Regulatory T cell development and T cell mediated tolerance.

Inauguraldissertation

Zur

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Prof. Dr. Eberhard Parlow

A mon père,

Summary

T cell tolerance is achieved through multiple mechanisms. In this study we have tried to characterize tolerance and T cell development in various situations. First, in the setting of bone marrow transplantation, we could show that radioresistant T cells from immunocompetent mice protect against the development of syngeneic graft-versus-host disease whereas immunodeficient mice succumb to autoimmunity. However, co-injection of sorted regulatory T cells is able to prevent the development of the disease. Second, by further investigating radioresistant T cells in the thymus of bone marrow chimera, we could show that a small population of host-derived DN1-2 pro-thymocytes showed similar properties of radioresistance. Moreover, this small population is able to generate a single wave of developing T cells, which participate in immune protection of the host before donor-derived T cells can provide protective immune reconstitution. Third we took advantage of the protective role of regulatory T cells during syngeneic bone marrow transplantation described above to study γ/δ T cell development and to investigate the role of the rearranged β chain found in 15% of γ/δ T cells. We could show that the γ/δ-derived β chain is actually indistinguishable from the β chain isolated in α/β T cells and is able to take part in the development of fully functional α/β T cells. Finally, we have generated double transgenic mice by expressing the agonist antigen ovalbumin in specific cell subsets concomitantly with OVA-specific TCR. Several similar models have been previously used to study tolerance and development of regulatory T cells. We characterized the tolerant status of these mice and showed that the choice of the agonist along with the TCR affinity for the same agonist is playing a significant role in the outcome of double transgenic mice.

Part I

Sublethally-irradiated, immuno-deficient, C57Bl/6 RAG-2 gene-deleted recipient mice reconstituted with T cell-depleted bone marrow grafts frequently developed diarrhea, lost weight and showed signs of auto-immunity, dying between four and seven weeks after reconstitution. Mice died despite evidence of efficient donor-derived hemato-lymphoid reconstitution and disease was associated with the presence of IgG anti-nuclear antibodies. Auto-immunity was initiated by T cells, but could be prevented by transfer of naturally arising regulatory T cells. In contrast, lethally-irradiated, bone marrow-reconstituted immuno-competent, C57Bl/6 mice survived without signs of auto-immunity. Survival of immuno-competent mice was shown to be due to the presence of residual, extra-thymically-located, radio-resistant, functional regulatory T cells. The importance of regulatory T cells was further shown by the reduced survival of immuno-competent BM recipients whose $CD25^+$ T cells had been depleted prior to bone marrow transplantation. The implications of these results in the context of syngeneic graft-versus host disease following BM transplantation will be discussed.

Part II

It has been known for more than thirty years that in lethally irradiated bone marrow chimeras, part of the reconstituted T cell compartment is derived from the irradiated host. However, the detailed origin and functional activity of these host-derived T cells has not been thoroughly analysed. Here, we generated bone marrow chimeras by reconstituting lethally irradiated C57BL/6 mice with either syngeneic RAG2-deficient or CD3-epsilon-deficient BM neither of which is capable of generating T cells and therefore, all surviving T cells were exclusively host-derived. We show that in the absence of donor-derived cells, host-derived T cells can reconstitute 35% of the normal T cell pool. By comparing thymectomized versus non-thymectomized host, we show that host-derived T cells comprised a major (70%) subpopulation of *de novo* generated, thymus-derived, polyclonal, naïve cells and a minor subpopulation of surviving, peripheral, oligoclonal, memory-like cells. Host-derived thymocytes regenerated from conventional DN1-2 prothymocytes and their differentiation recapitulated normal thymic ontogeny. Thus, host-derived T cells might provide a first line of defence against infections during recovery from lymphopenia after BMT. This conclusion is supported by the fact that host-derived T cells were fully functional.

Part III

Between 10 and 20% of the peripheral γδ T cells express cytoplasmic TCRβproteins, but whether such TCRβ chains can partake in αβ T cell development has never been systematically investigated. Therefore, we reconstituted the T cell compartment of CD3ε deficient mice with Pax5-TCRβ deficient pro B cells expressing, via a retroviral vector, TCRβ chains from either peripheral γδ or αβ T cells. The thymi of recipients reconstituted with pro B cells containing empty vector were small (10-15 x10^6 cells), contained few γδ T but no αβ T cells. In contrast, thymi from mice receiving pro B cells containing γδ or αβ T cell-derived β chains contained 80-120 x10^6 cells, and showed a normal CD4, CD8 and αβ TCR expression pattern. However, regardless of the source of TCRβ chain, 4 weeks after transplantation, mice developed diarrhea, lost weight and showed signs of autoimmunity dying 5 to 15 weeks following reconstitution. Autoimmune disease induction could be prevented by co-transfer of regulatory T cells thereby allowing the functionality of the generated T cells to be assessed. Results obtained show that TCRβ chains from γδ T cells can efficiently take part in αβ T cell development. The implications of these findings for γδ T cell development will be discussed.

Part IV

In order to avoid autoimmunity the T cell compartment has to be tolerant to self. In the thymus T cell tolerance (central tolerance) is established by deletion (negative selection) while peripheral T cell tolerance is mediated by the induction of anergy, by suppression through Tregs and by activation induced cell death. Recently we showed that transgenic mice expressing Influenza hemagglutinin (HA) under of the kappa light chain (KLC) promoter when crossed with the mouse expressing a CD4, HA specific TCR (TCR-HA) led to the formation of TCR-HA regulatory T cells. On the contrary when mice expressing HA under the control of the CD11c promoter were crossed with TCR-HA mice, these double transgenic mice developed systemic autoimmunity. Analysis of the T cell compartments in these mice revealed that TCR-HA T cells escape negative selection in the thymus by the expression of a second endogenous TCR alpha chain. Thus the systemic autoimmunity seems to be caused by T cells expressing two TCRs. In order to test whether the development of systemic autoimmunity is restricted to the HA system or more general we have now generated transgenic mice expressing OVA under control of the CD11c and KLC promoter. From both transgenic lines founders have been identified that show strong negative selection in the thymus and the periphery of OT1 (MHCI restricted OVA specific TCR) and OT2 (MHCII restricted OVA specific TCR) T cells.

Introduction

In order to face the multiple and various attacks coming from the environment, vertebrates have throughout evolution developed an immune system with different levels of complexity involving many different cell types with various degree of specialisation.

Haematopoiesis

Every cell of the immune system originates from a common precursor located in the bone marrow (BM): the haematopoietic stem cell (HSC). HSCs are defined by their ability to renew themselves and to give rise to all mature blood cell types. These unique properties of pluripotency and self-renewal capacity have been used for many years in clinical care through bone marrow transplantation for reconstituting a life-long complete haematopoietic system in immunodeficient patients.

Haematopoiesis begins in the mouse embryo as early as embryonic day E7.5 in the yolk sac (Figure 1) (Dzierzak and Speck, 2008). Around E10, haematopoietic stem cells migrate to the foetal liver where they undergo further differentiation. They colonise thymus and spleen around E11 and E12.5, respectively. Finally, starting at E15, the BM becomes the main site for haematopoiesis and ensures continuous production of 10^{11} to 10^{12} blood cells daily throughout life.

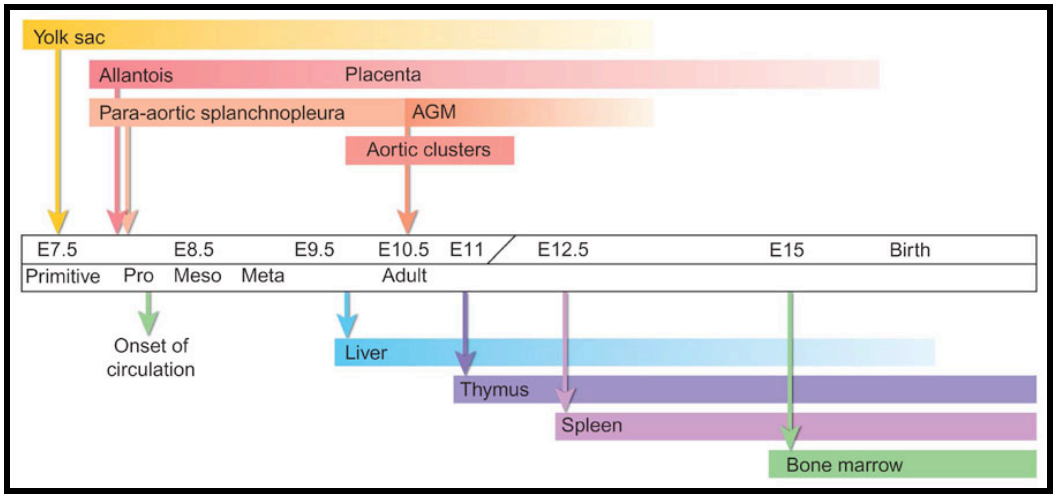


Figure 1: Haematopoiesis:

Arrows above indicate the onset of specific hematopoietic cell generation and/or appearance; arrows below indicate the earliest time of colonisation of the secondary hematopoietic territories. Adapted from Dzierzak and Speck, 2008.

Among the HSC population, a small fraction lacking expression of the cytokine receptor fms-related tyrosine kinase 3 (FLT3) is able to self-renew, whereas $FLT3^+$ population are non-renewing cells and are referred to as multipotential progenitors (MPP). Three different lineages arise from HSCs that are defined as Lineage negative (Lin^-) Stem-cell antigen 1 positive ($SCA1^+$) cKIT^{hi} (also called CD117) (LSK). The erythroid cell lineage is represented by red blood cells and megakaryocytes. These cells arise from megakaryocyte/erythroid progenitors (MEPs), which themselves originate from MPPs. The common myeloid progenitor (CMP), derived from MPPs, gives rise to granulocytes, megakaryocytes, and macrophages. Myeloid cells can play a role in innate, adaptive immunity and coagulation. Finally, the third lineage is comprised of lymphoid cells such as B and T lymphocytes that are key players in adaptive immunity. These cells develop from a Common lymphoid Progenitor (CLP) characterised by low expression of cKIT, high level of interleukin 7 receptor α-chain (IL-7Rα) and FLT3.

Along their differentiation towards committed precursors, HSCs express various key transcription factors such as Pax5 or Ikaros (Figure 2). These transcription factors are essential, as mutations or deletions of their genes result in a complete or partial block in cell lineage development. They have been used to define different developmental stages of haematopoiesis in that they reflect the commitment of a cell to a discrete cell lineage. In addition, these developmental stages can be characterized by surface expression of various receptors for cytokines, such as the receptor for IL-7 or growth factors like colony stimulating factor (CSF). Receptor expression is tightly regulated since it conditions the responsiveness of a stage-specific cell to receive a signal that allows it to further differentiate. Many of the ligands for these receptors, cytokines as well as growth factors, are provided by the microenvironment and more specifically by stromal cells that support HSC growth and differentiation. Hence, the HSC's fate is thought to be determined by the microenvironment, which provides the growth factors necessary for cell survival.

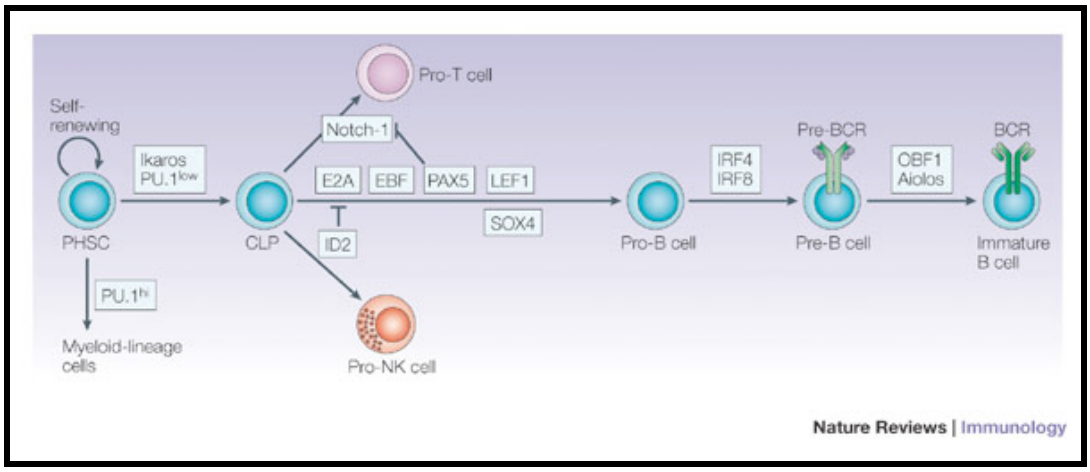


Figure 2: The genetic control of B-cell specification and commitment in the bone marrow.

This diagram shows a scheme of the genetic switch that regulates early B-cell specification. For simplicity, only some of the differentiation stages are shown. The developmental steps that are affected by the mutation of specific transcription factors are indicated. In some cases, the simultaneous mutation of two factors is required for a developmental phenotype to be observed. ID2 (inhibitor of DNA binding 2)-deficient mice have impaired natural killer (NK)-cell development and increased E2A activity, which correlates with an increased frequency of class switching to IgE. BCR, B-cell receptor; EBF, early B-cell factor; CLP, common lymphoid progenitor; IRF, interferon-regulatory factor; LEF1, lymphoid-enhancer-binding factor 1; OBF1, OCT (octamer-binding transcription factor)-binding factor 1; PAX5, paired box protein 5; PHSC, pluripotent haematopoietic stem cell; SOX4, sex-determining region Y (SRY) box 4 (adapted from Matthias and Rolink).

Different models have been proposed to describe haematopoiesis. Whereas it was originally thought that HSC differentiation was rather linear, with a progressive loss of lineage potential corresponding with increasing differentiation, recent studies have shown that committed progenitors may actually maintain the potential to differentiate into various lineages until late in development. As an example, pro B cells from Pax5-deficient mice can develop into myeloid, NK and T cells both *in vivo* and *in vitro* (Rolink et al., 2002). Among the different models that have been suggested, the widely accepted Weissman model is based on cell surface phenotype (Figure 3) (Kondo *et al*., 2003) A second model based on analyses of transcription factor mutant mice has been proposed by Singh *et al*. (Figure 4) (Medina and Singh, 2005). Finally, based on studies on *in vitro* proliferation of murine fetal liver progenitors, Katsura *et al.* proposed that all progenitors maintain a myeloid potential early into lymphoid and erythroid differentiation (Figure 5) (Katsura, 2002). It may be possible that these models are not mutually exclusive but rather complementary to one another. Indeed, Rolink *et al.* has proposed a fourth model integrating these previous models (Rolink *et al.*, 2006). We have chosen to base our work on the Rolink model of haematopoiesis, which results of the integration of the previous models and our experiments (Figure 6).

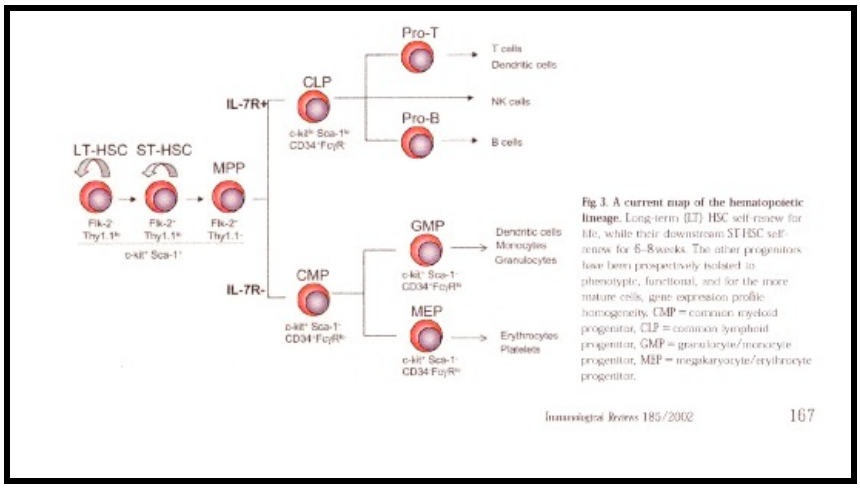


Figure 3: Weissman Model

Conceptual hematopoietic trees in adult mice: Indicated cell populations can be purified based on the cell surface phenotype. Not all of the linear relationships in this figure have been proven. Multipotent progenitors (MPPs), at least at the population level, can differentiate into all types of hematopoietic cells, but have no detectable self-renewal potential *in vivo*. Megakaryocyte progenitors have recently been identified. Pro T cells are present in the thymus (Adapted from Kondo *et al.*, 2003.).

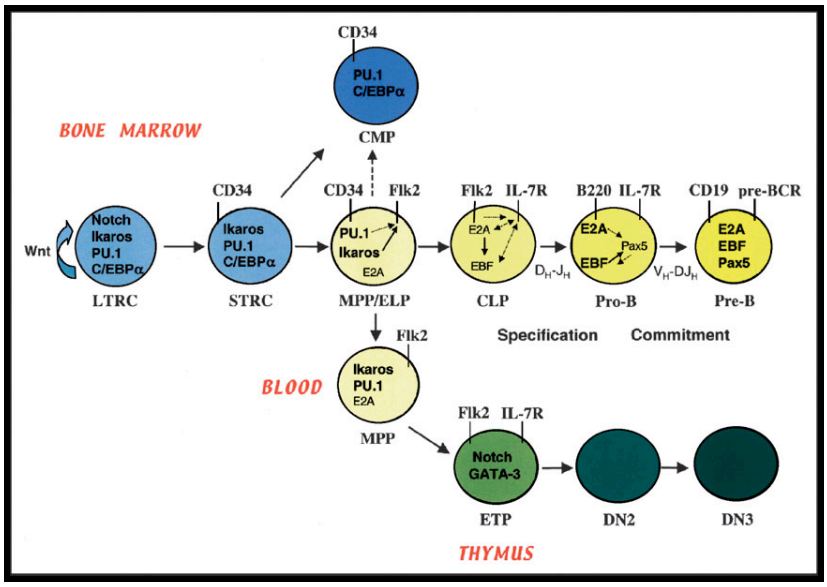


Figure 4: Singh Model

Developmental scheme for the generation of B cell precursors from multipotential hematopoietic progenitors: Alternate cell fate options (myeloid and T-lineage) are also shown. LTRC and STRC represent long- and short-term multilineage reconstituting cells. CMP, ELP, and ETP denote common myeloid progenitor, early lymphoid progenitor and early thymic progenitor, respectively. Regulatory molecules (signaling receptors and transcription factors) that are genetically demonstrated to be important for development are indicated. Bold case font is used to indicate changes in activity or expression state of the relevant transcription factor. Solid (experimentally based) and dotted arrows (proposed) highlight regulatory connections that can be assembled to form rudimentary networks. Cell fate specification and commitment are sequential transitions. CD19 is a B cell surface marker and pBCR refers to the pre-B cell receptor that is assembled after productive rearrangements of the IgH locus (Adapted from Medina *et al.*, 2005.).

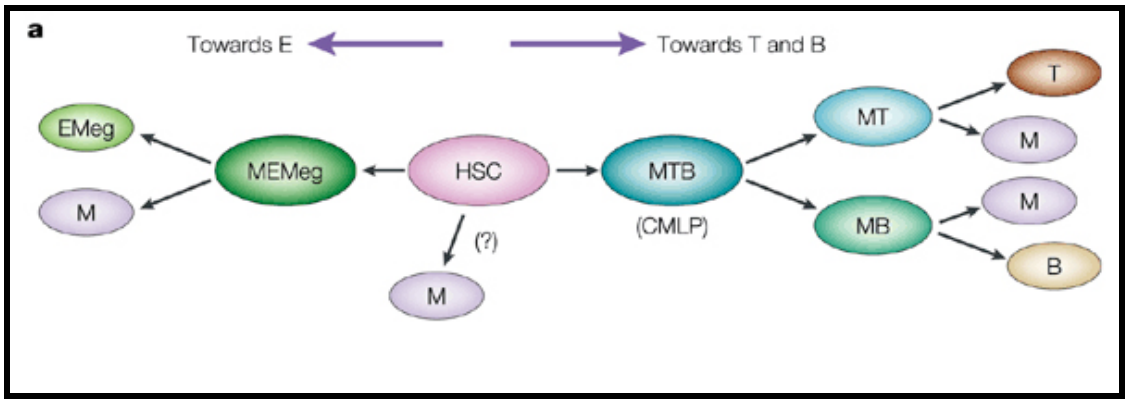


Figure 5: Katsura Model

Model of lineage commitment in hematopoiesis: A new model of hematopoiesis proposed from findings with the MLP–MTB and MLP–METB assays. Myeloid potential accompanies early stages of all T, B and erythroid (E) progenitors. T-cell and B-cell progenitors are produced from the common myelo-lymphoid progenitor (CMLP; p-MTB) through the intermediate p-MT and p-MB bipotent stages, respectively. It is still unclear whether a myeloid (M)-specific pathway independent of T, B or erythroid pathways exists. T, B, M, E and Meg (megakaryocyte) in this figure indicate the progenitor's name but not the name of mature cells (Adapted from Katsura *et al.*, 2002.).

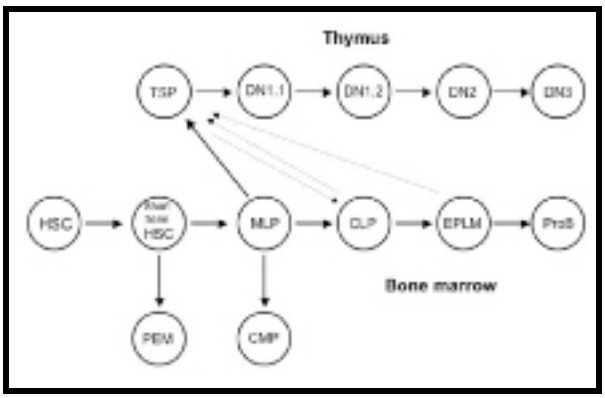


Figure 6 Current working model of hematopoietic development.

Figure 6 depicts our current working model of hematopoietic development. HSCs with long-term self-renewing capacity give rise to ones with only limited self-renewing potential. As proposed by the Katsura model mentioned above, the short-term self-renewing HSC gives rise to a progenitor with erythroid and myeloid (PEM) potential. The next cell in the scheme is a progenitor with a developmental potential restricted to the myeloid and lymphoid lineages (MLP). These cells are the direct precursors of the common myeloid progenitor (CMP), a cell type with a developmental potential restricted to the myeloid lineages. MLPs will moreover give rise the common lymphocyte progenitors (CLP), and some of the MLPs will migrate to the thymus (TSP: thymic seeding progenitor) and will there undergo the T cell differentiation program. Early stages of T and B cell development will be described in more detail below. The solid arrows in the model indicate the main developmental pathway. The broken arrows indicate the lymphocyte precursors in the bone marrow that still possess T cell developmental potential and thymocyte precursors that possess B cell developmental potential (Adapted from Rolink *et al.*, 2006).

Innate immunity

As a first line of defence against infection, the innate immune system mediates specific and direct responses against pathogens but does so with rather limited recognition capability and short-lived protective immunity. It includes four different levels of protection.

First, physical invasion of pathogens is prevented by an intact anatomical barrier. Thus, the skin and the mucosal surfaces of the gastrointestinal, respiratory and urogenital tracts are critical for efficient defence against pathogen attack. Second, regulation of pH and/or temperature maintains a physiological barrier that ensures further host protection. A third level of defence involves numerous soluble factors present in the blood or extracellular compartment. These include hydrolytic or digestive enzymes that degrade proteins of harmful microorganisms. In addition, antimicrobial substances such as α-defensins, interferon produced after viral infection, and complement components all play roles in fighting pathogen attacks. Finally, phagocytosis and endocytosis are used by multiple cell types and constitute the last degree of innate immune-mediated protection. Every cell can perform pinocytosis, on the other hand endocytosis is often mediated through a cell surface receptor. Phagocytosis is a property of specialized cell types such as macrophages, neutrophils, and dendritic cells; these cells can internalize and digest whole pathogenic microorganisms via specialized protein machinery.

Innate mechanisms are based on the detection of conserved molecular structures shared by a large group of pathogens, called pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors. As an example, lipopolysaccharide, a cell-wall component of all gram-negative bacteria, is recognized by Toll-like receptor 4 on the surface of dendritic and other cells. In this system, pattern recognition receptors signal the presence of infection and induce the production of antimicrobial proteins or peptides as well as pro-inflammatory cytokines. They also trigger the transcription of various gene products that control subsequent adaptive immune response.

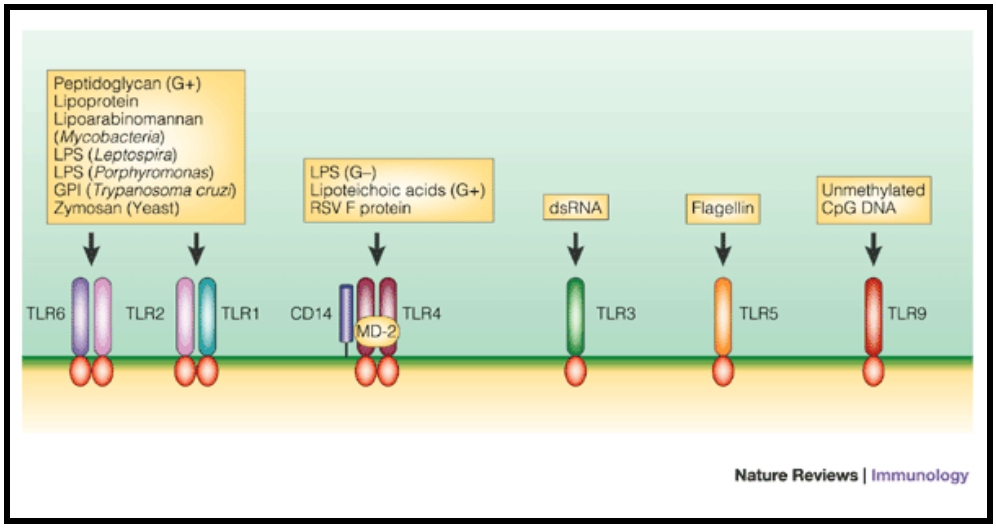


Figure 7: Ligand specificities of TLRs

Toll-like receptors (TLRs) recognize a variety of pathogen-associated molecular patterns (PAMPs). Recognition of lipopolysaccharide (LPS) by TLR4 is aided by two accessory proteins: CD14 and MD-2. TLR2 recognizes a broad range of structurally unrelated ligands and functions in combination with several (but not all) other TLRs, including TLR1 and TLR6. TLR3 is involved in recognition of double-stranded (dsRNA). TLR5 is specific for bacterial flagellin, whereas TLR9 is a receptor for unmethylated CpG motifs, which are abundant in bacterial DNA. G+, Gram-positive; G–, Gram negative; GPI, glycophosphoinositol; RSV, respiratory syncytial virus. Adapted from Medzhitov, 2001.

