anti-CD11-C (N418) antibody against DCs, and anti-FcR (2.4G2) mAbs, followed by streptavidin-conjugated dynabeads (Dynal, Meyerside, UK). Spleen cells isolated by enzyme digestion, as described in section (2.18), washed and re-suspended in 50\mu l ice-cold PBS containing 2\% FCS and an optimal dilution of the biotinylated Abs. For surface staining, cells were incubated for 30 minutes at 4°C. Subsequently cells were washed and incubated for 20 minutes at 4°C with 1X 10\textsuperscript{7} ml streptavidin M-280 Dynabeads (Dynal, Meyerside, UK). The amount of beads was calculated so as the beads to total cells ratio to be below 10 as recommended. After the incubation the rosetted cells were isolated by the use of a magnetic device (Dynal MCP- Magnetic Particle Concentrator). The negatively selected cells in the supernatant were collected and used for adoptive transfer.

2.18 Isolation of splenic DCs for antigen presentation assays.

Spleen single cell suspensions were prepared by digesting spleens from NP47 or NP47F5 or RagNegbb mice with a cocktail of 1.6mg/ml collagenase type IV (SIGMA), 0.1\% deoxyribonuclease (DNase-I, fraction IX, SIGMA) in RPMI medium for 45 min. at 37°C. Red blood cells were removed by incubation of the cell pellet with ACK lysis buffer (NH4Cl 0.15M: KHCO3 1.0M: Na2EDTA 0.1M pH: 7.2-7.4) for 5 minutes at room temperature. Following this the resulting cell suspensions were thoroughly washed and filtered and re-suspended in MACS buffer (5mM EDTA; 0.5\% BSA in PBS pH 7.2-7.4) at 400 \mu l per 1x10\textsuperscript{8} cells. Subsequently, anti-mouse CD11-c MicroBeads (Miltenyi Biotech Ltd.) were added at 100 \mu l per 1x10\textsuperscript{8} cells and incubated for 15 minutes at 4°C. Following this time, cells were washed in 10-20 X labelling volume for 10 minutes at 300g. Cells were re-suspended in MACS buffer as 500 \mu l per 1x10\textsuperscript{8} cells and were positively selected using autoMACS and Possel separation program. Both the CD11c positive and negative fractions were collected. Cells of the CD11 c negative fraction were re-suspended in MACS buffer (5mM EDTA; 0.5\% BSA in PBS pH 7.2-7.4) at 50 \mu l per 1x10\textsuperscript{6} cells and incubated with F4/80 Biotynylated antibody for 15 min at 4°C. Following this time, cells were washed in 10-20 X labelling volume for 10 minutes at 300g. Then cells were re-suspended in biotin free MACS buffer (5mM EDTA in PBS pH 7.2-7.4) at 400 \mu l per 1x10\textsuperscript{7} cells.
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Subsequently, streptavidin MicroBeads (Miltenyi Biotech Ltd.) were added at 100 μl per \(1 \times 10^8\) cells and incubated for 15 minutes at 4°C. Following this time, cells were washed in 10-20 X labelling volume for 10 minutes at 300g. Cells were re-suspended in MACS buffer as 500 μl per \(1 \times 10^8\) cells and were positively selected using autoMACS and Possel separation program. The resulting F4/80 as well as CD11c enriched cell populations were stained with F4/80 and MHCII or for CD11c and MHCII antibodies respectively and analysed by FACS. The purity of the selected populations, CD11-c positive and F4/80 positive was typically >70% as determined by flow cytometry.

2.19 Antigen presentation assays

1X10^5 F5 lymph node cells per well were cultured in triplicates, in round bottom 96 well plates, with different numbers of mixed CD11-c and F4/80 positive cells, isolated from NP47 or NP47F5 or from RagNegbb, as described previously (2.18). After 2,4,6 days of culture, IL-2 production in the culture supernatant was assessed with an Alamar blue-based CTLL assay. At the same time points (days 2, 4, 6) cultured cells were stained with anti CD8-APC, CD44-B10, CD69 FITC and CD25-PE mAbs and analysed by FACS for the expression of activation markers on F5 CD8 T cells.
3. RESULTS

3.1 Evidence for a peripheral tolerance mechanism in NP47F5 mice.

3.1.1.1 NP47F5 thymocytes develop normally.

The initial question was to determine the effect of NP antigen expression in NP47F5 mice on the development of F5 thymocytes. Although the antigen (NP), in the NP47F5 system, is expressed under the MHC-I H2-K^ promoter, which is expected to drive ubiquitous expression of the transgene in all MHC-I expressing cells, self-reactive CD8 T cells are present in the periphery of NP47F5 mice, and display an anergic phenotype. This indicates that cells escape deletion in the thymus, either because deletion does not operate or because deletion may occur but is incomplete and spares T cells that migrate in the periphery. Therefore the first issue to be investigated was at what site and at what stage of development tolerance is established in the CD8 compartment of the NP47F5 mice.

To assess whether tolerance is established during thymic development, the phenotype of NP47F5 thymocytes was compared with that of F5 single transgenic mice. In order to test whether central tolerance induction was age dependent or not, mice were divided into two groups. One group was composed of mice aged up to three weeks old and a second group included mice between three and fifteen weeks old. NP47F5 or F5 mice of the two age groups were analysed for the total thymus cellularity, and the absolute cell number and percentage of double negative (DN), double positive (DP), CD4 and CD8 single positive (SP), thymocyte sub-populations. F5 age matched female littermates of NP47F5 mice, were used as single F5 TCR transgenic controls in this experiment. That is because in NP47F5 mice NP transgene is incorporated to the Y chromosome and therefore female mice do not express the NP transgene, however they express normally the F5 TCR.

Comparing total thymic cellularity and the different thymic subpopulations of NP47F5 mice with those of age matched F5 mice, revealed no profound differences that would indicate negative selection. Both in young and older NP47F5 mice all thymic subpopulations were comparable to those in F5 mice of the same age (Fig.3). In some cases there was variation in the cell numbers and percentages within NP47F5 or F5 mice within the same age group. However statistical analysis did not show any significant differences either in the percentage or in the absolute cell numbers in all the different thymic
RESULTS

subpopulations between F5 and NP47F5 mice. This indicates normal T cell development in the thymus of NP47F5 mice. Therefore tolerance induction in the thymus by clonal deletion does not appear to operate in the NP47F5 model at any stage of thymic development either in young or in old mice.

3.1.1.2 NP47F5 thymocytes do not downregulate TCR or CD8 molecules to escape negative selection.

The similarity in the total cell numbers and percentages in the thymocyte populations between the NP47F5 and F5 mice shows that NP47F5 thymocytes undergo normal development and escape deletion by negative selection. If the cells encounter the antigen, in the thymus, then they must apply a mechanism to escape deletion and one potential mechanism is TCR or CD8 downregulation.

The avidity spectrum of TCR-ligand interactions has been shown to influence thymic tolerance induction and any means to reduce the avidity of the interaction, like expression of an endogenous TCRα chain, or TCR or CD8 downregulation could allow self-reactive T cells to escape thymus deletion (Legrand and Freitas, 2001; Zal et al., 1996). The deficiency in the Rag-1 gene in NP47F5 mice excludes the possibility of the expression of endogenous TCR chains. However TCR or CD8 downregulation is a possible way for self-reactive T cells to escape deletion. To test whether self reactive CD8 T cells in the NP47F5 mice can escape negative selection by downregulation of CD8 or TCR molecules or not, thymocytes of NP47F5 mice of different age groups were stained for CD4, CD8 and TCR, analysed and compared to the thymocytes from age-matched F5 mice. The CD4, CD8 profile of the developing thymocytes and the levels of CD8 and TCR expression in the CD8 SP population of the NP47F5 mice are similar to that of the F5 mice (Fig. 4), indicating that TCR or CD8 downregulation is not operating in these mice.

3.1.1.3 Do thymocytes of NP47F5 mice "see" the antigen during development?

Despite the fact that the NP transgene is expected to be expressed on all MHC class I expressing cells in the thymus, self-reactive NP47F5 thymocytes develop normally and escape negative selection without CD8 or TCR downregulation. Therefore the amount of
RESULTS

NP antigen expression may be below a threshold required for negative selection of developing NP47F5 thymocytes.

Messenger RNA for NP has been detected by RT-PCR in the thymus and spleen of NP47 transgenic mice. However the NP47 mice strain has lower levels of NP expression, as seen by reduced ability of NP47 DC and macrophages (Mφ), to activate F5 CD8 T cells, when compared to DC and Mφ of other H2NP transgenic strains (Mamalaki et al., 1996).

To test whether the NP47F5 thymocytes encounter the NP antigen during their development in the thymus or not, the expression levels of the CD44 marker were analysed on the SP CD8 population in the thymus. The NP47F5 CD8 mature SP thymocytes, ex-vivo have low levels of CD44 expression, similar to F5 mice that do not express the antigen (Fig.4 D). This indicates that they have not yet encountered their antigen and further supports the assumption that NP expression in the thymus is so suboptimal that it does not affect thymocyte maturation.

3.1.1.4 CD8 SP thymocytes of NP47F5 mice show normal reactivity to the NP68 peptide in- vivo.

Identical patterns in the cell numbers and TCR and CD8 expression between NP47F5 and F5 CD8 SP mature thymocytes do not necessarily prove that the NP47F5 thymocytes have the same functional capacity. Therefore CD8 SP thymocytes of the NP47F5 mice were analysed for their ability to respond to the NP peptide in vivo.

The expression of CD44 marker is tightly regulated during the maturation of thymocytes. Double positive thymocytes have high levels of CD44 expression and upon expression of TCR β chain the levels of CD44 marker expression drop and remain low, throughout further development (Fehling and von Boehmer, 1997; Killeen et al., 1998). CD44 is an activation marker for mature peripheral and memory T cells and generally upregulation of CD44 expression can be taken as indication of exposure of T cells to their cognate antigen (Budd et al., 1987). As fully mature single positive thymocytes can respond to anti-TCR stimulation similarly to peripheral T cells (Barthlott et al., 1997; Ramsdell et al., 1989; Volkmann et al., 1997), and CD44 marker characterises activated peripheral T cells, firstly it was investigated whether CD44 marker can be used as a reliable activation marker for mature thymocytes.
RESULTS

For that reason, F5 mice were injected intraperitoneally (i.p.) with 50 nmol of soluble NP68 peptide daily for 2 days and the thymus phenotype was analysed on day 4. Intraperitoneal injection of NP68 peptide into F5 mice has been shown to activate thymic and peripheral F5 T cells and to cause deletion of the DP thymic sub-population (Mamalaki et al., 1993b; Tarazona et al., 1998). In this experiment upon intraperitoneal injection of NP68 peptide into F5 mice, apart from deletion in the DP thymocyte sub-population, there was a drastic increase in the proportion of CD44 positive mature CD8 SP thymocytes. (Fig: 5 e, f, g, h). This shows that mature single positive thymocytes can react to the antigen by upregulation of CD44 marker, and that this marker is reliable for mature SP thymocyte activation. Having established that CD44 is a reliable marker for mature CD8 SP thymocyte activation, the same experiment was performed on NP47F5 mice to test whether the CD8 SP thymocytes of the NP47F5 mice are able to respond to the NP peptide in vivo or not.

For this purpose, soluble NP68 peptide (50 nmol) was injected i.p. for 2 days into NP47F5 mice and the CD44 level of expression on CD8 SP thymocytes was analysed. NP47F5 mice injected with the peptide showed deletion of the DP population and increased expression of CD44 marker in the CD8 SP population, similar to what was observed in F5 mice. In PBS injected mice there was no deletion or CD44 upregulation. The extent of deletion in the DP population and CD44 upregulation in the CD8 SP population was the same in the NP47F5 in comparison to the control F5 mice treated in the same way (Fig.5). This indicates that the NP47F5 mature SP CD8 thymocytes are able to respond to the cognate peptide in vivo and have not been anergized like the peripheral NP47F5 CD8 T cells, whose response after the same treatment will be discussed later in section 3.3.1.

3.1.1.5 CD8 SP thymocytes of NP47F5 mice show normal reactivity to the NP68 peptide in-vitro.

Upregulation of CD44 marker on NP47F5 CD8 SP mature thymocytes upon i.p. injection of NP68 soluble peptide, indicates that CD8 SP mature thymocytes are able to respond to cognate antigen in-vivo. However in order to investigate the extent of the functional capacity of these cells to respond to cognate antigen, the proliferative capacity upon antigenic stimulation in-vitro of the NP47F5 SP mature thymocytes was analysed.
RESULTS

Total thymocytes were isolated from NP47F5 or F5 control mice, and were enriched for CD8 SP thymocytes by depletion of DP and CD4 SP thymocyte subpopulations using CD4 Dynabeads. Following that the mature CD8 SP thymic subpopulation was sorted by FACS. Thymocytes were labelled with CD4-PE and HSA (Heat Stable Antigen) -FITC conjugated monoclonal antibodies and the CD4 negative HSAlow population was selected by FACS sorting. In that way a population of >95% pure CD8, HSAlow population was isolated, which comprised of the mature CD8 SP HSA low subpopulation (Bendelac et al., 1992) (Fig. 6, Fig. 7). The isolated mature CD8 SP thymocytes were then activated in-vitro for 48 hrs with H-2b DCs and different concentrations of NP68 peptide. In-vitro antigenic stimulation of NP47F5 CD8 SP mature thymocytes revealed no difference in their capacity to proliferate when compared with the single transgenic F5 CD8 SP mature thymocytes, isolated and treated in the same way (Fig. 8).

In addition, total NP57F5 or F5 control thymocytes were isolated and labelled with CFSE fluorescent dye and stimulated in-vitro with H-2b DCs and 100nM NP68 peptide. The function of the CFSE labelled cells can be assessed by their proliferative response, as measured by flow cytometry. CFSE fluorescent dye penetrates spontaneously cell membranes and upon coupling to intracellular proteins labels the cells stably. When cells divide CFSE labelling is distributed equally between daughter cells and therefore each division of CFSE labelled cells cause 2-fold reduction in mean fluorescence intensity of the daughter cells.

Two days later the cells were stained with CD8-APC mAb, H57-PE (anti-TCR) mAb, and the fluorescence intensity of the CFSE indicative of cell divisions was analysed on the living CD8, TCR positive population (Fig. 9). Also here NP47F5 thymocytes were shown to be able to respond to antigenic stimulation in-vitro in a similar way to F5 single transgenic thymocytes. NP47F5 thymocytes have undergone a similar number of divisions to F5 single transgenic thymocytes after 48 hours antigenic (NP68) stimulation in-vitro, although the kinetics of their response appear slightly slower.

These data collectively demonstrate that the presence of the NP peptide in the thymus does not affect the development of the NP47F5 thymocytes. Therefore NP47F5 CD8 SP thymocytes show normal development and are able to react to NP peptide in vivo and in-vitro. The phenotypic analysis and the functional capacity of the NP47F5 thymocytes
RESULTS

suggest strongly that the observed in-vivo tolerance in the NP7F5 transgenic system must be induced in the periphery.

3.1.2 What is the peripheral tolerance mechanism in NP47F5 mice?

3.1.2.1 Do NP47F5 CD8 T cells escape peripheral deletion?

Several peripheral T cell tolerance mechanisms have been described including deletion, ignorance, anergy, TCR or CD8 downregulation or exhaustion after repeated Ag stimulation. To test whether peripheral deletion sustains tolerance in the periphery or not, and if so to investigate the time of tolerance establishment after migration from the thymus, the phenotype of the peripheral NP47F5 CD8 T cells was characterised.

Two groups of NP47F5 mice, young (< 3 weeks) and old (> 3 weeks) were analysed for the absolute numbers and percentage of CD8 T cells in the spleen or lymph nodes, and compared to that of age matched F5 single transgenic control mice (F5 female littermates). A small but not statistical significant reduction in the peripheral CD8 T cell numbers of the NP47F5 male mice can be seen, in either young or old mice (Fig. 10). The only statistical difference affected the percentage of CD8 T cells in the lymph nodes between F5 and NP47F5 mice. However this difference was not statistical significant on the absolute cell number of CD8 T cells in the lymph nodes between F5 and NP47F5 mice. This small reduction in the percentage of peripheral NP47F5 CD8 T cells in the lymph nodes may indicate deletion but may also be attributed to the sex difference between the NP47F5 male and the female age matched littermates that were used as single TCR transgenic controls. Therefore the difference in the numbers of peripheral CD8 T cells between NP47F5 and F5 mice does not provide strong evidence for deletion operating as major tolerance mechanism in the periphery, although it cannot be completely excluded.

3.1.2.2 What is the function of the peripheral CD8 T cells?

In most cases where self-reactive T cells escape deletion in the thymus, peripheral self reactive T cells were found anergic to exposure to the antigen in-vitro under optimal conditions in the presence of co-stimulation. Since peripheral self-reactive NP47F5 CD8 T
RESULTS

cells were shown to escape deletion both in the thymus and in the periphery the functional status of these cells was analysed.

Total spleen or lymph node cells were isolated from NP47F5 or F5 control mice, and cultured in vitro for 48 hrs with H-2\(^{b}\) syngeneic DCs and different concentrations of NP68 peptide. In-vitro proliferation, IL-2 and IFN-\(\gamma\) cytokines production were severely impaired in NP47F5 peripheral CD8 T cells in comparison to F5 peripheral CD8 T cells (Fig.11). This functional unresponsiveness was evident on peripheral NP47F5 CD8 T cells irrespectively of the age of the mice (data shown only for mice aged between 5 and 8 weeks old). As reported before (Mamalaki et al., 1996), the peripheral T cells of the NP47F5 mice are unable to respond in vitro to their cognate antigen, and display all the typical characteristics of being anergic.

3.1.2.3 Is down regulation of TCR or CD8 a mechanism of peripheral tolerance in the NP47F5 system?

To investigate the time point for establishment of anergy in NP47F5 peripheral CD8 T cells, and to test if downregulation of any of the TCR or CD8 markers after antigenic stimulation is the mechanism of tolerance induction, the following experiment was performed.

Peripheral T cells from the spleen or lymph nodes of NP47F5 of different ages and of age matched F5 control (F5 female littermates) were analysed for CD8, TCR and CD44 levels of expression. F5 mice irrespective of age have peripheral CD8 T cells that express high levels of CD8 and TCR molecules, and low levels of CD44 marker. However, peripheral NP47F5 CD8 T cells are heterogeneous and differ according to the age of the mice.

The peripheral CD8 T cell population of old (> 4 weeks) mice can be divided into two different sub-populations. One is characterised by TCR high and CD44 intermediate phenotype and the other displays a TCR intermediate CD44 high phenotype. The peripheral CD8 T cell population of young mice (< 2 weeks) mice can be also divided into two sub-populations according to the expression of the CD44 activation marker, but is homogeneous as far as TCR expression is concerned. Young NP47F5 mice have peripheral
RESULTS

CD8 T cells that express high levels of TCR but half of the cells display a CD44 high phenotype (Fig. 12).

Therefore in the NP47F5 system early contact with the antigen in the periphery leads to activation of CD8 T cells as judged by high levels of expression of the CD44 surface marker. TCR downregulation is a possible mechanism of tolerance induction that requires longer time of exposure to the antigen. The heterogeneity in the CD8 peripheral population shows that there are cells in an activated status (CD44 high, TCR int.), but also that there are cells not fully activated yet (CD44 int. TCR high). The latter population may be a population of cells that have recently migrated from the thymus, and not been exposed to antigen for a long time. The appearance of such a population of recent thymic emigrants in old NP47F5 mice is not surprising as in F5 transgenic mice there is no age associated thymic atrophy (Aspinall, 1997).
RESULTS

3.1.3 Summary

We have made use of a double transgenic (Ag x TCR) NP47F5 mouse model to examine the physiology of CD8 T cells anergised in vivo. The aim of the experiments described in this part of the result section was to determine whether tolerance in the NP47F5 system is induced in the thymus or in the periphery. In summary we have found the following.

Thymus
- NP47F5 thymocytes undergo normal development.
- Escape from deletion of self-reactive NP47F5 thymocytes is not due to down regulation of TCR or CD8 molecules.
- Mature SP CD8 thymocytes display a naïve phenotype, which indicates that they do not recognise antigen in the thymus. NP expression in the thymus may be suboptimal to induce deletion or anergy.
- The function of mature CD8 SP thymocytes is normal as they show normal response to antigenic stimulation both in vitro and in vivo, which shows that tolerance is not induced in the thymus.

Periphery
- Peripheral NP47F5 CD8 T cells escape deletion.
- NP47F5 peripheral CD8 T cells have impaired ability to respond to antigenic stimulation in vitro by proliferation and cytokines production.
- The majority of peripheral NP47F5 CD8 T cells display an activated phenotype showing that they encounter antigen in the periphery.
- Peripheral CD8 T cells in NP47F5 mice become tolerant by inducing unresponsiveness to self-antigen.
RESULTS

Figure 3 Thymus subpopulations.

The absolute cell numbers and percentage of total thymocytes (A) and of each phenotypically defined subpopulation within the thymus of NP47F5 and F5 (B-G) at different ages are presented. Each point is the result of a single animal and between three and nine animals are used in each group. Mean values ± standard error and P values are displayed.
Figure 3
Figure 4: TCR and CD44 phenotype of CD8-SP mature thymocytes of NP47F5 mice.

Thymocytes of NP47F5 mice were isolated and triple labelled with m-Abs for CD8, CD4 and TCR (Vβ11) or CD44. Dot plot analysis represent CD4, CD8 expression of thymocytes from NP47F5 (A) and F5 (B) mice. The levels of TCR (C) and CD44 (D) expression of CD8-SP thymocytes are presented as mean fluorescence histogram overlays between NP47F5 (blue line) and F5 (red line).
RESULTS

Figure 5 CD8 thymocytes from NP47F5 mice show normal reactivity to the NP peptide in-vivo.

NP47F5 or F5 mice were injected intraperitoneal for 2 days with PBS or 50 nmol NP68 peptide. On day 4 thymocytes were isolated and stained with m-Abs for CD4, CD8 and CD44 molecules. Two-colour dot plot analysis shows CD8-CD4 expression of NP47F5 or F5 thymocytes injected with PBS alone (a, e) or with NP68 (b, f) respectively. Mean fluorescence histograms show expression levels of CD44 on CD8 SP thymocytes of NP47F5 or F5 mice injected with PBS alone (c, g) or NP68 (d, h) respectively.
Figure 5
Figure 6: Experimental approach for isolation of CD8 mature SP thymocytes and in-vitro antigenic stimulation.

Total thymocytes were depleted from DP and CD4 SP thymic subpopulations using CD4 Dynabeads. The remaining thymocytes mainly DN and CD8 SP were double labelled with anti-CD4 PE mAb and anti-HSA-FITC mAb and the mature CD8 SP subpopulation was sorted by FACS selecting for CD4 negative, HSA low cells. Sorted cells were then cultured in vitro with H-2b DCs and NP68 peptide for 48 hours.
Figure 7: Isolation of CD8 mature SP thymocyte populations.

This figure shows the efficiency of the approach for the isolation of SP mature CD8 thymocytes, after the first step, which is the depletion of DP thymocytes, and after the FACS sorting for CD4 negative HSA low population. Dot plots represent CD4 and CD8 T cells after each step and the percentage of SP CD8 T cells is displayed.
Figure 8: **NP47F5 thymocyte in-vitro reactivity.**

NP47F5 (blue line) or F5 (red line) SP mature CD8 sorted thymocytes were cultured with H-2\textsuperscript{b} DCs and NP68 peptide for 48 hours. Proliferative response was measured by \textsuperscript{3}H thymidine incorporation. Each point represents the mean of triplicate samples.
Figure 9: **Normal proliferation of NP47F5 total thymocytes upon in-vitro Ag challenge.**

Total NP47F5 (A) or control F5 (B) thymocytes were labelled with CFSE and stimulated in-vitro with H-2\(^{b}\) DCs and 100nM NP68 peptide for 48 hours. Thymocytes were then labelled with m-Abs for CD8 and TCR (H57). CD8 and TCR positive living cells were analysed with FACS for CFSE fluorescence intensity.
RESULTS

Figure 10 NP47F5 peripheral CD8 T cell numbers.

The absolute CD8 T cell numbers and percentage of total spleen or lymph nodes of NP47F5 and F5 (A-E) at different ages are presented. Each point is the result of a single animal and between three and nine animals is used in each group. Mean values ± standard error and P values are displayed.
Figure 10

Mice > 3 weeks  Mice < 3 weeks  Mice > 3 weeks  Mice < 3 weeks

TOTAL PERIPHERAL CELLS

LYMPH NODES

SPLNEE
Figure 11: **Function of peripheral CD8 T cells of NP47F5 mice.**

Spleen cells of NP47F5 (blue line) or F5 (red line) mice were cultured with H-2b DCs and NP68 peptide for 48 hours.

- Proliferative response was measured by ³[H] thymidine incorporation.
- IL-2 response was assayed by proliferation of the IL-2 dependent CTLL line.
- IFN-γ cytokine secretion was detected by ELISA of culture supernatant after 48 hours stimulation.

Each point represents the mean of triplicate samples.
Figure 12: TCR and CD44 phenotype of CD8-SP peripheral CD8 T cells of NP47F5 mice.

Spleen cells from young (A, B, C) or old (D, E, F, G) NP47F5 or F5 mice were isolated and stained with m-Abs for CD8, TCR (Vβ11) and CD44. Dot plots analyses represent CD8 and TCR expression levels. The levels of CD44 expression on total CD8 T cells are presented as mean fluorescence histogram overlays for NP47F5 (blue line) and F5 (red line).

Panel G represents the levels of CD44 expression on different subpopulations of total CD8 T cells in old NP47F5 mice, as defined by the levels of TCR expression (D). R1 region (grey filled line) defines total CD8 T cell population, R2 region (purple line) shows TCR highCD8 T cells and R3 region (yellow line) shows TCR intermediate CD8 T cell population. The histogram shows mean fluorescence intensity of CD44 expression.
3.2 Ag in the NP47F5 system is presented by bone marrow derived APC.

3.2.1 Introduction.

Different types of APC play an important role in tolerance induction, as they carry and present information on their surface that guide T cell development in the thymus and initiate T cell responses in the periphery. Expression of various surface molecules on APC subsets, such as co-stimulatory molecules, fine-tunes a T cell response.

In the thymus the fate of a developing T cell and the choice between positive or negative selection may depend on the nature of the stroma cell that present a T cell ligand. DCs and medullary epithelial cells can induce negative selection but the role of cortical epithelium in that process is unclear. Self-reactive T cells that display low avidity of TCR-MHC-peptide ligand interaction may escape deletion in the thymus.

According to the two-signal model a T cell will become activated when its TCR recognises the cognate MHC-peptide ligand on the surface of a professional APC that also express a second co-stimulatory signal. Such types of APC are bone-derived cells including DCs, B cells, macrophages and T cells. However TCR engagement in the absence of co-stimulation can result in anergy. Consistent with this prediction is the observation that transgenic expression of an allo-MHC II molecule on pancreatic islet β cells, which do not provide co-stimulatory signals, induces anergy in the cognate CD4 T cell population (Burkly et al., 1990). However this is not always the case as even when Ag is expressed on professional APCs anergy can still be induced by other mechanisms such as CTLA-4 signalling. This raises the question whether or not in the NP47F5 system self-reactive T cells escape from negative selection and are rendered unresponsive in the periphery due to antigen presentation by non-professional APC. The following experiments were set up to address the question which type of APC express and present antigen in the NP47F5 system.
3.2.2 Experimental approach.

To ascertain whether NP47F5 CD8 T cells escape deletion in the thymus and are being tolerised in the periphery as a result of NP expression and presentation by BM-derived cells or epithelial cells or both, a set of BM chimeras were established. These chimeras were constructed so that F5 CD8 T cells would develop in mice where NP antigen presentation would be restricted to either bone marrow derived or non-BM derived cells.

To target NP antigen expression to non-BM-derived APC, bone marrow from GFPF5 animals was used to reconstitute lethally irradiated NP47F5 transgenic animals (I: GFPF5 (BM) → NP47F5). Conversely to follow the development of F5 T cells in mice with NP expression targeted to BM derived cells; bone marrow from NP47F5 mice was used to reconstitute lethally irradiated GFPF5 transgenic animals (II: NP47F5 (BM) → GFPF5). (Fig. 13)

The advantage of this approach is the fact that the efficiency of the chimerism can be assessed with the use of GFP-F5 mice, as BM donors in the first case or as host mice in the second case. GFP-F5 mice are F5 TCR transgenic and transgenic expression of green-fluorescent protein (GFP) driven by the human CD2 promoter labels all CD8 T cells permanently green (unpublished data). Therefore in both cases the green fluorescence provides a tool to discriminate between donor and host cells.

In the case where NP47F5 mice were lethally irradiated (1000 Rad) and reconstituted with GFPF5 BM cells, to target expression of NP antigen on non-BM derived APC, efficient reconstitution required 8 weeks after the transfer of GFPF5 BM cells. At that time the developing GFPF5 T cells comprised around 83% of the total peripheral CD8 population. These cells displayed a naïve phenotype judged by the characteristic expression of the low levels of CD44 activation marker. (Fig. 14 A, C, E). When these cells were recovered and activated in vitro for 2 days with H-2<sup>b</sup> DCs and different concentrations of NP68 peptide, they showed normal reactivity in proliferation and cytokines production (Fig. 14 G, H). This demonstrates that NP antigen expression on non-BM derived cells does not lead to T cell recognition. That could be either because there is no expression at all, or because antigen expression and presentation by these cells is below a threshold required for the activation of the donor T cells. The function of the donor T cells is not affected, as they are able to respond to antigenic stimulation in vitro.
RESULTS

On the other hand, where GFPF5 mice were lethally irradiated and reconstituted with NP47F5 BM cells, to target antigen expression to BM cells, efficient reconstitution also required 8 weeks, as judged by the fact that the developing NP47F5 T cells comprised around 95% of the peripheral T cell population. In this situation the developing peripheral CD8 T cells displayed an activated phenotype, as judged by the characteristic upregulation of CD44 activation marker. (Fig. 14 B, D, F) When these cells were recovered and restimulated in vitro for 2 days with H-2b DCs and different amounts of NP68 peptide they had impaired ability to respond to antigenic stimulation by proliferation and IL-2 cytokine production. (Fig. 14 G, H)

Therefore, the activated phenotype and the functional unresponsiveness to antigenic stimulation in vitro that characterises the peripheral CD8 T cells in NP47F5 system is displayed only in chimeric mice that express the antigen on BM derived APCs. In conclusion, the cells responsible for antigen expression and presentation in the NP47F5 system, are BM derived APCs such as DCs and macrophages, or T cells, since there are no B cells in Rag-/- mice.

3.2.3 Is there T-to-T cell antigen presentation in the NP47F5 system?

Although DCs are the most efficient BM derived type of APC for antigen presentation, T cells that also originate from the BM could potentially act as antigen presenting cells. CD8 T cells are non-professional APC and in vitro studies have shown that T-to-T cell antigen presentation can induce unresponsiveness (Lamb et al., 1983). For that reason we investigated whether unresponsiveness of peripheral NP47F5 CD8 T cells is the result of T-to-T cell antigen presentation.

To test that, CD8 T cells were isolated from the spleen and lymph nodes of NP47F5 and F5 mice using fluorescent cell sorting. CD8 positive T cells from F5 mice were labelled with CFSE dye and transferred alone or with the same number of CD8 positive T cells from NP47F5 mice, into β2MNegRagNeg bb hosts. These hosts were chosen because they do not have endogenous T cells and because their deficiency in MHC I expression does not allow cross presentation of the antigen by endogenous DCs to the naïve injected CD8 T cells (Zijlstra et al., 1990). Thus, in this case the F5 CD8 T cells, co-injected with NP47F5 CD8
RESULTS

T cells could only be activated if they recognised antigen presented by NP47F5 CD8 T cells.

Thirteen days after injection, CFSE labelled F5 CD8 T cells from spleen and lymph-node cells of the hosts were analysed for activation, as judged by up-regulation of the CD44 marker and the number of divisions. F5 CD8 T cells could be isolated, on the basis of high CFSE fluorescence intensity, to distinguish them from the NP47F5 CD8 T cells that were not CFSE labelled. F5 CD8 T cells, co-transferred with NP47F5 CD8 T cells, had undergone the same number of divisions (1-2 divisions) and displayed the same low levels of CD44 expression as F5 CD8 T cells that were transferred alone in the same host (Fig.15). This indicates that there is no T-to-T cell antigen presentation between NP47F5 and F5 CD8 T cells. The limited number of divisions of F5 CD8 T cells seen in MHC I deficient hosts are possibly cytokine (IL-7) and not TCR mediated.
3.2.4 Summary

Using BM chimeras to target expression of NP antigen to bone marrow or non-bone marrow derived cells, we found that an activated phenotype and *in vitro* unresponsiveness to Ag stimulation was displayed only on T cells developing in mice that express the antigen on BM derived APC.

- Therefore we conclude that NP antigen in the NP47F5 system is functionally expressed only on BM derived APC like DCs and Mφ.

This excludes the possibility that escape from negative selection of self reactive NP47F5 CD8 T cells in the thymus is due to Ag presentation on non-BM derived stromal APCs.

Also in the NP47F5 system unresponsiveness induction in the periphery is not due to Ag presentation by non-professional, non-BM derived APC that lack the expression of co-stimulatory molecules.

Using adoptive co-transfer of F5 and NP47F5 CD8 T cells into β2MnegRagNegbb lymphopenic hosts to exclude the possibility for Ag cross-presentation by endogenous DCs, it was shown that there is no T-to-T cell antigen presentation from NP47F5 to F5 CD8 T cells, possibly because NP47F5 CD8 T cells express suboptimal amounts of antigen.

- Therefore also T to T cells antigen presentation in the NP47F5 system is excluded from being a mechanism that renders peripheral NP47F5 CD8 T cells unresponsive.
Figure 13: **Experimental approach to follow F5 T cell development in BM chimeras.**

Generation of BM chimeras to target expression of NP antigen to different types of APC to follow the development and function of F5 T cells when they meet antigen on different cell types.
RESULTS

Figure 14 Analysis of peripheral CD8 T cells of chimeric mice.

Peripheral T cells from type I or II chimeric mice described at the top of the figure (one out of 5 mice of each type is shown) were isolated and stained with anti-CD8 APC, anti-H57-PE and anti-CD44 BIO mAbs. Dot blot analyses represent CD8 TCR expression levels (A, B) or CD8 GFP levels (C, D). The levels of CD44 expression on donor CD8 T cells are presented as mean fluorescence histograms.

Peripheral CD8 T cells from each type (I, II) of chimeric mice were isolated and activated in vitro for 2 days with H2b DCs and different amounts of NP68 peptide.