Innate immunity offers the advantage of triggering an immediate response before activation of the adaptive immune system. However, it displays a limited repertoire of recognition molecules and lacks the properties of memory or long-lasting immunity. The interaction of different cell types of the innate immune response with the lymphocytes of adaptive immunity allows for a coordinated response that is both immediate and powerful in the elimination of foreign pathogens.

Adaptive immunity

In parallel with innate immunity, vertebrates have developed an adaptive immune system to respond more efficiently to immune challenges. Adaptive immune responses allow the host to respond in a highly specific manner to a broader range of antigens, to develop memory responses, and to discriminate between self and non-self.

Key players of adaptive immunity are lymphocytes. There are two major types of lymphocytes, namely B cells and T cells. These are very specialized cells that display each of the specific features mentioned above. They are highly specific via cell surface expression of the T cell receptor (TCR) or B cell receptor (BCR). Moreover, they encompass a highly diverse repertoire of specificity through gene recombination, and/or somatic hypermutation in the case of B cells. They are also able to differentiate into long-lived memory cells. Finally, through continual clonal selection throughout their development, T cells are able to distinguish non-self antigen presented exclusively by major histocompatibility complexes (MHC).

B lymphocytes

B cells initially develop from a common lymphoid progenitor within the bone marrow and further differentiate into mature B cells within secondary lymphoid organs.

B cells are specialized in triggering a humoral immune response specifically aimed at eradicating extracellular pathogens. They respond to antigen stimulation by differentiating into plasmocytes [plasma cells?], which produce a large quantity of antibodies directed against

specific antigenic epitopes. One cell is able to produce only one specific type of antibody able to bind one particular epitope of a given antigen. Their diversity potential is so high that B cells can together produce up to 10^{11} different antibodies.

B cell differentiation and germinal center reaction

Progenitor B cells, the first cells of the B lymphocyte lineage, develop within the bone marrow. They proliferate and differentiate into precursor B cells by influence of stromal cell products like stem cell factor (SCF) and interleukin 7 (IL-7) (Figure 8).

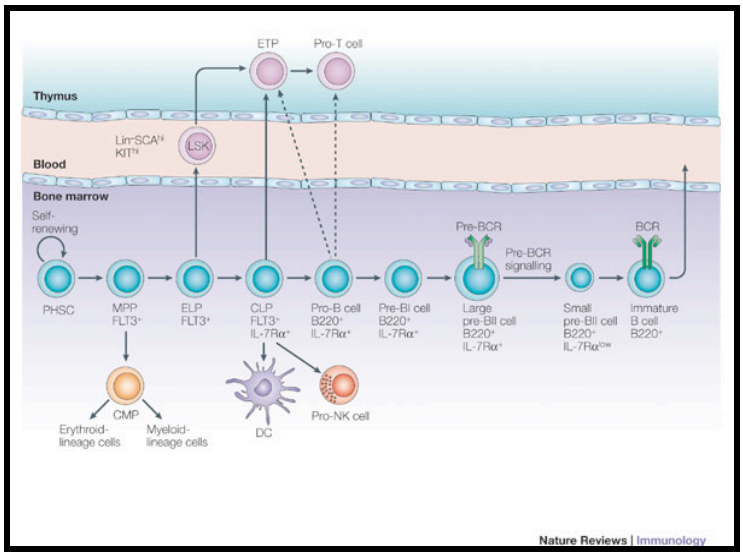


Figure 8: B cells develop from early haematopoietic progenitors.

The main compartments — bone marrow, thymus and blood — are shown. The various developmental stages that have been defined are indicated, as well as their relative order. Dashed arrows show pathways that are not yet firmly established. Pluripotent hematopoietic stem cells (PHSCs), multipotential progenitors (MPPs), common myeloid progenitors (CMPs), early lymphoid progenitors (ELPs) and common lymphoid progenitors (CLPs) are known as lineage $(Lin)^-$ cells; these cells lack detectable expression of any of the markers that are associated with cells of the mature blood lineages or their committed progenitors -- that is, CD3, CD8, B220, CD11b, CD19, GR1 and TER119. Cells that are defined as LSK are Lin^- stem-cell antigen $(SCA)^{hi}KIT^{hi}$, which is a possible precursor stage to early T-cell-lineage progenitors (ETPs) (Schwarz and Bhandoola, 2004) Using the Hardy classification (Hardy and Hayakawa, 2001), pro-B cells (also known as fraction B/C) are defined as $B220^+CD43^+$, and pre-B cells (also known as fraction C'/D) are defined as $B220^+CD43^-$ surface IgM^-. BCR, B-cell receptor; DC, dendritic cell; FLT3, fms-related tyrosine kinase 3; NK, natural killer; IL-7R, -chain of the interleukin-7 receptor (Adapted from Matthias and Rolink, 2005.).

B cell maturation involves immunoglobulin (Ig) gene rearrangement, leading to extraordinary antibody diversity. This process consists of somatic rearrangement of germline-encoded immunoglobulin segments, the V(D)J genes, creating a new sequence encoding for a unique BCR molecule (Figure 9). One single productive rearrangement occurs in an individual lymphocyte due to the mechanism of allelic exclusion: as soon as a productive rearrangement is accomplished, any further rearrangement is blocked. Recombination activating gene products

RAG-1 and RAG-2 are essential for the gene recombination process to occur. During the process of recombination, the terminal deoxynucleotidyl transferase (TdT) enzyme provides further junctional diversity by adding a few nucleotides at the gene segment junctions.

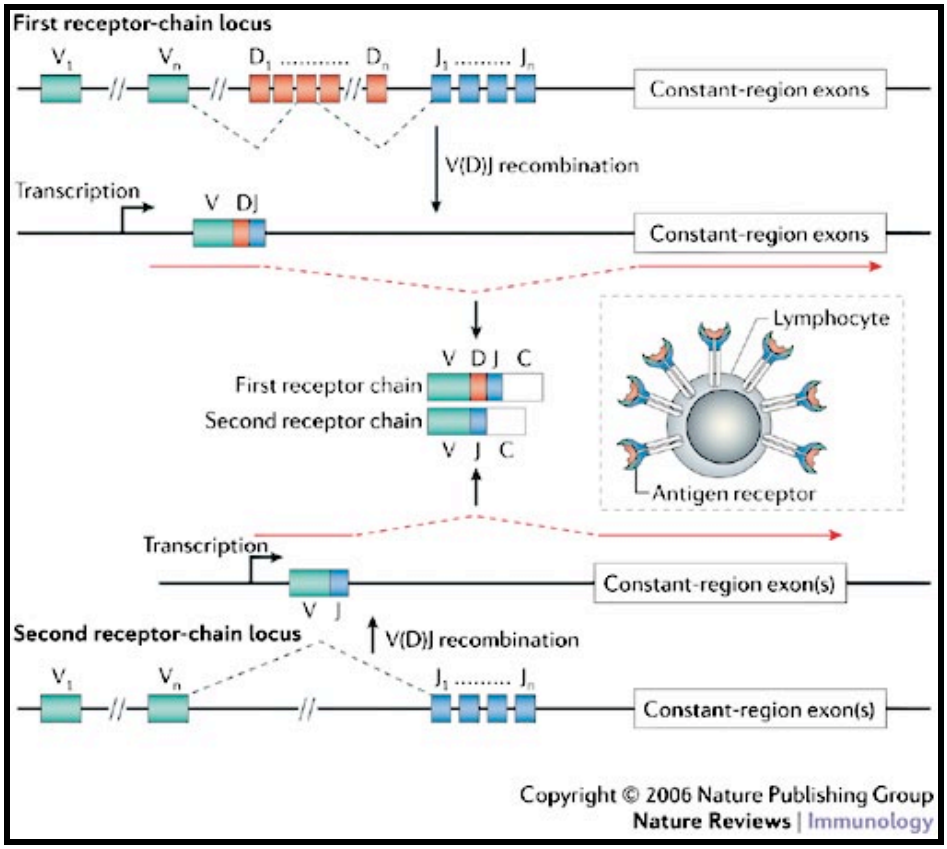


Figure 9: General scheme of V(D)J recombination for assembly of antigen-receptor genes.

Antigen receptors consist of two protein chains that are encoded by independent loci. One locus (the first receptor-chain locus) is generated by the assembly of individual germline variable (V), diversity (D) and joining (J) minigene elements from among multiple minigene elements. The other locus (the second receptor-chain locus) lacks D elements and is assembled by direct V-to-J joining. V, D and J elements are recombined through the activity of recombination-activating gene 1 (RAG1)–RAG2 protein complexes and the non-homologous end-joining machinery to generate VDJ or VJ combinations. Upon transcription, mRNAs that encode the protein chains are generated and spliced as indicated by the red lines. D-element-encoded regions, and junctions between V, D and J elements, are centrally displayed in the antigen-receptor antigen-combining site, as depicted in the inset. C, constant (Adapted from (Nemazee, 2006).

Upon completion of the heavy chain rearrangement, a pro B cell becomes a pre B cell. Further rearrangement of the Ig light chain provides the immature B cell with a given antigenic specificity. When they leave the bone marrow en route to the spleen, B cells are still immature and are characterized by membrane-bound immunoglobulin IgM that together with heterodimers of Ig-α and Ig-β forms the B cell receptor (BCR). They also express on their surface flow-cytometric markers B220 (CD45R) and CD19. Throughout the maturation process, if an immature B cell encounters a self-antigen, it becomes anergic or dies by apoptosis. This

process is known as negative selection. However, there is also evidence that at least some immature B cells can be rescued from apoptosis by receptor editing. In other words, B cells are able to express a second light chain to form a novel non-autoreactive BCR (Nemazee, 2000; Tiegs *et al.*, 1993).

Once in the periphery, activation, proliferation and differentiation of a given mature B cell requires an encounter with its specific antigen (Figure 10). These steps occur in the lymph nodes or in the spleen, depending on whether the antigen originates in the lymphatic vessels or blood, respectively. A B cell binds the antigen, internalizes it, and presents it on its MHC II surface molecules. The initial activation of B cells takes place in the paracortex, where T and B cells interact, via CD40, the MHC II-Ag complex and cytokines, to form a B-T conjugate. This interaction leads to proliferation of B cells to form primary foci. B cells then differentiate into plasma cells that secrete IgM isotypes. Subsequently, a few activated B cells along with some T_{helper} cells migrate to primary follicles, which becomes secondary follicles. Subsequently proliferation of activated B cells forms a germinal center. Activated B cells undergo clonal expansion and somatic hypermutation; they are then called centroblasts and constitute the so-called dark zone of the germinal center. The selective survival of high-affinity centroblasts leads to their differentiation into centrocytes. Centrocytes move to the light zone where they encounter antigen presented by follicular dendritic cells. Subsequently, centrocytes will form two kinds of progeny: small memory B cells and large plasmablasts. The first population of cells will go back to the bone marrow and recirculate in the periphery. Plasmablasts remain predominantly in the lymph nodes as short lived memory cells, but may also be found in the bone marrow as long-lived memory cells.

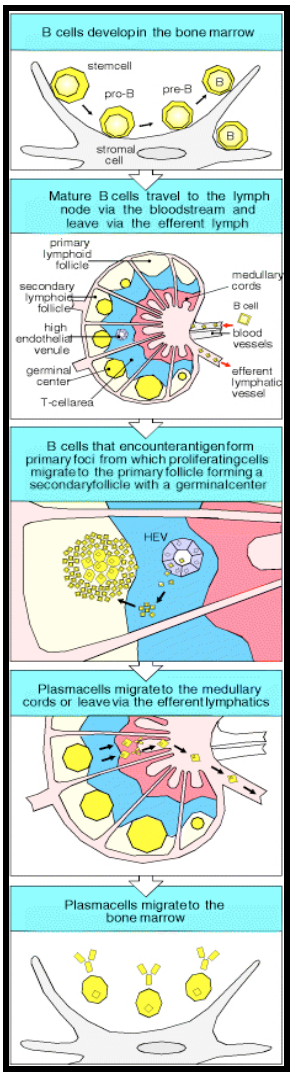


Figure 10: Activated B cells form germinal centers in lymphoid follicles

Some B cells activated in the primary focus migrate to form a germinal center within a primary follicle. Germinal centers are sites of rapid B-cell proliferation and differentiation. Follicles in which germinal centers have formed are known as secondary follicles. Within the germinal center, B cells commence their differentiation into either antibody-secreting plasma cells or memory B cells. Plasma cells leave the germinal center and migrate to the medullary cords or leave the lymph node altogether via the efferent lymphatics and migrate to the bone marrow. Memory B cells continue to recirculate through the B-cell zones of secondary lymphoid tissue (not shown) and some may preferentially reside in the splenic marginal zone (Adapted from Immunobiology, Janeway, Charles A.; Travers, Paul; Walport, Mark; Shlomchik, Mark New York and London: Garland Science ; 2001.).

T lymphocytes

T lymphocytes are thymus-derived and are specialized in immunity against intracellular microbes such as viruses or intracellular bacteria and provide help to B cells in the generation of antibodies.

The Thymus

The thymus is a bilobed organ located in the upper anterior thorax where T cell development occurs. Each lobe is comprised of an outer cortex, which contains a dense collection of thymocytes, and an inner medulla, which contains fewer thymocytes. Three major cell types of distinct developmental origin are found in the thymus, mesenchymal cells and thymic epithelial cells constituting the stroma and bone marrow-derived T lymphocytes. Mesenchymal components of the stroma derive from the neural crest; they are a constituent of the thymic capsule and septae but can also be found in the cortex. Mesenchymal cells have been shown to influence thymic development in two different ways. First, they influence the initial stage of thymic formation by regulating thymic epithelial cell differentiation and proliferation through various interactions and production of fibroblast growth factor (FGF). Second, mesenchymal fibroblasts support T cell precursor survival and early maturation by secreting IL-7. Thymic epithelial cells (TEC) are stromal cells of endodermal origin. They originate from the third pharyngeal pouch to form a thymic anlage, which attracts cells of haematopoietic origin at day E11.5. TECs constitute a complex network that provides an optimal microenvironment for the development of bone marrow-derived cells, namely thymocytes, macrophages and dendritic cells (DC). During development, there is mutual interaction between thymic epithelial cells and thymocytes such that the latter influence the development, survival and organization of the thymic epithelial network, which in return supports thymocyte maturation by providing cytokines essential for thymocyte proliferation.

The rate of T cell production is not constant throughout lifetime. Production is highest during younger years and drastically slows down in adults to a level just sufficient for maintaining a constant peripheral pool of T cells. This age-related thymic involution is responsible for the absence of new T cell production in older individuals.

In the mature thymus, TECs are subdivided into medullary and cortical (mTEC and cTEC, respectively). TECs mediate central tolerance along with BM derived thymic DCs (BMdDCs).

Uncommitted lymphoid precursors coming from the BM enter the thymic cortex via blood vessels at the cortico-medullary junction. The most immature thymocytes do not express antigen receptors or T cell markers such as CD4 or CD8, and are hence called double-negative (DN $CD4^-CD8^-$) thymocytes. They represent about 5% of the total thymocyte population. In the cortex, thymic precursors undergo intense proliferation and differentiation for about one week. Thymocytes migrate from the cortex, transitioning from DN to double-positive (DP $CD4^+CD8^+$), then through the medulla, where the vast majority of thymocytes become single-positive (SP $CD4^-CD8^+$ or $CD4^+CD8^-$). Along the way, they interact with various cell types, namely cTEC and macrophages in the cortex and mTECs, thymic DCs and macrophages in the medulla (Figure

11). En route to the medulla, thymocytes rearrange their TCR, first the β chain followed by the α chain, to become $CD4^+CD8^+$ DP cells. A particular population of DN cells do not rearrange their β and α TCR genes, but rather rearrange their γ and δ genes. These cells acquire only the CD3 surface marker, do not transition through the $CD4^+CD8^+$DP stage, and become γ/δ T cells. Specifics of the development of this particular population will be discussed in a specific chapter later. $CD4^+CD8^+$DP thymocytes undergo positive selection in the cortex, which allows for the selection of cells able to recognize self-MHC molecules. This is followed by negative selection, which eliminates cells recognizing self-antigens. These two processes will be discussed in more detail later.

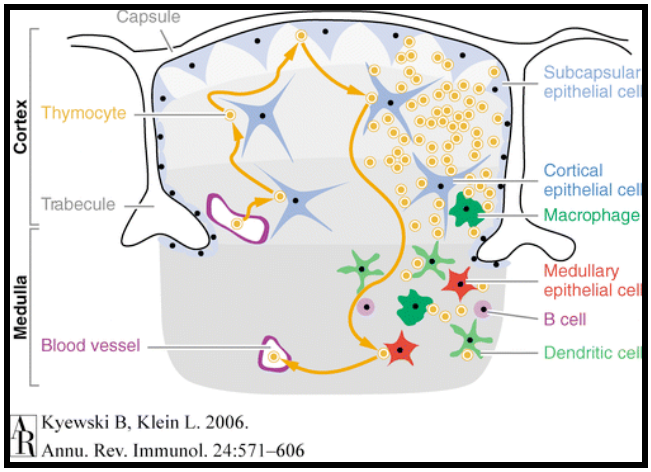


Figure 11: Cellular composition of the thymus.

The major cell types and the sequential cell-cell interactions along the migratory route of developing thymocytes are depicted here. The different APCs are color-coded. mTECs, highlighted in red, play an essential role in self-tolerance induction toward tissue-restricted self-antigens. Shaded areas depict functionally distinct stratified microenvironments as recently proposed (Petrie, 2003). (Adapted from Kyewski, 2006.)

More than 95% of thymocytes will die by apoptosis in the thymus throughout the maturation process, either because they were not able to produce a functional TCR or failed to receive a survival signal after positive selection or rearrangement of their TCR genes or because they received an apoptotic signal during negative selection. The dying cells are ingested by macrophages present in the cortex as well as in the medulla.

After positive and negative selection, thymocytes downregulate one of their co-receptors, either CD8 or CD4, and become a SP $CD4^+CD8^-$ or SP $CD4^-CD8^+$. αβTCR $CD4^+CD8^-$ T cells represents about 12% of the adult thymocyte population. These are MHCII-restricted and have a helper activity function. On the other hand, αβTCR $CD4^-CD8^+$ T cells display a cytolytic activity and are MHCI-restricted. Once these two mature populations reach the periphery, they are called $CD4^+$ helper (Th) and $CD8^+$ cytolytic (CTL) T lymphocytes.

Early T cell development

As mentioned above, MLP give rise to B cells as well as T cells. Although it has been shown that other progenitors retain T cell lineage potential – i.e., they are able to initiate T cell development when artificially injected intravenously - MLP are considered to be the most probable physiological originator of the early T-cell progenitor. Since there are no HSCs within the thymus, there is a constant need for BM progenitors to colonize the thymus in order to maintain a constant rate of T cell development. It is thought that thymus-settling progenitors (TSPs) characterised by a $ckit^{int}CCR9^{+}CD44^{+}CD25^{-}Flt3^{+}$ phenotype migrate to the thymus to undergo T cell differentiation.

One very important determinant for T cell commitment is Notch1 receptor signaling, as the thymus of Notch1 deficient mice is colonised by B cell precursors (Radtke *et al*., 1999). Many studies have been trying to characterise the earliest TSP, which retains B cell potential and loses it upon Notch signaling as shown by Radtke *et al*. It is well established that these cells belong to the DN $CD4^{-}CD8^{-}$ population.

The DN population can be subdivided into four different subsets according to CD25 and CD44 markers, corresponding with four successive developmental stages (Figure 12). T cell progenitors enter the thymus via the blood as $Lin^{low}ckit^{high}CD25^{-}$ cells and then develop into DN1 cells, which are $CD25^{-}CD44^{+}$. When they acquire CD25 cell surface marker to become DN2, they start to rearrange the β chain locus. DN3 thymocytes downregulate CD44 and c-kit to become $CD25^{+}CD44^{-}$. They are arrested at this stage until they productively rearrange the β chain locus of the TCR; this checkpoint is called β selection. TCR β chain pairs with a surrogate pre-Tα chain, and the whole pre-TCR is expressed on the cell surface. This pairing signals through the cytoplasm and triggers entry into the cell cycle. Surface expression of the pre-TCR is associated with the DN4 stage: low level of surface CD3, loss of CD25, completion of β rearrangement, cell proliferation, and finally acquisition of CD4 and CD8. Once proliferation is over, DP decrease in size and start to rearrange the α locus gene segment, which results in DP cells expressing low levels of $\alpha\beta$TCR on their surface along with the CD3 complex. These cells are ready to undergo positive and negative selection.

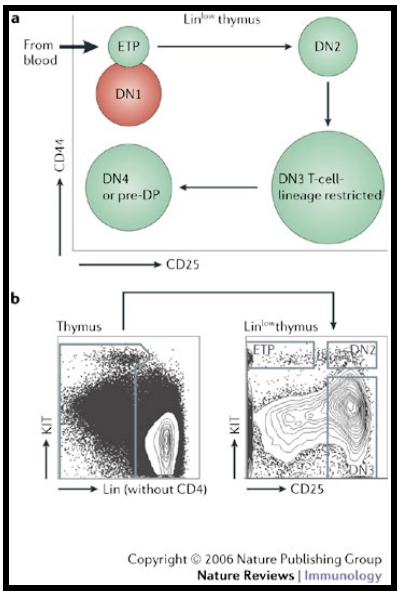


Figure 12: Immature thymocyte progenitor subsets.

a. Subsets of double-negative (DN) thymocytes, based on their expression of CD25 and CD44 and their sequential development through the DN1 ($CD25^{-}CD44^{+}$), DN2 ($CD25^{+}CD44^{+}$) and DN3 ($CD25^{+}CD44^{-}$) stages, are shown. $CD25^{-}CD44^{-}$ cells, which are also called DN4 cells, express *Cd4* and *Cd8* mRNA and are termed pre-double-positive (pre-DP). Early T-cell progenitors (ETPs), which are c-$kit^{hi}CD25^{-}CD44^{hi}$, are efficient T-cell progenitors that are transcriptionally equivalent to DN1 cells.

b. Defining ETPs. C57Bl/6 total thymocytes ($25x10^{6}$ cells) were stained with antibodies specific for c-kit and CD25, as well as a cocktail of antibodies specific for the following lineage (Lin) markers: NK1.1, T-cell-receptor -chain (TCR), TCR, macrophage receptor 1 (MAC1), B220, TER119 (also known as Ly76), CD3, CD8, CD8, CD11c and CD19, but not CD4 because some ETPs express of CD4 on the cell surface. Gating for lineage markers is adjusted so as not to exclude c-kit^{hi} cells that are expressing low levels of Lin (left panel). ETPs are defined as Lin^{low}c-$kit^{hi}CD25^{-}$ (right panel), (Adapted from Bhandoola and Sambandam, 2006).

TSPs are included in the DN1 population and can be distinguished from B, NK and myeloid thymic cells by expression of the c-kit marker. In addition, it has been recently shown that the DN1 population can be further subdivided into DN1.1 and DN1.2. DN1.1 cells are characterised by Flt3 ligand receptor (CD135) and CC-chemokine receptor 9 expression, whereas DN1.2 cells, which are the direct downstream progeny of DN1.1 cells, do not (Sambandam et al., 2005). DN1.1 cells are most probably the earliest thymic progenitors since they retain a B cell potential. It has to be noted that, while these DN1.1 cells are in a very low number in adult mouse thymi, they are much more numerous and hundred times more potent for B cell development in newborn mice.

Table 1 shows the main markers used to distinguish between early thymocyte populations.

	c-kit CD117	FLT3 (CD135)	CD93	B220	IL-7Rα (CD127)	CD44	CD25
TSP	+++	+	ND	-	-	+++	-
DN1.1	+++	+	-	-	-	+++	-
DN1.2	+++	-	-	-	-	+++	-
DN2	+++	-	-	-	+	++	++
DN3	+	-	-	-	+	+/-	++

Table 1: Cell-surface markers expressed by early thymocytes.

It has been previously shown that DN1 and DN2 require Notch signaling, IL-7 and c-kit, which is under the control of Notch signaling. These cells are not yet committed to the T cell lineage; but while TSP can still generate B cells, DN cells have lost B cell potential. On the other hand, DN3 and DN4 lose c-kit expression, DN3 showing intermediate levels and DN4 being negative for c-kit. They are unable to differentiate into anything other than T cells. DN3 still require Notch signaling to continue T cell development, but their growth is independent of IL-7 and c-kit.

TCR gene rearrangement in αβ T cells

During development from DN to DP, thymocytes undergo TCR gene rearrangement in a similar manner as the immunoglobulin gene rearrangement occuring in B cells.

The TCR molecule is a membrane-bound antigen receptor made up of two different polypeptides, either αβ or γδ. We will mainly talk in this section about αβ TCR; γδ TCR will be the subject of a separate chapter. The TCR molecule displays high structural and sequence similarity with Ig from B cells and is indeed encoded by homologous genes. Each chain is composed of a variable and a constant region, the variable part containing the antigen-binding cleft.

Germline TCR genes are organised similarly as Ig genes: V and J segments form the α and γ chains whereas V, D and J segments form the β and δ loci (Rowen et al., 1996). The same enzymatic machinery as in B cells, RAG1 and RAG2, performs this gene segment recombination (Shinkai *et al*., 1992). As a result, a genetic defect affecting the control of V(D)J recombination will equally affect T and B cells and leads to lack of functional lymphocytes in the deficient individual. The high diversity of TCR sequences is a result of somatic recombination within sets of gene segments. It is also a result of junctional variability through the addition of P

and N nucleotides at the junctions between V and J segments or V, D and J segments of the α or β chain (Abbey and O'Neill, 2008; Nemazee, 2006). In contrast to that of the BCR, TCR gene rearrangement displays lower diversity within the constant regions, which encode for the transmembrane polypeptides. Since the TCR is not secreted like immunoglobulins, the constant region does not mediate any particular functions and only the variable region is important in recognition of Ag presented by MHC molecule. In addition, the variability of the TCR has to be restricted to some extent in order to ensure binding with the MHC molecule. Although controversial, it is thought that the highest degree of diversity is focused on the variable part of the α chain. Indeed, more than 60 J gene segments can be found in the TCRα locus, and the CDR3s of the α and β chains show a great degree of hypervariability. These two regions take part in the formation of the centre of the TCR, the portion that binds the Ag. Finally, somatic hypermutation does not occur during TCR gene recombination unlike during BCR gene recombination.

T cells, like B cells, have a high degree of allelic exclusion, meaning that one specific cell is carrying only one specific TCR. However, whereas it is true concerning the β chain, it appears to be not as stringent for the α chain. It has been reported that one TCR β chain can actually pair with two different α chains on the same cell. This phenomenon might allow some autoreactive T cells to escape negative selection by downregulating the autoreactive α chain and expressing a new α chain, thereby acquiring a new non-autoreactive TCR (Figure 13). Indeed it has been reported by Sarukhan *et al.* that once they reach the periphery, these dual TCR-expressing T cells carry the two different TCRs on their surface and trigger autoimmune disorders (McGargill *et al.*, 2000; Sarukhan *et al.*, 1998; Zal *et al.*, 1996).

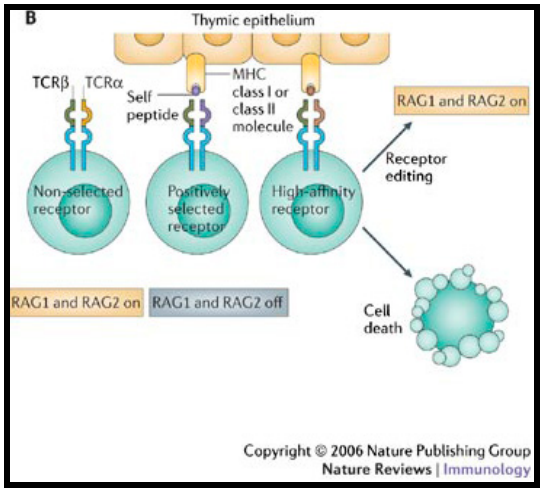


Figure 13: Editing versus positive selection as mutually exclusive aspects of receptor selection.

In double-positive ($CD4^+CD8^+$) thymocytes, which express T-cell receptors (TCRs), the threshold for positive selection requires weak signaling after recognition of self-peptide–MHC complexes. Agonists, by contrast, trigger tolerance by inducing developmental arrest and either apoptosis or, possibly, receptor editing.

Altogether, the TCR coupled with the CD3 molecule forms the TCR receptor complex. CD3 is in charge of transmitting the signal sensed by the TCR, which has short cytoplasmic tails (Figure 14). Ag binding of the TCR leads to cellular activation by phosphorylation of ITAM sequences of the CD3 intracytoplasmic tail. This is followed by release of second messengers such as inositol tri-phosphate (IP3) and Ca^{++}. This leads to NFκB (Nuclear factor kappa B) as well as NF-AT (Nuclear Factor of Activated T cells) nuclear translocation. In parallel MAPK (Mitogen Activated Protein Kinase) cascade is triggered and induce translocation of various transcription factors in the nucleus, which set off transcription of specific genes. It should be noted that monoclonal anti-CD3 antibody can actually bypass the TCR Ag sensing requirement and lead to a similar activation effect (Cantrell, 1996).

CD4 and CD8 function as co-receptor molecules by transducing TCR signals and stabilising interactions with MHC I or II. Intracellularly, these two molecules interact with leukocyte specific tyrosine kinase (lck).

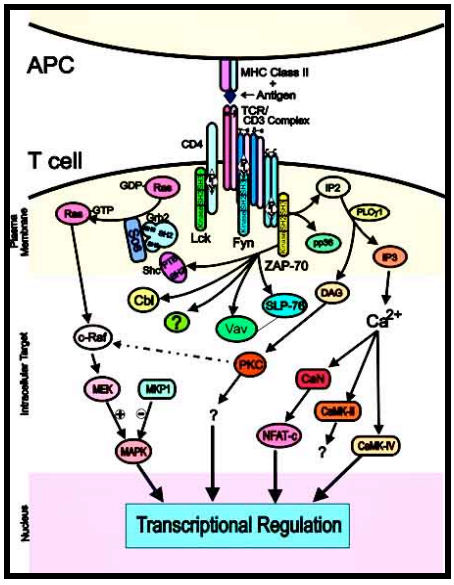


Figure 14: A current model for TCR-mediated signal transduction.

Interaction of antigen with TCR/CD3 complex initiates a series of biochemical events, of which the earliest is phosphorylation of various proteins on tyrosine residues. Stimulation of PTKs is coupled to the hydrolysis of PLCγ1, which results in a rise in intracellular Ca2+, and activation of PKC through IP3 and DAG pathways, respectively. Activation of Lck, Fyn, and ZAP-70 induces localization of adaptor proteins Shc, Grb2, and Sos to the cytoplasmic membrane. Associations of these adaptor proteins with Ras, allows the rapid conversion of Ras from the inactive form (GDP-Ras) to the active form (GTP-Ras). Activation of Ras results in sequential phosphorylation and activation of a series of enzymes involved in MAPK cascade that eventually transmit the stimulatory signal received from cytoplasmic membrane into the nucleus. Abbreviations: TCR, T

cell receptor; APC, antigen presenting cell; MHC, major histocompatibility complex; PLCg1, phosphlipase C-gamma 1; DAG, diacylglycerol; IP2, phosphoinositol biphosphate; IP3, inositol 1,4,5-triphosphate; SH2, Src-homology-2; PTB; phosphotyrosine binding; PKC, protein kinase C; NFATc, nuclear factor of activated T cell; CaN, calcineurin; CaMK, calcium calmodulin-dependent protein kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MKP1, MAPK phosphatase-1 (Adapted from Pahlavani, 1998).

Selection of MHC-restricted αβTCR specific for foreign antigens

As discussed earlier, antigen receptor gene recombination guarantees antigen recognition with a high degree of diversity and specificity. Nevertheless, the TCR has to be tested for its antigen recognition properties, in terms of affinity and specificity. The interaction of the TCR with the thymic environment will condition the fate of the TCR-bearing thymocyte, in other words, survival or death. Ultimately, thymocytes must be simultaneously MHC-restricted and specific for foreign Ag but tolerant to self-antigen. Subsequent developmental steps involve peptide-MHC complex interaction with the TCR of stromal cells.

Developing thymocytes whose receptor interacts weakly with self-peptide-MHC complex will survive; this process is called positive selection. The lymphocytes that do not receive any signal die by neglect since they are of useless specificity. This default fate occurs for the majority of thymocytes. Additionally, negative selection will ensure that lymphocytes whose receptor binds strongly to self–antigen receive an apoptotic signal. This prevents any auto-reactive T cells from joining the mature T cell repertoire.

Positive selection

Small DP quiescent thymocytes, which have passed the βselection checkpoint, actively rearrange the α TCR locus, but unlike for the β chain, productive α chain rearrangement is not sufficient to downregulate recombination machinery expression and to stop further rearrangement. Only after MHC restriction of the αβ TCR has been checked does the cell trigger termination of the recombination process (Brandle *et al.*, 1992). Although the α and β chains display, as mentioned above, an inherent propensity to bind to MHC molecules, TCR-MHC matching is actually quite rare due to the high degree of polymorphism of MHC molecules (Zerrahn *et al*., 1997). That is why the majority of DP thymocytes maintain an elevated level of RAG expression and stay undifferentiated. One thymocyte can perform multiple recombinations at the same allele, which starts at the 5' end of the J locus to end at the 3' end. This increases the probability for a cell to get an MHC- restricted TCR (Petrie *et al.*, 1993). This process is, however, limited in time by the life span of a DP thymocyte, estimated as no more than 3 to 4 days. RORγ and TCF1 are thought to be the transcription factors regulating the Bcl-X_L gene

expression, allowing the DP cell to survive (Ioannidis et al., 2001; Sun et al., 2000). If a cell fails to receive a signal induced by weak recognition of the TCR and peptide-MHC within a time frame of 3 to 4 days, RORγ and TCF1 will not trigger Bcl-X_L expression and the cell will consequently die by neglect.

In addition, due to incomplete allelic exclusion of the α locus, one single developing lymphocyte has the ability to express two different rearranged α chains during positive selection; this "TCR editing" process enhances the yield of positive selection (Nemazee, 2006). It is estimated that about one-third of the mature T cell population will possess two α chains on their surface.

The nature of the specific ligand for positive selection is still unclear. Whereas it was originally thought that most of the peptides displayed by MHC molecules during positive selection are self-peptides, the situation regarding this statement seems to be more complex. It is clear that the TCR affinity for positively selecting ligands is much lower than for negative selection (Liu *et al.*, 1998). In fact, Naeher *et al*. identified a constant affinity threshold for negative versus positive selection in MHCI-restricted thymocytes and demonstrated that there is clear correlation between selection potential and apparent affinity, defined as the strength of interaction between MHC ligand and the TCR and its co-receptor (Naeher *et al.*, 2007). Previously, this question has been addressed mainly through the study of transgenic TCR systems, which represent forced TCR expression; these models have a different kinetics of TCR expression than WT mice. It has been shown that it is not mandatory for the positively selecting peptide presented by MHC to have structural homology to the antigenic peptide ligand (Ignatowicz *et al.*, 1997). In addition, these peptides have been shown to be non-stimulatory in *in vitro* assays (Berg *et al.*, 2000). In conclusion, positive selection seems to be mediated through the presentation of rare, low affinity self-peptide ligands with more or less structural homology to the antigenic peptide (Starr *et al.*, 2003).

All of the physical components of the TCR complex (α and β chain, CD3γ and CD3ε) and of its signaling pathway (src and syk kinases, ZAP 70, LAT or PLCγfor example) are required for positive selection to occur, but a few other transcription factors are suspected to play a role as well in this process. Helix-loop-helix family members like E proteins seem to play an important role of positive selection probably through their capacity to regulate RAG gene expression and TCR locus accessibility (Quong *et al.*, 2002). In addition, a mouse deficient in Schnurri-2, a zinc finger transcription factor, has been found to have a block in positive selection (Takagi *et al.*, 2001).

Importantly, positive selection is carried out within an intact three-dimensional (3D) thymic microenvironment formed by a network of cTECs, which are the second key cellular component

in positive selection (Hare *et al.*, 2001). This has been shown by performing bone marrow chimera experiments from mice with distinct MHC haplotypes. The 3D architecture is of great importance since a monolayer culture of cTECs is unable to complete positive selection (Sato *et al.*, 2001). In addition to presenting the positive selection ligand by MHC, cTECs provide the specialized accessory interaction necessary for positive selection. Advanced microscopy techniques have allowed visualization of thymocytes migrating through the 3D network of the thymic cortex, sampling cTEC for recognition of peptide/MHC complex and stopping for a couple hours when finding the corresponding match (Bousso *et al*., 2002).

Positive selection coordinates expression of CD4 and CD8 molecules according to the TCR specificity for MHCI or MHCII and potential effector functions, helper or cytotoxic, respectively. It is generally admitted that positive selection depends on the engagement of both the TCR and its co-receptor, but the link between this engagement and the lineage commitment of thymocytes towards CD8 or CD4 T cell is not quite clear.

Negative selection

In order to eliminate any potential autoreactive T cells from the mature T cell repertoire, developing thymocytes undergo negative selection. The thymocyte engages its TCR with a high affinity ligand in the thymus, which leads to apoptosis.

Various models have been used to study clonal deletion, but the differences between these models have led to very little consensus concerning the mechanisms underlying negative selection. Classical models in the field are based on TCR transgenic mice that concomitantly express self-antigen where both TCR and Ag are expressed as transgenes. Transgenic TCR expression kinetics in these systems are all different, with some being expressed much earlier than in physiological conditions. The affinity between the TCR and its specific ligand may also greatly differ between one system and another (Pircher *et al.*, 1989). Finally, the cell type expressing transgenic self-antigen might also play a significant role in the TCR-ligand interaction outcome.

Clonal deletion can also be induced by using cross-linking antibodies, which present the drawback of generating huge T cell activation. This induces the production of inflammatory cytokines and steroid hormones, and leads to nonspecific death of DP thymocytes (Page *et al.*, 1998; Xue *et al.*, 1996). *In vitro* assays in which thymocytes are incubated with cross-linking antibodies also show a high rate of non-specific apoptosis among thymocytes. Additionally, this method does not consider the possible requirement for costimulatory factors. Finally, Ab crosslinking might induce a qualitatively distinct signal through the TCR than the one induced by peptide-MHC ligand interaction.

A third technique to study clonal deletion has used direct injection of peptides of interest. Again, this has led to overwhelming activation in the thymus involving cytokine-mediated stromal cell activation, recruitment of eosinophils, and finally collapse of thymic architecture (Martin and Bevan, 1997; Murphy *et al.*, 1990).

In conclusion, the most physiologic model for studying negative selection seems to use TCR transgenic mice where the TCR$\alpha\beta$ molecule is not expressed earlier than normal and where the frequency of the responder T cell is not artificially high (Starr *et al.*, 2003).

It is thought that about 5% of the total thymocytes undergo negative selection, and a similar proportion of cells undergo positive selection (Palmer, 2003). Despite the sequential location of DN to SP cells from the cortex to the medulla, respectively, the two events seems to be rather independent of each other since negative selection can happen either before or after positive selection and T cells seem to be able to undergo negative selection at all stages of development.

Heterogeneous TCR transgenic models have been used to characterise thymocytes undergoing negative selection. In some cases, clonal deletion appears to occur early during the transition from DN to DP stage, whereas in other models, it occurs later at the DP stage (Stockinger, 1999). The first situation, however, is believed to be a transgenic artefact; normal mice are indeed thought to undergo negative selection at the DP stage. Nevertheless, it is possible that two different molecular mechanisms govern clonal deletion, one mechanism for early clonal deletion in transgenic mice and another mechanism driving late negative selection in WT mice. This is further supported by the fact that DP T cells are found in the cortex whereas SP thymocytes reside in the medulla. Physiologically, clonal deletion is thought to happen more efficiently at later stages, during the DP$\rightarrow$SP transition, since it requires intact and strong surface expression of TCR$\alpha\beta$ (Sant'Angelo and Janeway, 2002). It has been shown to involve MHCII-restricted TCR T cells displaying a $CD24^{high}$ phenotype. This subpopulation of SP thymocytes is called semi-mature and becomes apoptotic in response to antigenic stimulation. After clonal deletion, SP CD4 T cells down regulate CD24 (Kishimoto and Sprent, 1997).

The location of negative selection has been controversial as some experiments indicate that it occurs in the cortex (von Boehmer, 1990), while others suggest the medulla (Burkly *et al.*, 1993). TCR transgenic mice expressing high-affinity self antigen show atrophy of the cortex along with a high rate of clonal deletion among DP thymocytes, but again, the abnormally high expression of transgenic TCR on DP cells might introduce a bias in the negative selection in those mice (Sprent and Kishimoto, 2001). In addition, mice with MHCII expression restricted to the cortex area show an increase in mature auto-reactive CD4 T cells, proving that indeed some negative selection occurs outside of the cortex (Laufer *et al.*, 1996). Another observation

favouring this idea is the lack of expression of the co-stimulatory molecules CD80 and CD86 on the surface of cTEC although they are thought to play a role in clonal deletion (Degermann *et al.*, 1994). In contrast, in the medulla, two types of antigen presenting cells (APC) are present and crucial for negative selection -- bone marrow-derived dendritic cells (BMdDCs) and mTECs. Medullary TECs are central players in negative selection in that they are responsible for promiscuous gene expression (PGE) (Klein *et al.*, 2000). PGE refers to the ability of these cells to express and present nearly the entire peripheral self-peptide repertoire of an individual. PGE is, at least partially, under the control of the autoimmune regulator transcription factor (AIRE); and AIRE deficiency in patients leads to a multi-organ autoimmune syndrome known as Autoimmune Polyendocrine Syndrome type 1 (APECED) (Anderson *et al.*, 2002). mTECs have been shown to be efficient APCs and capable of mediating negative selection of CD4 auto-reactive T cells (Laufer *et al.*, 1999). DCs present in the thymus are thought to be very important for central tolerance and are able to acquire self-antigen from mTECs by the mechanism of cross-presentation and induce clonal deletion of both developing CD4 and CD8 T cells (Gallegos and Bevan, 2004).

The question of whether the TCR signal is by itself sufficient to induce negative selection or if a second signal is required is still unresolved. The first insight into this issue has been the observation that antibodies against the TCR added to thymocytes *in vitro* is not sufficient to promote clonal deletion while the use of APCs restores negative selection (Punt *et al.*, 1994). Two co-stimulatory molecules, namely B7-1 and B7-2, have been proposed to provide a second signal to DP thymocytes, inducing apoptosis (Page *et al.*, 1993). In fact, anti-CD28 mAbs used *in vitro* have been shown to have a similar effect on DP thymocytes. Similarly, the addition of anti-CD43 and anti-CD5 mAbs *in vitro* has been reported to provoke an increased death rate among $CD4^{+}CD8^{-}CD24^{hi}$ (Kishimoto and Sprent, 1999). Surprisingly, gene knock-out (KO) or mutant mice for these co-stimulatory molecules, such as $CD28^{-/-}$ animals, show normal thymopoiesis (Walunas *et al.*, 1996). CD40L deficiency in thymocytes results in impaired negative selection, although this block is incomplete and CD40L deficiency might only delay negative selection (Foy *et al.*, 1995). LIGHT, a TNF receptor family member which binds to lymphotoxin β receptor, has likewise been proposed to provide a second signal for negative selection, but again the corresponding KO mouse model does not show any deficiency in thymopoiesis (Wang *et al.*, 2001). It could well be that co-stimulatory function is so important for negative selection that several molecules are responsible for it; this redundancy among co-stimulatory molecules would explain the lack of an observed phenotype in single KO mice. On the contrary, high-affinity interactions involved in clonal deletion do not require co-stimulation. This difference between high-affinity versus low-affinity interactions may underlie the differences in negative selection between whether an endogenous or an exogenous ligand is used

Unlike positive selection, it appears that the CD4 and CD8 co-receptors do not participate in clonal deletion, perhaps due to the high-affinity of the interaction which makes it less dependent on any co-receptor involvement.

A simplistic view in the field presumes that recognition of low-affinity peptide-MHC complex ligand mediates positive selection whereas high-affinity interactions lead to clonal deletion. However, the mechanisms behind these events are not clear. Understanding the mechanisms of positive versus negative selection is further complicated by the paradox that the same TCR binding has to result in two different downstream signaling events leading to opposite cell fates. There are two ways of explaining the outcome of thymic selection. The first one considers the rate of occupancy of TCR molecules at a given time point, the cell being able to count how many TCRs are engaged. It is assumed that the higher the affinity, the more TCRs are interacting with peptide-MHC ligands. A certain threshold number of occupied TCRs could trigger negative selection. This interpretation, however, has not been verified consistently by experiments (Sebzda *et al.*, 1994). Another explanation would consider the sensing of differential durations of the TCR/peptide-MHC interaction. If the length of TCR engagement with peptide/MHC complex is short, the cell will receive only an early TCR signal; this is the case with low-affinity-mediated positive selection. If the TCR forms a sustained interaction with its ligand due to high affinity, both early and late TCR signals would be triggered, followed by clonal deletion. This version has been supported by surface plasmon resonance studies showing a slow off rate for high-affinity ligands and fast off rate corresponding to low-affinity ligands (Alam *et al.*, 1996). This is known as kinetic proofreading. Some of the problems raised with Surface Plasmon Resonance are that it does not account for the role of co-stimulatory molecules and does not explain what happens when the TCR binds an intermediate-affinity ligand.

Along with this kinetic differentiation in thymic selection outcome, a similar kinetics of extracellular signal regulated kinase (ERK) activation has been observed. In the presence of positively selecting ligands, a slow and sustained accumulation of ERK has been reported whereas negatively selecting ligands induce a strong but transient burst of ERK activity (Werlen *et al*., 2000).

The signaling pathway and transcription factors involved in negative selection are a vast area of investigation that will not be extensively discussed herein, but will just be mentioned for completeness. As in positive selection, all TCR complex components are necessary for negative selection. The MAPK pathway seems to be involved in a differential kinetics-related manner, involving the same molecules as positive selection, namely p38, Jun N-terminal Kinase (JNK) and ERK. Linker for activation of T cells (LAT) acts upstream of the MAPK pathway to activate it, and the recruitment of the adaptor GRB2 to LAT seems also to be of primary importance.

Phosphatase and tensin homologue (PTEN), the nuclear orphan steroid receptor (NUR77), and NFκB are also implicated in negative selection, as in positive selection. On the other hand, the FAS apoptotic pathway seems to be dispensable for deletion of self-reactive thymocytes. Rather, pathways mediated by B-cell lymphoma 2 anti-apoptotic protein (BCL-2) family members have been shown to be more relevant, via involvement of the pro-apoptotic factor BCL-2 interacting mediator of cell death (BIM) (Werlen *et al.*, 2003).

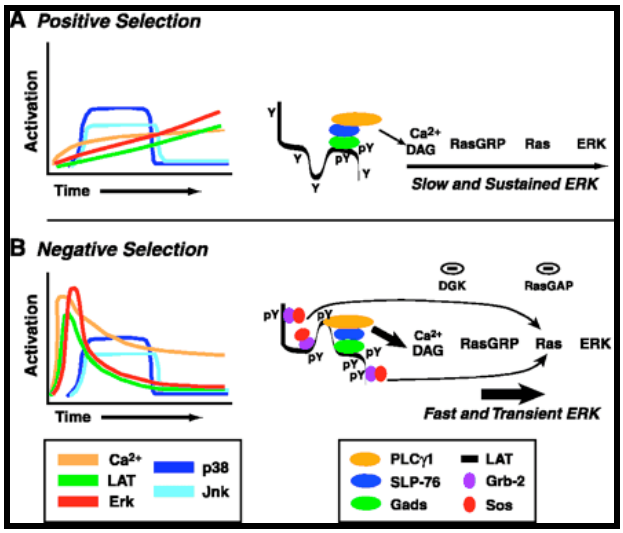


Figure 15: Kinetics of positive and negative selection signals.

(**A**) During positive selection, LAT phosphorylation, Ca^{2+} mobilization, and ERK activation are slowly induced over a sustained period of time. This contrasts with the induction of p38 and JNK, which are activated with different kinetics. Low-affinity pMHC ligands, which occupy the TCR for a relatively short time, could induce the phosphorylation of Tyr^{175} and Tyr^{195} on LAT. This might recruit Gads, SLP-76, and PLC-1 and lead to the slow production of DAG and the slow activation of RasGRP, Ras, and ERK. (**B**) During negative selection, LAT phosphorylation, Ca^{2+} mobilization, and ERK activation are rapidly and transiently induced. High-affinity ligands occupy the TCR for longer times and might induce the phosphorylation of Tyr^{175}, Tyr^{195}, and Tyr^{136}. The phosphorylation of the Tyr^{136} residue might stabilize PLC-1 within the growing TCR signalosome, leading to the rapid activation of Ras and ERK. High-affinity ligands could also lead to the phosphorylation of Tyr^{113}, Tyr^{132}, and Tyr^{235}, which recruit Grb-2 and the guanine nucleotide exchange factor, Sos. This could increase the speed of ERK activation. Diacylglycerol kinase (DGK) and RasGAP might terminate ERK activity. Interestingly, the kinetics of p38 and JNK activation during negative selection are the same as during positive selection (Adapted from Werlen, 2003).

Agonist selection

As previously mentioned, thymocyte fate is thought to be conditioned by TCR affinity. DP thymocytes encountering a low-affinity peptide-MHC complex are positively selected, thymocytes encountering high-affinity ligands undergo clonal deletion, and DP cells unable to display a functional TCR or with no affinity for MHC peptide die by neglect. A fourth pathway of differentiation has been described whereby thymocytes can actually be selected following encounter with a high-affinity TCR agonist ligand (reviewed in Baldwin et al., 2004). Agonist

selection has been shown to drive the development of three mature T cell populations, namely NK T cells, CD8αα intraepithelial cells T lymphocytes (IEL) of the gut, and $CD4^+CD25^+$ regulatory T cells. These populations are self-reactive and share common phenotypic and functional properties. They are all involved in the regulation of the immune response.

NK T cells are CD1d-restricted, CD1 molecules being non-classical MHC I molecules. The majority of NK T cells express invariant rearranged TCR Vα14 chain, which recognises an unidentified agonist endogenous glycolipid associated with CD1d molecules. Development of NK T cells requires an encounter with the CD1d-glycolipid complex exclusively on BMdDCs of the thymus (Kronenberg and Gapin, 2002). They are thought to arise from DP thymocytes as shown by transfer experiments (Gapin *et al.*, 2001). At the end of their thymic development, they acquire the marker NK1.1, as well as other activation markers such as CD44. Finally, they are specialised in regulating immune responses through the secretion of cytokines.

CD8αα intraepithelial lymphocytes also have regulatory functions. They are numerous in the gut and represent about 50% of all intraepithelial T cells. They are MHC I-restricted and, like NK T cells, display an activated phenotype. Several studies have suggested that they arise after agonist selection; in fact, they are increased in TCR transgenic mice expressing agonist ligand (Leishman *et al.*, 2002). Whether these cells originate within or outside the thymus has been the subject of controversy. An experiment using fetal thymic organ culture (FTOC) in combination with high-affinity agonist ligand suggests that the development site of these CD8αα T cells is within the thymus (Cheroutre and Lambolez, 2008; Hogquist and Bonnevier, 1998). It is furthermore unclear whether CD8αα are derived from DP or DN T cells. One argument in favour of the latter hypothesis is the expression of the Fc fragment of IgE, high-affinity I receptor γ polypeptide (FcεR1γ) signal transducer, within the TCR complex of this lymphocyte population (Heiken *et al.*, 1996). On the other hand, Eberl and Littman showed that CD8αα IEL express a reporter gene that is only activated after β selection (Eberl and Littman, 2004).