Proliferative response was measured by $^3$H thymidine incorporation (G) and the IL-2 response was assayed by proliferation of the IL-2 dependent CTLL line using the Alamar-blue assay (H). The results of in vitro stimulation are shown for cells of two out of five chimeric mice and each point represents the mean of triplicate samples.
CHIMERAS

IRR. RECIPIENTS
DONORS
Ag EXPRESSION

<table>
<thead>
<tr>
<th>CHIMERAS</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRR. RECIPIENTS</td>
<td>NP47F5</td>
<td>GFP F5</td>
</tr>
<tr>
<td>DONORS</td>
<td>Ag expression on NON-BM derived APC (Epithelial cells)</td>
<td>Ag expression on BM derived APC (DC, Macrophages)</td>
</tr>
</tbody>
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A

CD8

TCR

B

CD8

GFP

CD44 ON DONOR CD8

C

D

R1 79.44%

R1 74.94%

R3 15.49%

R3 94.71%

R4 83.28%

R4 2.64%

E

R4 MEAN 4.83

F

R3 MEAN 43.71

G

PROLIFERATION

H

IL-2

Proliferation

CD44

Figure 14
Figure 15: **NP47F5 CD8 T cells do not present the NP peptide to normal naïve F5 CD8 T cells.**

F5 sorted CD8 T cells were CFSE labelled and transferred alone or with the same number of NP47F5 sorted CD8 T cells into β2MnegRagNegbb hosts. Thirteen days after transfer, lymph node cells of the host were harvested and stained with m-Abs for CD8, TCR and CD44 molecules, and analysed with FACS for CFSE fluorescent intensity. Histogram overlays represent CD44, levels of CD8 CFSE positive cells (C). Histograms represent CFSE levels of CD8 T cells (A, B).
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3.3 Peripheral CD8 T cells from NP47F5 are heterogeneous with respect to antigen reactivity.

3.3.1 Evidence for the presence of Ag-reactive T cells

The fact that only a fraction of the peripheral NP47F5 CD8 T cells have an activated phenotype (CD44^high) and that there are some non-activated CD8 T cells (CD44^int.) as shown in section 3.1.2.3, shows that the peripheral NP47F5 CD8 population is phenotypically heterogeneous. This raises the question whether or not this phenotypic heterogeneity reflects to heterogeneity of the tolerance status of these cells as well. Therefore, we investigated whether or not there are any peripheral NP47F5 CD8 T cells that are still able to respond to the antigen.

NP47F5 or F5 control mice were injected i.p. for 2 days with NP68 peptide or PBS. Peripheral T cells from these mice were analysed 4 days later for CD8, TCR and CD44 markers. Upon injection of the peptide all peripheral CD8 T cells from the F5 mice were activated and had upregulated CD44 marker. NP47F5 mice as evident in the PBS injected control group, have some CD8 T cells in the periphery with high CD44 levels, but they also contain CD8 T cells with low CD44 levels. Upon injection of the peptide, within 4 days, all the CD8 T cells of the NP47F5 mice had upregulated CD44 marker (Fig. 16). Therefore peripheral CD8 NP47F5 T cells that displayed a non-activated phenotype (CD44^int.) were able to respond to in vivo antigenic stimulation upon injection of soluble NP68 peptide by further upregulation of the CD44 activation marker.

In order to further investigate the functional status of the different peripheral CD8 subpopulations of the NP47F5 mice, and to investigate whether or not there are effector cells among the anergized ones, peripheral NP47F5 CD8 T cells were assessed for their capacity to produce IFN-γ ex vivo, as indication of their functional status.

As this analysis aimed to investigate whether or not there is any functional difference between the peripheral NP47F5 CD8 sub-populations, an intracellular cytokine staining method to detect cytokine expression in individual cells was applied, which would allow us to compare the different CD8 sub-populations for cytokine production. Intracellular
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cytokines staining in contrast to any methods detecting cytokines in the supernatant would give information about the identity of the cytokine producing cells.

Peripheral lymphocytes from NP47F5 or F5 as control were isolated and restimulated in vitro for 4 hours with PDBU, and Ionomycin in the presence of Brefeldin-A; The latter prevents exit of newly synthesised proteins from the Golgi compartment. Following restimulation, the cells were fixed and permeabilized and stained with anti-CD8 APC, anti-TCR FITC anti-CD44 BIO, and anti-IFN-γ PE m-Abs.

Peripheral T cells from F5 mice were negative for IFN-γ expression after a 4 hours PDBU pulse, (Fig.17 B) as expected because this type of restimulation would allow expression of IFN-γ only on already Ag-experienced cells.

A proportion of peripheral T cells from NP47F5 mice (3.7%) were positive for IFN-γ expression after 4 hours restimulation, indicative of being antigen experienced cells (Fig.17 A). When the cytokine producing cells were analysed for the expression of CD44 and TCR molecules, it was revealed that they belong to the CD44 high, TCR int. sub-population (Fig.17 C). However not all of the cells in that sub-population are positive for IFN-γ expression, but rather a small proportion of these cells express IFN-γ cytokine under these conditions.

This finding indicates that when NP47F5 CD8 T cells come in contact with the cognate antigen in the periphery, they get activated and pass through an effector phase, where they can functionally respond and produce cytokines like IFN-γ. Prolonged exposure to antigenic stimulation renders NP47F5 CD8 T cells tolerant by the induction of unresponsiveness to self-peptide. The fact that there are some NP47F5 CD8 T cells able to produce cytokines shows that there are antigen experienced cells in the periphery that are not tolerised yet, and are possibly still in the effector phase.

3.3.2 Generation of anergic F5 CD8 T cells without thymus output.

In the NP47F5 system continuous Ag presence in the periphery and continuous export of naïve T cells from the thymus generates a population of peripheral CD8 T cells that are heterogeneous. The majority of the cells are in an unresponsive state, however there are cells recently activated, still in the effector phase, able to respond to the antigen. As there are no phenotypic means to distinguish between the cells that are tolerised and the
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effector counterparts, it is difficult to study the anergic population directly in the NP47F5 system. To overcome this problem and to generate a more homogeneous population of anergic T cells, an adoptive transfer approach was undertaken. Peripheral F5 CD8 T cells were isolated and adoptively transferred into single transgenic NP47 hosts that express the cognate antigen. As these mice are RagNegbb, they have no endogenous T cells. Such an approach has previously been used as a way to generate anergic T cells \textit{in vivo}. (Rocha et al., 1993; Tanchot et al., 2001).

The response of naïve T cells to Ag \textit{in vivo} entails an initial expansion phase, followed by deletion of a significant proportion of the responding cells (Kurts et al., 1997; McCormack et al., 1993; Rocha and von Boehmer, 1991; Webb et al., 1990). Once antigen is eliminated, only a small colony of T cells remain as memory pool, but under conditions of Ag persistence surviving T cells appear functionally unresponsive (anergic) as assessed by proliferation and cytokine production following restimulation \textit{in vitro} (Bhandoola et al., 1993; Pape et al., 1998; Rocha et al., 1993). Therefore the first issue that was investigated in the adoptive (F5-NP47) transfer were the kinetics of the response to the antigen \textit{in vivo}.

F5 CD8 T cells were collected from the spleen and lymph nodes of naïve mice and were transferred i.v. into NP47 mice. 2 x mice were sacrificed 24 hours later to determine the numbers of cells that could be detected in spleen and lymph nodes. These were typically between 5 and 10% of the injected inoculum.

Between day 1 and day 10 cells began to proliferate and enter an expansion phase characteristic of effector cells. However after day 10 T cell recovery began to decline, suggesting either the onset of apoptosis due to antigen induced cell death, or migration to other tissues. By day 20 the cell loss in this second phase of the response was completed and thereafter the recovery of cells remained constant, indicative of a stable peripheral CD8 T cell population (Fig.18).
3.3.3 Phenotypic and functional analysis of peripheral CD8 T cells after transfer into NP47 Ag expressing hosts.

Having analysed the response to the antigen upon transfer of naïve F5 Tg T cells to NP47 Ag expressing hosts, the phenotype and the function of the cells in the stable stage 20 days after transfer, was studied. Peripheral cells from spleen and lymph nodes from naïve F5, as well as NP47F5 were isolated and analysed for the expression of activation markers and compared to F5 T cells anergised after transfer to NP47 mice for at least 20 days.

Twenty days after transfer into NP47 mice peripheral F5 CD8 T cells display an activated phenotype CD44 high, CD62-L low, but in comparison to NP47F5 CD8 peripheral T cells, the transferred cells form a homogeneous population where all the cells have high levels of CD44 expression. Also 20 days after transfer F5 CD8 T cells have downregulated the expression of CD69 early activation marker (Fig.19). This indicates that the cells have been exposed for long time to antigenic stimulation.

Characteristically F5 peripheral CD8 T cells after transfer into NP47F5 mice express low levels of CD25 (IL-2 R) surface marker similarly to peripheral NP47F5 CD8 T cells. F5 peripheral CD8 T cells after transfer into NP47 mice were also analysed for the expression of CD5 surface marker. CD5 is a monomeric glycoprotein, member of the cystein rich receptor family, constitutively expressed on mature T lymphocytes. This surface molecule has been reported to regulate negatively naïve CD4 T cell responses to peptide MHC ligand (Smith et al., 2001) and to regulate negatively Ig receptor signalling in anergic B cells (Hippen et al., 2000). Interestingly after transfer to NP47 mice F5 CD8 T cells express higher levels of CD5 surface marker compared to naïve F5 CD8 T cells (Fig.19).

When the F5 CD8 T cells after transfer into NP47 mice were compared with naïve F5 peripheral CD8 T cells for the expression of TCR and CD8 molecules, the surface levels of expression were comparable, with only a few (≤ 5%) transferred cells to have lower surface expression of TCR molecules. (Fig.20 A, B).

To characterise the function of peripheral CD8 T cells after transfer into NP47 mice, total spleen or lymph node cells from NP47 host mice were isolated at different time points after injection of F5 CD8 T cells and cultured in vitro for 48 hrs with H-2b syngeneic DCs and different concentrations of NP68 peptide. Upon in vitro antigenic stimulation, the
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recovered F5 T cells after transfer into NP47 mice, showed the characteristic unresponsive condition of strongly impaired proliferation, IL-2, IFN-γ cytokines production, when compared to naïve F5 CD8 T cells (Fig. 20 C, D, E).

To further investigate the functional status of the peripheral CD8 upon transfer into NP47 mice, cells were assessed for their capacity to produce IFN-γ upon in vitro antigenic stimulation. As control the same experiment was performed on peripheral CD8 cells from NP47F5 mice or on control naïve F5 and on memory F5 peripheral CD8 T cells.

Analysis of the ability of peripheral NP47F5 CD8 T cells to express IFN-γ cytokine per cell basis by intracellular staining, confirmed that there was a small population (9.93% IFN-γ positive cells) (Fig. 21 C) of NP47F5 CD8 T cells able to express IFN-γ cytokine in response to a 19 hours NP68 in vitro antigenic stimulation In comparison to naïve or memory cells that are able to respond to such an antigenic stimuli (32% IFN-γ positive cells) (Fig. 21 A, B) the percentage of NP47F5 CD8 T cells, able to respond under these conditions by expression of IFN-γ was greatly reduced.

In vitro antigenic stimulation, of F5 CD8 T cells recovered from NP47 hosts, showed that these cells had impaired capacity (3% IFN-γ positive) (Fig. 21 D) to express IFN-γ in comparison to naïve or memory F5 cells, as judged by intracellular staining. The slightly increased capacity of NP47F5 CD8 T cells to express IFN-γ upon in vitro antigenic stimulation, in comparison to F5 CD8 T cells recovered from NP47 hosts, may be due to peripheral NP47F5 CD8 T cells that appear as naive and are not tolerised yet. These data indicate that in the adoptive transfer system there are no cells able to respond to in vitro antigenic re-stimulation and that all cells are in the same functional status. That further supports that chronic exposure to antigen in the absence of thymic export generates homogeneous T cell population that appears anergic.
3.3.4 Summary

NP47F5 peripheral CD8 T cells were shown to be heterogeneous and contain a subpopulation of cells, that are able to respond to antigenic stimulation \textit{in vivo}, possibly recent thymic emigrants that were not tolerised yet.

An adoptive transfer approach of peripheral F5 CD8 T cells into NP47 Ag-expressing host without endogenous T cells was applied in order to generate a homogenous anergic peripheral CD8 T cell population. Peripheral CD8 T cells in that system, display an activated phenotype and have all the functional characteristics of being unresponsive to antigenic stimulation \textit{in vitro}, and therefore are characterised as being anergic.

Analysis of the response of naïve F5 CD8 T cells after adoptive transfer to NP47 hosts show that upon antigenic stimulation \textit{in vivo} cells pass through an effector expansion phase, followed by a short deletion phase after which a population of cells that has escaped deletion remains in an unresponsive state.
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Figure 16 NP47F5 CD 8 peripheral lymphocyte reactivity to soluble NP68 peptide in-vivo.

NP47F5 or F5 mice were injected intraperitoneal for 2 days with PBS or 50 nmol NP68 peptide. On day 4 peripheral lymphocytes were isolated and stained with m-Abs for TCR, CD8 and CD44 molecules. Two-colour dot plot analyses shows CD8-TCR expression of NP47F5 or F5 thymocytes injected with PBS alone (A, E) or with NP68 (B, F) respectively. Mean fluorescence histograms show expression levels of CD44 on CD8 peripheral lymphocytes of NP47F5 or F5 mice injected with PBS alone (C, D) or NP68 (G, H) respectively.
Figure 17: A subpopulation of NP47F5 peripheral CD8 T cells can express INF-γ cytokine.

NP47F5 or F5 peripheral lymphocytes were isolated and re-stimulated in vitro with PDBU and Ionomycin in the presence of Brefeldin-A for 4 hours. Following that time surface staining for CD8, TCR and CD44 surface markers was performed. Afterwards the cells were fixed and permeabilised and intracellular staining for INF-γ expression was performed. Dot blots represent TCR and INF-γ expression on NP47F5 or F5 cells (A, B respectively), or on NP47F5 TCRint. CD44high cells E. TCR and CD44 expression is represented by dot blots on NP47F5 INF-γ positive cells (panel C), or on total NP47F5 CD8 T cells (panel D).
Figure 18: **Kinetics of response of F5 CD8 T cells after transfer to Ag bearing NP47 host**

A total of 10 x10⁶ F5 CD8 T cells were transferred into NP47 mice. Results represent the absolute F5 CD8 T cells recovered from NP47 mice evaluated as described at section (2.4), at different time points after transfer starting at day 1.
Figure 19: **Phenotypic analysis of F5 CD8 T cells after transfer to Ag bearing NP47 hosts.**

Expression of CD44, CD25, CD62-L, CD69, CD5 cell surface markers on F5 CD8 T cells after transfer to NP47 mice (yellow line) in comparison to naïve F5 (red line) or NP47F5 (blue line) or F5 memory (green line) CD8 T cells are shown as of mean fluorescent overlays gated on CD8 T cells.
Figure 20: Phenotypic (TCR, CD8) and functional analysis of F5 CD8 T cells after transfer to Ag bearing NP47 host.

Expression levels of CD8, TCR molecules are shown as density plots of naïve F5 peripheral T cells or of F5 T cells after transfer to NP47 mice for at least 20 days (A, B respectively). The response of transferred F5 T cells (blue line) or control naïve F5 T cells (red line) to different concentrations of NP68 antigenic stimulation in vitro and in the presence of H-2b DCs, for 48 hours is shown as the result of proliferation assessed by ³H-thymidine incorporation and as the result of IL-2 and IFN-γ secretion in the culture supernatant.
Figure 21: IFN-γ expression of F5→NP47 CD8 T cells upon antigenic stimulation.

Peripheral CD8 cells from NP47F5 (C) mice or on control naïve F5 (A) and on memory F5 (B) or after transfer into NP47 hosts (D), were isolated and activated in vitro for 19 hours with H-2b DCs and 1µM NP68 peptide. For the last 4 hours of stimulation Brefeldin-A was added to the cultures. Cells after culture were stained with anti-CD8, TCR CD44 and IFN-γ mAbs. Dot plots represent IFN-γ and CD8 levels of expression of CD8 T cells.
3.4 Functional characteristics of anergic T cells.

3.4.1 Life span, and survival characteristics of anergic T cells.

The main part of this study was focused on the systematic characterisation of the functional capacities of peripheral CD8 T cells anergised in vivo. The hypothesis that anergic T cells are biologically significant, and that they may have a functional role would require that the cells have a long life span, and that T cell anergy is not a last step before deletion as originally believed. Recent studies indicate that anergic T cells can persist for long a time and that anergy is a mechanism independent of deletion (Mondino et al., 1996; Pape et al., 1998; Rocha et al., 1995).

In the NP47F5 system, mice contain peripheral CD8 T cells, that do not decline with age, indicating that anergic T cells can be maintained for long time, and there is no evidence of deletion taking place in the periphery. However this may be because there is continuous export of T cells from the thymus and therefore deletion is not apparent. In the F5 into NP47 adoptive transfer system cells are maintained for long time, indicating that CD8 T cells anergised in vivo are not on the verge of deletion.

For the assessment of survival characteristics of in vivo anergised CD8 T cells, peripheral lymphocytes from spleen and lymph nodes of NP47F5 or F5 control mice were isolated, labelled with CFSE fluorescent dye and transferred into syngeneic RagNegbb mice (Fig.22). RagNegbb MHC compatible, lymphopénie host mice allow the study of the survival of anergic T cells in the absence of the antigen and in the absence of other competing T cells. The CFSE fluorescent dye in addition allows the assessment of any cell division after transfer.

At three time points after transfer (day 10, 23, 6 weeks) the transferred lymphocytes were recovered from RagNegbb host mice, were stained with anti-CD8, Vβ11 mAbs and analysed by FACS for the presence of CFSE positive CD8 transferred T cells. NP47F5 anergic T cells can persist for at least 6 weeks when transferred into lymhopenic host as seen by the presence of CD8 T cells with high fluorescence intensity, even after 6 weeks of transfer (Fig. 23).
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When T cells are transferred into “empty” lymphopenic hosts undergo a number of cell density dependent homeostatic divisions. These are an active response to cytokines such as IL-7 and to contact with syngeneic-MHC ligands, both of which are available in increased amounts in “empty” hosts. After transfer into RagNegbb host, naïve F5 CD8 T cells undergo a number of homeostatic divisions as indicated by the gradual loss of CFSE fluorescent intensity. Although NP47F5 peripheral T cells have impaired ability to proliferate to antigenic stimulation in vivo, surprisingly they are able to undergo homeostatic divisions when transferred to lymphopenic host in the absence of the antigen, similarly to naïve F5 CD8 T cells (Fig.23).