Finally, naturally thymic-derived $CD4^+CD25^+$ regulatory T cells (Tregs), which will be discussed in further detail in a later chapter, are also selected through agonist selection. Tregs are able to inhibit T cell proliferation *in vitro* and to prevent many autoimmune disorders *in vivo*, such as colitis or diabetes (Mottet *et al.*, 2003; Salomon *et al.*, 2000). Caton *et al* have shown that haemagglutin (HA)-specific TCR transgenic mice expressing the agonist ligand HA under the control of various promoters display various levels of deletion but a striking increase in the $CD4^+CD25^+$ T cell population (Apostolou *et al.*, 2002; Jordan *et al.*, 2001). This subset carries particular surface activation markers on their surface, such as CD25, GITR and CD5. They are generated in the thymus where they are presumed to be selected by radioresistant APCs presenting high-affinity self-antigen. It is still under debate whether thymic epithelial cells or

thymic BMdDCs are the physiologic cell type supporting their development (Apostolou *et al*., 2002). They have been shown to be self-reactive since they expand rapidly after adoptive transfer into lymphopenic hosts (Hsieh *et al.*, 2004). Tregs can also arise from $CD4^+CD25^-$ peripheral T cells through specific conditions of antigen encounter. Thymic derived regulatory T cells cannot be distinguished from their peripheral counterparts since they are phenotypically identical.

All of the above three subsets share common features such as partially activated phenotype, regulatory function, and a dominant role for agonist interaction during their development. However, this does not imply that they share a similar development pathway. The discovery of FoxP3 repressor and its close association with regulatory T cell phenotype and function, as well as the reporter gene knock-in mouse generated by Rudensky *et al.*, have proven the existence of a unique regulatory T cell lineage (Fontenot *et al.*, 2003; Fontenot *et al.*, 2005a; Fontenot *et al.*, 2005b). Nevertheless, it is not yet clear whether the other populations represents a unique lineage or merely a specific activation state of T cells.

It is also unclear whether agonist selection is a variation of positive or negative selection or if it represents a particular type of antigen reactivity. These cell subsets might develop at a specific stage of development through interaction with MHC-peptide complex or react to agonist stimulation in a different way than clonal selection. It appears that both selection features may be involved (ref. Baldwin TA). Another question raised is whether their specific phenotype allows them to survive clonal deletion or if the agonist encounter drives their development. It seems that although TCR affinity seems to dictate the fate of these cells, upregulation of costimulation or signaling molecules are necessary for these cells to develop (Baldwin *et al*., 2004).

It has been hypothesized that different degrees of avidity would allow agonist-selected T cells to avoid deletion; however, despite indications that avidity might play a role, direct proof has not been provided (Jordan *et al*., 2000). Another possibility is that agonist presentation occurs through different APC types. For example, it is not quite clear which cell type induces regulatory T cell development. Laufer *et al*. have shown that cortical epithelial expression of MHCII could support the differentiation of Tregs (Bensinger *et al*., 2001) whereas studies by Aschenbrenner *et al.* argue in favour of a role for $Aire^+$mTECs in the selection of Tregs (Aschenbrenner *et al.*, 2007).

Receptor editing

As discussed previously, quasi-random gene rearrangement ensures a high diversity of TCR specificities, while the risk of generating autoreactive thymocytes is limited through elimination of cells carrying unsuitable Ag receptors. According to the Burnett model, this is accomplished

through the control of lymphocyte survival and proliferation illustrated best as clonal deletion.

Recently, increasing evidence has suggested the existence of alternative regulatory mechanisms, such as receptor editing. This is defined as a feedback mechanism by which the antigen receptor could signal either to cease or to continue recombination, thereby controlling the maintenance or the alteration of Ag receptor specificity (Nemazee, 2006). It basically decides to turn the recombination machinery on or off depending on the nature of the signal received. In this manner, receptor editing excises a rearranged Ag receptor gene segment by producing a secondary rearrangement and results in alteration of the original receptor specificity. Receptor editing has been shown to play a major role in central tolerance by replacing auto-reactive receptors of developing thymocytes with innocuous ones, preventing subsequent autoimmune disease from developing in the periphery (Santori *et al.*, 2002). As mentioned previously, this phenomenon is well established in B cells and is thought to happen in an analogous mode in T cells (Nemazee, 2000).

There is essentially no editing at the level of the TCR β chain. After a proliferative burst of pre-T cells along with the first chain rearrangement, thymocytes modify DNA accessibility for RAG1 and 2 proteins in order to maintain allelic exclusion. This is done by deacetylation of the Vβ gene during the transition form DN to DP stage (Tripathi *et al.*, 2002). On the other hand, the α chain locus is more prone to receptor editing. In fact, as mentioned earlier, the recombination of the α chain is less stochastic than the β chain recombination due to 5' to 3' sequential recombination attempts at the *TCRA* locus.

Rearrangement events occur in the thymic cortex. It has been known for a while that TCR cell surface expression is not sufficient to stop recombination events; rather, only successful positive selection leads to termination of RAG protein expression (Brandle *et al.*, 1992; Brandle *et al.*, 1994). In fact, recombination is ongoing at the DP stage. As reported earlier, high-affinity interaction presumably leads to clonal deletion but negative selection studies have been limited by the wide use of TCR transgenic models, which in the vast majority present a forced expression of the TCR and subsequently promote a distinct form of central tolerance. Wang *et al*. first showed that negative selection might actually lead to receptor editing (Wang *et al*., 1998). At the same time, Mc Gargill *et al.* showed that receptor editing represents a major developmental mechanism in OT1 transgenic mice expressing OVA as a self-Ag on the surface of cTECs, and they proposed that auto-reactive DP thymocytes can undergo receptor editing in the cortex upon encountering their Ag provided that TCR expression is not abnormally premature (McGargill *et al.*, 2000). The OT1 transgenic mice demonstrate decreased deletion and activation of auto-reactive T cells, as well as internalisation of the TCR, with maintained RAG expression. An increase in endogenous rearrangements at the TCR α locus leads to higher numbers of cells expressing the endogenous α chain pairing with the transgenic β chain.

This Ag-induced TCR internalization provides thymocytes with the opportunity to rearrange other receptors and edit their specificity. Contrary to these findings, another study by Buch *et al.* argues that receptor editing might not be a governing principle in thymic development since their HY-I, a VαJα knock-in combined with a cre-lox system of TCRδ mice, produce a negligible amount of receptor-edited T cells (Buch *et al.*, 2002). They interpret these results as sequential gene rearrangement being a direct consequence of positive selection screening.

It is not clear what the factors promoting receptor editing are. The avidity, the stage of deletion during negative selection, either early or late, or the tissue specificity have been proposed to influence the occurrence of receptor editing (McGargill and Hogquist, 2000). It seems that the type of cells presenting the Ag would also be a major determinant since, according to McGargill and Hogquist, cTECs are not able to provide any costimulation factor to developing T cells nor are they able to provoke apoptosis in the same cells. It has to be mentioned though that a double transgenic mouse model developed in our lab co-expressing HA-specific TCR and HA agonist in DCs displays a high rate of TCR editing despite the presence of costimulation molecules on the surface of the DCs.

When secondary gene rearrangement of the TCR occurs at a different allele than the primary gene rearrangement, it does not lead to excision of the first TCR gene rearrangement, such that one T cell then carries two different receptor specificities. This dual TCR receptor expression has generally been considered as a potential hazard for the peripheral immune system since T cells with an auto-reactive TCR can escape negative selection and reach the periphery (McGargill and Hogquist, 2000). Another group challenged this idea by considering dual TCR T cells as a way to expand the TCR repertoire for foreign antigens (Gavin and Rudensky, 2002; He *et al.*, 2002). Nevertheless, autoimmunity due to dual receptor expressing T cells has only been described in TCR transgenic contexts and are likely overtly exaggerated in these systems compared to wild-type situation (McGargill and Hogquist, 2000).

In conclusion, the importance of TCR editing in the pathway of central tolerance in physiological conditions is unknown at present. It may concern only a minority of developing self-reactive T cells but cannot be overlooked.

T cell tolerance

The immune system faces the dual challenges of mounting an adequate immune response against an incredibly high range of pathogens while at the same time limiting any reaction against a large variety of self-antigen. The latter process is called tolerance. A fine-tuned balance between these two processes prevents the two pathological extreme situations of immunodeficiency and autoimmunity. Central tolerance refers to the mechanisms preventing the development of auto-reactive immune cells in primary sites of lymphocyte development.

Although this is an efficient process, it does not eliminate all self-reactive lymphocytes. Thus, peripheral tolerance also exists to control the activation and proliferation of self-reactive mature cells in lymphoid and non-lymphoid organs. Induction of T cell anergy, T cell deletion, and immunological ignorance are some mechanisms of peripheral tolerance.

Central tolerance

In the thymus, central tolerance is achieved through selection and development of a T cell repertoire targeting foreign antigens and devoid of any self-reactivity. An immature T cell encountering its cognate antigen in the thymus becomes self-tolerant through induction of anergy, a state of non-responsiveness to Ag stimulation, clonal deletion, receptor editing, or the development of regulatory T cell population as described earlier. This results in the impairment or elimination of high-affinity self-reactive T cells.

One longstanding question was how developing T cells encounter peripheral antigens in the thymus. It had been hypothesised for a long time that Ag could access the thymus via blood vessels, either in a soluble form or carried by recirculating cells such as immature DCs. In fact, Le Douarin and colleagues were the first to show that tolerance was mediated by the thymus. They transplanted thymectomised birds with thymic anlage and rendered them tolerant (Ohki *et al.*, 1987). Almost ten years later, Asano *et al* showed that neonatal thymectomy of mice up to 3 days after birth resulted in multi-organ autoimmunity (Asano *et al.*, 1996). They were the first to identify a $CD4^{+}CD25^{+}$ regulatory T cell population. Finally, when generating a transgenic mouse model using tissue specific promoter elements, unexpected expression of the transgenic gene product was reported in the thymus in addition to targeted organ specific expression (reference rudensky GFP knock in).

This led to the discovery years later of promiscuous expression of tissue-restricted self-antigens (TSA) specifically by thymic stromal cells (Derbinski *et al.*, 2001). Derbinski *et al.* identified a variety of peripheral antigens expressed specifically in mTEC that are often targets of autoimmune disorders. They also showed that promiscuous gene expression is a property conserved in the thymus throughout life, even during age-related thymic involution, and that it is independent of any developmental regulation. Interestingly, TSA genes do not show any structural or functional commonalities but have been shown to organise in genetic clusters (Derbinski *et al*., 2005; Gotter *et al.*, 2004). Other studies have suggested a link between thymic promiscuous gene expression and tolerance induction (Klein *et al*., 1998; Klein *et al.*, 2000).

The identification of the autoimmune regulator AIRE, which in humans is associated with the multiorgan autoimmune disorder known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (Bjorses *et al.*, 1998), and the generation of AIRE KO mice (Anderson *et al.*, 2002) revealed new insights into this field. AIRE is a transcription factor

displaying a nuclear localisation signal and several DNA binding domains. It is expressed specifically in MHCII^{high} CD80^{high} mTECS in the thymus (Derbinski *et al.*, 2005) and controls promiscuous gene expression (PGE) in the thymic medulla (Anderson *et al.*, 2002). Liston *et al.* directly demonstrated that AIRE is indeed responsible for negative selection of organ-specific T cells through regulation of TSA expression (Liston *et al.*, 2003).

Although it acts in a gene-specific manner, how AIRE controls PGE is still unclear. AIRE has been shown to co-localize and function in cooperation with CREB binding protein (CBP), which controls expression of a wide range of genes, but there is no experimental evidence yet for direct binding of AIRE to CIS-acting sequences of target genes (Pitkanen et al., 2000; Pitkanen et al., 2005). The genetic clustering of PGE suggests that molecular regulation might be dictated by epigenetic mechanisms to ensure that TSA expression mirrors practically all tissues in the body irrespective of developmental or spatio-temporal expression patterns.

PGE is not restricted to mTECs, but rather can also occur in cTECs as well as thymic DCs. Furthermore, TSA expression is not always controlled by AIRE; C-reactive protein (CRP) and glutamic acid decarboxylase 67kDa (GAD67) are two examples of peripheral antigens whose expression is not regulated by AIRE (Anderson *et al.*, 2002). In addition, there is phenotypic heterogeneity upon AIRE loss: APECED can be sporadic and slow to develop; the phenotype of AIRE KO mouse differs according to genetic background; and finally, disease onset is actually much accelerated after bone marrow transplantation (BMT) of WT into AIRE$^{-/-}$ hosts (Anderson et al., 2002). These observations led to the conclusion that some other genes might compensate for lack of AIRE and sustained PGE may play a role in tolerance independent of AIRE.

Given the very low concentration of TSA synthesized by mTECs and the rare number of mTECs displaying a given peripheral antigen (1-3%), T cells have to scan extensively the stromal cell compartment for expression of self-antigen, necessitating a very high degree of motility during their time of residence in the medulla (Kyewski and Klein, 2006). In addition, considering the low efficiency of mTECs in inducing apoptosis, it is quite possible that thymic DCs help mTECs in inducing clonal deletion through cross-presentation. Thymic DCs can indeed acquire TSA via the presence of apoptotic mTECs; they have been described to have a high turnover of about 3 weeks. Antigen spreading can also be mediated by secreted exosomes or by gap junction's transfers shown by *in vitro* data (Harshyne *et al.*, 2001; Neijssen *et al.*, 2005; Thery *et al.*, 2002). This would increase the availability of tolerizing antigen for T cells undergoing negative selection. In fact, Bevan and Gallegos have reported such a mechanism in the thymus (Gallegos and Bevan, 2004).

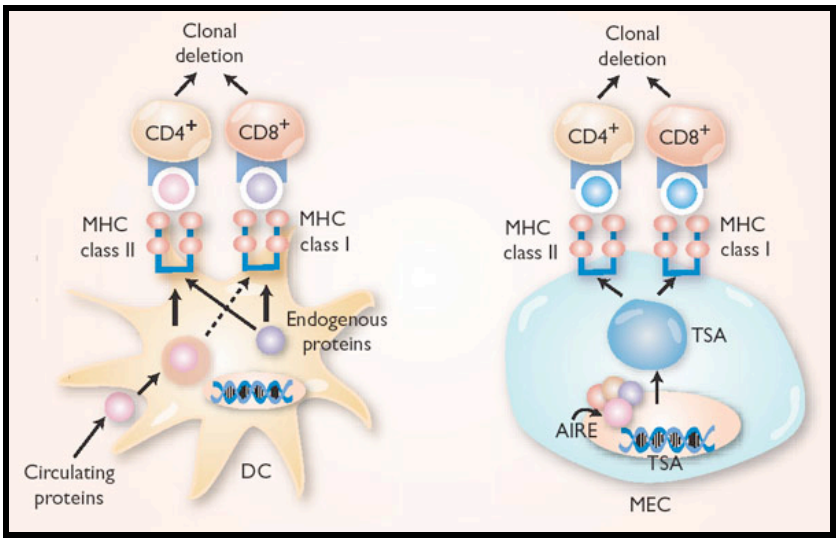


Figure 16: Presentation of self-proteins by bone marrow_derived APCs versus mTECs (MEC) for tolerance induction in the thymus.

APCs, as represented by dendritic cells (DCs), efficiently present endogenously synthesized proteins to both $CD4^+$ and $CD8^+$ T cells and circulate proteins to $CD4^+$ T cells. APCs thus induce tolerance (clonal deletion) to ubiquitous proteins, whether they are secreted or arise intracellularly. Tolerance to ubiquitous antigen may also be mediated by MECs (Medullary Epithelial Cells, not illustrated). However, the principal role of MECs may be to purge T cells that are reactive to tissue-specific antigens (TSAs). Such deletion is the result of ectopic expression of TSAs by MECs. How MECs are able to synthesis TSAs is still unknown, but recent work has indicated that AIRE, a putative transcription factor, is centrally involved in this process. In direct support of this idea, Liston *et al* show that clonal deletion of T cells reactive to a neo-TSA is abolished or greatly reduced in the absence of AIRE (Adapted from Sprent 2003).

Regulatory T cells

As mentioned earlier, despite negative selection, some autoreactive T cells reach the periphery and represent a potential hazard for the individual. Regulatory T cell subsets are supposed to control the proliferation and effector functions of these harmful autoreactive T cells. There are numerous subpopulations of regulatory T cells, in addition to $CD8\alpha\alpha^+$ and NK T cells mentioned earlier. The CD4 T cell subset includes IL-10-producing Tr1 regulatory T cells (Groux *et al.*, 1997) and transforming growth factor β (TGF-β)-producing T helper type 3 cells (Chen *et al.*, 1994) that are confined in the periphery. We will mainly focus here on the natural thymus-derived $CD4^+CD25^+FoxP3^+$ population and peripheral induced Tregs, which altogether represent about 10% to 15% of the peripheral CD4 T cell population.

Phenotype and Function

Recent studies have implicated so-called naturally-arising regulatory T cells (Treg) as key components controlling autoimmunity (Kronenberg and Rudensky, 2005; Sakaguchi *et al.*, 1995; Shevach, 2004). Tregs were first described as a population of $CD5^{high}CD4^+$ cells that upon transfer could protect mice from autoimmune disease caused by neonatal thymectomy (Sakaguchi *et al.*, 1982). Later, it was shown that during mouse ontogeny, Tregs appeared

shortly after birth and that CD25, the alpha chain of the IL-2R complex, could be used as a surrogate marker for these cells (Fontenot *et al.*, 2005a; Sakaguchi *et al.*, 1995). More recently, the transcription factor FoxP3 has been shown to be critically associated with Treg function (Hori *et al.*, 2003); and indeed a knock-in transgenic mouse line containing a GFP-FoxP3 construct indicated that FoxP3 may be used as a lineage marker for Tregs (Fontenot *et al.*, 2005b). Many phenotypic features of Tregs, namely spontaneous CD25 expression, glucocorticoid-induced tumour necrosis factor receptor (GITR) (Shimizu *et al.*, 2002) and cytotoxic T lymphocyte antigen 4 (CTLA-4) constitutive expression (Read *et al.*, 2006), as well as down-regulation of CD4, CD3 and TCRβ transcripts and surface antigen expression are similar to those of activated T cells (Bosco et al., 2006b; Gavin et al., 2002b; Kasow et al., 2004). Natural Tregs are anergic: they respond very poorly to TCR stimulation and do not produce IL-2 *in vitro* although their survival is critically dependent on this specific cytokine. However, this anergy can be overcome by adding anti-CD28 mAb and IL-2 to the culture, although at the expense of their suppressive capacity. Surprisingly, they proliferate extensively *in vivo* and more specifically in lymphopenic conditions (Cozzo *et al.*, 2003). The repertoire of TCR expressed by Treg is generally thought to be broad; however, it would seem that their TCR have a relatively high affinity for self antigens (Hsieh *et al.*, 2004). Thus, their $CD5^{high}$ phenotype may have protected them from negative selection in the thymus (Azzam *et al.*, 2001).

Functionally, Tregs inhibit the proliferation and cytokine production of naïve responder T cells *in vitro* (Thornton and Shevach, 1998). Their main functional role *in vivo* (Asano *et al.*, 1996) appears to be in preventing the activation and reducing the expansion of activated T cells (Sakaguchi, 2004). Tregs are part of the natural T cell repertoire and suppress harmful immunopathological responses to self or foreign antigen. Indeed, several experiments have shown that regulatory T cells exert dominant extrathymic immune regulation. $CD4^+CD25^+$ Tregs have been shown to inhibit experimental autoimmune encephalomyelitis (EAE) (Olivares-Villagomez *et al.*, 1998) as well as spontaneous immune diabetes in susceptible mouse strains (Salomon *et al.*, 2000; Tang *et al.*, 2006). Another model of natural Treg-mediated immune regulation is the inflammatory bowel disease (IBD) model reported by Powrie *et al.*, whereby the severe colitis in SCID mice reconstituted with $CD4^+CD45Rb^{hi}$ can be inhibited by cotransfer of $CD4^+CD45RB^{lo}$ containing the $CD25^+$ subset. Finally, natural Tregs mediate tolerance to alloantigen in transplantation experiments, impede anti-tumour immunity, and control homeostasis (Sakaguchi *et al.*, 2001).

Natural Treg-mediated inhibition of T cell expansion can be seen as advantageous in situations of autoimmunity, but they may be disadvantageous in situations of lymphopenia-induced proliferation or anti-tumour immunity (Dolnikov *et al.*, 2003; Nomura and Sakaguchi, 2005).

Foxp3

Mice carrying the spontaneous X-linked Scurfy mutation and humans affected by the rare Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome share similar symptoms and suffer from autoimmunity (Wildin *et al.*, 2001). Their disease is characterised by chronic polyclonal activation and hyperproliferation of $CD4^+$ T cells, which produce a variety of pro-inflammatory cytokines, display decreased activation threshold, and show diminished dependence on co-stimulation signals leading to multi-organ lymphocytic infiltrations. Brunkow *et al.* in 2001 were the first to clone the gene responsible for the disease, namely *Foxp3* (Brunkow *et al.*, 2001). It belongs to the forkhead/winged helix family of transcriptional repressors and carries a zinc finger as well as a leucine zipper motif. The forkhead domain has been recently shown to be required for both DNA binding and nuclear localization whereas the leucine zipper motif mediates homodimerization. The less characterized N-term domain has been shown to mediate suppression by binding to NFAT/AP-1 sites of 5' regulatory sequences of cytokine genes and more specifically the IL-2 promoter, explaining the anergic phenotype of regulatory T cells (Lopes *et al.*, 2006).

Foxp3 is a critical element for the differentiation and function of mouse regulatory T cells (Hori *et al.*, 2003; Khattri *et al.*, 2003), and adoptive transfer of natural regulatory T cells is able to prevent disease in scurfy mice (Fontenot *et al.*, 2003). In addition, ectopic expression of *Foxp3* in $CD4^+$T cells endows non-regulatory T cells with regulatory properties such as anergy and suppressive ability (Khattri *et al.*, 2003; Khattri *et al.*, 2001). Based on these studies, *Foxp3* has been well established as a mediator of genetic mechanisms governing dominant tolerance and a Treg-lineage specification marker. It has to be noted, however, that *Foxp3* seems to behave differently in humans as activation-mediated *Foxp3* induction has been reported in $CD4^+CD25^+$ T cells (Walker *et al*., 2003).

The generation of a knock-in allele encoding a *Foxp3-gfp* fusion product has provided many insights into FOXP3 biology (Fontenot *et al.*, 2005a) and Treg function in general. This new tool allows for tracing much more faithfully regulatory T cell fate *in vivo* and facilitates sorting of natural Tregs. It is now clear that FOXP3 is highly restricted to T cells.

Regulatory T cell development

$CD4^+CD25^+$ Tregs represent 5% of thymic CD4 SP T cells and are capable of suppressive activity *in vitro*. It has been reported that, despite their presence in neonates, they are not functional before 3 to 4 days after birth (Asano *et al.*, 1996). $CD25^+$ is acquired relatively late during T cell development, during transition from the DP to SP stage. Moreover, *Foxp3-gfp* mice have allowed identification of GFP-positive cells preferentially within the CD4 SP thymocyte population, although about 11% of $FOXP3^+$ cells are also found within the DP $CD24^{hi}$ subset. It

has not been clearly demonstrated that the former is the progeny of the latter, but this demonstrates that commitment to Treg occurs during late stages of development (Fontenot *et al*., 2005a).

For years, the origin of regulatory T cells had been a subject of intense debate, some considering Tregs as products of effector T cell differentiation and others favouring the existence of an independent thymic-derived Treg lineage. It appears today that both of these hypotheses are correct: a unique thymic-derived $FOXP3^+$ $CD4^+$ T cell population is well established as an independent lineage, but generation of FOXP3-expressing Tregs from effector T cells also occurs in the periphery. In this section, we will mainly focus on thymic development of natural Tregs. Multiple studies have reported increased generation of natural Tregs in double transgenic mice co-expressing a specific TCR and its agonist in the thymus (Apostolou *et al.*, 2002; Jordan *et al.*, 2001). From these studies, it was concluded that the choice of Treg lineage is induced upon binding of TCR to its cognate Ag. It was hypothesized that this interaction, at an avidity just below the threshold required for negative selection, would promote development of regulatory T cells (Maloy and Powrie, 2001). Indeed, Jordan *et al.* showed that the affinity of the TCR for its antigen determines the T cell developmental outcome, namely regulatory T cell commitment versus negative selection (Jordan *et al.*, 2000). An alternate view on regulatory T cell development is defended by van Santen *et al.,* who argue that T cells are stochastically selected towards the Treg lineage (van Santen *et al.*, 2004). They found no increase in absolute numbers of Tregs in a similar model of double transgenic mice, but rather only an increase in the percentage of Tregs. They explained this observation by the fact that Tregs are merely more resistant to induced apoptosis. As a result, preferential survival of Tregs would inflate their percentage within the total thymic cell population while $CD4^+CD25^-$ are eliminated by negative selection. These two models are not mutually exclusive, and further studies using the KI *Foxp3-gfp* model have helped reconcile the two hypotheses. Lin *et al.* have shown that when *Foxp3* is replaced by a non-functional copy of the gene fused with *egfp*, a true population of Tregs with classical Treg phenotype develops, although these Treg-like cells do not show suppressive capacity either *in vivo* or *in vitro* (Lin *et al.*, 2007). It seems that the high-affinity TCR-Ag/MHC interaction initiates the development of a Treg cell-like precursor, which upon TCR signalling induces FOXP3 expression. In addition to TCR signaling, other costimulatory molecules influence Treg commitment. For example, CD28-deficient ($CD28^{-/-}$) as well as B7-deficient (B7 being a ligand for CD28 expressed by medullary DCs) mice have decreased numbers of regulatory T cells, and $CD28^{-/-}$ mice do not show an increase in Treg percentage in the double transgenic condition (Salomon *et al.*, 2000). TSLP-differentiated DCs, which are known to upregulate B7 expression, have also been reported as capable of inducing regulatory T cells (Watanabe *et al.*, 2005). Other molecules, such as CD40 or CTLA-4, have

been shown to be involved in Treg development. However, although a decrease in Tregs has been reported in the corresponding deficient animals, the remaining Tregs are functional and capable of suppression, suggesting that these molecules are not absolutely necessary for Treg development. On the other hand, TGF-β and IL-2 have been shown to be required for Treg survival and maintenance in the periphery but not for development (Buhlmann *et al.*, 2003; Furtado *et al.*, 2002; Kumanogoh *et al.*, 2001; Marie *et al.*, 2005).