A difference in the kinetics of homeostatic proliferation can be seen between the NP47F5 and F5 cells upon transfer into RagNegbb Ag free hosts. At 10 days after transfer NP47F5 CD8 T cells have undergone less divisions than F5 control cells. However 23 days after transfer NP47F5 CD8 T cells have undergone even more divisions than F5 controls. 6 weeks after transfer, although the majority of NP47F5 CD8 T cells display kinetics of homeostatic expansion similar to F5 control cells, there is a population of NP47F5 CD8 T cells that has divided many times and has lost all CFSE. Such a population is not apparent in F5 control cells. Comparing the kinetics of the homeostatic expansion between NP47F5 and F5 CD8 T cells, one can see that some of the NP47F5 cells after an initial lag phase are able to undergo the same number of homeostatic divisions as F5 cells. However at later time points NP47F5 cells contain a population of fast dividing cells. This suggests that after an initial lag phase, long exposure to Ag-free lymphopenic environment increase their ability of NP47F5 CD8 T cells for self-renewal. The fast dividing population of NP47F5 CD8 T cells could either be a population of cells that acquired the ability for fast division or it could indicate the presence of a heterogeneous population of NP47F5 CD8 T cells as far as their ability for homeostatic expansion is concerned. However one can not exclude the possibility that the fast dividing cells are cells that respond to some antigen co-transferred by other NP47F5 cells.

The fact that anergic T cells are able to undergo homeostatic divisions indicates that they have not lost their proliferative capacity and they may retain some of their functions. Additionally the fact that after long time in Ag-free lymphopenic condition NP47F5 CD8 T cells acquire increased ability for homeostatic expansion suggests that they may restore some of their functions.
3.4.2 Can anergic cells restore their function in the absence of the antigen?

The finding that anergic T cells can survive for long time and are able to undergo homeostatic proliferation when transferred into syngeneic antigen free lymphopenic hosts, raised the question whether the anergic state can be reversed in the absence of the antigen, as seen in some cases (Alferink et al., 1995; Mondino et al., 1996; Pape et al., 1998; Rocha et al., 1993).

In order to study the functional status of NP47F5 cells after transfer into antigen free lymphopenic hosts, CD8 T cells from NP47F5 or F5 mice that had been adoptively transferred to RagNeg bb host for different time periods, were recovered and activated in vitro with H-2b DCs and different concentrations of NP68 peptide. Proliferation, IL-2 and IFN-γ production were tested 48 hours later as a means of effector function. Twenty-three days after transfer the anergic cells still showed impaired ability to antigenic stimulation. However 6 weeks after transfer to RagNeg bb host the recovered cells had restored their ability to proliferate and secrete IL-2 and IFN-γ to almost the same level as the recovered F5 cells, after in-vitro antigenic stimulation (Fig. 24).

3.4.3 Survival of NP47F5 CD8 T cells in lymphopenic hosts that do not support thymus development.

A potential caveat in the studies showing recovery of function following transfer was the fact that total spleen cells rather than purified CD8 T cells were transferred. Spleen is a peripheral lymphoid tissue that contains many cell types apart from lymphocytes, amongst them also stem cell lymphoid progenitors, which can support thymic T cell development (Hamad et al., 1995; Katsura et al., 1988).

This raised the possibility that spleen cells from the NP47F5 mice contained T cell precursor cells, that after transfer to RagNeg bb host, might have reconstituted the thymus and developed into mature naïve T cells which were not anergic since there was no source of antigen in this host. Upon examination of the thymi of adoptive hosts at the time of analysis I did not detect any DP thymocytes, indicating that there was no thymic
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reconstitution. However to further exclude this possibility the same transfers were performed using as hosts B10 nude mice that have an inherited thymus hypoplasia, preventing T cell development and maturation.

Anergic NP47F5 CD8 T cells showed long-term survival up to 6 weeks and were able to undergo homeostatic divisions similarly to normal naïve F5 CD8 T cells also in B10 nude hosts (Fig.25). This result confirms that the long term survival of peripheral NP47F5 T cells represents indeed the survival of mature NP47F5 CD8 T cells and is not due to the development of donor precursor stem cells in the host mice. In this approach recovery of NP47F5 CD8 T cell function after 6 weeks of transfer was also detected similarly to the previous approach (data not shown).

3.4.4 Survival and function of homogeneous population of anergic CD8 T cells after transfer to Ag free lymphopenic host.

As shown earlier, peripheral NP47F5 CD8 T cells are heterogeneous both in their phenotype and functional status. Antigen reactive NP47F5 CD8 T cells that exist among the anergic ones, may preferentially be selected for survival and display homeostatic expansion into Ag free lymphopenic hosts. In order to exclude this possibility, in the assessment of the survival of anergic NP47F5 CD8 T cells, the same approach was followed using as a source of anergic CD8 T cells, F5 CD8 T cells that had been rendered anergic after transfer into NP47 hosts for at least 20 days. As shown earlier this method generates a phenotypically and functionally homogenous anergic population.

Spleen and lymph node cells from NP47 host mice that had been injected with peripheral F5 CD8 T cells 20 days earlier were isolated and stained with anti CD8 mAb. In order to exclude the possibility of transfer of NP47 host cells that express the antigen, the *in vivo* anergised F5 CD8 T cells were FACS sorted, then labelled with CFSE and transferred into RagNegbb host mice. At three time points after transfer (day 10, 20, 6 weeks) peripheral lymphocytes from RagNegbb host mice were isolated and stained with anti-CD8, anti-Vβ11 mAbs and analysed by FACS for the presence of CFSE positive CD8 transferred T cells. Homogeneous anergic CD8 T cells were shown to persist for at least 6 weeks when transferred in lymphopenic hosts (Fig. 26), and similarly to the previous results, anergic T cells can undergo homeostatic divisions into lymphopenic hosts. However the kinetics of
the homeostatic divisions of the anergic F5 CD8 T cells were slower, when compared with naïve F5 CD8 T cells. At the latest time point of 6 weeks after transfer, naïve F5 CD8 T cells have undergone so many divisions that already all have lost CFSE expression. In contrast the majority of the anergic cells are still CFSE positive.

### 3.4.5 Recovery of function of homogeneous anergic T cells after transfer into Ag free lymphopenic host

After the assessment of the survival of homogeneous anergic T cell population in Ag-free lymphopenic host, we went on to study the functional status of this population. At different time points after transfer into RagNegbb host as described in section 3.4.4. CD8 anergic or control naïve T cells were recovered and activated in vitro with H-2^b^ DCs and different concentrations of NP68 peptide. Proliferation, IL-2 and IFN-γ production were tested 48 hours later as a means of effector function. Two days after transfer the anergic cells showed impaired ability to antigenic stimulation. However 6 weeks after transfer to RagNeg bb host the anergic cells had restored their ability to proliferate and secrete IL-2 and IFN-γ to almost the same level as the recovered F5 cells, after in-vitro antigenic stimulation (Fig. 27). This result confirmed that in order for the anergic state to be maintained, continuous presence of the antigen is required.

### 3.4.6 Do anergic CD8 T cells compete with normal T cells for survival and space in the lymphoid niches?

The experiments described before addressed the question of what is the intrinsic ability of anergic T cells for survival. It was shown that anergic CD8 T cells have the ability to survive for long time after transfer into lymphopenic hosts, suggesting that anergic T cells are not predisposed for apoptosis. However the question is whether or not anergic T cells can have a long life span in a more physiological situation where they would have to compete for survival factors and space in the lymphoid organs with other naïve or memory T cells. Such a situation of reduced survival is shown on anergic B cells. Failure of anergic B cells to compete with normal cells for entry into follicles excludes them for...
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survival factors, resulting to their short-term survival (Cyster et al., 1994; Goodnow et al., 1995).

In order to address the question whether anergic T cells can compete with other normal cells for survival factors and space in lymphoid niches, the following approach was taken. Firstly in order to generate a homogeneous anergic CD8 T cell population marked by a fluorescent marker, peripheral lymphocytes from GFP F5 mice were isolated and transferred into NP47 mice. Twenty days after transfer peripheral lymphocytes from NP47 mice were isolated. In order to exclude the possibility that any NP47 cells that would express the antigen were contaminating the anergic CD8 T cell population, the anergised GFP F5 CD8 T cell population, was sorted by FACS selecting, for GFP positive cells. As controls GFPF5 naïve CD8 T cells were isolated by FACS sorting, selecting for GFP positive cells as well. 0.5x10⁶ isolated GFPF5 anergic or naïve CD8 T cells were then co-injected with a population of peripheral lymphocytes of syngeneic B10 polyclonal mice containing the same number of CD8 T cells, into RagNegbb recipient mice. The use of GFPF5 anergised or naïve CD8 T cells in this approach allows the distinction of TCR specific F5 CD8 T cells from the co-injected polyclonal CD8 T cells, due to the expression of high levels of GFP fluorescent intensity.

At two different time points (day 10, day 30) the RagNegbb recipient mice were analysed for the presence of GFP positive anergic or control naïve GFPF5 CD8 T cells. Lymphocytes from spleen or lymph nodes of the host RagNegbb hosts were re-isolated at these time points and stained with anti-CD8 APC, anti-TCR (H57) PE mAbs, and analysed by FACS. Anergic CD8 GFP positive T cells were detected at both time points after transfer with polyclonal CD8 T cells into RagNegbb hosts similarly to control GFP positive F5 naïve CD8 T cells (Fig.28). This result shows that in contrast to anergic B cells anergic CD8 T cells can compete for survival factors and space with normal T cells. Moreover, anergic CD8 T cells have the ability to survive in the presence of polyclonal T cells for as long as naïve T cells under the same conditions.

3.4.7 Survival of anergic T cells in polyclonal hosts.

In the previous approach we could see that anergic T cells could compete with normal polyclonal T cells for survival in lymphopenic RagNegbb host. In most cases of
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Lymphopenia T cells have the ability to undergo homeostatic expansion and anergic T cells, as shown earlier when transferred into lymphopenic hosts are also able to divide and are rescued from the anergic state. This raises the question whether survival of anergised CD8 T cells and effective competition with polyclonal normal T cells is the outcome of their homeostatic expansion and recovery of function in the absence of the antigen.

In order to test whether anergic CD8 T cells can survive and equally compete normal polyclonal T cells without undergoing homeostatic expansion, the following approach was undertaken. Lymphocytes from spleen and lymph nodes of NP47F5 mice were isolated and positively selected using anti-CD8 MACS beads, in order to exclude the presence of antigen expressing NP47F5 cells within the anergic CD8 T cell population. The selected NP47F5 CD8 T cell population (purity ≥ 95%) was labelled with CFSE fluorescent dye and adoptively transferred into B10 syngeneic polyclonal host mice. The same experiment was followed with naïve F5 CD8 T cells, as control.

At different time points after transfer (day 2, day 18, day 48) lymphocytes from spleen and lymph nodes from B10 recipient mice were isolated and stained with anti-CD8-APC, and anti-TCR (Vβ11) BIO mAbs and analysed by FACS for the detection of transferred cells.

Using this approach the anergic population can be distinguished from the endogenous CD8 T cells by the high CFSE fluorescent intensity, which does not diminish as the cells do not divide in “full” hosts in the absence of antigen. Anergic NP47F5 CD8 T cells could be detected in the polyclonal recipients up to 48 days, which is as long as the control naïve CD8 T cells. Although there is a difference in the percentage between NP47F5 and F5 CD8 T cells at days 18 and 48, this could be the reflection of the difference in the starting number cells that could be detected in spleen and lymph nodes of the host mice on day 2, however one should be careful to directly compare the percentage of cells as one mouse was analysed per time point.

The presence of NP47F5 cells into B10 hosts after 48 days of transfer, confirms that anergic CD8 T cells can compete with normal polyclonal T cells for survival for as long as naïve CD8 T cells with the same specificity. This result also demonstrates that the survival of anergic CD8 T cell population does not depend on homeostatic expansion, as anergic CD8 T cells do not divide in “full” hosts as shown by the retention of high CFSE fluorescence intensity (Fig.29).
3.4.8 What are the functional capacities of "anergic" F5 CD8 T cells?

Anergy defines a state of unresponsiveness of antigen-experienced cells to antigenic stimulation as judged by the ability for proliferation and production of effector cytokines. However, in some situations of in vivo induced anergy, although anergic T cells fail to respond to antigenic stimulation in vitro, the degree of unresponsiveness in vivo can vary and may affect only some but not all effector functions.

Usually unresponsiveness restricts the expansion of cells after antigenic stimulation and the production of effector cytokines however in some cases cytolytic activity and production of cytokines are unaffected. (Ehl et al., 2000; Malvey et al., 1998; Vezys et al., 2000; Zajac et al., 1998). When F5 CD8 T cells anergised in vivo, were assessed for effector function upon in vitro restimulation with NP68 peptide in the presence of DC as APC, they show impaired ability for expansion and production of cytokines. However their survival characteristics, long term survival, ability of homeostatic expansion and recovery of function in the absence of the antigen, suggested that these cells might retain some of their functional capacities.

To examine this possibility, that anergic F5 CD8 T cells may maintain some of their effector functions in vivo, their cytotoxic capacity was tested. Most of the assays described to test cytotoxic capacity of T cells like $^{51}$Cr release assay or JAM assay (Matzinger, 1991) assess cytotoxicity in vitro and usually require antigenic in vitro restimulation of the effector population. In order to test the cytotoxic ability of F5 anergic CD8 T cells in vivo an in vivo cytotoxicity assay described by S.Oehen et al was used (Oehen and Brduscha-Riem, 1998).

Adjusting this assay for our system, normal B10 syngeneic spleen cells serving as target cells were pulsed with 1μM NP68 peptide, and were labelled with a high CFSE (CFSE high) intensity. To control for any unspecific elimination of target cells unpulsed B10 spleen cells were labelled with a low (CFSE low) intensity. CFSE high and CFSE low target cells were mixed in a ratio 1:1 and transferred into different type of recipients containing the T cell population tested for cytotoxic capacity. CTL effector function was measured by monitoring the killing of CFSE high cells by flow cytometry 20 hours after transfer (Fig.30).
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In order to check whether anergic F5 CD8 T cells can have any effector function this in vivo cytotoxic test was performed on anergic mice, which were prepared by adoptively transferring $5 \times 10^6$ F5 CD8 T cells into NP47 recipients 20 days before the transfer of the target population (Fig. 31 E). Effector control mice were prepared by adoptively transferring $5 \times 10^6$ F5 CD8 T cells into NP47 recipients for 3 days before target cells injection (Fig. 31 C). At this time point the F5 CD8 T cell population in NP47 host is still at the effector-expansion phase as shown earlier (3.3.2, Fig 18).

Effector mice were also generated by immunization of naïve F5 mice i.p. with $10^6$ PFU of A/NT/60/68 influenza virus 7 days before the target cells injection (Fig. 31 B). The time point of 7 days after immunisation with influenza-A virus was chosen in order to have maximal cytolytic activities, as it has been shown that at days 6-8 post infection maximal cytolytic activity of F5 Tg CTL correlate with reduction of virus in the lungs (Moskophidis and Kioussis, 1998).

It has been reported that environmental pathogens or infectious agents such as viral infections, can break tolerance to peripheral autoantigens, and result in autoimmunity. (Ohashi et al., 1991; Rocken et al., 1992). In order to test whether virus infection could break tolerance and allow previously anergic CD8 T cells to become CTL we tested the cytotoxic ability of F5 CD8 T cells anergised in vivo, after transfer in NP47 mice.

Anergic mice were prepared by adoptively transferring $5 \times 10^6$ F5 CD8 T cells into NP47 recipients 20 days before the transfer of the target population, and were immunised by i.p. injection of $10^6$ PFU of A/NT/60/68 influenza virus. Seven days after immunisation mice were injected with the target population (Fig. 31 F). As effector control mice, NP47 mice, transferred with $5 \times 10^6$ F5 CD8 T cells 3 days earlier (Fig. 31 D), or naïve F5 mice (Fig. 31 B), were immunised i.p. with $10^6$ PFU of A/NT/60/68 influenza virus 7 days before the target cells injection. As negative control, naïve F5 mice were used and injected with the target population (Fig. 31 A).

Figure 31 shows that F5 naïve mice (Fig. 31 A) do not display any cytotoxicity and the ratio between CFSE Low (B10 unpulsed Cells) / CFSE high (B10 NP68 pulsed) remains low ~1. However F5 immunised mice (Fig. 31 B) or NP47 mice containing F5 T cells in the effector phase, immunised or not (Fig. 31 C, D), contained cells that displayed high cytotoxic capacity evident by the increased ratio of target cells. Surprisingly, F5 CD8 T cells “anergised” in vivo after transfer into NP47 Ag expressing hosts, retain their lytic
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capacity in vivo, since they killed peptide pulsed targets not only after virus immunisation but in an unsensitised state (Fig. 31 E, F) as well.

This sensitive in vivo cytotoxicity assay demonstrated that F5 CD8 T cells after chronic exposure to endogenously expressed low levels of antigenic stimulation, although they appear unresponsive in vitro, they retain their capacity in vivo to lyse target cells expressing high amounts of cognate antigen. Therefore the term anergy is not accurate to describe these cells because unresponsiveness is detected only on the particular readouts of proliferation and cytokines (IL-2 and IFN-γ) production after in vitro antigenic restimulation in the presence of costimulation.