The thymic cells responsible for Treg selection have also been a matter of debate. Long ago, it was shown that radio-resistant thymic elements of the thymus were responsible for T cell-mediated tolerance (Ohki *et al.*, 1987). It is now widely accepted that radio-resistant elements can mediate selection of $CD4^+CD25^+$ Tregs. According to Jordan and Apostolou, BM-derived DCs expressing self-Ag induce anergy of $CD4^+CD25^-$ Tregs whereas TECs mediate development of regulatory T cells. Again, there is always the possibility of cross-presentation between the two cell types (Gallegos and Bevan, 2004). In fact, it has been suggested that thymic DCs can also mediate Treg selection (Graca *et al.*, 2006). Some studies argue for a major role for cTEC whereas others favour the medullary component of thymic epithelium (Aschenbrenner *et al.*, 2007; Bensinger *et al.*, 2001). In support of the latter, deficiencies in genes playing a key role in the mTECs, such as NFκB, show an impaired regulatory T cell compartment. In addition, green cells of the *Foxp3-gfp* KI mice generated by Rudensky's laboratory are found as a majority in the medulla (Fontenot *et al.*, 2005a). Moreover, mTECs express all of the costimulatory molecules that may influence Treg development, such as CD80 and CD86, MHCII, and specific cytokines. However, it is not clear if only the medullary component is required or if Tregs are generated in the cortex with the medulla being necessary for further development.

What are the peptide ligands presented to Treg precursors? Based on the increase in Tregs generated in double transgenic mice, the fact that they proliferate robustly in lymphopenic hosts, and the lack of protection against a specific organ when the Tregs originate from an animal lacking this organ (Seddon and Mason, 1999; Taguchi and Nishizuka, 1980), it is natural to hypothesise that presentation of tissue-restricted self Ag would select for regulatory T cells. It is also tempting to suggest a role for TSA, promiscuously expressed by mTEC, in the selection of Tregs. However, the Treg compartment is normal in AIRE-deficient mice, suggesting that AIRE is not needed for Treg generation. Hsieh *et al.* have shown that the TCR repertoire of Tregs partially overlaps with the repertoire of conventional T cells and, in fact, that the same TCR could be expressed by both self-reactive T cells and Tregs (Hsieh *et al.*, 2004; Hsieh *et al.*, 2006) A recent study showed selection of Tregs specific for self antigen by $AIRE^+$ mTECs presenting that Ag, along with the concomitant deletion of self-reactive conventional T cells by thymic DCs cross-presenting TSA (Aschenbrenner *et al.*, 2007). $AIRE^+$ mTECs seem to be

sufficient for Treg generation, but it remains uncertain whether they are the only cells capable of supporting Treg selection. In contrast, the same study showed that Ag presentation by DCs is dispensable for agonist-specific T cells. It may be that the two cell types cooperate with each other, perhaps through cross presentation. It remains obscure how thymic DCs and mTEC manage to elicit negative selection and Treg production at the same time.

Homeostasis of Tregs in the periphery and *de novo* generation

As mentioned previously, TGFβ and IL-2 have been shown to be necessary for the peripheral maintenance of natural Tregs (D'Cruz and Klein, 2005; Fontenot *et al.*, 2005a; Li *et al.*, 2006; Marie *et al.*, 2005), although this has been recently challenged by Liu *et al.* (Liu *et al.*, 2008). The partially activated phenotype of Tregs suggests continuous stimulation of these cells. In addition, their repertoire is thought to tend towards self-reactivity. The source of IL-2 is presumed to be $CD4^+CD25^-$ naïve T cells. The presence of cognate antigen in the periphery is also thought to be necessary for the maintenance of natural Tregs. Indeed, adoptive transfer of peripheral Tregs from athyroid rats are unable to inhibit thyroiditis, whereas thymocytes isolated from the same donor are effective in doing so. This last experiment argues in favour of organ-specific, antigen-driven peripheral maintenance of Tregs. On the other hand, adoptive transfer of Ag-specific Tregs generated from double transgenic Ag-specific TCR/ agonist mice can survive for several weeks in a host lacking this agonist: they are able to proliferate and to suppress Ag-driven CD4 and CD8 responses *in vivo* against Ag expressing tumour cells after priming (Klein *et al.*, 2003). The discrepancy between the two studies may lie in the use of lymphopenic mice in earlier studies, which do not reflect physiologic homeostatic conditions. It is also quite possible that the priming of Ag-specific Tregs by immunization enhanced their suppressive potential in Klein's studies whereas such priming does not occur in the Le Douarin model of organ-specific tolerance. It would be interesting to use *Foxp3-gfp* knock-in Treg cells and physiologic non-lymphopenia driven autoimmune models, such as diabetes, to better define the factors driving homeostatic proliferation of Ag-specific Tregs. If not agonist-driven, Treg homeostatic proliferation may alternatively be driven by MHCII molecules, as suggested by Gavin *et al.* (Gavin et al., 2002a). However, it has been quite challenging to prove a direct effect of MHCII deficiency on Tregs and to distinguish between MHCII-mediated effects on development versus on homeostasis of Tregs (Bochtler *et al*., 2006; Shimoda *et al.*, 2006)

Despite the major role of natural thymic-derived regulatory T cells, it is well established now that other regulatory T cells can be induced from naïve T cells in the periphery, under specific conditions. Their phenotype and function are indistinguishable from those of thymic-derived Tregs: they are FOXP3-positive and capable of suppression (Walker *et al.*, 2003). Three examples support this concept. Apostolou and Von Boehmer reported that the generation of

$CD4^+CD25^+$ suppressor T cells is a normal component of the host response to the introduction of low concentrations of antigen (Apostolou and von Boehmer, 2004). Furthermore, specific tolerogenic conditions also promote differentiation of naïve T cells into regulatory T cells, which is mediated by a specific DC subset called tolerogenic (Mahnke and Enk, 2005). Finally, TGFβ has been shown to induce a regulatory phenotype in naïve T cells (Chen *et al*., 2003). However, it is not clear whether these induction protocols genuinely educate naïve T cell to display a regulatory phenotype and to acquire regulatory function or if they merely induce proliferation of a previously undetectable Treg population.

Mechanisms of suppression

The mechanism underlying the suppressive function of regulatory T cells has been the subject of a substantial number of studies, which have suggested involvement of many alternate molecules and/or pathways. This might be due to the variety of models used to characterise Treg-mediated suppression. For one, the *in vitro* setting might be quite different from the *in vivo* setting. Even among *in vitro* suppression assays, conditions may vary depending on the source of Tregs used – whether taken directly *ex vivo* or expanded through IL-2 and anti-CD28 mAb stimulation, in the presence of APCs or TGFβ in the culture medium. *In vivo*, the use of different models of autoimmunity for studying Treg-mediated suppression might further account for the discrepancies in identification of suppression mechanisms. In addition, before the development of the *FoxP3-gfp* mouse tool, the definition of regulatory T cells by the CD25 marker or various markers shared by other activated Teffs might have led to isolation of heterogeneous or distinct Treg populations acting by different mechanisms.

In vitro data support a mechanism based on cell-cell contact. Studies have also shown that TCR activation of regulatory T cells is necessary for suppression, although once activated, Tregs can inhibit Teff proliferation in a manner independent of TCR specificity. *In vitro* data have further shown that this suppression mechanism is insensitive to TGFβ or IL-10 blockade, which does not seem to be the case for *in vivo* experimental models. Barthlott *et al.* have suggested a mechanism based on competition for IL-2 between Teffs and Tregs since the survival and proliferation of both of these subpopulations are highly dependent on this cytokine (Barthlott *et al.*, 2003; Barthlott *et al*., 2005). Tregs would consume the IL-2 available in the local environment, which would then inhibit Teff proliferation. This hypothesis has been questioned by the fact that addition of IL-2 in so-called classical inhibition assays does not overcome suppression (Duthoit *et al.*, 2005). A role for CTLA-4 as mediator of suppression has also been suggested as CTLA-4 is well known for its inhibitory effect. However, peripheral Tregs of CTLA-4-deficient mice display normal suppressive function, albeit reduced numbers (Kataoka *et al.*, 2005; Read *et al.*, 2006). In humans, Grossman *et al.* have shown a role for perforin- and

granzyme-dependent pathways in Treg-mediated suppression, mediating apoptosis of Teffs. However, no evidence for killing has been found in Teffs undergoing Treg-mediated suppression in mice (Grossman *et al*., 2004). *In vivo,* some cytokines have been shown to play a role in Treg-mediated suppression. For example, Treg-mediated suppression in IBD involves IL-10 and TGFβ (Annacker *et al.*, 2001). Recently, a new molecular target, cyclic adenosine monophosphate (cAMP), has been proposed as a mediator for suppression. cAMP is a second messenger, able to inhibit T cell proliferation and IL-2 production when added *in vitro*. It is proposed that this molecule is transmitted via gap junctions formed between effector and regulatory T cells (Bopp *et al*., 2007). In conclusion, several molecules seem to be involved in the suppression mediated by Tregs, but none of these mechanisms have been found to be absolutely essential for this inhibitory function. This suggests that perhaps several mechanisms are involved and that the cell might be able to adapt its suppression mechanism depending on its local environment or the conditions of suppression.

In addition, some molecules can regulate the suppressive function of Tregs. These include cytokines like IL-2 and TNFα, as well as costimulatory factors like CD80 and CD86. For example, ligation of CD40 or activation of TLRs on DCs appears to abrogate the effect of Tregs (Pasare and Medzhitov, 2003; Serra *et al.*, 2003).

Therapeutic perspectives in Humans

The enthusiasm raised by Tregs also lies in their great therapeutic potential to control physiological and pathological immune responses. In cancer patients, depletion of Tregs leads to an increased T cell response against tumour-associated Ag when stimulated *in vitro* with tumour antigen (Danke *et al.*, 2004; Nishikawa *et al.*, 2005). In addition, $FoxP3^+$ Tregs are often found in tumours and are suspected to impede immunosurveillance by reducing responsiveness of Teffs towards tumour cells. Promoting the depletion of Tregs specifically in the tumour might be a promising approach for cancer immunotherapy to enhance the anti-tumour immune response.

During infection, depletion of natural Tregs enhances the immune response to pathogenic microbes, as reviewed by Belkaid and Rouse (Belkaid and Rouse, 2005). Tregs have even been shown to be responsible for long-term persistence of *Leishmania major* in chronically infected mice (Belkaid *et al*., 2002). They serve to control the quality and the magnitude of anti-microbial immune responses and are thought also to play a role in termination of the immune response.

Finally, one can use the ability of Tregs to expand *in vitro* or *in vivo* and their stable suppressive property to induce tolerance in autoimmune diseases, to treat allergy, or to suppress graft rejection (Kang *et al*., 2007). Moreover, techniques aimed at clonally expanding Ag-specific

natural Tregs are of great potential since Ag-specific Tregs have been shown to be more effective in models of autoimmune diabetes, BMT, and organ transplantation. However, the need for more specific and reliable surface molecular markers is necessary to discriminate between Tregs and Teffs. CD127, the IL7 receptor α chain, has been found to correlate with both FoxP3 and regulatory activity and will likely be of great help in isolating true human Tregs. It is also possible to take advantage of the conversion of adaptive T cells into Tregs under specific conditions of antigenic stimulation to enhance transplant tolerance since Tregs are suspected to limit the inflammatory response during organ rejection. Additionally, treatments that preferentially dampen effector T cell function while preserving and even enhancing Treg function may prove beneficial. One example is anti-CD3 Mab therapy administered to NOD mice, which reverses diabetes due to Treg induction (You *et al.*, 2007). Finally, the induction of pDCs may promote tolerance since pDCs are able to induce Tregs in the periphery (Ochando *et al*., 2006).

γ/δ T cells

γ/δ T cells are non-MHC-restricted, nonconventional T cells. They are located in the epithelia of various tissues constituting the internal and external surfaces of the body, such as the tongue, skin, reproductive tract, and lung. In addition, other γ/δ T cells can be found in secondary lymphoid organs, representing 1 to 5% of circulating T cells. They are characterized by a limited repertoire due to unique invariant γ and δ chain combinations corresponding to each specific tissue. They are thought to act as a first line of defence in primary immune responses and also play roles in immunoregulation, tumour surveillance, and wound healing (Hayday and Tigelaar, 2003). γ/δ T cells are the first T cells to develop during ontogeny. Specific subsets of γ/δ T cells expressing unique TCR chain combinations develop in waves, with increasing TCR complexity, during the transition from foetal to adult thymus; and they sequentially populate the different tissues mentioned above. Each wave of γ/δ T cell development corresponds to different hematopoietic progenitor cohorts with distinct developmental potential (Ikuta *et al*., 1990). In addition, foetal or newborn stroma may provide specific properties favouring the development of specific γ/δ T cell subsets since the adult thymus is unable to support differentiation of those γ/δ T cell subsets developing during foetal life. These early developing γ/δ T cell subsets are tissue-specific and are characterized by a very simple TCR composition. For example, dendritic epidermal T cells (DETCs) present in the skin carry invariant Vγ and Vδ gene segments, specifically Vγ5Vδ1, without any junctional diversity since thymic TdT activation occurs later in their development. In contrast, γ/δ T cells developing in the adult thymus present a higher variability of Vγ, being Vγ2, Vγ1.1 or Vγ1.2, and in addition possess highly diverse junctional

sequences; these T cells will migrate to secondary lymphoid organs. Lastly, Vγ5 T cells constitute intestinal intraepithelial lymphocytes. Some γ/δ T cells have also been reported to develop extrathymically (Lefrancois and Puddington, 1995).

γ/δ T cells develop from a common DN progenitor giving rise to either α/β or γ/δ T cells. The molecular events leading to lineage bifurcation between the two populations has not been fully resolved but this lineage decision is thought to be determined at the DN2 stage, despite the fact that DN2 precursors of γ/δ T cell are indistinguishable from α/β DN2 precursors. γ/δ T cells never go through a DP stage, and signals through the γ/δ TCR regulate differentiation into mature γ/δ T cells, characterized by down-regulation of CD24. Following the DN3 stage, γ/δ T cells do not undergo a β selection checkpoint-induced proliferative burst like α/β T cell precursors as they do not express pre-TCR but rather the definitive form of the γ/δ TCR. Therefore, major clonal expansion of γ/δ T cells occurs after completion of their maturation.

To account for γ/δ development, two models have been proposed. One is a selective model whereby lineage specification is thought to be mainly determined in thymocyte precursors before TCR gene rearrangement starts. The adequacy of the subsequent random TCR rearrangement would allow for survival or death. The other is an instructive model whereby the signal originating from the TCR influences uncommitted progenitors towards either α/β or γ/δ fates. As detailed below, various studies have supported these models, but to date, none can entirely explain the physiological situation and fully validate one of the models.

The selective model is supported by the observations of Durum *et al.* that a pro-T cell population carrying high levels of IL-7Rα is more prone to develop towards the γ/δ T cell lineage whereas IL-7 Rα low or negative pro-T cells will preferentially differentiate into α/β (Durum et al., 1998; Kang et al., 2001). Further support for this hypothesis was provided by Maki *et al.*, who showed that IL-7Rα-deficient mice are devoid of γ/δ T cells (Maki *et al.*, 1996). The theory of TCR-independent lineage specification is also supported by the fact that some DN2 cells display a bias in lineage potential before the TCR is expressed on their surface, as shown by the clonal analysis potential of DN2 in Notch ligand-dependent cell culture (Ciofani *et al.*, 2006). Garcia-Peydro *et al.* also suggested a positive role for Notch in the development of γ/δ T cells from very early precursors (Garcia-Peydro *et al.*, 2003). Surprisingly, a deficiency in Notch specifically at the transition from DN2 to DN3 stage favours an increase in γ/δ T cell production. These two seemingly contradictory results have been reconciled by Ciofani *et al.,* who showed that Notch is essential for early γ/δ T cell development up to the DN2 stage but not beyond the DN3 stage (Ciofani *et al.*, 2006). This is in contrast to the continuous requirement of Notch throughout α/β T cell development up to the DP stage.

The instructive model, on the other hand, proposes that signals from the γ/δ TCR regulate γ/δ cell development. It is based on the observation that pre-Tα–deficient mice show a great increase in γ/δ T cell differentiation. In addition, γ/δ TCR transgenic mice show inhibition of α/β T cell development. Moreover, developing T cells expressing γ/δ TCR are excluded from the α/β lineage (Kang *et al*., 1995; Livak *et al*., 1995). These findings support the conclusion that γ/δ TCR expression is not neutral, but rather drives T cell progenitors towards the γ/δ T cell pathway. In addition, it was previously argued that the γ/δ TCR signal is stronger than the signal delivered by pre-TCR (Hayes *et al*., 2003). Nevertheless, these experiments do not directly prove that TCR dictates lineage decision as its expression may simply allow for survival of cells that are already committed. In addition, this theory is challenged by the fact that γ/δ TCR chain rearrangement occurs in α/β T cell progenitors and that intracellular β chain rearrangement has been reported in about 15% of γ/δ T cells. Ultimately, it has been suggested that lineage decision might be conditioned by the quality or the strength of the TCR signal, and indeed, despite a high similarity between the two TCR signaling pathways, γ/δ TCR have been shown to induce a stronger signal than α/β TCR (Haks *et al*., 2005; Hayes *et al*., 2005). Through the use of γ/δ TCR transgenic mice, Hayes *et al.* were able to alter γ/δ versus α/β T cell numbers by adjusting the strength of TCR signaling. The stronger the TCR signal, the more γ/δ and the fewer α/β T cells that developed. More recently, Kreslavsky *et al.* showed through single cell analysis that there is no commitment to either the α/β or γ/δ lineage before TCR expression and that modification of TCR signaling can change lineage commitment (Kreslavsky *et al*., 2008). These reports provide new insights in favour of this quantitative model of commitment.

In addition to the two models described above, Pennington *et al.* have proposed an alternate possibility. They defined a γ/δ-biased gene expression profile to identify γ/δ precursors and found them to be absent in pre-Tα mice and RAG-deficient mice. Based on this, they proposed that failure of γ/δ T cell development was due to the lack of DP T cells by a so called “trans-acting” feedback via the LTβ receptor.

Thymic ligands for positive selection of γ/δ T cells have been investigated for years before Boyden *et al.* identified one of them recently. It was previously thought that γ/δ T cells might be selected by encountering their cognate antigen in the peripheral tissues where they migrate. This encounter with self-antigen would induce their survival. Indeed, by mapping and genetic complementation of a mouse strain devoid of Vγ5Vδ1 epidermal T cells, Boyden *et al.* showed that *Skint1*, expressed in the thymus and skin, positively selects epidermal γ/δ T cells (Boyden *et al*., 2007).

Aim of the study:

We propose to study tolerance and T cell development throughout different models. We first observed that immunodeficient mice reconstituted with bone marrow from syngeneic host suffer from autoimmunity whereas immunocompetent mice stayed healthy. We have tried to identify the radioresistant cell subset responsible for this protection. In a second study we investigated further on radioresistant thymocytes to identify the origin of the single wave of differentiation of host derived T cells observed in mice reconstituted with bone marrow. In a third part we propose to explain the presence of β chain in the cytoplasm of γ/δ T cells by reconstituting immunodeficient mice with Pax5 KO cells transfected with β chain isolated from either α/β or γ/δ T cells. Finally we have developed a new double transgenic system by crossing mice expressing OVA antigen under the control of KLC and CD11c promoter and OT1 and OT2 mice. This will allow us to study T cell tolerance through regulatory T cell development and anergy induction.

Results

Part I: Regulatory T cells control auto-immunity following syngeneic bone marrow transplantation.

Angèle Bénard, Rhodri Ceredig, and Antonius G Rolink.

Introduction

Reconstitution of the lympho-hemopoietic system by bone marrow (BM) transplantation is a frequently used treatment modality for various haematological abnormalities, including anaemia, leukaemia and lymphoma (Nakase et al., 2005) and more recently fulminant auto-immunity (van Bekkum, 2004). Although this is frequently carried out with BM from haplo-disparate donors, even in situations where the BM graft is haplo-identical, patients can develop syngeneic graft versus host disease (GVH) (Adams et al., 2004; Latif et al., 2003; Spaner et al., 1998). Disease can be acute, starting at day 8 (Latif et al., 2003) or more chronic starting at about 5 weeks (Adams et al., 2004) following BM transplantation, a time when lymphoid reconstitution has begun, but when the patient is still relatively lymphopenic. Many of the features of syngeneic chronic GVH, including diarrhoea, weight loss, cutaneous and lymphocytic infiltrations in multiple organs and the presence of auto-antibodies are also seen in systemic auto-immune diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis, systemic lupus erythematosis and systemic sclerosis (Lyons et al., 2005)

Recent studies have implicated so-called naturally-arising regulatory T cells (Treg) as key components controlling auto-immunity (Kronenberg and Rudensky, 2005; Sakaguchi et al., 1995; Shevach, 2004). Treg were first described as a population of $CD5^{high}CD4^{+}$ cells that upon transfer could protect mice from auto-immune disease caused by neonatal thymectomy (Sakaguchi et al., 1982). Later it was shown that during mouse ontogeny, Tregs appeared shortly after birth and that CD25, the alpha chain of the IL-2R complex, could be used as a surrogate marker for these cells(Fontenot et al., 2005a; Sakaguchi et al., 1995). More recently, the transcription factor FoxP3 has been shown to be critically associated with Treg function (Hori et al., 2003) and indeed a knock-in transgenic mouse line containing a GFP-FoxP3 construct indicated that FoxP3 may be used as a lineage marker for Treg (Fontenot et al., 2005b). Many phenotypic features of Treg, namely spontaneous CD25 expression, down-regulation of CD4, CD3 and TCRβ transcripts and surface antigen expression are similar to those of activated T cells (Bosco et al., 2006b; Gavin et al., 2002b; Kasow et al., 2004). The repertoire of TCR expressed by Treg is generally thought to be broad; however, it would seem

that their TCR have a relatively high affinity for self antigens (Hsieh et al., 2004). Thus their $CD5^{high}$ phenotype may have protected them from negative selection in the thymus (Azzam et al., 2001). Functionally, Treg inhibit the proliferation of naïve responder T cells in vitro (Thornton and Shevach, 1998). Their main functional role in vivo (Asano et al., 1996) appears to be in preventing the activation and reducing the expansion of activated T cells (Sakaguchi, 2004). This inhibition of T cell expansion can be seen as advantageous in situations of auto-immunity, but may be disadvantageous in situations of lymphopenia-induced proliferation or anti-tumor immunity (Dolnikov et al., 2003; Nomura and Sakaguchi, 2005). It is therefore rather paradoxical that auto-immunity is controlled in a dominant fashion by a population of T cells which is itself intrinsically auto-reactive (Kronenberg and Rudensky, 2005).

In the mouse, syngeneic BM transplantation of immuno-competent recipients rarely results in disease. In contrast, in man, syngeneic GVH has been reported to develop with a cumulative incidence of 18% among syngeneic hematopoeitic cell transplant recipients (Adams et al., 2004). This difference could be due to the relatively faster kinetics of reconstitution in mice, which is of the order of weeks compared with months in man. However, in analyzing the reconstitution potential of sub-lethally irradiated immuno-deficient recipient mice with in vitro-generated, T cell-restricted, BM-derived progenitor cells, recipient mice frequently died between four and six weeks after reconstitution (A. Rolink unpublished observations). Mice died despite evidence indicating that the T cell compartment was being well reconstituted by donor-derived cells. In this report we describe results of experiments where the outcome of syngeneic BM transplantation in sublethally-irradiated immuno-deficient C57Bl/6 RAG-$2^{-/-}$ was compared with that in immuno-competent WT C57Bl/6 recipients. Whereas immuno-competent mice survived, immuno-deficient mice succumbed to auto-immunity with diarrhea and weight loss beginning at three weeks following BM transplantation. Disease was associated with IgG anti-nuclear antibodies. Auto-immunity was initiated by T cells but could be prevented by co-transferring naturally-arising regulatory T cells with the BM inoculum. Survival of immuno-competent recipients was shown to be due to the presence of residual, extra-thymically-located, radio-resistant, functional regulatory T cells in the lethally-irradiated host. Moreover depletion of regulatory T cell in wild type mice prior to bone marrow transplantation resulted in a phenotype similar to that of immuno-deficient hosts although with milder clinical symptoms. The implications of these results in the context of BM transplantation will be discussed.

Results

RAG-2$^{-/-}$ mice show symptoms of autoimmune disease following transplantation of syngeneic RAG proficient BM.

When sublethally-irradiated Bl/6 Ly5.2 RAG-2$^{-/-}$ mice were reconstituted with C57Bl/6 wild type (WT) Ly5.1 T-cell depleted BM and despite successful reconstitution of T lymphopoiesis in the thymus and B lymphopoiesis in the BM, most animals died between four and seven weeks later. Figure 1.A shows the survival curve of a group of 25 RAG-2$^{-/-}$ mice from 5 individual experiments reconstituted with T cell-depleted BM from WT syngeneic donors. Until about 33 days, 100% mice survived, but from then on there was a progressive decrease in survival with only 20% of mice surviving beyond 50 days.

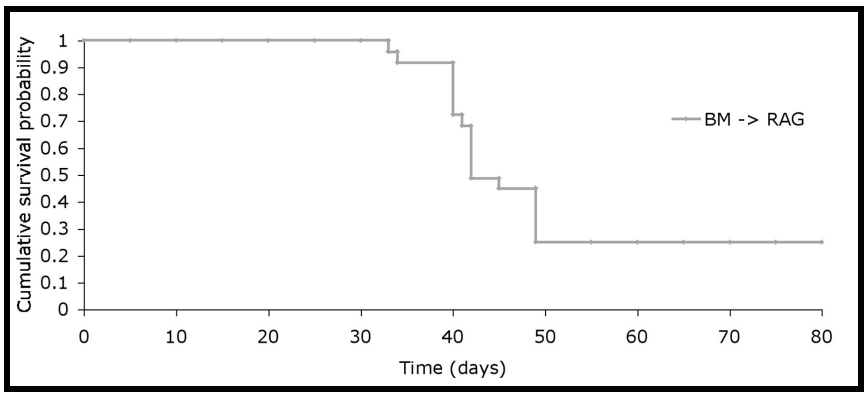


Figure 17: A. Survival curve of C57Bl/6.Ly5.1→C57Bl/6.RAG-2$^{-/-}$.Ly5.2 BM chimeras.

Sub-lethally irradiated Ly5.2 RAG2$^{-/-}$ mice were transplanted with T cell depleted bone marrow from Ly5.1 C57Bl/6 mice. Shown is the pooled survival curve of 25 mice from four different experiments. Survival did not differ between individual experiments.

Prior to day 33, mice began having diarrhoea and when groups of five to six mice were individually weighed following BM transplantation (Figure 1B) there was progressive weight loss from day 21 with mice loosing approximately 40% of their initial body weight by day 42. Thus, diarrhoea and weight loss preceded death of the mice.

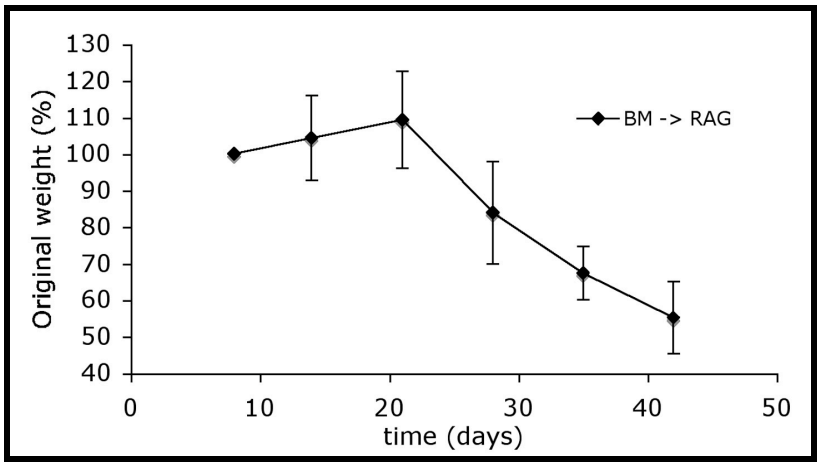


Figure 1: B. Weight loss among BM chimeras.

Shown is the kinetic of mean weight loss in one group of six BM chimeras. Mice were weighed every three days over a six-week observation period.

Inspection of the intestines from mice with diarrhoea showed that the lower third of the colon was dilated with thickening of the wall. Histological analysis of this region (Figure 1C1) showed characteristic features of IBD (Maloy et al., 2005), namely epithelial hyperplasia, extensive lymphocytic infiltration of the lamina propria, crypt abscesses and destruction of the mucous membrane with prominent ulceration.

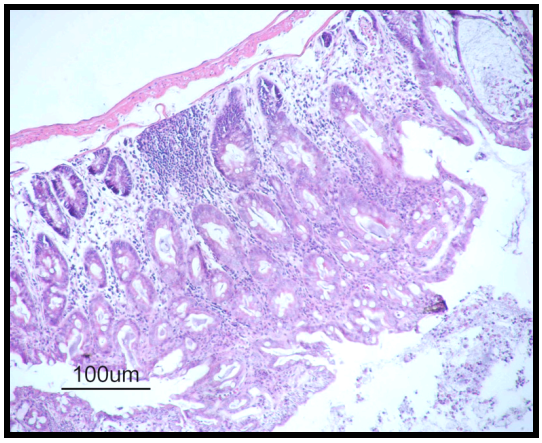


Figure 1C1. Widespread lymphocytic infiltration in non-lymphoid organs of immuno-deficient mice after bone marrow transplantation.

The upper picture is a photograph of H+E stained 3_m paraffin sections of the lower third of the colon from Bl/6→RAG2$^{-/-}$ BM chimeras (Original magnification x100).

Histological analysis of other organs, for example the liver (Figure 1C2), also showed evidence of lymphocytic infiltration.

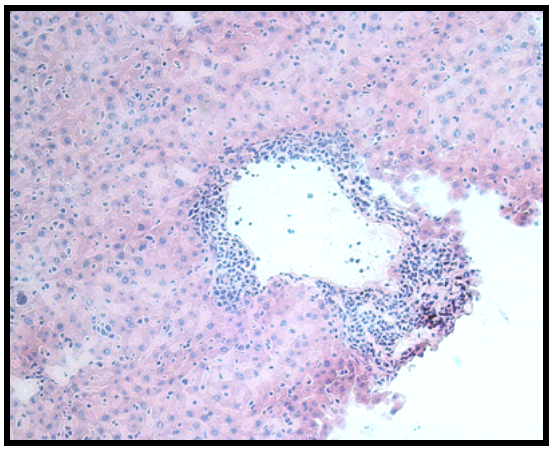


Figure 1C2. Widespread lymphocytic infiltration in non-lymphoid organs of immuno-deficient mice after bone marrow transplantation.

The upper picture is a photograph of H+E stained 3_m frozen sections of liver from Bl/6→RAG2$^{-/-}$ BM chimeras (Original magnification x400).

Moreover the architecture of secondary lymphoid organs was disturbed in that discrete lymphoid follicles were absent (Figure 1C3).

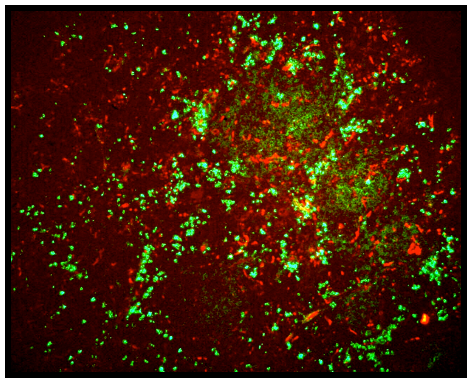


Figure 1C3. Disorganised cellular architecture in lymphoid organs of immuno-deficient mice after bone marrow transplantation.

The upper picture is a photograph of immunohistochemical analysis of 5_m frozen sections of spleen from Bl/6→RAG2$^{-/-}$ BM chimeras (Original magnification x100). Sections were stained for peanut agglutinin (red) and IgM (green).

Flow cytometric analysis of LN cells from mice with diarrhoea was carried out. Results obtained (Figure 2) showed that whereas there was clear evidence of B cell (left cytogram) and T cell (middle cytogram) reconstitution, further analysis of gated CD4$^+$ cells (right cytogram) showed that many had up-regulated CD69 and CD25 expression. Two color analysis of gated CD4$^+$ T cells showed that ~19% were CD69$^+$CD25$^-$, 18% CD69$^+$CD25$^+$ and 12% CD69$^-$CD25$^+$. Thus, CD4$^+$ T cells from affected mice showed an activated phenotype.

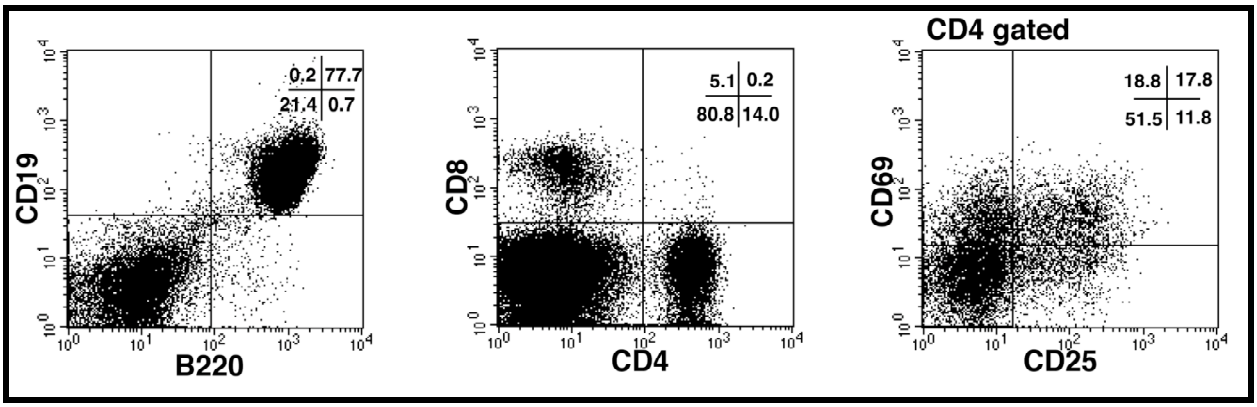


Figure 18: Lymphocyte reconstitution in sub-lethally-irradiated C57Bl/6.Ly5.1_C57Bl/6.RAG-2$^{-/-}$.Ly5.2 BM chimeras.

Shown are two-color cytogram displays of LN cells from BM chimeras stained with the indicated mAb. In each panel, quadrants are placed so that 100% unstained cells were contained in the lower left quadrant. In the upper right panel, the % positive cells in each quadrant is indicated. The left panel shows B cell reconstitution, the middle panel that of CD4 and CD8 T cell subpopulations and the right panel expression of CD69 and CD25 on gated CD4^{+} T cells. See text for details.

Onset of disease in RAG-2$^{-/-}$ recipient mice is mediated by T cells.

In RAG-2$^{-/-}$ mice reconstituted with syngeneic WT BM, onset of diarrhoea and weight loss coincided with a state of relative lymphopenia during the initial phase of lymphocyte reconstitution. In addition, CD4^{+} T cells from affected mice showed evidence of extensive activation. Therefore, to see whether T cells were responsible for initiating disease, RAG-2$^{-/-}$ mice were reconstituted with BM from donors incapable of reconstituting the T cell compartment, namely Ly5.1 CD3ε$^{-/-}$ mice. In six Ly5.2 RAG-2$^{-/-}$ mice reconstituted with Ly5.1 CD3ε$^{-/-}$ BM and in which the BM B cell compartment was fully reconstituted with Ly5.1 B cells, none developed signs of diarrhoea or weight loss with all mice surviving until six months following reconstitution (Figure3). Thus, T cells were responsible for initiating disease in BM-transplanted mice.

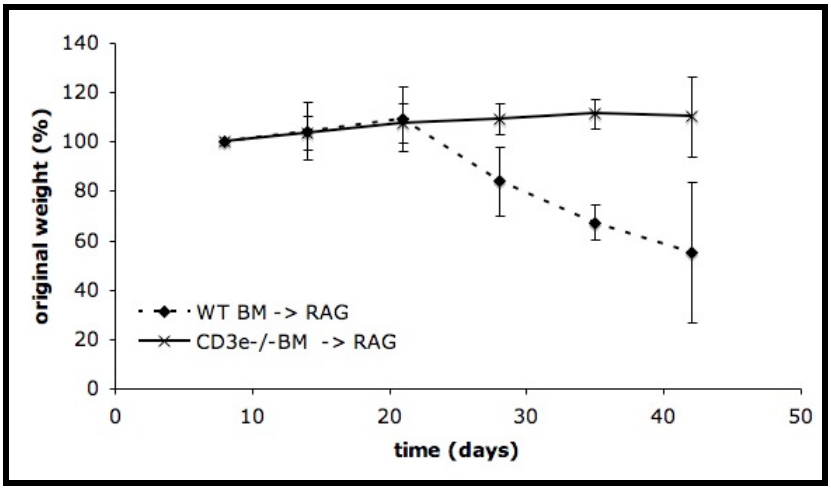


Figure 19: Absence of weight loss among BM chimeras transplanted with CD3ε$^{-/-}$ bone marrow.

Shown is the kinetic of mean weight loss in two groups of BM chimeras. The first group (n=6) was RAG$^{-/-}$ mice transplanted with wild type bone marrow and the second group (n=5) were RAG$^{-/-}$ mice transplanted with CD3ε$^{-/-}$ bone marrow Mice were weighed every three days over a six-week observation period.

Co-transplantation of regulatory T cells protects RAG-2$^{-/-}$ recipient mice from disease.

RAG-2$^{-/-}$ mice reconstituted with syngeneic BM manifested signs of IBD and this was initiated by T cells. Previous experiments have shown that naturally arising regulatory T cells could protect lymphopenic mice from IBD (Martin et al., 2004). Therefore, to see if naturally arising regulatory T cells could protect BM transplanted mice from IBD, Ly5.2 RAG-2$^{-/-}$ recipient mice were reconstituted with a mixture of T cell-depleted Ly5.2 BM together with 1-3x10^5 sorted Ly5.1 CD4$^+$CD25$^+$ LN cells. In three groups of experimental mice, no diarrhoea or weight loss was noted. Gross inspection of the intestines showed them to be normal and histological analysis of the lower third of the colon confirmed this (Figure4) showing a completely normal picture.

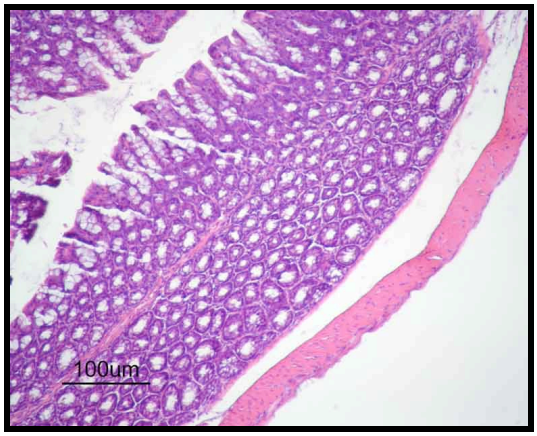


Figure 20A

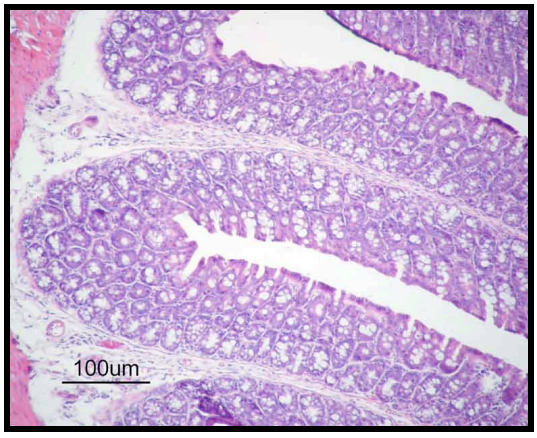


Figure 20B

Absence of lymphocytic infiltration in non-lymphoid organs of bone marrow transplanted, regulatory T cells coinjected immuno-deficient mice.

Hematoxylin and eosin staining of the gut in $RAG2^{-/-}$ mice transplanted with bone marrow cells along with $CD4^{+}CD25^{+}$ T cells. The luminal epithelial is intact and thin, there is no lymphocytic infiltration detectable, and the crypt size is limited. (Original magnifications, x100)

Histological analysis of other organs from these mice showed the total absence of lymphocytic infiltration. Moreover secondary lymphoid organs showed a normal architecture. However, relatively large numbers of germinal centers were present in the spleen. This finding strongly suggests that, although not showing signs of disease, an immune response was ongoing in these mice.

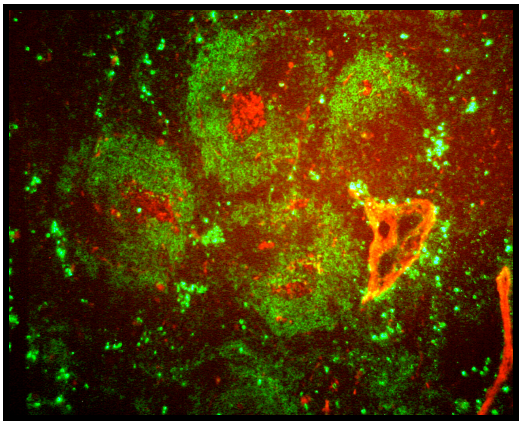


Figure 21: cellular architecture in lymphoid organs of immuno-deficient mice after bone marrow transplantation and co-injection of Treg.

The upper picture is a photograph of immunohistochemical analysis of 5_m frozen sections of spleen from Bl/6 + Treg→$RAG2^{-/-}$ BM chimeras (Original magnification x100). Sections were stained for peanut agglutinin (red) and IgM (green)

The phenotype of the transferred $Ly5.1^{+}$ $CD4^{+}CD25^{+}$ T cells as well as that of the cohort of T cells derived from the BM donor was analyzed by three-color flow cytometry. Figure 6 shows the results of a typical experiment of spleen cells seven weeks after BM transplantation. Thus, 0.2% of gated lymphocytes expressed Ly5.1, the Ly5 allotype of the injected $CD4^{+}CD25^{+}$ LN T cells (Figure 6.A, histogram). Further analysis of gated $Ly5.1^{+}$ spleen cells (right cytogram displays) showed that they were practically all $CD4^{+}CD8^{-}$ cells (right upper cytogram) with the vast majority (80%, lower right cytogram) retaining the $CD25^{+}$ phenotype.

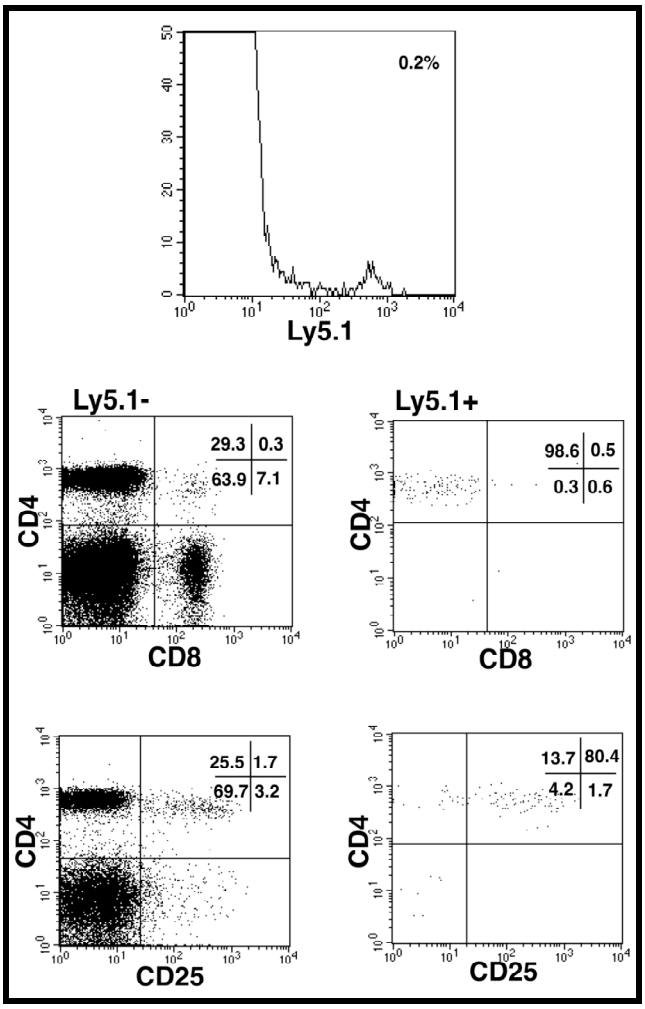


Figure 22A. $CD4^+CD25^+$ T cells co-injected into recipient mice survive for up to 9 weeks following BM reconstitution

Sub-lethally-irradiated (500Rad) Ly5.2.$RAG2^{-/-}$ mice were reconstituted with T cell-depleted Ly5.2.Bl/6 WT BM together with sorted $Ly5.1^+CD4^+CD25^+$ T cells. The top histogram is of spleen cells from one of four similar experiments stained with FITC-labeled anti-Ly5.1mAb. and shows the presence of residual $Ly5.1^+CD4^+CD25^+$ regulatory T cells at this time. Cytogram displays show the two color staining profiles of gated donor-derived (left, $Ly5.1^-$) and injected $Ly5.1^+CD4^+CD25^+$ (right) T cells stained for CD4 and CD8 (upper cytograms) or CD4 and CD25 (lower cytograms). Figures in the upper right quadrant of each panel show the % of positive cells in each quadrant. See text for details.

Thus, the transferred $Ly5.1^+$ cells were not contaminated by cells capable of reconstituting the host thymus and they persisted in recipient mice for up to ten weeks after BM transplantation.

When gated on Ly5.1-negative (namely $Ly5.2^+$) cells, the spleen contained the expected subpopulations of donor-derived $CD4^+$ and $CD8^+$ T cells, with in this case a high CD4/CD8 T cell ratio (~4) (left upper cytogram). However, the proportion of $CD25^+$ cells among the $CD4^+$ subpopulation was relatively low (~7%, left lower cytogram). As is the case for naturally-arising $CD25^+$ regulatory T cells, donor-derived $CD4^+CD25^+$ cells had a slightly decreased CD4 expression (Bosco et al., 2006b). Additional staining of LN cells from such chimeras with CD4, CD25 and CD69 showed that co-transplantation of Treg prevented, to a large extent, the

activation of BM donor-derived T cells. Thus only 11.6% of BM donor CD4 cells expressed the early activation marker CD69 (Figure 6B) compared with 36.6% in the chimera without co-transferred Tregs (Figure 2). Moreover, most gated $CD25^+$ cells were now CD69-negative (Figure 3.B), confirming that they were not simply activated $CD25^+$ T cells. Thus, co-transfer of naturally arising $CD25^+$ regulatory T cells to BM transplanted mice inhibits the activation of BM-derived T cells and also protected mice from IBD.

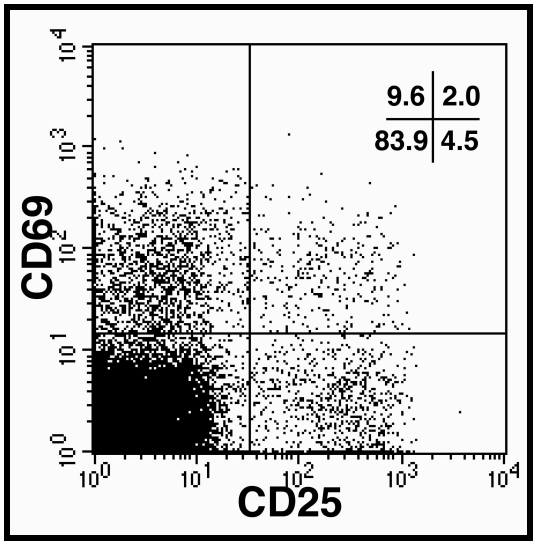


Figure 6B. Donor-derived CD4+ T cells retain a naive phenotype.

The two-color cytogram shows the CD69 versus CD25 staining profile of Bl/6 WT-derived CD4 cells in C57Bl/6.Ly5.2→C57Bl/6.RAG-$2^{-/-}$.Ly5.2 BM chimeras co-injected with sorted Ly5.1.$CD4^+CD25^+$ T cells.

Co-transplantation of regulatory T cells prevents the formation of auto-antibodies in RAG-$2^{-/-}$ recipients.

Given the fact that BM transplanted mice showed signs of systemic auto-immunity, we decided to investigate whether the serum from such mice contained auto-antibodies. For this, frozen sections of kidneys from RAG-$2^{-/-}$ mice were incubated with serum from BM transplanted RAG-$2^{-/-}$ mice reconstituted with or without Tregs and the presence of autoantibodies determined by indirect immunofluorescence.

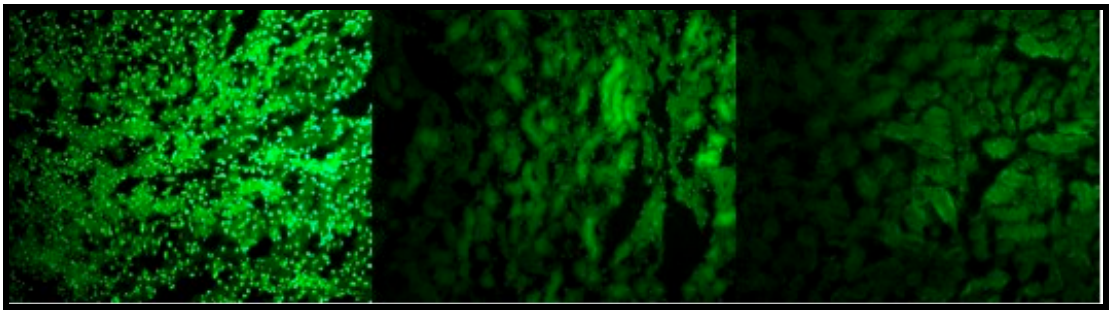


Figure 23A: Immunofluorescent detection of anti-nuclear autoantibodies

A. Kidney sections of RAG$^{-/-}$ mice were incubated with serum samples of RAG$^{-/-}$ mice transplanted with bone marrow cells alone (left picture), or co-injected with CD4^{+}CD25^{+} T cells (middle picture) and goat anti-mouse IgG FITC labeled antibody was used to detect anti-nuclear autoantibodies The right panel shows a negative control without the addition of serum. (original magnification, x200)

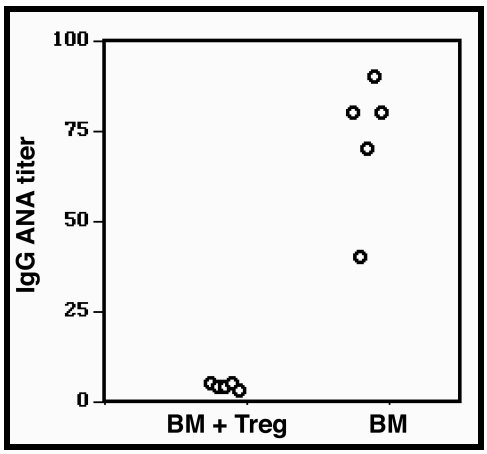


Figure 7B: Co-injection of regulatory T cells inhibits the formation of antinuclear auto-antibodies in BM chimeras

Shown are the titers of anti-nuclear auto-antibodies, detected by indirect immunofluorescence as described, in sera from BM chimeras co-injected (left, BM+Treg) or not (right, BM) with sorted CD4^{+}CD25^{+} regulatory T cells.

As shown in Figure 7B, whereas the serum of BM-transplanted mice contained readily-detectable anti-nuclear antibodies, they were undetectable in BM recipients co-transferred with Tregs.