Although these cells have the ability in vivo to lyse target cells expressing high amounts of antigen (1μM NP68) the question remains whether or not NP47F5 CD8 T cells are able to lyse endogenous cells expressing very low amounts of antigen. Also another important question regarding NP47F5 CD8 T cells that appear “anergic” in vitro is whether they have any other function in vivo, apart from cytotoxic ability, such as the ability to produce cytokines or participate in an immune response in a viral infection or whether they display a negative regulatory role on other T cells.
RESULTS

3.4.9 Summary

Systematic characterisation of the functional capacities of peripheral F5 CD8 T cells of the NP47F5 system or after transfer into NP47 hosts, showed the following:

Survival characteristics

- F5 CD8 cells “anergised” in vivo have an intrinsic capacity for long-term survival, which shows that the cells are not pre disposed for apoptosis and that anergy is not a step before deletion.
- These CD8 cells can efficiently compete with other T cells for survival factors and space in the lymphoid niches, and survival is not dependent only on homeostatic expansion.

Role of antigen in the maintenance of anergy.

- Continuous presence of the antigen is required for the maintenance of anergy on peripheral CD8 T cells.

Effector functions

- F5 CD8 T cells after chronic low-level antigenic stimulation appear unresponsive to in vitro antigenic stimulation, assessed by proliferation and cytokines production. However in vivo they maintain some effector function and are able to kill target cells expressing high amounts of antigen.
Assessment of survival

Adoptive transfer of CD8 sorted and CFSE labelled F5 or Anergic F5 T cells into “empty” syngeneic hosts

Rag-/- host, MHC identical to donor cells
No other lymphocytes
No antigen

Figure 22: Experimental approach for the assessment of survival of anergic T cells.
Figure 23: **Survival and homeostatic divisions of anergic CD8 T cells after transfer into antigen free RagNegbb hosts.**

Total spleen cells from NP47F5 or F5 mice containing 20 X10^6 CD8 T cells were labelled with CFSE and injected intravenously into RagNegbb mice. Lymph node cells from the host mice were isolated at different time points after transfers and stained with m-Abs for CD8, TCR (Vβ11), and analysed with FACS for CFSE fluorescence intensity. Histograms represent CFSE fluorescence intensity of CD8 positive T cells.
Figure 2.4: Function of anergic T cells after transfer to RagNegbb, Ag free hosts.
NP47F5 (blue line) or F5 (red line) cells were adoptively transferred into RagNegbb hosts. Spleen cells were harvested 23 days (A, B, C) or 6 weeks (D, E, F) later. Equal numbers of CD8 T cells were cultured with H-2^b DCs and NP68 peptide for 48 hours.
Proliferative responses were measured by ^[3]H thymidine incorporation. IL-2 responses were assayed by proliferation of the IL-2 dependent CTLL line and IFN-γ cytokine secretion in culture supernatant was detected by ELISA.
Each point represents the mean of triplicate samples. One representative experiment of two is shown.
Figure 25: **Survival and homeostatic divisions of anergic CD8 T cells after transfer into antigen free B10 nude hosts.**  

8 X10^6 peripheral CD8 T cells of NP47F5 or F5 mice were labelled with CFSE and injected intravenously into B10 nude mice. Lymph node cells from the host mice were isolated at different time points after transfers and stained with m-Ab's for CD8, TCR (H57) or CD44 and analysed with FACS for CFSE fluorescence intensity. Histograms represent CFSE fluorescence intensity of CD8 T cells and dot blots show CFSE and CD44 levels of CD8 T cells of NP47F5 (A, C) and F5 (B, D) cells respectively, after 6 weeks transfer into B10 nude mice.
Figure 26: Survival and homeostatic divisions of homogeneous anergic CD8 T cells after transfer into antigen free RagNegbb hosts. F5 peripheral T cells were anergised in vivo after transfer into NP47 mice. Peripheral anergic CD8 T cells were isolated from NP47 mice after 20 days by FACS sorting. 5X10^6 anergic CD8 T cells or control F5 naive CD8 T cells were labelled with CFSE and injected intravenously into RagNeg bb mice. Lymph node cells from the host mice were isolated at different time points after transfers and stained with m-Abs for CD8, TCR (Vβ11), and analysed with FACS for CFSE fluorescence intensity. Histograms represent CFSE fluorescence intensity of CD8 positive cells, the mean of the number of divisions is shown.
Figure 27: **Function of homogenous anergic T cells after transfer to RagNeg bb host.**

F5 T cells anergised in vivo after transfer into NP47 mice (blue line) or F5 (red line) cells were adoptively transferred into RagNegbb host. Spleen cells were harvested 2 days (A, B) or 6 weeks (C, D) later. Equal numbers of CD8 T cells were cultured with H-2^b^ DCs and NP68 peptide for 48 hours.

Proliferative responses were measured by ^3[H] thymidine incorporation, IL-2 responses were assayed by Alamar Blue based proliferation of the IL-2 dependent CTLL line and IFN-γ cytokine secretion was detected in culture supernatant by ELISA.
Anergic F5 CD8 T cells can compete with polyclonal T cells for survival.

0.5x10^6 GFPE5 CD8 T cells anergised in vivo after transfer into NP47 mice were co-transferred with B10 polyclonal lymphocytes that contained the same number of CD8 T cells into RagNegbb recipient mice. The same experiment was performed with GFPE5 naïve CD8 T cells. At day 10 and day 30 after transfers cells from spleen and lymph nodes of RagNegbb hosts were isolated and stained with anti-CD8 APC and anti-TCR (H57) PE mAbs. The survival of GFPE5 anergic or GFPE5 naïve CD8 T cells after co-transfers with polyclonal B10 lymphocytes into RagNeg host mice is shown by density plots of expression of CD8 and GFP on spleen and lymph node cells of RagNegbb host mice after day 10 and day 30 of transfer. The percentage of transferred GFP positive cells within the CD8 compartments is displayed, as well as the ratio between GFP positive and B10 GFP negative CD8 T cells.
Figure 29: Survival of anergic F5 CD8 T cells in polyclonal recipients.

4x10^6 peripheral NP47F5 or control F5 CD8 T cells were positively selected with anti-CD8 MACS beads and after labelling with CFSE fluorescent dye, were transferred into B10 syngeneic recipient mice. At different time points after transfer spleen and lymph nodes of B10 hosts were isolated and stained with anti-CD8 APC and anti-TCR (Vβ11) BIO mAbs. The survival of NP47F5 or F5 naïve CD8 T cells after transfer into polyclonal B10 host mice is shown by density plots of expression of CD8 and CFSE on spleen and lymph node cells of B10 host mice at day 2, day 18 and day 48 after transfer. The percentage of transferred CFSE positive cells within the CD8 compartment is displayed, as well as the ratio between CFSE positive and B10 CFSE negative CD8 T cells.
Figure 30: **Description of in vivo cytotoxicity test.**

B10 syngeneic target cells were pulsed with 1µM NP68 peptide, and were labelled with a high CFSE (5µM CFSE high) intensity. B10 unpulsed cells as control were labelled with a low (0.5 µM CFSE low) intensity. CFSE high and CFSE low target cells were mixed in a ratio 1:1 and transferred into different type of recipients containing the T cell population tested for cytotoxic capacity. CTL effector function was measured by monitoring the killing of CFSE high cells by flow cytometry 20 hours after transfer.
Figure 31: F5 CD8 T cells after transfer into NP47 host retain their cytotoxic capacity.

Figure 28 shows the result of in vivo cytotoxicity test performed on F5 naïve (A), F5 immunised (B), F5 effector mice (C), F5 effector immunised (D), F5 “anergic” (E), and F5 “anergic” immunised (F).
3.5 What is the function of NP47F5 CD8 T cells in vivo?

3.5.1 Are NP47F5 CD8 T cells auto-reactive in vivo?

Up to now we found that F5 CD8 T cells that are chronically exposed to low levels of endogenously expressed antigen, retain their ability to lyse cells expressing high amounts (1μM NP68) of antigen. Since they can have cytotoxic function in vivo raises the question why no sign of autoimmunity is evident in the NP47F5 mice.

The fact that NP47F5 CD8 T cells can have cytotoxic capacity does not mean that these cells are autoreactive in vivo. One possibility is that in the NP47F5 system expression of self-antigen might be below a threshold required for effector lytic function of CD8 T cells. In that case NP47F5 CD8 T cells would not have any autoreactivity in vivo, and cytotoxicity would be displayed only on syngeneic cells pulsed with antigen, which have artificially high levels of antigen expression.

If the NP47F5 CD8 T cells are autoreactive in vivo, one might expect that they would be able to kill bone marrow derived cells, expressing high amounts of antigen. For that reason we compared the antigen presentation capacity of NP47F5 and NP47 bone marrow derived cells, mainly dendritic cells and macrophages, which seem to be the only cells in NP47F5 mice expressing recognisable levels of antigen.

Since the only difference between NP47F5 and NP47 mice is the presence of CD8 T cells in the former mice, this comparison would give us information whether in the presence of CD8 T cells in the NP47F5 system, cells that express high amounts of antigen are killed or not. Such a comparison would indirectly address the question whether catatonic F5 CD8 T cells are autoreactive in vivo.

Spleen cells from NP47F5 or from NP47 or from RagNeggbb mice as negative control were isolated by enzyme digestion. The purification procedure for the isolation of bone marrow derived cells involved two steps of magnetic cell sorting (MACS) as outlined in the scheme in Fig.32. Dendritic cells were isolated by MACS from total spleen cells on the basis of the expression of CD11-c marker, using anti-CD11-c magnetic beads. The depleted fraction was then used as a source of macrophages. Cells of this negative fraction were labelled with F4/80-BIO mAb and positively selected by MACS using streptavidin
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beads. The purity of bone marrow derived cells, was assessed by staining the positive fractions for the selecting markers or for the expression of MHC class II and for the expression of TCR to test for the presence of contaminating NP47F5 CD8 T cells (Fig.32). The APC populations isolated from NP47 or NP47F5 mice were adjusted to the same concentration and tested for their capacity to present NP endogenous antigen in vitro, using naïve F5 lymph node cells as responders.

F5 CD8 responder T cells were analysed for their ability to upregulate CD69 (early activation marker), CD25 and CD44 activation markers, and for their ability to secrete IL-2 after stimulation for 2, 4 or 6 days, with different amounts of bone marrow derived cells from NP47F5 or NP47 mice or from RagNegbb negative control mice not containing NP expressing cells.

On day 4 and 6 upregulation of activation markers such as CD69, CD25 and CD44 was clearly visible in F5 cells stimulated with DC and Mφ as antigen presenting cells from NP47 mice. There was some activation markers upregulation in cultures stimulated with DC and Mφ from NP47F5 mice, but this as well as IL-2 production was well below the levels seen in NP47 stimulated cultures. However, it was above background levels compared with absence of antigenic stimulation by APC from antigen negative RagNegbb mice (Fig.33)

This result clearly indicated that NP47F5 mice contain fewer DC and Mφ with high levels of NP antigen, and could be an indication that these cells, which are continuously produced by the bone marrow, with highest levels of antigen expression are killed whereas the ones with lower antigen levels seem to be spared. It is therefore likely that there is a degree of autoreactive killing ongoing in NP47F5 mice, which is however matched by the continuous output of new DC and Mφ from the bone marrow.

Another possibility would be that autoreactive CD8 NP47F5 T cells may affect the presentation capacity of these APCs, not by eliminating the cells expressing higher amounts of antigen but by instructing the NP47F5 antigen presenting cells to lower the NP antigen expression or/and presentation. Therefore “anergic” NP47F5 T cells may inhibit the antigen presentation capacity of APCs, a function that has been referred to in vitro anergised T cells (Chai et al., 1999; Taams et al., 1998).
3.5.2 Summary

Comparative analysis of the antigen presentation capacity between APC from NP47F5 and NP47 cells showed that NP47F5 bone marrow derived cells expressing high amounts of antigen are absent from NP47F5 mice. The absence of NP47F5 cells expressing high amounts of antigen must be attributed to the NP47F5 CD8 T cell population, as this is the only difference between NP47F5 and NP47 mice. This indicates that NP47F5 CD8 T cells are possibly autoreactive \textit{in vivo} against cells that express high amounts of antigen. NP47 BM-derived cells were shown to be able to highly activate naïve F5 CD8 T cells, and the fact that these cells are missing from the NP47F5 mice indicates that endogenous expression of antigen on some cells can reach the levels required for effector lytic function.

If that is the case the fact that there is no apparent autoimmune phenotype on NP47F5 mice, must be attributed to the site that antigen is expressed. Expression of self-antigen on a “non-dangerous” site, like bone marrow derived cells in the NP47F5 system, would avoid measurable tissue damage caused by self reactive CD8 T cells, as the affected tissue (BM-derived cells) is continuously regenerated. In that case self-reactivity would cause no measurable damage, which would explain the lack of an autoimmune phenotype.
Total spleen cells were isolated with collagenase/Dnase enzyme digestion for 1 hour.

Figure 32: Purification of BM-derived cells for the assessment of their antigen presentation capacity.

BM-derived cells (dendritic cells and macrophages) were purified from spleens from NP47F5, NP47 or RagNegbb mice by MACS separation as illustrated. Cells were stained for expression of CD11-c, MHC II, F4/80 molecules and TCR using N418-BIO, M514-BIO, F4/80-BIO and H57-FITC mAb respectively. Histograms show expression of CD11-c, MHC II or F4/80 molecules on total spleen cells (grey filled line) and on purified cells (black line).
Figure 33: Antigen presentation capacity of NP47F5 cells.

1x10^4 responder F5 CD8 lymph node cells were cultured with titrated number of mixture of DC and Mø as antigen presenting cells purified from NP47 (plum circles), NP47F5 (blue triangle), or RagNegbb (black diamond) mice. On days 2, 4, 6 of culture responder F5 CD8 T cells were stained with anti-CD8-APC, CD69-FITC, CD25 PE, CD44-BIO mAbs, and analysed by FACS. At the same time points culture supernatant was measured for the amount of IL-2 secreted by the responder cells as measured by CTLL assay. The response of F5 CD8 T cells after culture with NP47 or NP47F5 or RagNegbb antigen presenting cells is shown as the percentage of F5 CD8 T cells that are positive for the expression of CD69, CD25, CD44 activation markers, and as the amount of IL-2 they secrete at days 2, 4, 6 of culture.
RESULTS

3.6 What is the biological significance of “anergic” CD8 T cells?

3.6.1 Do “anergic” F5 CD8 T cells negatively regulate the responses of normal T cells of the same specificity?

Previously it was shown that “anergic” F5 CD8 T cells either in the NP47F5 system or after transfer into NP47 host, have a long life span and they can compete with other T cells for survival and space in the peripheral T cell pool. Their survival characteristics, together with the fact that these cells, although unresponsive in vitro, retain their capacity to lyse target cells expressing high amounts of antigen, raises the question why are these self-reactive T cells maintained although they are potentially autoreactive.

Cumulative data suggest that anergic T cells are not functionally inert, but may develop regulatory functions. A regulatory role of anergic T cells has been linked with suppression of effector function of other T cells, either by the release of regulatory cytokines such as IL-10 (Buer et al., 1998) or by inhibition of the antigen presenting function of APCs (Chai et al., 1999; Taams et al., 1998). Recent data also support the existence of regulatory T cells capable of inhibiting autoimmune diseases. Types of regulatory T cells that have been described are usually antigen-experienced cells partially unresponsive in vitro to TCR stimulation.

The possibility that “anergic” F5 CD8 T cells can negatively regulate in vivo the effector function of normal T cells with the same specificity was investigated. To test this hypothesis an experimental system was set up, to follow the influence of “anergic” T cells on the response of normal T cells against a physiological immune challenge, that was a virus infection.

2x10^6 normal naïve GFP F5 CD8 T cells were co-transferred into RagNegbb mice, with the same number of F5 CD8 T cells that had been transferred into NP47 mice several weeks before to induce “anergy”. In order to exclude the possibility of any antigen carriage by NP47 cells, the “anergic” CD8 T cell population was depleted of APC by Dynabeads negative selection for cells expressing MHC class II and CD11-c molecules. On the day of the co-transfers of the two T cell populations, the host mice were immunised i.p. with 10^6 PFU of A/NT/60/68 influenza virus. A control group received naïve F5 CD8 T cells instead
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of "anergic" T cells. GFP F5 CD8 T cells were used to distinguish between the effector and the "anergic" population.

Fourteen days after transfer, host mice were analysed to detect whether normal GFPF5 CD8 T cells had any difference in the response to virus immunisation in the presence of anergic T cells. To test that, spleen and lymph node cells isolated from the host mice, and positively sorted for the GFP positive F5 population. The sorted cells were then stained with m-Abs for cell surface expression of CD8, TCR and CD44 or Fas. Sorted cells were also stained for intracellular expression of IFN-γ. In the presence of "anergic" T cells the response of normal GFPF5 CD8 T cells to viral infection was the same when compared to the response of normal GFPF5 CD8 T cells to viral infection in the presence of naïve F5 CD8 T cells. This was judged by the ability of normal GFPF5 CD8 cells to upregulate the expression of CD44 and Fas surface markers, and to express IFN-γ intracellularly after viral infection in the presence of either naïve or “anergic” T cells (Fig. 34). This result indicates that F5 “anergic” CD8 T cells do not have a negative regulatory role on the response of normal F5 T cells to viral infection.

3.6.2. Do “anergic” F5 CD8 T cells have a regulatory role through an antigen non-specific mechanism?

Studies in a number of experimental models of organ specific autoimmune diseases, which usually involve induced lymphopenia, show that there is a dominant control mechanism of tolerance, where certain T cells actively downregulate the activation and expansion of self-reactive T cells (Mason and Powrie, 1998; Shevach, 2000). An experimental model of inflammatory bowel disease (IBD) can be induced in SCID immunodeficient mice by transfer of CD4 CD45RB high (naïve) T cells from normal mice and induce colitis. However co-transfer of CD4 CD45RB low cells protect these mice from the disease. The regulatory cells capable of inhibiting the development of IBD that have been described in this model are found within the CD4 CD45RBlow CD25 population (Groux et al., 1997; Powrie et al., 1994). CD4 CD25 cells that have been described in this and in other models usually show little proliferation in response to TCR stimulation in vitro, therefore they appear anergic (Maloy and Powrie, 2001).
The fact that regulatory cell subsets appear anergic, together with data that demonstrate that anergic T cells can regulate immune responses in vivo, through antigen non-specific mechanisms, raise the possibility that "anergic" F5 CD8 T cells could have developed such a regulatory role. "Anergic" F5 cells, similar to regulatory T cell subsets, are antigen experienced cells and appear hyporesponsive in vitro to antigenic stimulation, however they do not express CD25. The majority of the cells able to display a regulatory role belong to the CD4 T cell subset and there are a few cases where CD8 T cells have shown such a regulatory activity. (Gilliet and Liu, 2002). Regulatory cells have been shown to downregulate the expansion of activated CD4 T cells through the release of regulatory cytokines like IL-10 and TGF-β (Mason and Powrie, 1998). However it has been proposed that the mode of action of these cells could be the result of a homeostatic control mechanism. Regulation in that case could be the result of competition for survival and expansion between regulatory cells and self-reactive cells. “Anergic” F5 CD8 T cells have the ability to undergo homeostatic expansion and to compete with other cells for survival in the peripheral T cell pool. These properties of F5 “anergic” CD8 T cells raise the possibility to have a regulatory role inhibiting the expansion of other T cells through a homeostatic mechanism rather than an antigen-specific mechanism.

In order to test this hypothesis, a model similar to IBD, was used. In that model it was investigated whether or not NP47F5 CD8 T cells can have a regulatory role against the expansion of CD4 CD45RBhigh T cells upon transfer into RagNeggb lymphopenic hosts. Also the ability of “anergic” NP47F5 CD8 T cells to protect the host mice from the T cell mediated wasting disease, through this homeostatic mechanism was analysed.

Peripheral T cells of normal BIO polyclonal mice were isolated from spleen and lymph nodes and enriched for the CD4 population by MACS selection using CD4 magnetic beads. After this enrichment cells were labelled with anti-CD4-APC, anti CD45RB-FITC, and anti- CD25-PE mAbs. CD4⁺CD45RBhigh CD25⁺, and the CD4⁺CD45RBlow CD25⁺ populations were then purified by FACS sorting. CD8 T cells from F5 or NP47F5 mice were positively selected by MACS, using CD8 magnetic beads (Fig.35 I).

RagNeggb host mice were divided into 5 groups of 6 mice per group, and were treated as follows: The first group of RagNeggb host mice were transferred with 2x10⁵ CD4⁺CD45RBhigh CD25⁻ B10 cells. RagNeggb host mice of the groups 2,3,4 were co-transferred with 2x10⁵ CD4 CD45RBhigh CD25⁻ cells together with the same number of
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either CD4^CD45RB^{low} CD25^* or F5 CD8 or NP47F5 CD8 T cells respectively. The fifth group of RagNegbb host mice were transferred only with 2x10^5 F5 CD8 T cells as control (Fig.35II).

Host mice of each group were analysed regularly for the expansion of transferred CD4 CD45RB^{high} CD25^- B10 cells as measured by the percentage of CD4 T cells in the blood. Host mice were also monitored for the development of the wasting disease as measured by incidence curves, which present the percentage of total mice that either lost more than 20% of their weight or developed severe diarrhoea at that time after cell transfer. As seen in (Fig. 36) by the incidence curves of RagNegbb mice that received CD4^CD45RB^{high} CD25^- cells only (group 1), all mice developed the disease by day 40. On the contrary when CD4^CD45RB^{high} CD25^- cells are co-transferred with the CD4^CD45RB^{low} CD25^* regulatory population, the majority of the mice did not develop the disease (group 2). Similarly RagNegbb mice that received F5 CD8 T cells only, which do not cross-react with enteric antigens, are healthy (group 5). However mice that are co-transferred with CD4^CD45RB^{high} CD25^- pathogenic population together with F5 CD8 T cells, rapidly lose weight and develop the disease (group 3). The same situation describes RagNegbb mice that are co-transferred with CD4^CD45RB^{high} CD25^- and NP47F5 CD8 T cells (group 4). Therefore the NP47F5 CD8 T cell population did not protect from the wasting disease induced by the CD4^CD45RB^{high} CD25^- population (Fig.36).

Upon analysis of the expansion of CD4 T cells in the periphery of the host mice of each group, at different time points after transfer, it is clear that the only population that was able to control and negative regulate the expansion of CD4 SCD45RB^{high} CD25^- population in the RagNegbb host was the CD4 CD45RB^{low} CD25^- NP47F5 CD8 T cells did not have the ability to negatively regulate the expansion of CD4 CD45RB^{high} CD25^- population in the RagNegbb host and a small decrease in the percentage of CD4 T cells in the blood seen in groups 3 and 4 was not significant and did not prevented the onset of the disease (Fig.37).

Therefore NP47F5 CD8 T cells are not able to negatively regulate CD4^CD45RB^{high} CD25^- T cells and prevent from the wasting disease through a non Ag-specific mechanism. Although NP47F5 CD8 T cells are able to expand homeostatically and compete with other cells for survival factors and space in the peripheral niches, this expansion may not be sufficient to out-compete and suppress the rapid expansion of CD45RB^{high} CD25^- cells that
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cause the disease. Other factors like cytokines such as IL-2 and IL-7 may also regulate the size of each T cell population, and competition between different T cell populations for cytokines such as IL-2 may also be a means for the regulation of the expansion of CD45RB\textsuperscript{high} CD25\textsuperscript{+} population. The fact that NP47F5 CD8 T cells are CD25 negative may indicate that one reason why they are not able to regulate the expansion of CD45RB\textsuperscript{high} CD25\textsuperscript{+} is lack in competition for IL-2, although expression of CD25 marker is not indispensable for regulatory function.

3.6.3 Summary

In this section the hypothesis that NP47F5 CD8 T cells may play a regulatory role and actively participate in the maintenance of tolerance, was investigated. Although NP47F5 CD8 T cells retain some of their functions \textit{in vivo}, have a long life span and are able to undergo homeostatic expansion, they do not show any negative regulatory role in the systems used. NP47F5 CD8 T cells did not affect the effector function of CD8 T cells of the same specificity, and failed to prevent the expansion of pathological gut-reactive polyclonal CD4\textsuperscript{+}CD45RB\textsuperscript{high}CD25\textsuperscript{+} population upon transfer into lymphopenic host. These results show that NP47F5 CD8 T cells do not have developed into a regulatory population, that would control the expansion and effector function of other T cells through an antigen specific mechanism or through competition for cytokines, survival factors and space in the peripheral lymphoid niches. Maintenance of tolerance in the NP47F5 system is unlikely to be the result of a regulatory role of NP47F5 CD8 T cells as it had been hypothesised. Instead it is possible that the site that the antigen is expressed may play a key role in the avoidance of autoimmunity.
Figure 34 Is there a regulatory role of “anergic” T cells on normal T cells of the same specificity?

GFP F5 T cells were transferred into RagNegbb host, together with the same number of either normal F5 cells or with F5 cells that have been rendered “anergic” by transfer into NP47 mice. The host mice were immunised with A/NT/0/68 influenza A strain virus the same day of the injections. Fourteen days after immunisation peripheral T cells from the host mice were harvested and the GFP positive cells were sorted by FACS sorting. GFP positive cells were stained with m-Abs for CD8, TCR, CD44 and Fas cell surface expression or for CD8, TCR surface expression and IFN-γ intracellular expression. Dot blots show CD8-CD44 (A, D), CD8-Fas (B, E), and CD8- IFN-γ (C, F) expression on GFP F5 CD8 T cells.
Figure 35: I

B10 TOTAL

CD4 CD45RB high
CD25 negative

CD4 CD45RB low
CD25 positive

CD25

CD45RB

CD4

CD8

NP47F5

CD8 POSITIVE

F5

CD8 POSITIVE

GROUPS | HOSTS | TRANSFERED CELLS
---|---|---
I | RagNegbb | CD4CD45RBhighCD25−
II | RagNegbb | CD4CD45RBhighCD25− CD4CD45RBlowCD25+
III | RagNegbb | CD4CD45RBhighCD25− F5 CD8
IV | RagNegbb | CD4CD45RBhighCD25− NP47F5 CD8
V | RagNegbb | F5 CD8
Figure 36: **NP47F5 CD8 T cells do not prevent the wasting disease on RagNegbb mice mediated by the CD4CD45RB high CD25 neg. population.**

The health status of the host mice of each different group is presented as incidence curves. Incidents are defined as either more that 20% of weight loss or development of diarrhoea.
Figure 37: NP47F5 CD8 T cells do not regulate the expansion of CD4CD45RBhigh CD25 neg. pathogenic population upon co-transfer into RagNegbb hosts.

At different time points after transfer of the different T cell populations host mice of each group as described in the table, were analysed for the expansion of transferred CD4 T cells. Peripheral blood lymphocytes (PBL) were labelled with anti-CD8 APC, anti-CD4 PE, anti-H57F6TC and anti-CD44 BIO mAbs. The expansion of the transferred CD4 T cells in each group is shown as the mean of % of CD4 T cells in the PBL at different time points after transfer.
DISCUSSION

4. DISCUSSION

The aim of this study was to investigate aspects of peripheral tolerance induction. Of particular interest was the physiology of self-reactive cells that escape negative selection and are rendered unresponsive upon encounter of self-antigen in the periphery. For that reason the double transgenic, NP47F5 non-deletional mouse model system was chosen. In that system transgenic CD8 T cells express F5 TCR specific for an influenza virus peptide NP that is also present and expressed under the MHC class I H2-K^b promoter. Peripheral NP47F5 CD8 T cells escape deletion and reach the lymphoid periphery, where they come in contact with cognate antigen.

Up to date in the majority of the cases where antigen is expressed ubiquitously, antigen specific T cells are deleted in the thymus. If central deletion is incomplete T cells are subsequently subjected to peripheral tolerance mechanisms such as TCR or CD8 downregulation that leads to the induction of their functional unresponsiveness (Lanoue et al., 1997; Teh et al., 1988). Although the NP transgene is driven off the MHC class I H2-K^b promoter that is expected to drive ubiquitous expression of the transgene in all MHC-I expressing cells, there is no involvement of the thymus in the tolerance induction in the NP47F5 system. As most of the stromal cells in the thymus express MHC-I, in principle all APC in the thymus should be capable of expressing the NP transgene and therefore induce negative selection to developing NP specific NP47F5 thymocytes.

As negative selection is determined by the overall avidity of the interaction of TCR and its ligand MHC/self-peptide, a possible way that NP47F5 CD8 T cells may escape deletion would be to apply a mechanism to reduce the avidity of their interaction with the ligand. Such a mechanism that has been reported in other systems of double transgenic mice, where self-reactive T cells have escaped deletion, is TCR or CD8 down-regulation (Mamalaki et al., 1996; Schonrich et al., 1991).

Partial deletion due to TCR downregulation can also be the outcome of expression of a second endogenously rearranged TCR α chain, due to incomplete allelic exclusion in the TCR α chain locus. Escape from negative selection due to expression of endogenous TCR α chain, that as a result decreases the avidity of the TCR-ligand interaction has been demonstrated in several models (Girgis et al., 1999; Lanoue et al., 1997; Legrand and Freitas, 2001; Zal et al., 1996). However expression of endogenous TCR α chains, as a
mechanism to lower the avidity or the TCR-MHC-peptide ligand interaction was directly excluded in the NP47F5 system because Rag-1 gene deficiency does not allow such an expression. Analysis of the cell surface expression of TCR and CD8 molecules on NP47F5 thymocytes showed normal levels of expression, excluding the possibility that NP47F5 thymocytes escape negative selection due to reduced TCR affinity.

The role of antigen density in negative selection.

Another parameter that controls the extent of clonal deletion in the thymus is the density of the antigen. In a study of a TCR transgenic model, with T cells specific for MHC class I alloantigen, differences in the antigen density determined whether thymic tolerance was achieved by deletion or by clonal anergy. High antigen density in this model, in H-2\textsuperscript{kb} homozygous mice totally deleted T cells from the periphery, whereas in low antigen density situations of heterozygous H-2\textsuperscript{kb} or H-2\textsuperscript{kd} mice, tolerance was maintained by modulation of CD8 expression, and anergy induction (Auphan et al., 1992).

In the NP47F5 system, although NP transgene expression was below a threshold required for clonal deletion, the possibility remained that the levels of antigen expression in the thymus may still be high enough to induce tolerance by rendering cells unresponsive. In contrast to other systems, the levels of NP expression in the NP47F5 system do not induce anergy in the thymus, as NP47F5 SP mature thymocytes showed normal reactivity to NP68 antigenic stimulation both \textit{in vivo} and \textit{in vitro}. When SP mature NP47F5 thymocytes were phenotypically characterised, they displayed a naïve phenotype (CD44 low), in contrast to the peripheral NP47F5 T cells the majority of which are activated cells. Collective evidence suggests absence of antigen recognition in the thymus.

Negative selection is a very sensitive procedure and several studies have shown that deletion of antigen specific thymocytes require a lower threshold than antigenic stimulation of mature T cells in the periphery (Lucas et al., 1999; Pircher et al., 1993 Vasquez, 1994 #344). Taking into account the sensitivity of negative selection, NP antigen expression in the thymus appears to be too sub-optimal to be recognised by developing thymocytes and to induce tolerance either by deletion or by the induction of unresponsiveness. Given that MHC class I H2-K\textsuperscript{b} drives NP antigen transgene expression, it is a paradox that the level of NP antigen expression in the thymus is too sub-optimal to be recognised by developing
thymocytes, but in the periphery the levels NP expression are high enough to activate T cells and induce tolerance by paralysis.

Differences in the levels of a transgene expression can occur when a transgene is subjected to position effect variegation (PEV). A characteristic feature of PEV is cell-to-cell variegation of gene expression within a cell lineage. This feature is correlated with insertion of a normally eucaryotic gene within centromeric heterochromatin. It has been shown that PEV of the human CD2 gene as a consequence of deletion of a Locus Control Region (LCR) manifested a mosaic expression pattern of the transgene, rather than a lower expression in all cells of the same lineage (Festenstein et al., 1996). LCRs are powerful genetic regulatory elements that control tissue-specific and physiological levels of expression on linked genes. Furthermore they activate the transcription of transgenes in a copy dependent, but position independent manner (Li et al., 1999). When such regulatory elements are present, they can overcome position effect variegation of a transgene.

Therefore expression of NP under the MHC class I H2-Kb promoter may variegate, due to lack of such a control element (LCR). Variegation may result in silencing of the NP gene, in a proportion of cells of the same lineage. In that case the NP transgene may be expressed by different cell types in the thymus than in the periphery, which could explain why there is lack of tolerance in the thymus, but activation and tolerance induction in the periphery of NP47F5 mice. Equally possible is that variegation may affect the levels of expression of the NP transgene. Reduced levels of NP expression in the thymus could explain why there is no deletion or recognition of NP transgene in the thymus, even if the sensitivity of negative selection is increased in comparison to the sensitivity of activation of mature T cells in the periphery.

The role of thymic APC in negative selection.

Another parameter that can influence negative selection is the nature of thymic APC that express the antigen. Redundancy or complementation in the functional properties of the different thymic APC with regard to their ability to present self-antigens and their tolerogenic competence has been proposed.

Several piece of evidence suggest that medullary thymic epithelial cells can drive negative selection of CD4 (Burkly et al., 1993) or CD8 (Sprent, 1993) autoreactive T cells.
DISCUSSION

The findings that medullary thymic epithelial cells can utilise MHC class II endogenous pathway to induce tolerance to a nuclear protein (Oukka et al., 1996) and their unique role to express tissue specific antigens (Derbinski et al., 2001), enhance the role of these cells in negative selection. The role of thymic cortical epithelium however in negative selection is still unclear. Some reports support that thymic cortical epithelial cells can induce deletion (Laufer et al., 1996; Volkmann et al., 1997), at least to those T cells that display high avidity for TCR-MHC-peptide ligand. However other reports show that thymic cortical epithelial cells are less efficient in negative selection than medullary epithelial cells or than cells of haematopoietic origin (Roberts et al., 1990). When bone-marrow chimeric mice were generated to target expression of NP antigen on BM-derived or non-BM-derived cells, it was shown that NP antigen is expressed only on cells of haematopoietic origin. This finding excludes the possibility that NP47F5 thymocytes escape negative selection because of antigen presentation on thymic cortical epithelial cells.

The cellular composition of haematopoietic origin in the thymus of a normal mouse is diverse and includes macrophages some B cells and DCs. However Rag-1 deficiency in the NP47F5 mice restricts this composition to only macrophages and DCs. Thymic DCs are highly efficient at taking up and processing exogenous protein antigens in vitro and in vivo and at the same time are able to present these antigens to developing T cells (Kyewski et al., 1986). These properties qualify them as the main cell type to be involved in negative selection. On the other hand, thymic macrophages which also originate from the bone marrow, have primarily been attributed a role in the removal of apoptotic cells, that is dying thymocytes, whereas they fail to contribute to negative selection. Thymic macrophages are the only cell type unable to induce negative selection to MHC class II restricted C5-specific T cells due to failure to present C5 antigen of an extracellular source (Volkmann et al., 1997). If the main cell type of haematopoietic origin able to express NP transgene in the NP47F5 system, are macrophages, then a possible reason why NP47F5 thymocytes escape negative selection may be that this cell type serves only as scavenger for apoptotic cells in the thymus and does not contribute to negative selection.
Peripheral T cells in NP47F5 are heterogeneous, but chronic exposure to antigen in the absence of thymic export generates a homogeneous T cell population that appears anergic.