Normal Bl/6 mice do not develop symptoms of auto-immunity following BM transplantation

Auto-immunity was only seen when RAG-2$^{-/-}$ recipients were reconstituted with T cell-depleted BM. Normal Bl/6 mice reconstituted with T cell depleted BM did not suffer from IBD. To see whether radio-resistant, host-derived, naturally-arising CD25^{+} regulatory T cells were responsible for protecting mice from IBD, a series of chimeras was established in which normal Ly5.1^{+} Bl/6 mice were reconstituted with T cell-depleted Ly5.2^{+} Bl/6 BM alone. As expected, no diarrhoea or weight loss was seen in normal Bl/6 recipients. Again, histological analysis of intestines and other organs showed no abnormality (not shown). Flow cytometric analysis of LN cells at six weeks following BM transplantation showed the presence of a significant (14%) population of host-derived (Ly5.1^{+}) cells (Figure 8.A, histogram) and further analysis of these gated host-derived LN cells (right cytograms) showed that most (93.5%) were T cells, being 74% CD4^{+} and 19.5% CD8^{+}. Further analysis of the CD4^{+} subpopulation (lower right cytogram) showed that 18.7% (13.9/74.5) were CD25^{+} cells, the latter again having a slightly reduced CD4 expression as is characteristic of Treg. Parallel analysis of the donor-derived (Ly5.1-negative)

population showed that, as expected, the majority (67%, lower left quadrant) expressed neither CD4 nor CD8 and were mostly $CD19^+$ B cells. Indeed, after six weeks, analysis of the thymus and BM in such chimeras showed that 98.8% ± 0.7 (n=5) of thymocytes and 100% BM $CD19^+$ cells were donor-derived. This result showed that with this irradiation dose, the progenitor T and BM B cell lineage was fully reconstituted with donor-derived cells and that there was no detectable population of surviving host-derived B cells (not shown). Donor-derived T cells (23.4% CD4 and 9% CD8) had a normal CD4:CD8 ratio and additional analysis of the $CD4^+$ cells (lower cytogram) showed a distinct population of 7.5% $CD25^+$ cells.

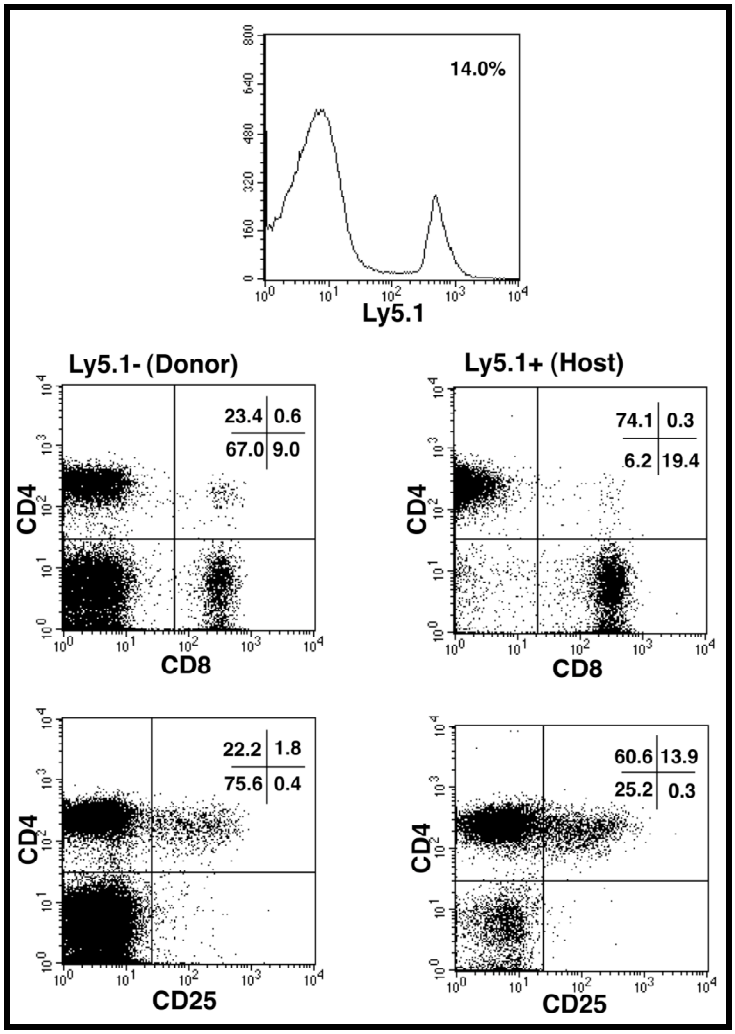


Figure 8A: Radio-resistant, host-derived $CD4^+CD25^+$ regulatory T cells protect WT recipients from autoimmune disease.

The top histogram is of LN cells from C57Bl/6.Ly5.2→C57Bl/6. Ly5.1 BM chimeras stained with FITC-labeled anti-Ly5.1 mAb. and shows the presence of 14% residual, host-derived, cells. Below are cytogram displays of cells stained for CD4 and CD8 (upper cytograms) or CD4 and CD25 (lower cytograms) and gated for either donor ($Ly5.1^-$, left) or host ($Ly5.1^+$right) derived cells. C57/Bl6 $Ly5.1^+$ mice were irradiated with 9.5 Gy before being reconstituted with T cell-depleted BM cells from Bl/6 $Ly5.2^+$ mice.

To show that radio-resistant, host $CD4^+CD25^+$ T cells possessed regulatory function, we carried out a classical T cell co-culture assay (Thornton and Shevach, 1998) using naïve

$Ly5.1^+CD4^+CD25^-$ responder T cells and as potential inhibitory cells host-derived $Ly5.2^+CD4^+CD25^+$ T cells from Ly5.1_Ly5.2 BM chimeras six weeks after BM transplantation. Results obtained (Figure 8B) show clearly that when stimulated with anti-CD3ε mAb in the presence of irradiated APC, naïve T cells alone proliferated normally (left stippled bar) but that addition of $Ly5.2^+CD4^+CD25^+$ host-derived T cells from chimeras inhibited this proliferation (right filled bars). Thus, the host-derived $CD4^+CD25^+$ subpopulation that survived lethal irradiation possessed classical T regulatory activity.

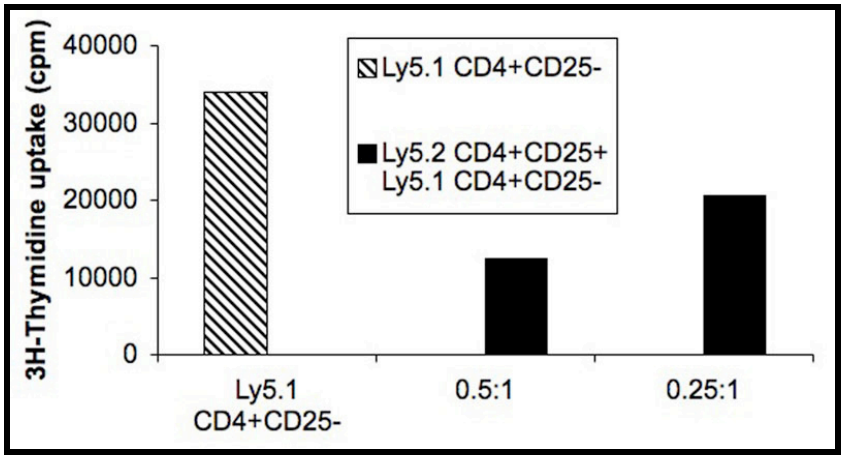


Figure 8B. Host-derived radio-resistant $CD4^+CD25^+$ T cells retain regulatory T cell function.

Shown are the ^3H thymidine counts from cultures containing $Ly5.1^+CD4^+CD25^-$ responder naive T cells cultured either alone (left column) or together with sorted $Ly5.2^+CD4^+CD25^+$ host-derived cells from C57Bl/6.Ly5.→C57Bl/6. Ly5.2 BM chimeras at the indicated ratios. Cells were cultured as described in Methods for 72 hours with anti-CD3 mAb and irradiated spleen cells as APC. Each column represents the mean of ^3H-thymidine incorporation from triplicates wells.

Compared with donor-derived cells, the host-derived $Ly5.1^+CD4^+$ subpopulation was relatively enriched in $CD25^+$ cells (Fig 8A, bottom cytograms). This suggested that $CD25^+$ T cells might preferentially survive a lethal dose of irradiation. The possibility also existed that host-derived $CD4^+CD25^+$ T cells were the progeny of the transient, host-derived, cohort of thymocytes generated following irradiation and BM reconstitution (Hori et al., 2003). To determine whether the thymus was required for the appearance of these peripheral $CD4^+CD25^+$ cells, recipient adult mice were first thymectomized and one week later, irradiated and reconstituted with T-cell-deficient RAG-$2^{-/-}$ BM. In these thymectomised mice, any surviving T cells must have been in an extra-thymic location at the time of irradiation. Flow cytometric analysis of LN cells from such a chimera at 5 weeks following reconstitution showed that about 3% were $CD4^+$ $TCR\beta^+$ T cells and about 38% of these expressed CD25 (Figure 9).

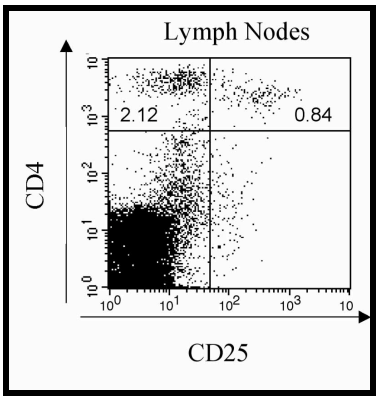


Figure 24: Preferential survival of $CD4^+CD25^+$ host T cells following irradiation.

Shown is a cytogram display of LN cells from a six week-old B/6 mouse that had been thymectomised, one week later 4.5 Gy body-irradiated, reconstituted with BM from Bl/6.RAG2$^{-/-}$ donors and stained with the indicated markers. As shown, about 35% (0.84/2.96) of surviving host-derived CD4 cells expressed CD25.

However, it should be noted that the number of host-derived T cells in thymectomized chimeras was dramatically lower than in euthymic controls. Thus the majority of host peripheral T cells in BM chimeras are the progeny of the cohort of thymocytes generated following irradiation and BM reconstitution.

Regulatory T cell depleted Bl/6 mice suffer from IBD following BM transplantation

To check whether among radio-resistant cells, regulatory T cells were the ones responsible for protecting the mice against IBD, we depleted mice of Tregs prior to irradiation and BM reconstitution. It has been previously shown that injection of anti-CD25 mAb *in vivo* depletes the regulatory T cell compartment (Oldenhove et al., 2003), and several groups have used this approach to boost anti-tumor immune responses (Dannull et al., 2005). Anti-CD25 mediated depletion might not eliminate all regulatory T cells (Fontenot et al., 2005b; Kohm et al., 2006), nevertheless so far it is the only method of eliminating the majority of regulatory T cells *in vivo*. Therefore, groups of 5 Ly5.2$^+$Bl/6 mice received either a single 0.5mg dose of PC61 anti-CD25 mAb intra-venously or PBS as control. After 5 days, mice were lethally irradiated (8Gy) and subsequently reconstituted with T cell depleted Ly5.1$^+$Bl/6 wild type BM. Three weeks after reconstitution, mice started losing weight and by 6 weeks anti-CD25-treated mice had lost 34% of their initial body weight (Figure10), accompanied by extensive diarrhea. Out of 5 mice, 1 died after 4 weeks and the other 4 were sacrificed 7 weeks after transplantation according to Institutional guidelines due to their deteriorating health.

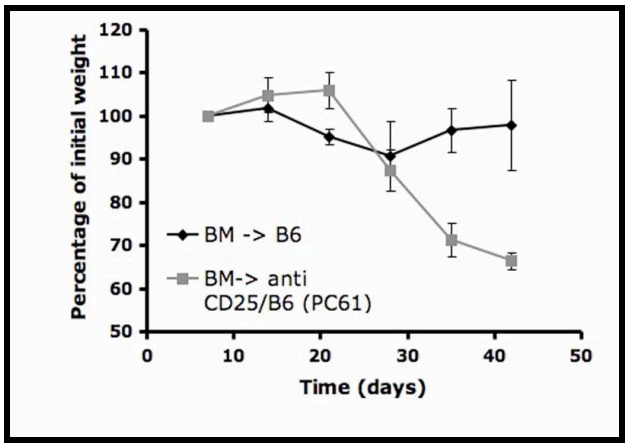


Figure 25: Mice depleted of regulatory T cells suffer weight loss after BM reconstitution.

Ly5.2^{+}Bl/6 mice were lethally-irradiated with 9.5Gy and then transplanted with T cell depleted bone marrow cells from Ly5.1^{+}C57Bl/6 mice. Bone marrow transplanted Bl/6 mice were weighted every 7 days for 6 weeks. The above figure represents the mean weight variation from one group of five mice.

Flow cytometry analysis with an anti-CD25 mAb recognizing a CD25 epitope distinct from that recognized by PC61, the CD25 mAb used for *in vivo* depletion, showed that CD25^{+} cells had been reduced by between 92.5% and 99.9%. In addition, in the absence of CD25^{+} cells, surviving CD4^{+}CD25^{-} cells were in a more activated state as shown by enrichment for CD69^{+} cells (Figure 11).

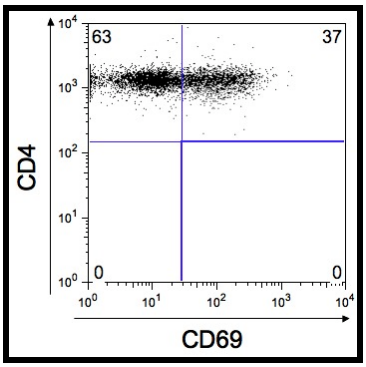


Figure 26: Donor-derived CD4^{+} T cells show an activated phenotype when mice are depleted from regulatory T cells prior to bone marrow transplantation.

The two-color cytogram shows the CD69 versus CD4 staining profile of lymph nodes Bl/6 WT-derived CD4 cells in C57Bl/6.Ly5.1→C57Bl/6.Ly5.2 BM chimeras treated with anti-CD25 MAb prior to bone marrow transplantation.

However, clinical parameters were not as severe as in RAG2$^{-/-}$ recipients presumably because, as shown by others, some regulatory T cell activity still remains following anti CD25 mAb depletion (Fontenot et al., 2005b; Kohm et al., 2006).

Discussion

Bone marrow transplantation, whether by haplo-identical or non-identical grafts, is a frequently used clinical procedure for the reconstitution of the immune system following chemotherapeutic and irradiation treatment (Van Bekkum, 2003). One of the major complications of such a procedure is the appearance of GVH especially in cases were patients are transplanted with BM from haplo-disparate donor. However, and thus far for largely unknown reasons, a similar type of GVH is also seen even when a haplo-identical graft is used. Clinically, this is marked by cutaneous, hepatic and gastrointestinal infiltrations (Nakase et al., 2005). In this report, we have investigated this phenomenon in a mouse model of syngeneic BM transplantation. These studies were initiated because lethally or sub-lethally irradiated immuno-deficient RAG-2$^{-/-}$ mice reconstituted with either T cell-depleted BM or *in vitro*-generated T cell-committed lymphoid progenitors developed diarrhoea and weight loss, frequently dying four to seven weeks after reconstitution (Balciunaite et al., 2005b). Here, we show that so-called naturally arising regulatory T cells, either transferred together with the bone marrow graft, or derived from endogenous radio-resistant T cells of the host, protect recipient mice from developing auto-immunity. In corollary, depletion of Treg in WT mice prior to BM transplantation resulted in the appearance a relatively mild, non-lethal, disease.

Reconstituted mice developed diarrhoea and weight lost. Histological analysis of the lower third of the colon showed the classical signs of inflammatory bowel disease (IBD), namely epithelial hyperplasia, extensive lymphocytic infiltration of the lamina propria, crypt abscesses and destruction of the mucous membrane with prominent ulceration (Figure 1). Initiation of this disease was T cell-dependent as shown by absence of disease in mice reconstituted with CD3e$^{-/-}$ BM (Figure 2). Transfer of naturally arising regulatory T cells prevented the appearance of IBD in this model (Figure 2). Auto-immunity was only seen when RAG-2$^{-/-}$ recipients were reconstituted with T-cell depleted BM and onset of IBD could be prevented by transferred naturally arising CD25^{+} regulatory T cells. However, WT Bl/6 mice reconstituted with BM did not suffer from IBD. One clear difference between RAG-2$^{-/-}$ and Bl/6 mice is the presence of a mature T cells in the latter prior to irradiation. It is known that some thymocyte progenitors (Ceredig and MacDonald, 1982) and peripheral T cells (Lowenthal and Harris, 1985) can survive a lethal dose of irradiation. Indeed, it was shown some time ago that mitogen-activation of normal mouse peripheral T or B cells rendered them more resistant to irradiation in vitro (Lowenthal and Harris, 1985). In addition it as been recently reported that recipient CD4^{+} T cells surviving irradiation can regulate chronic graft-versus-host disease in a B10.D2 (H-2^{d})→BALB/c (H-2^{d}) MHC-compatible, multiple minor histocompatibility antigen-incompatible BM transplantation model(Anderson et al., 2004).

Functional tests indicated that host-derived $CD4^+CD25^+$ cells inhibited the proliferation of anti-CD3e-activated naïve responder T cells *in vitro*, thereby satisfying the criterion that they were genuine regulatory T cells (Thornton and Shevach, 1998). Moreover, depletion of $CD25^+$ cells in lethally-irradiated Bl/6 WT mice prior to reconstitution led to development of IBD as observed when using immuno-deficient hosts, although to a milder extent. Interestingly, naturally arising $CD25^+$ regulatory T cells (Treg) share many features with activated T cells, namely down-regulation of CD4, CD3 and TCRb as well as expression of CD25(Bosco et al., 2006a; Kasow et al., 2004). In addition, transcriptome analysis has shown that Treg contain abundant transcripts of pro-survival genes, for example GITR (Nocentini et al., 1997) and more recently Bcl-x_L has been suggested to be up-regulated in Tregs(Minamimura et al., 2006). They are thought to be constantly encountering their cognate self-antigen in the periphery and therefore to be in a semi-activated state (Hsieh et al., 2004). However by comparing thymectomized mice transferred with $RAG^{-/-}$ BM with WT recipients transplanted with WT BM, we could show that most of the host radio-resistant regulatory T cells were probably derived from the thymus.

Anderson et al. recently reported that radio-resistant Tregs could prevent chronic GVH in a mouse model of BM transplantation across multiple minor histocompatibility loci (B10.D2 (H-2^d)→BALB/c (H-2^d)) (Anderson et al., 2004). Thus BALB/c $RAG2^{-/-}$ mice transplanted with BM from B10.D2 develop a severe GVH whereas the same recipients transplanted with BM from WT BALB/c, used as controls, stayed healthy. Unlike what we report here, the authors did not describe any signs of syngeneic GVH. However, it should be noted that the mice in their experiments were treated with antibiotics throughout the time course of the experiment. When we treated our $RAG2^{-/-}$ BM recipients with antibiotics, we observed milder disease with more animals surviving. These findings strongly suggest that the antigenic load, and more specifically the gut flora, seem to play important roles in determining disease onset and evolution in this experiment setting. In this context, it is worthwhile noting that mice prone to IBD do not develop this disease when kept under germ-free conditions (Contractor et al., 1998; Madsen et al., 2000; Mahler and Leiter, 2002; Panwala et al., 1998). We conclude that the syngeneic GVH described in the present paper might be strongly influenced by antigen-driven T cell responses. This is substantiated by the activated phenotype of T cells in $RAG2^{-/-}$ recipients. The fact that Tregs can prevent both the activated phenotype and disease onset might suggest that they are controlling the strength of this immune response.

Another parameter worthy of consideration is the age of the recipient mice. No sign of disease was observed in sub-lethally irradiated neonatal $RAG2^{-/-}$Bl/6 mice transplanted with WT syngeneic BM. At least two mutually non-exclusive explanations could explain this. Firstly, the antigenic load in neonatal mice may be dramatically lower than in adults and secondly the neonatal thymus might be more efficient at generating Tregs.

The T cell-induced auto-immune disease seen in immuno-deficient mice reconstituted with syngeneic BM resembles, in many ways, the so-called syngeneic GvH disease seen in a clinical setting. In our mouse model, Treg of either host or donor origin play a key role in controlling both the severity of IBD and the appearance of auto-antibodies. Using reconstitution of the lympho-hemopoietic system, after BM transplantation, development of autoimmunity is clearly dependant upon the number of regulatory T cells. This leads us to propose that infusion of such cells into BM transplant recipients should be considered as a treatment modality for the prevention of syngeneic GVH.

Material and methods

Mice.

Ly5.1 and Ly5.2 C57BL/6 (Bl/6), Ly5.1 Bl/6 CD3ε gene deleted ($CD3\varepsilon^{-/-}$) (Malissen et al., 1995) and Ly5.2 Bl/6 RAG-$2^{-/-}$ (Shinkai et al., 1992) mice were maintained in our own animal SPF facilities. Male and female mice were used at 8 weeks of age and all experiments carried out according to Institutional guidelines. Adult thymectomy (ATx) was performed on six week-old C57Bl/6 mice and mice were used 7 weeks later and absence of residual thymus tissue verified at the time of autopsy.

Reagents and antibodies.

The following mAb were purchased from PharMingen (San Diego, USA): anti-$CD25^{FITC}$ (7D4), anti-$CD62L^{FITC}$ (MEL-14), FITC or biotin-conjugated anti-CD8α (53-6.7), anti-$CD69^{PE}$ (H1.2F3), anti-$TCR\beta^{PE}$ (H57-597). Anti-$CD4^{PE}$ (RM4-5) antibody was purchased from eBioscience (San Diego, USA). The anti-$Ly5.1^{FITC}$ (A20) and anti-$Ly5.2^{FITC}$ (104) antibodies were produced and labeled in our laboratory according to standard techniques. To reveal biotin-labeled antibody, Streptavidin-APC (Becton Dickinson, San Diego, USA) was used. Anti-CD25 mAb PC61 was purified from hybridoma culture supernatant by standard procedures.

Flow cytometric analysis and sorting.

Single-cell suspensions of thymus, spleen and lymph nodes were prepared in PBS supplemented with 2% FBS and 0.2% sodium azide as described previously (Balciunaite et al., 2005b). Cells were adjusted to 20–10 _ 10^6 cells/ml and 0.5–1 _ 10^6 cells incubated for 30 min at 4°C with the indicated reagents at saturating concentrations as previously described. Stained cells resuspended in PBS 2% FBS 0.2% azide containing propidium iodide (PI) were analyzed using a FACSCalibur (Becton Dickinson) and data analyzed using CellQuest (Becton

Dickinson). Viable lymphoid cells were defined by a combination of FSC, SSC and PI fluorescence. Stained cells were sorted on a on a FACSAria (Becton Dickinson).

Bone marrow transplantation and adoptive transfer of regulatory T cells:

Bone marrow cell suspensions from 3 to 5 Bl/6 donor mice were prepared by flushing femurs and tibias with PBS using a 23g needle. After red blood cell lysis, T cells were depleted by re-suspending cells in a mixture of rat IgM anti-CD90 (AT83) anti-CD4 (RL172) and anti-CD8α (31M) monoclonal antibody (mAb) hybridoma supernatants and incubated for 20 min at 4°C (Ceredig and Rolink, 2002). Following a washing step, antibody-coated cells were lysed by adding rabbit complement (Low-Tox, Cedarlane, Canada) dissolved in serum-free Dulbecco's Modified Eagle's Medium (DMEM). After incubation for 45 min at 37°C, cells were washed and resuspended in DMEM prior to injection. To obtain naturally-arising regulatory T cells, spleen cell suspensions from Ly5.2$^+$ Bl/6 mice were stained with anti-CD4^{PE} (RM4-5) and anti-CD25^{FITC} (7D4) antibodies and CD4$^+$CD25$^+$ cells sorted on a FACSAria. The purity of sorted cells was always > 98%.

For *in vivo* regulatory T cell depletion, mice received one injection of anti-CD25 mAb (PC61; 0.5mg/injection i.v.) 5 days prior to irradiation and BM transplantation.

Viable BM and sorted cells were counted and resuspended in DMEM. Mice were reconstituted with 200μl containing 3 x 10^6 T cell-depleted BM cells alone or together with 1-3x10^5 sorted CD4$^+$CD25$^+$ cells. Recipient mice were γ-irradiated using a Cobalt source (Gammacell 40, Atomic Energy of Canada, Ltd) four hours prior to reconstitution. RAG-2$^{-/-}$ mice were irradiated with 4Gy and Bl/6 mice with 9.5Gy. Chimeric mice were weighed once a week for up to 12 weeks and analyzed between 4 and 12 weeks. The origin and composition of lymphoid cells was determined by means of the Ly5.1 and Ly5.2 markers.

Functional regulatory T cell assay:

The ability of CD4$^+$CD25$^+$ T cells from chimeras to inhibit the in vitro proliferation of naïve T cells was carried out as previously described (Thornton and Shevach, 1998). Briefly, CD4$^+$CD25$^-$ naïve T cells and CD4$^+$CD25$^+$ isolated from chimeras were purified by sorting. Antigen-presenting cells (APC) were T cell-depleted, irradiated syngeneic spleen cells from Bl/6 mice. Control cultures contained 2.5x10^4 CD4$^+$CD25$^-$ naïve responder T cells, 5x10^4 APC and 0.1μg anti-CD3ε mAb. To test for inhibition, 2.5x10^4 CD4$^+$CD25$^+$, obtained from the indicated chimeric mice were added. Cells were cultured in round-bottomed 96-well plates for 72 hours and 1μCi/well of ^{3}H-thymidine was added for the last 8 hours prior to harvesting.

Immunohistochemistry and histological staining:

Organs were snap frozen in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and 5 _m sections cut on a cryostat. Sections were then fixed for 10 min in acetone and stored at _20 °C. For staining, sections were covered with antibody solution at saturating concentrations and incubated for 30 min at RT. For the second step, sections were washed in PBS and incubated with a Neutralite Avidin-TXRD/PBS (Southern Biotech) solution for 15 min at RT. Primary antibodies used included anti-CD90^{FITC}, anti-IgM^{Cy5} (M41) and biotinylated peanut agglutinin (Vector laboratories). Slides were washed; one to two drops of a 1:1 mixture of PBS and glycerin were placed onto the slide and covered with a coverslip (Harfst et al., 2005). A 10_ or 5_ objective was used for magnification. Intestines were fixed in 4% par formaldehyde, embedded in paraffin, cut in 3 µm sections and stained with haematoxylin/eosin.

For detection of serum anti-nuclear antibodies, snap-frozen sections of kidneys from RAG-2$^{-/-}$ mice were incubated with sera diluted 1:10 to 1:10,240 and bound antibodies revealed with a 1:50 dilution of FITC-labeled, mouse IgG subclass-specific secondary antibodies (Jackson ImmunoResearch Laboratories). The pattern and titer of anti-nuclear antibodies were assessed using an incident-light fluorescence microscope (Zeiss axioskope) and serum titer defined as the highest dilution showing specific nuclear staining.