Peripheral NP47F5 CD8 T cells were found to be a heterogeneous population, both phenotypically in respect to activation status and functionally in the response to antigenic stimuli. Phenotypic analysis of peripheral NP47F5 CD8 T cells revealed that a sub-population of CD8 T cells exhibited an activated phenotype (CD44 int. TCR high), whereas other cells were not fully activated (CD44int. TCR high). This phenotypic heterogeneity reflected a functional heterogeneity with respect to antigenic reactivity in-vivo and in vitro. Injection of NP47F5 mice with soluble NP68 peptide i.p. resulted in further up-regulation of CD44 marker, indicative of the presence of peripheral NP47F5 CD8 T cells able to respond to antigenic stimulation in vivo. Similarly upon in vitro antigenic stimulation there was a population of NP47F5 CD8 T cells able to express IFN-γ cytokine.

The appearance of a peripheral NP47F5 CD8 T cell sub-population with a “naïve” phenotype that shows normal reactivity to antigenic stimulation could be due to cells that have recently migrated from the thymus. These cells would not have been exposed to the antigen for a sufficient time period in order to be activated or tolerised. Thymic involution in non-transgenic (C57BL/10) mice normally leads to a reduction in the numbers emerging from the thymus, to join the peripheral T cell pool, with the greater rate of loss to occur in the period between 3 and 5.6 months of age. F5 transgenic mice do not display age-associated thymic atrophy in any of the thymic subsets as shown in a recent study (Aspinall, 1997). Age associated thymic atrophy was associated thus with a deficiency affecting rearrangements of the TCR genes during intrathymic T cell development. It is conceivable therefore that continuous export from the thymus of naïve NP47F5 CD8 T cells is the explanation to the heterogeneous peripheral NP47F5 CD8 T cell compartment.

Adoptive transfer of F5 peripheral CD8 T cells into NP47 mice generated a homogeneous peripheral CD8 T cell population where all cells displayed an activated phenotype (CD44 high) and had an impaired ability to respond to in vitro antigenic stimulation by proliferation and cytokines production. In vitro antigenic stimulation of F5 CD8 T cells recovered from NP47 hosts showed that the cells were unable to express IFN-γ.
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as judged by intracellular staining. These data indicate that in the adoptive transfer system there are no cells able to respond to *in vitro* antigenic re-stimulation and that all cells are in the same functional status. This further supports that chronic exposure to antigen in the absence of thymic export generates a homogeneous T cell population that appears anergic.

The data collectively show that when NP47F5 CD8 T cells come in contact with antigen in the periphery, they get activated, evident in the up-regulation of CD44 marker followed by downregulation of TCR surface expression, and pass through an effector stage. This effector stage is followed by induction of tolerance. Chronic antigenic stimulation renders peripheral NP47F5 CD8 T cells unresponsive as judged from their impaired capacity for proliferation and cytokines production to further *in vitro* antigenic stimulation.

What cell type mediates peripheral unresponsiveness in the NP47F5 system?

Since central tolerance in the thymus is incomplete, mature T cells that escape tolerance in the thymus are still susceptible to tolerance induction in the periphery. Different mechanisms of tolerance operate in the periphery from ignorance, to anergy, to clonal deletion, that may reflect different levels of peripheral tolerance. Different peripheral tolerance mechanisms or different levels of peripheral tolerance may depend on signals qualitatively or quantitatively distinct, transmitted to a self-reactive mature T cell upon ligand recognition. Parameters that determine peripheral tolerance induction relate either to the T cell itself (affinity of the TCR, level of TCR expression and accessory molecules) or relate to properties of the tissue bearing the self-antigen.

The two signal model predicts that a T cell will become activated when its TCR recognises the cognate MHC-peptide ligand on the surface of a professional, BM-derived APC, that also express a second co-stimulatory signal, whereas TCR engagement in the absence of co-stimulation results in anergy. In line with this model qualitative difference in antigen presentation by different subsets of APC may determine whether or not tolerance is induced. An alternative possibility is that quantitative differences such as ligand density, or in the frequency, length and intensity of contacts between T cells and tolerizing tissue are crucial. The more recent observations that CTLA-4 regulates anergy induction *in vivo* by controlling cell cycle progression (Fecteau et al., 2001; Greenwald et al., 2001; Perez et al.,
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1997), suggests that counter-regulatory pathways such as signalling through alternative receptors rather than lack of co-stimulation, influence the ultimate outcome of TCR engagement and therefore the induction of anergy.

Tolerance to antigens expressed on extrathymic non-lymphoid tissues was demonstrated by expression of Kb antigen under the control of tissue specific promoters (albumin, keratin, glial fibrillary acidic protein promoter) crossed with Kb specific TCR transgenic mice (Arnold et al., 1993). All types of double transgenic mice were tolerant, however the basis for the observed tolerance was different. Central deletion followed by peripheral anergy induction as a result of TCR and CD8 downregulation operated when antigen was expressed on cells of neuroectodermal origin or on hepatocytes. However when antigen was expressed on keratinocytes no deletion was evident although peripheral CD8 T cells were tolerant. Later studies showed that early migration of recent thymic emigrants to non-lymphoid sites (skin expressing MHC I antigen) only during neonatal period resulted in tolerance induction in these mice. Blocking E- and P selectin on these T cells prevented migration of these cells to neonatal skin and thus tolerance induction to Kb antigen. This indicated that T cell trafficking through non-lymphoid tissues in the neonate may be important for the establishment of peripheral tolerance to tissue expressed antigens, especially in situations where Ag expression is too low for cross presentation to take place. (Alferink et al., 1998).

Induction of tolerance in the periphery could be determined by the nature of the cell type presenting self-antigen. In the NP47F5 system self-reactive T cells develop normally and leave the thymus unaffected. The tissue that expresses the NP transgene could affect peripheral tolerance by induction of unresponsiveness in the NP47F5 system, and it could be the result of antigen presentation on non-professional APC. Our experimental approach to this issue was to construct radiation BM chimeras targeting expression of NP transgene exclusively to BM-derived cells or to cells of non-haematopoietic origin. In GFPF5 (BM)\(\rightarrow\)NP47F5 chimeras, antigen expression on non-BM-derived cells did not induce activation of peripheral F5 CD8 T cells. However NP-specific F5 CD8 T cells retrieved from NP47F5 (BM)\(\rightarrow\)GFPF5 chimeras were activated and exhibited impaired proliferative response and cytokine production in response to (NP68) antigenic re-stimulation in vitro. Therefore the only source of APCs able to present recognisable levels of NP antigen in the NP47F5 system is cells of haematopoietic origin. In the NP47F5 system tolerance is induced by NP
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presentation on haematopoietic cells, which in this model include DCs, macrophages or T cells.

Although antigen expression on cells of haematopoietic origin is thought to be immunogenic, several models have described T cell tolerance to self-antigens expressed or presented on haematopoietic cells. HA-specific TCR transgenic T cells in double transgenic mice where HA was expressed under the Ig kb promoter, were tolerised by the combined mechanisms of central deletion and induction of unresponsiveness in the periphery (Lanoue et al., 1997). The NP47F5 system differs from this model in that tolerance is induced only in the periphery and does not affect thymic development. In another study CD4 cells specific for HA, were rendered anergic after transfer into mice expressing HA as parenchymal self-antigen. Analysis of tolerance induction in parent→F1 chimeras, demonstrated that tolerance was not induced by antigen expression on parenchymal cells, but it rather required antigen transfer to BM-APCs, a cross presentation pathway (Adler et al., 1998). Like CD8 T cells, CD4 T cells specific for SV40 T antigen have been reported to be tolerised by a cross presentation pathway. In this case proliferation of T cells in the draining lymph nodes, was followed by deletion although some remaining cells exhibited properties of anergy (Forster et al., 1995).

All three types of "professional" APCs B cells (Fuchs and Matzinger, 1992), macrophages (Miyazaki et al., 1993) and dendritic cells (Hawiger et al., 2001), have been shown in various systems to be capable of inducing T cell tolerance. This may be due to the fact that APCs require activation to become immunogenic, and in resting conditions are tolerogenic.

Transgenic expression of I-E molecules restricted to macrophages by the use of a fragment of colony stimulating factor-1 as promoter, has shown that antigen presentation and T cell priming by Mø were impaired. I-E reactive T cells containing Vβ gene segments, were anergised in the periphery in that model but were not clonally deleted in the thymus. This suggested that interaction of T cells with antigens on macrophages leads to tolerance possibly due to lack of co-stimulatory signals or due to low levels of MHC molecules surface expression (Miyazaki et al., 1993).

Studies were also focused on the role of B cells in antigen presentation and tolerance induction. Antigen experienced female T cells were able to respond to H-Y antigen expressed on B cells, whereas naïve T cells were rendered tolerant. Tolerance of naïve T
DISCUSSION

cells in that case was also the result of antigen presentation on activated B cells (Fuchs and Matzinger, 1992). Other studies however indicate that T cell tolerance may be partially overcome by high potent Ag presenting capacity of activated Ab expressing B cells (de Vos et al., 2000). DCs can express constitutively co-stimulatory molecules and MHC molecules and they have the unique ability to acquire antigens from apoptotic cells and efficiently present the to T cells, by a cross presentation mechanism. However the capacity of DCs to induce T cell stimulation is strictly dependent on their maturation status. In the absence of inflammatory stimuli, immature/resting DCs are relatively poor T cell stimulators and can presumably induce tolerance. In that line, co-injection of DC-targeted antigen and anti-CD40 agonistic antibody was shown to change the outcome of T cell response from tolerance to immunity (Hawiger et al., 2001). Therefore current knowledge suggests that the induction of tolerance or immunity could be determined by the ratio of resting/immature to activated/mature DCs (Jonuleit et al., 2001).

T-to-T cells antigen presentation due to the absence of co-stimulation has been proposed to induce tolerance, based on studies on human T cell clones after culture in vitro with antigen in the absence of APC (Sidhu et al., 1992). Although the mechanism responsible for this type of T cells unresponsiveness is still unknown, expression of both MHC II and B7 molecules on activated human T cells (Azuma et al., 1993; Wyss-Coray et al., 1993) makes it unlikely to be due to lack of co-stimulatory signals. Other studies suggest that unresponsiveness induced by T-to-T cells antigen presentation may be due to altered TCR-CD3 signalling (Lombardi et al., 1996).

In the NP47F5 system the only potential cell type of haematopoietic origin other than DC and Mφ that could present NP antigen in a tolerogenic way is the T cell population. However when NP47F5 CD8 T cells were investigated for their capacity to present NP antigen on naïve F5 T cells in vivo, in a model that does not allow cross presentation (β2MnegRagNeg mice), NP47F5 CD8 T cells were incapable of antigen presentation. Therefore T-T antigen presentation as a mechanism to induce peripheral unresponsiveness in the NP47F5 system was ruled out.

Although T-T antigen presentation in vitro has been shown to induce tolerance, such a mechanism is rather unlikely to take place in vivo, where the location of naïve T cells (lymph node paracortex) and stromal interactions optimise contact with DCs. The architecture of lymph nodes where T cell priming mainly takes place, is designed such that
lymph borne antigens will percolate through the densely clustered naïve T cells and interdigitating DCs that mostly make up the central paracortex. Such architecture optimises the chances for effective antigen presentation (Mondino et al., 1996). \textit{In vivo} histology tracking on CD4 T cells has shown, that few if any interactions between T cells and B cells in the T cell rich area of paracortex after infection occur. The lack of physical interactions between T and B cells after infection suggest that T cell interactions with DCs precede any other interactions. This may indicate that order antigen presentation would ensure that T cells are always activated by a DC first avoiding initial Ag presentation by other cells, such as B cells, that would delete or inactivate T cells (Garside et al., 1998).

**Fate of unresponsive CD8 T cells.**

The fate of anergic T cells \textit{in vivo} is still elusive. Until recently anergy was believed to be a functional stage before deletion and that anergic T cells are programmed for cell death (Sprent and Tough, 1994). However recent studies show that anergy and exhaustion are independent mechanisms of peripheral tolerance (Rocha et al., 1995), indicating that anergic T cells can be long lived. In a study on BM-chimeras expressing different amounts of male Ag, it was shown that at high concentrations of persisting antigen, T cells did not differentiate into effector functions and persist as anergic cells. At low concentrations of persisting antigen, however, differentiation into effector functions did occur, but was followed by deletion (Rocha et al., 1995).

F5 CD8 T cells anergised \textit{in vivo} either directly into NP47F5 mice or after transfer into NP47 hosts, display long-term survival. Long-term survival in this model is unlikely to account for selection of a sub-population of NP47F5 cells that have not been tolerised, as the same survival characteristics were displayed on a homogeneous population of F5 CD8 T cell population tolerised in NP47 hosts. However the possibility was raised that survival is due to selection of T cell donor precursors repopulating the thymus of lymphopenic hosts and developing into naïve F5 CD8 T cells. Firstly analysis of the thymi of Rag Negbb host mice showed no SP donor NP47F5 CD8 T cells, making such a possibility highly unlikely. The fact that \textit{in vivo} anergised F5 CD8 T cells showed the same pattern of survival and recovery of function after transfer into athymic B10 nude mice finally excluded such a possibility.
DISCUSSION

In contrast to other model systems, F5 CD8 T cells pass through an effector stage and proliferate upon self-antigen recognition, before they become unresponsive. This shows that even cells that pass through an effector stage and survive AICD could persist for long time in an unresponsive stage. Survival of anergic T cells has also been shown to occur in vivo for long time points after injection of the tolerising antigen (Pape et al., 1998) and for TCR Tg T cells specific for K\(^{b}\) antigen in mice expressing K\(^{b}\) on keratinocytes (Alferink et al., 1995). In the majority of the cases investigating the fate of anergic T cells, long term survival was reported in lymphopenic hosts, that is, either persistence of cells in irradiated, thymectomised mice (Rocha et al., 1995) or after transfer into RagNeg or SCID hosts. These studies show the intrinsic properties of anergic T cells for survival, but such conditions do not address the question whether or not anergic T cells can survive in a more physiological situation where they would have to compete for survival factors and space in the peripheral niches. Such a situation of short-term survival has been described for anergic B cells. Failure to compete with normal B cells for entry into follicles resulted in short term survival of anergic B cells (Cyster et al., 1994).

In this study, in contrast, unresponsive F5 CD8 T cells were able to compete for expansion and survival with polyclonal T cells. Unresponsive F5 CD8 T cells were found to survive after transfer into polyclonal mice as long as naïve cells of the same specificity. Therefore, unlike anergic B cells unresponsive CD8 T cells may have the intrinsic capacity for long-term survival and can compete with other cells for survival factors and space in the peripheral niches.
DISCUSSION

Homeostasis

Transfer of unresponsive F5 CD8 T cells into lymphopenic Ag-free hosts (RagNeg or B10 nude), showed that although these cells are unable to proliferate after antigenic stimulation they are capable to undergo a series of homeostatic divisions similarly to naïve T cells. CD4 CD25 regulatory T cells that are unresponsive to antigenic or mitogenic stimuli and therefore appear anergic were also shown to be able to undergo homeostatic expansion upon transfer into lymphopenic hosts (Gavin et al., 2002). Homeostatic expansion of these cells did not abrogate their unresponsive status, which was shown to involve proximal blockade of Ca\(^{2+}\) mobilisation nor their suppressive capacity.

One possible explanation for the difference in the capacity of these cells to undergo homeostatic versus antigenic proliferation could be that TCR- and cytokine- derived signals that result in homeostatic proliferation may be distinct from those that are downstream antigenic stimuli, which are blocked in unresponsive T cells. A molecule that is downstream of TCR signalling that may play a role in aspects of T cell anergy is Fyn. This molecule is a member of the Src-kinase family, which is involved in TCR stimulated signalling and proliferation of T cells. Upregulated expression of Fyn was demonstrated on anergic versus naïve CD4 T cells using gene array technology (Lechner et al., 2001). In another study CD4\(^+\) CD8\(^-\) TCR\(^+\) Tg cells specific for p2Ca antigen rendered anergic in mice expressing this peptide demonstrated elevated levels of Fyn expression. Loss of Fyn in these DN αβTCR\(^+\) anergic T cells led to a substantial recovery of their proliferation but heightened their survival ability (Utting et al., 2001). These suggest that Fyn may play a role in anergy induction by negatively regulating aspects of TCR signalling, but on the other hand may promote cell survival.

Reversal of anergy

Unresponsiveness of self-reactive T cells as a peripheral tolerance mechanism, is a dynamic process, and under certain circumstances the unresponsive state can be reversed. In vitro induced anergy due to TCR stimulation in the absence of professional APC, can be reversed either by antibody mediated cross-linking of the common γ chain which is shared
by the receptors for IL-2, IL-4 and IL-7 (Boussiotis et al., 1994a), or by addition of IL-2, (DeSilva et al., 1991). These data led to the suggestion that anergy is induced as a consequence of TCR occupancy in the absence of cell division.

In accordance with this, cyclin inhibitor p27 is over-expressed on anergic T cells, indicating that cell cycle regulators might collaborate in controlling T cell induction of unresponsiveness (Balomenos and Martinez, 2000). In other studies that were focused to test whether co-stimulation was able to prevent anergy by promoting T cell proliferation, an immunosuppressive drug, rapamycin was used. This drug is a potent inhibitor of IL-2 induced proliferation. Block of proliferation by this drug induced anergy on cells that had received both TCR and co-stimulatory signals (Powell et al., 1998; Vanasek et al., 2001). However, although \textit{in vitro} induced unresponsiveness can be reversed by IL-2 or common \( \gamma \) chain signalling, T cells rendered unresponsive \textit{in vivo} usually do not recover their function by addition of IL-2. On the other hand, T cells rendered anergic \textit{in vivo} can recover their function in the absence of tolerising antigen. Requirement of continuous presence of the antigen for maintenance of the anergic state was demonstrated in many systems where T cells were anergised \textit{in vivo}. Transfer of these cells into Ag free hosts allowed the recovery of their function to antigenic stimulation (Alferink et al., 1995; Rocha et al., 1993; Tanchot et al., 2001).

Maintenance of the anergic state of F5 CD8 T cells anergised \textit{in vivo} also required continuous presence of the antigen. When these cells were transferred into antigen free RagNegbb hosts they recovered from their unresponsive state, although this required extensive time periods, longer than 4 weeks. Since recovery of function was observed in lymphopenic hosts in which also underwent homeostatic expansion, one could pose the question of whether or not reversal of the anergic state is dependent on homeostatic expansion and progression through the cell cycle. Homeostatic expansion is linked to increased effector function in some cases (Surh and Sprent, 2000), which could be a possible means for the recovery of function of anergic T cells. We are currently addressing this issue after transfer of anergic F5 T cells into “full” polyclonal hosts.

As far as the induction of unresponsiveness is concerned, it has to be mentioned that F5 CD8 T cells are rendered unresponsive after an initial effector stage where cells in response to antigenic stimulation proliferate extensively before they become unresponsive. Such an initial expansion phase argues against the possibility that unresponsiveness is due
DISCUSSION

to lack of proliferation when Ag is encountered. In another model where HA specific T cells are rendered anergic after transfer into mice expressing HA in the periphery, also unresponsiveness followed a rapid initial proliferative response against HA. In that study tolerising stimulus could induce similar T cell mitotic rates to a priming stimulus (Adler et al., 2000).
Do anergic F5 T cells have a regulatory role?

The fact that F5 CD8 T cells tolerised in the periphery, have a long life span and in the absence of the antigen their functional capacities can be restored, raises the possibility that these cells are maintained because they have developed a functional role. Thymic selection of self-reactive thymocytes, bearing high affinity TCR for a self-peptide that develop into regulatory CD4+ CD25+ subset (Jordan et al., 2001), would suggest that a selection of self-reactive T cells that develop regulatory function could exist in the periphery as well. Development of unresponsive T cells into regulatory cells could explain the significance behind the maintenance of a T cell population that is potentially autoreactive in the periphery.

Immune regulatory function of unresponsive T cells has been suggested in different studies, where anergic T cells were found to be able to suppress the response of normal naïve T cells (Jordan et al., 2001). Such a suppressive role of unresponsive T cells has been attributed to an active mechanism through the release of cytokines such as IL-10 (Becker et al., 1994; Buer et al., 1998; Jooss et al., 2001), or IFN-γ (Blish et al., 1999; Cauley et al., 1997). Another mechanism by which anergic T cells have been suggested to mediate immune regulation is by inhibition of the antigen presentation capacity of APC. Such a mechanism that has been reported in studies on anergic T cell clones is thought to require direct contact between T cells and the affected APCs (Chai et al., 1999; Taams et al., 1999; Taams et al., 1998; Vendetti et al., 2000).

In the NP47F5 system, CD8 T cells rendered unresponsive in vivo did not appear to influence immune response of other T cells in vivo. The fact that naïve F5 CD8 T cells showed normal response to virus infection in vivo in the presence of unresponsive NP47F5 CD8 T cells showed that these cells did not display negative regulatory function against naïve T cells of the same specificity.

CD4 CD25 regulatory cells have the ability to suppress immune responses of other T cells in an antigen unspecific manner. Down-regulation of the expansion of activated CD4 cells was shown to depend on the release of regulatory cytokines, such as IL-10 and TGF-β from the regulatory cell subset. However another antigen non-specific means of immune regulation has been proposed to be the result of the homeostatic control mechanism that
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takes place in the periphery. Immune regulation in that way would be the result of competition for survival and expansion between regulatory cells and self-reactive cells.

Although NP47F5 T cells are able to compete with other cells for survival and can undergo homeostatic expansion, they were unable to out-compete and suppress the rapid expansion of CD45RB^{high} CD25^{−} pathogenic population upon co-transfer into lymphopenic host. Therefore NP47F5 T cells do not display any regulatory function neither antigen specific nor antigen non-specific based on the competition for homeostatic expansion. It has to be mentioned though that in comparison to NP47F5 T cells, which are CD8 cells, the majority of the cells able to display a regulatory role belong to the CD4 T cell subset. It therefore appears that maintenance of tolerance in the NP47F5 system is unlikely to be based on immune regulation, displayed by NP47F5 CD8 T cells.

Functional capacities of F5 CD8 T cells rendered unresponsive in vivo.

Interaction between self-reactive T cells and cognate antigen in the periphery can have different outcomes from ignorance, to unresponsiveness, to deletion or to activation and autoimmunity. The functional stages of APC and the responding T cells and the signals they exchange are considered key factors in determining whether contact with antigen leads to activation or unresponsiveness.

Analysis in the kinetics of the response of F5 CD8 T cells upon transfer into NP47 mice has shown that after an initial expansion phase that is followed by AICD, a population of T cells that have escaped peripheral deletion persist for long time. Phenotypic analysis of this population showed that these cells express high levels of CD44 activation marker and decreased levels of CD62-L surface expression. This phenotype "CD44^{high} CD62-L^{low}\), is characteristic of activated, memory T cells and further support that the cells are antigen-experienced cells. Despite the expression of Ag-induced activation markers and on an initial effector phase, these cells differ from effector and memory cells, as they display a profoundly defective response in proliferation and cytokines (IL-2, IFN-\(\gamma\)) production after antigenic stimulation in vitro. In this context it is interesting that non-responsive F5 T cells expressed increased levels of negative regulator CD5 when compared with naïve or memory F5 T cells.
CD5 is a monomeric cell surface glycoprotein expressed on thymocytes, all mature T cells and a subset of B cells (B-1). The cytoplasmic domain of CD5 contains three potential tyrosine phosphorylation sites, including ITAM (immunoreceptor tyrosine-based activation motive) and ITIM (immunoreceptor tyrosine-based inhibition motive) and multiple potential Ser/Thr phosphorylation sites. After TCR engagement CD5 is tyrosine phosphorylated and becomes associated with a multi molecular complex that may include TCR-ζ, CD2, CD8, CD4, p56 lck, p59fyn and Zap 70 (Davies et al., 1992). In CD5 deficient mice it has been shown that CD5 can influence the fate of developing thymocytes by acting as a negative regulator of TCR signal transduction (Tarakhovsky et al., 1995). Developmentally regulated expression of CD5 has been proposed to be significant for the establishment of tolerance via fine-tuning of thymocytes to self-peptide MHC complexes (Azzam et al., 1998).

CD5 has also been found to negative regulate TCR signalling on mature peripheral T cells. CD5 expression on naïve CD4 T cells was shown to be regulated by TCR-MHC contact. Reduced CD5 levels in T cells deprived of MHC contact are associated with elevated Ca\(^{2+}\) to TCR signalling, suggesting that CD5 expression can fine-tune TCR responses to peptide-MHC contact of peripheral T cells (Smith et al., 2001). Furthermore CD5 has been shown to play a role in the maintenance of B cell tolerance in a transgenic model. In that model CD5 expression was higher on HEL-Ig/sHEL anergic B cells than on naïve single transgenic HEL-Ig B cells. Anergic B cells deficient of CD5 showed enhanced proliferation in vitro and elevated Ca\(^{2+}\) levels at rest and IgM cross-linking. These data suggested that CD5 might negatively regulate IgM receptor signalling in anergic B cells (Hippen et al., 2000). Similar to that system, increased CD5 expression on F5 CD8 T cells as a consequence of chronic TCR stimulation in NP47F5 mice or in NP47 hosts, may negative regulate TCR signalling and retain these cells in an unresponsive state. Future analysis of expression of CD5 on NP47F5 CD8 T cells upon transfer into Ag free hosts that results to the recovery of their function may establish whether or not this molecule is critically involved in the induction of unresponsiveness in this model.

Other molecules that negatively regulate T cell responses have been suggested as candidates to regulate the response of a T cells upon self-antigenic stimulation in the periphery. These molecules include CTLA-4, PD-1 and Tob. CTLA-4 has been shown to have as essential role in determining the outcome of the T cell encountered with a
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tolerogenic stimulus, and to regulate anergy in vivo by blocking of IL-2 expression and by controlling cell cycle progression (Coyle and Gutierrez-Ramos, 2001; Greenwald et al., 2001; Walunas and Bluestone, 1998). PD-1, a member of the B7 gene family has been shown to inhibit TCR-mediated lymphocyte proliferation. In addition PD-1 signalling has been shown to inhibit at least sub-optimal levels of CD28-mediated co-stimulation. Increased levels of PD-1 expression among anergic T cells led to the suggestion that this molecule may be important in induction of unresponsiveness (Lechner et al., 2001). Finally Tob, a member of BTG anti-proliferative protein family, was shown to be constitutively expressed in primary human blood T cells and in T cells anergised in vitro, and its downregulation was found necessary for T cells activation (Tzachanis et al., 2001).

Despite the fact that peripheral NP47F5 CD8 T cells are unresponsive to antigenic stimulation in vitro, in terms of proliferation and cytokines production, in vivo they retain some of their functions. Sensitive in vivo cytotoxicity assay showed that chronic stimulation of F5 CD8 T cells after transfer into NP47 hosts allows these cells to retain their cytolytic function against self-antigen pulsed cells. The fact that antigen-expressing cells, expressing high amounts of antigen were absent from NP47F5 mice, argues that self-reactive NP47F5 CD8 T cells may be cytolytic in vivo, against normal tissue. Therefore the cytolytic capacity may only be displayed against APC pulsed with artificially high amounts of NP68 antigen.

Peripheral T cells partially inactivated have been reported in other systems. In one of them where high avidity CD8 T cells specific for K^b antigen were allowed in the periphery of manipulated chimeric mice expressing K^b antigen in islet β cells, self-reactive T cells ignored islet antigen. However increase in the avidity after priming of these cells with professional APC expressing K^b enabled them to cause autoimmunity and infiltrate and destroy islet β cells (Heath et al., 1995). Also in a study where self-reactive OVA specific CD8 T cells were transferred into mice expressing OVA antigen in intestinal epithelium, cells were activated in draining lymph nodes and after an initial deletion, cells that remained infiltrated epithelium without causing any damage. Upon viral infection these cells destroyed tissue and remained in the epithelium as partially anergic, retaining their cytolytic activity but without producing any cytokines (Vezys et al., 2000). The anatomical location of antigen was important in the avoidance of autoimmunity in that system. Cross
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activation of self-reactive T cells in lymphoid tissues resulted in deletion whereas partial inactivation was induced in the mucosa.

Peripheral self-reactive NP47F5 CD8 T cells on the contrary do not require priming with virus to become cytolytic. This may be explained by the fact that antigen is expressed only on BM-derived cells, capable of providing co-stimulation and initiate full response. It has to be mentioned that in the assessment of in vivo cytotoxicity (section 3.5.1), viral infection for 7 days of NP47 mice containing self-reactive F5 CD8 T cells, did not cause any apparent autoimmunity, and therefore it seems unlikely that such a treatment can break tolerance in these mice. In that case the question remains how is peripheral tolerance maintained in NP47F5 system where self-reactive peripheral CD8 T cells can be cytolytic without a need for inflammation?

Several studies show that tolerance versus activation or memory stages fail to account for all stages or properties of antigen-experienced cells. Intermediate stages of differentiation may occur depending on the priming conditions and the properties of the tissue expressing the antigen. Maintenance of peripheral tolerance, by inactivation at the level of expansion and autocrine growth rather than at the level of effector function has been shown for both CD4 and CD8 self-reactive T cells. Transgenic CD4 T cells specific for OVA peptide rendered tolerant in irradiated hosts after several rounds of antigenic stimulation in vivo, were found unable to proliferate and produce IL-2 upon antigenic stimulation. However these cells were capable to produce other effector cytokines and to provide CD4 help to B cells for the production of IgG antibodies (Malvey et al., 1998). Similarly CD8 transgenic T cells specific for a tumour-associated antigen, in mice expressing this antigen in hepatocytes, were found partially inactive. They showed impaired proliferation and IL-2 production against in vitro antigenic stimulation, however retained their ability to lyse target cells in vitro upon stimulation in the presence of IL-2 (Ohlen et al., 2002). This raises the question of whether or not control of peripheral autoreactive T cells at the level of expansion can be efficient even if control at the level of effector functions is incomplete. In other words how efficiently can tolerance be maintained if some amount of autoreactivity is permissive in a very low frequency of autoreactive T cells?

T cells partially tolerant to organ specific antigens in some cases can be activated upon encounter of high-localised expression of antigen but without subsequent tissue
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destruction. Such a case was reported in a study where mice with transgenic CD4 T cells specific for hen egg lysozyme were crossed with mice expressing cognate antigen on thyroid epithelium, or on pancreatic islet β cells, or systemically. Depending on the pattern of antigen expression deletion of thymocytes ranged from minimal to complete and peripheral CD4 cells exhibited grated *in vitro* unresponsiveness and *in vivo* helper activity. Interestingly the least tolerant cells were found in mice expressing antigen on thyroid epithelium or on islet β cells. These self-reactive cells showed reduced proliferation to antigenic stimulation *in vitro* and this status was not reverted by addition of IL-2. However cells were able to provide help to B cells and mice developed subclinical autoimmune inflammation to tissues expressing antigen. Sub clinical thyroiditis or insulitis nevertheless did not eliminated antigen expressing endocrine cells (Akkaraju et al., 1997).

NP47F5 mice, containing self-reactive peripheral T cells, in comparison to single transgenic NP47 mice, contain fewer BM-derived cells (DC and Mφ) with high levels of NP antigen. This indicates that there is a degree of autoreactive killing ongoing in NP47F5 mice, against cells expressing high amounts of antigen. It is therefore possible that low degree of autoreactivity is permitted in this system because the affected tissue DC and Mφ is always regenerated from the BM. Antigen expression in a “non-dangerous” site, such as BM derived cells, can activate self-reactive T cells without causing measurable tissue damage. Once high antigen expressing cells are eliminated, continuous low-level antigenic stimulation may render cells in a state where expansion and release of cytokines are abrogated, but cytolytic activity is not. The fact that DCs can be targets of CD8 CTL cells *in vivo* was shown in a study of chronic LCMV infection in mice, caused by a specific LCMV clone, which infects DCs. In this study, virus induced immuno-suppression was associated with CD8 CTL-mediated destruction of virus-infected DCs in the spleen (Borrow et al., 1995).

In line with this hypothesis antigen persistence in viral immune invasions has been reported to sustain activated cells in an unresponsive state (Ehl et al., 2000; Zajac et al., 1998). In these cases the same type of APC delivering similar signals induced activation and unresponsiveness, but the outcome was depended on the dose and duration of persistence of these cells. Also a similar to NP47F5 antigen induced unresponsive state has been reported for CD8 transgenic T cells (OT-1) specific for OVA peptide. When OT-1 Tg mice were injected i.p. with OVA expressing tumour cells, T cells migrated to the
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peritoneal cavity where they expanded and controlled tumour growth. These cells had direct ex-vivo lytic activity but were not able to proliferate in response to full antigenic stimulation in vitro. However their proliferative capacity upon antigenic stimulation was rescued with exogenous IL-2 (Tham et al., 2002). It was suggested in this study that induction of partial unresponsiveness upon continuous antigenic stimulation may serve as a regulatory check point for a potentially autoreactive CTL response, keeping the cells at low frequency, ensuring that expansion is CD4 helper (IL-2) dependent.

A similar finding was reported in a study where a single clone of transgenic male specific CD8 T cells was stimulated in vivo in male/female chimeras. In that study it was found that priming in the absence of CD4 help, rendered CD8 T cells partially inactive. These cells had impaired ability to proliferate and produce cytokines upon antigenic stimulation, however they were able to eliminate antigen-expressing B cells in vivo. When these cells received CD4 help during secondary responses eventually recovered their functions and behaved as memory cells. (Bourgeois et al., 2002). Although even in the presence of CD4 help anergy induction was not prevented. Antigen induced unresponsiveness was reverted in the presence of CD4 help.

Since in the NP47F5 system antigen expression on BM-derived cells allows some amount of autoreactivity, the question is raised whether or not peripheral tolerance can be maintained if antigen is expressed on a "dangerous" site where autoreactivity can cause measurable tissue damage. In order to test this NP47F5 mice are currently crossed with 48RagNeg mice. In the latter mice, the human growth hormone promoter, which includes an LCR control element, drives expression of influenza nucleoprotein peptide (NP) specifically in the anterior pituitary gland. When 48RagNeg mice are crossed with F5 mice, double transgenic 48RagNeg X F5 mice develop a dwarf autoimmune phenotype, due to migration of self-reactive T cells into anterior pituitary and destruction of growth hormone, NP antigen expressing cells (Jersey et al., 2002). Using this model NP47F5 X 48RagNeg, the in vivo function and autoimmune potential of self-reactive NP47F5 T cells can be assessed by measuring the effect of these cells on growth hormone producing cells. This can give information on whether or not autoreactivity can be detected on in the NP47F5 system dependent on the site of antigen expression.

Considering the fact that NP47F5 T cells retain some functional activity, which appears to even persist in vivo (e.g. killing of APC with high levels of NP expression), we
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feel the term “anergic” is inadequate to describe their functional state. A better expression would be the term “catatonic” which describes a state of partial paralysis that follows initial activity.

In conclusion peripheral tolerance induction is a complicated procedure as it is based on highly dynamic events. The variables that determine such multi-parametric events include not only the nature and the state of cells and the signals that they exchange, but also other factors such as the dose, localisation, distribution kinetics and persistence of the antigen. Unravelling these variables requires further investigation in order to have a clearer picture of the events that lead to the maintenance of peripheral tolerance.
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