Part II: Auto-reconstitution of the T cell compartment by radio-resistant hematopoietic cells following lethal irradiation and bone marrow transplantation

Nabil Bosco, Lee Kim Swee, Angèle Bénard, Rhodri Ceredig and Antonius Rolink

Introduction

In man, syngeneic or allogeneic bone marrow (BM) transplantation (BMT) with BM donors is a frequently used treatment modality, which has curative results for a variety of haematological disorders such as malignant or genetic-based immune deficiencies. Nevertheless, the restoration of an adaptive immune system (with both B and T cells) in transplanted patients is a very slow process and during the initial reconstitution phase, patients are lymphopenic. T cell recovery plays a key role in the clinical recuperation of patients post-BMT as they lack adequate T cell-mediated immunity: they often succumb to one of several life-threatening infections, especially cytomegalovirus (CMV) infections (reviewed in Gress et al., 2007; Hakim and Gress, 2005). Roosnek and colleagues recently showed that the presence of residual host- (surviving the conditioning regimen) or donor-derived (persisting and expanding from the BM inoculum) CMV-specific $CD8^+$ memory T cells could provide efficient anti-viral immunity and help patients combat CMV infections in the lymphopenic period following BMT (Chalandon et al., 2006). Therefore, in man, it seems that donor and recipient immune infection history should be taken into account in designing the best BMT protocol strategy including whether the donor BM should be T cell depleted and how recipients should be conditioned. The ability of patients, especially adults, to regenerate T lymphocytes after BMT or conditioning therapy-related depletion of the host mature T lymphocyte compartment has emerged as a critical problem in clinical medicine. However, studies in man are difficult to control experimentally and animal models are required to dissect and improve BMT outcomes.

We and others have shown that host-derived T cells are present in lethally irradiated mouse BM chimeras (Anderson et al., 2004; Benard et al., 2006; Komatsu and Hori, 2007). However, the origin and function of these cells were not fully-characterized. This prompted us to examine more carefully the host-derived T cell pool in chimeric mice. We recently showed that host-derived T cells were enriched in extra-thymically located, radio-resistant, functional $CD4^+CD25^+$ regulatory T cells which could prevent a syngeneic graft-versus-host (GvH) disease following BMT (Benard et al., 2006).

The object of the current study was to characterize further host-derived T cell development and function in chimeric mice. To this end, we generated chimeras by

reconstituting lethally-irradiated C57BL/6 mice with either syngeneic RAG2$^{-/-}$ or CD3ε$^{-/-}$ BM. In such chimeras, donor-derived BM progenitors are not able to generate T cells and surviving T cells will be exclusively host-derived. We showed that host T cells contain a mixture of *de novo*-generated naïve T cells and surviving peripheral memory-like cells. We found that host-derived thymopoiesis was initiated by DN1-2 prothymocytes having a conventional (CD44^{high}, CD117^{high}, CD25$^{-/+}$) phenotype and we further demonstrate that T lymphopoiesis recapitulates normal thymic ontogeny after BMT. Additionally, by comparing host-derived T cell numbers in non-thymectomized (NTX) versus thymectomized (TX) hosts, we observed that the differentiation of host-derived thymocytes provided an important cohort of naïve, functional, mature T cells having a large TCR repertoire and accounting for up to 35% of the total T cell number found in a control chimeric or unmanipulated mice. Moreover, by using TX hosts, we could show that there was a second population of extra-thymically located, CD44^{high}CD62L^{-} memory-phenotype, functional T cells having an oligoclonal TCR repertoire. These host-derived T cells might provide a first line of defence against infections during recovery from lymphopenia after BMT.

Results

Host-derived T cells following BMT in lethally-irradiated mice.

More than 30 years ago, it was already recognised that in lethally irradiated BM chimeras, part of the T cell compartment was of host origin (Ceredig and MacDonald, 1982; Hirokawa et al., 1985; Kadish and Basch, 1975; Lesley et al., 1990; Sharrow et al., 1983). However, the precise origin and functional activity of these host-derived T cells has not been thoroughly analysed. In order to study host-derived lymphocytes, we reconstituted lethally-irradiated C57Bl/6 CD45.2 mice with T-cell depleted (TCD) BM from C57Bl/6 CD45.1 donors. After six months, to characterise the host- and donor-derived lymphoid compartments of chimeric mice, we stained splenocyte suspensions with combinations of CD45 allotype-specific antibodies, CD3 for T cells, and CD19 for B cells and analysed them by FACS. As expected, in these syngeneic bone marrow transplantation (BMT) experiments, all recipient mice survived (10/10) indicating a good BM engraftment and there was no evidence of Graft-vs-Host disease (GvHD). However, a sizeable host-derived CD45.2^{+} lymphocyte population survived even as long as 6 months after BMT. Host-derived lymphocytes in chimeric mice were exclusively CD3^{+} and represented about 5% total splenic T cells (Figure 1A).

Figure 1: Host-derived T cells are present in lymphoid and peripheral organs of mouse BM chimeras.

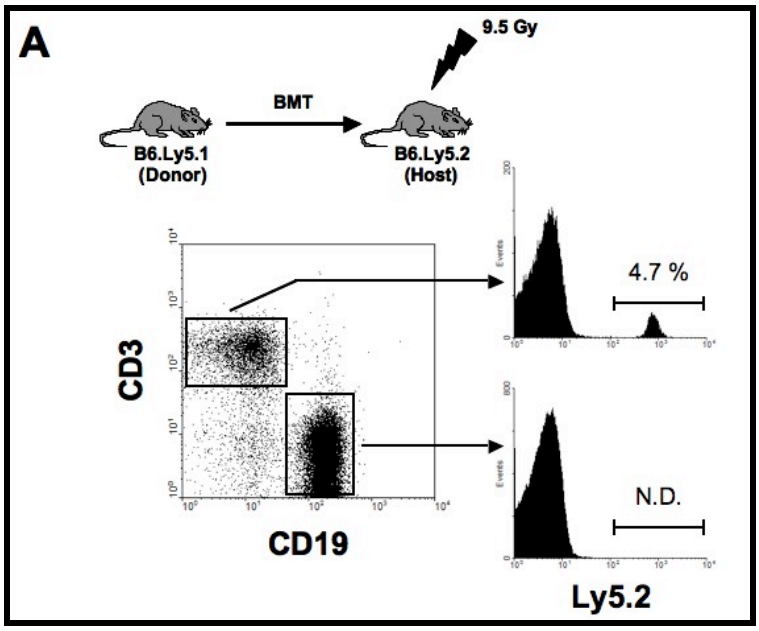


Figure 1A

Experimental design. Lethally-irradiated C57Bl/6 CD45.2 mice were reconstituted with T-cell depleted (TCD) BM from C57Bl/6 CD45.1 donors.

After six months, splenocyte suspensions were stained for CD3, CD19 and CD45 allotype. A typical result of FACS analysis is shown. $CD3^+$ T cells and $CD19^+$ B cells were gated as shown in left dot-plot and the proportion of $CD45.2^+$ (host-derived cells) was quantified as shown in the right histograms (N.D., not detected).

Importantly, the absence of host-derived $CD19^+$ B cells ruled out the possibility that host-derived multi-potent cells had survived in our chimeras; this was further confirmed by analysis of the BM where all progenitor compartment were donor-derived (data not shown). Additional phenotypic analysis of host-derived T cells showed that both $CD4^+$ and $CD8^+$ cells were present and that they were enriched in $CD44^{high}$ $CD62L^{+/-}$ cells compared with their donor-derived partners (Figure 1B).

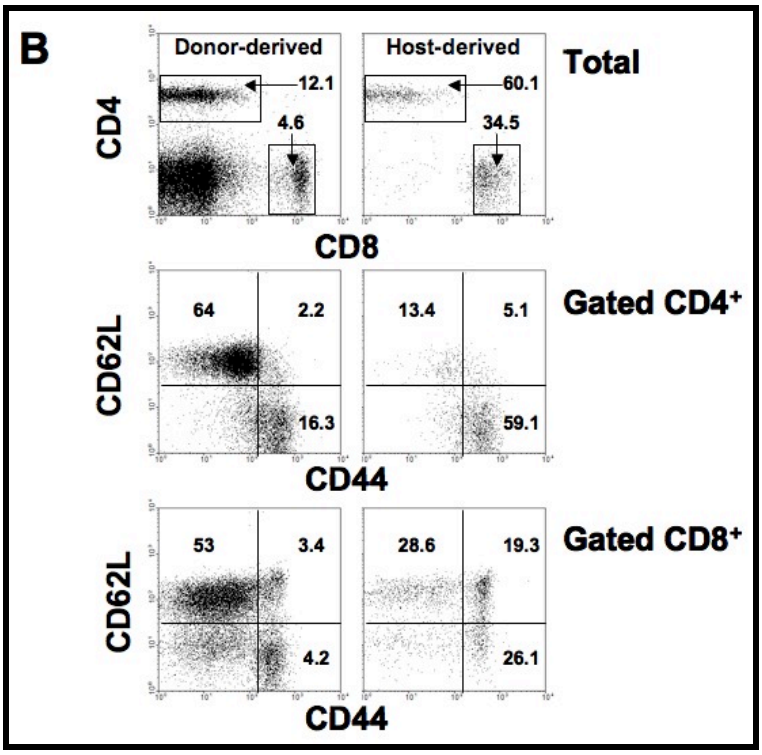


Figure 1B

In the upper dot-plots, shown are CD4 and CD8 distribution in splenocytes. In the middle and lower dot-plots data shown are CD44 and CD62L expression on gated $CD4^+$ (middle dot-plots) or $CD8^+$ (lower dot-plots). Cells were either $CD45.1^+$ donor-derived (left dot-plots) or $CD45.2^+$ host-derived (right dot-plots). Shown in B-C are representative results of 5-10 mice analyzed. Numbers in the quadrants indicate percentages of cells.

Thus, host-derived T cells are still present in chimeric mice and are mostly composed of $CD44^{high}$ memory-like cells (Tough and Sprent, 1994). To evaluate whether host-derived T cells could be found in other locations, we studied lymphocytes isolated from the blood, gut, liver, lung, lymph nodes and skin of chimeric mice 6 months after reconstitution. Again, none of these organs contained host-derived B cells (data not shown) but did contain host-derived T cells in different proportions. As shown in figure 1C, about 3-5% of blood, LN and spleen, and 10-20% of gut, liver, lung and skin $CD3^+$ T cells were host-derived. Together, these data revealed the presence of a substantial pool of host-derived mature T cells in chimeric mice despite lethal irradiation preceding BMT.

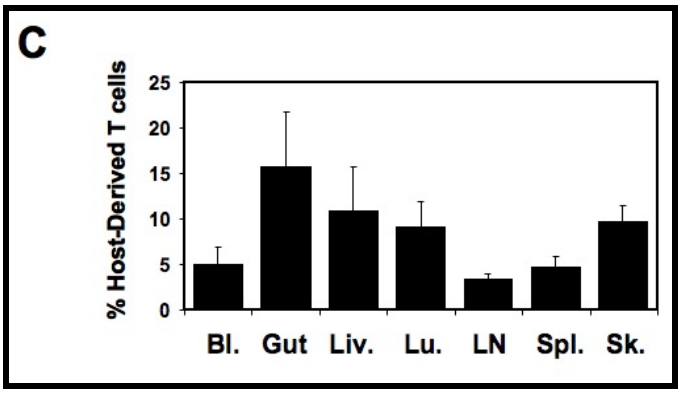


Figure 1C

T cells chimerism in blood (Bl.), gut, liver (Liv.), lung (Lu.), lymph nodes (LN), spleen (Spl.) and skin (Sk.) was analysed. Bar histogram represents the mean ± SD of host-derived T cells ($CD3^+CD45.2^+$) from 3-5 mice.

Following BMT most host-derived T cells are derived from a single wave of thymic T cell differentiation.

Multiple origins could explain the persistence of host-derived mature T cells in chimeric mice. Firstly, host-derived T cell progenitors could survive and generate T cells. These progenitors could reside either within the thymus or elsewhere and generate either thymus-derived T cells or extrathymic-derived T cells respectively. Secondly, some host-derived mature T cells could represent radio-resistant, resident T cells. These hypotheses are not mutually exclusive and to discriminate between them, we generated several chimeric mouse combinations as described below.

To demonstrate that the host thymus is still able to produce T cells following lethal irradiation and BMT, chimeras were generated by reconstituting lethally irradiated C57Bl/6 mice with C57Bl/6.$RAG2^{-/-}$ BM. In such chimeras, donor-derived BM progenitors are not able to generate T cells and surviving T cells will be exclusively host-derived. We tracked host-derived T cells in the thymus and spleen from 1 to 6 weeks following BMT. The total number of thymocytes rapidly decreased and reached about 2 x 10^6 cells one week post-BMT. Reduction of cell number occurred in each CD4 and CD8-defined thymocyte subset, but the degree of reduction varied among the subsets, possibly reflecting their relative radio-sensitivity ($CD4^+CD8^+$ double positive (DP) > $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP)). Figure 2A shows the changes in proportion of each T cell subsets in thymus and spleen.

Figure 2: Host-derived T cells in lethally irradiated chimeras are mainly coming from a transient wave of T cell differentiation within the thymus.

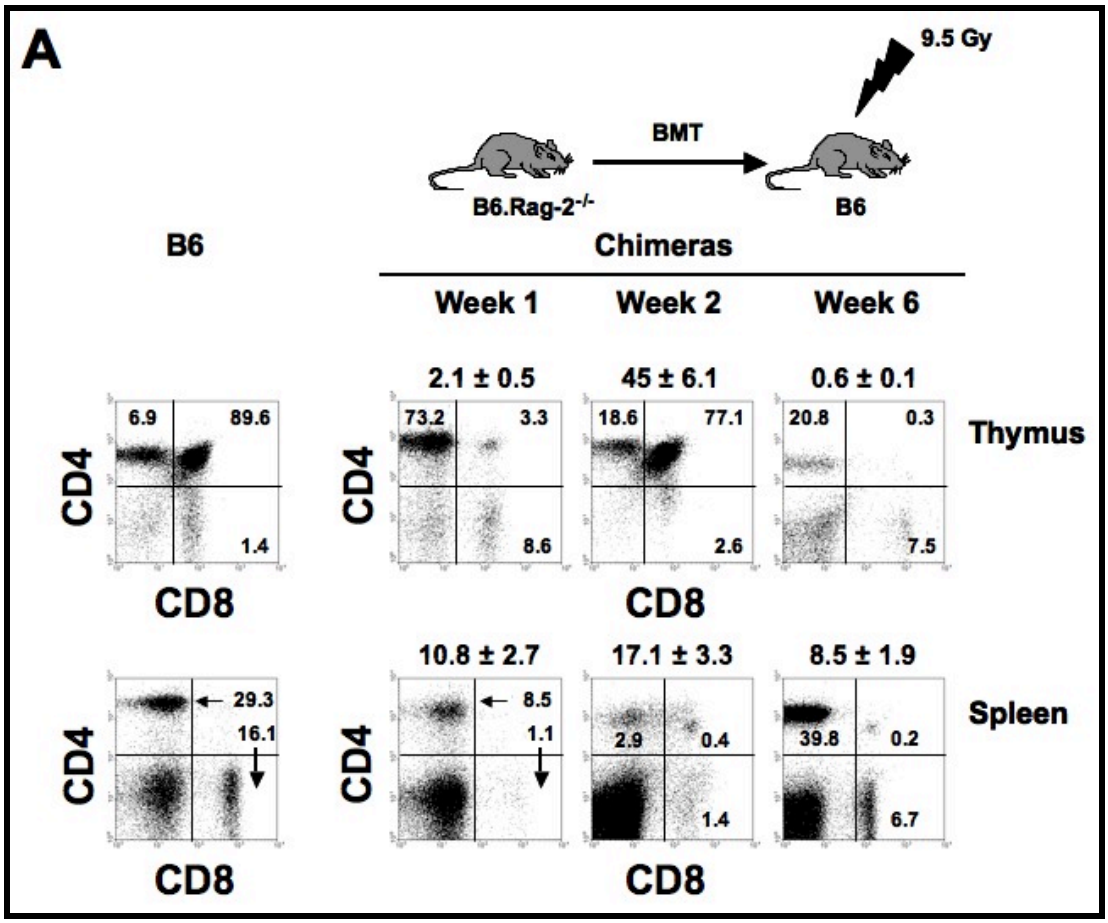


Figure 2A

Shown in the upper part is the experimental design. Lethally-irradiated C57Bl/6 mice were reconstituted with total BM from C57Bl/6.RAG2$^{-/-}$ donors. Thymus and spleen were analyzed at week 1, 2 and 6 post-BMT. Shown are CD4 versus CD8 staining on viable lymphocyte gate. Numbers on the top of the quadrants and in the quadrants indicate mean organ cell numbers ± SD and percentages of cells respectively.

As reported by others (Penit and Ezine, 1989), DP thymocytes are highly susceptible to irradiation and, in the first week following BMT, decreased rapidly from 89.6% in control non-irradiated mice to 3.3%. In contrast, at one week post-BMT, we observed a relative enrichment of CD4$^+$ SP cells, representing 73.2% of total thymocytes whereas they represented only about 10% of thymocytes in control mice. At two weeks post-BMT, thymocyte number had increased to reach a maximum of 45 x 10^6 cells, representing about 50% of control thymus cellularity. At that time, the CD4 and CD8 distribution was very similar to controls. Additionally, phenotypic analysis revealed that SP thymocytes in chimeric mice were indistinguishable from controls, expressing high level of CD3 and TCRβ, and about 5% of CD4$^+$ SP cells were FoxP-3$^+$ (data not shown). At six weeks, thymus cellularity had decreased to less than 1 x 10^6 cells. At this time, the thymic rudiment contained exclusively host-derived SP cells.

In the spleen of these chimeras, results reflected what we observed in the thymus. Thus, as early as one week post-BMT, there was a dramatic loss of cellularity and spleens contained a

mean of only 10.8 x 10^6 cells. $CD4^+$ T cells represented almost all radio-resistant T cells. At 2 weeks post-BMT, splenocyte number peaked at 17.1 x 10^6 cells, finally decreasing to ~8-10 x 10^6 cells at 6 weeks. Throughout this time, the numbers of $CD4^+$ T cells exceeded that of $CD8^+$ T cells. The percent $CD4^+$ T cells at 1, 2 and 6 weeks post-BMT were 8.5, 2.9 and 39.8 whereas those of $CD8^+$ cells were 1.1, 1.4 and 6.7 respectively. Interestingly and as already documented by others, host splenocytes contained about 0.4 % DP cells; this could reflect some degree of so-called extra-thymic T cell differentiation (Allman et al., 2001; Antica and Scollay, 1999; Garcia-Ojeda et al., 1998; Terra et al., 2005). Thus, as we (Ceredig and MacDonald, 1982) and others (Kadish and Basch, 1975; Penit and Ezine, 1989; Sharrow et al., 1983) have previously shown, following lethal irradiation and BMT, a single wave of host-derived reconstitution occurred (herein referred as *auto-reconstitution*), but the nature of the radio-resistant T cell precursors responsible for this remained undefined. Additionally, we observed a significant number of mature T cells in the periphery as long as 6 weeks post-BMT. It should be noted that this pool of host T cells could comprise a mixture of (i) radio-resistant mature peripheral T cells that had survived and expanded (ii) T cells derived *de novo* from the host thymus and (iii) some not well-characterized T cells generated via extra-thymic differentiation.

Next, to clarify the relative contribution of these processes to the host-derived T cell pool, we generated chimeras where C57Bl/6.CD3$\varepsilon^{-/-}$.CD45.1 BM was transferred into lethally irradiated C57Bl/6.CD45.2 hosts which were either thymectomized (referred herein as TX) or not thymectomized (referred herein as NTX). In these chimeras, all mature T cells would be host-derived with those in TX recipients being of only extra-thymic origin and those in NTX recipients a mixture of extra-thymically and thymus-derived cells. Thus, the difference in T cell numbers between these two groups of chimeras would reveal the contribution of thymic-derived T cells to the overall pool. As shown in figure 2B, the donor-derived B cells in the spleen of NTX and TX were very similar to controls and represented 50-60 x10^6 cells or 70-87% splenocytes respectively 2-3 months post-BMT. In contrast, the mean number of host-derived T cells was about 20 x10^6 in control chimeras, decreasing to ~7 x10^6 cells in NTX and to only about 2 x10^6 cells in TX chimeras.

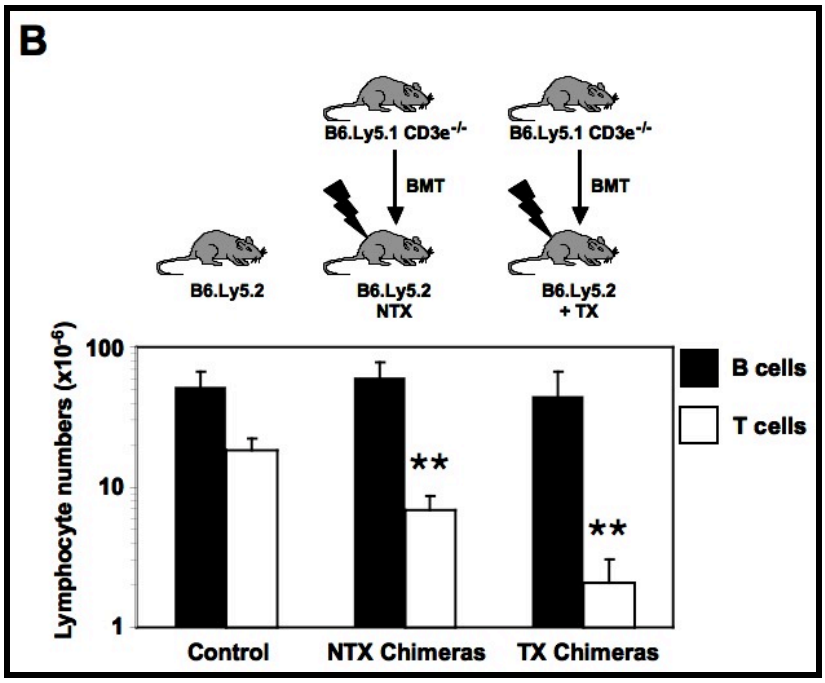


Figure 2B

Shown in the upper part is the experimental design. Lethally-irradiated C57Bl/6.CD45.2 mice were thymectomized (TX) or not (NTX) and reconstituted with BM from C57Bl/6.CD3$\varepsilon^{-/-}$.CD45.1 donors. Chimeras were analyzed 8-12 weeks post-BMT. Bar histograms represent total B (black bars) and T (white bars) lymphocyte numbers ± SD (n=10) in controls and chimeras. ******, *P* <0.01.

Thus, in the spleen, 5 x10^6 host-derived T cells were thymus-derived whereas only 2 x10^6 were from an extra-thymic origin. In TX chimeras, histological analysis of spleen sections revealed abnormal follicular architecture with small, scattered, T cell areas reflecting their severe lymphopenia. In contrast, spleen sections from NTX animals displayed normal features (figure 2C).

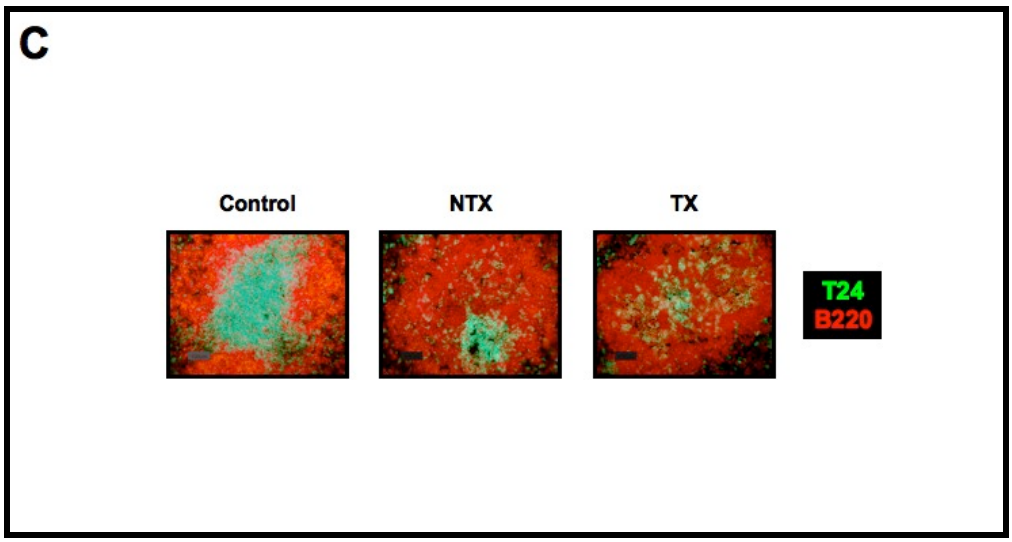


Figure 2C

Frozen spleen sections from chimeras described in figure 2B were prepared and stained as described in materials and methods. B cells appear in red and T cells in green. Scale bar, 100 μm.

Our results show that (i) in chimeras where all T cells are host-derived, about 35% [(7/20) x100] of the normal T cell pool could be formed and maintained and (ii) more importantly, at least 71% [100 – (2/7 x100)] of host-derived T cells are derived from the thymus. This value may be an underestimate because it assumes that the extra-thymically derived T cell pool remained constant in TX versus NTX recipients.

Thymus-derived host T cells originate from radio-resistant DN1-2 like thymocytes.

The above results demonstrated that most (>70%) host-derived mature T cells were derived from a single wave of thymocyte differentiation. The presence of a host-derived thymocyte precursor has been proposed (Ceredig and MacDonald, 1982; Kadish and Basch, 1975; Penit and Ezine, 1989; Sharrow et al., 1983), however, whether it is an intra-thymic "classical" progenitor or an "atypical" cell was not resolved. Indeed, it had been proposed recently (Maillard et al., 2006) that reconstitution of the thymus in the BM chimeras was derived directly from an "atypical" $CD25^{+}CD44^{-}CD117^{-}$ DN3-like precursor. Therefore, complementary approaches were undertaken to further characterise host-derived thymocyte precursors.

As host-derived mature lymphocytes were almost exclusively T cells, we concluded that this radio-resistant precursor should reside within the thymus where T cell precursor commitment is thought to take place. In order to demonstrate that a pool of intra-thymic T cell precursors survive lethal irradiation and can differentiate into mature thymocytes, we carried out foetal thymus organ culture (FTOC) experiments with foetal thymus lobes from lethally irradiated pregnant mice. As shown in figure 3A (and data not shown), one week after FTOC, irradiated thymi contained $TCR\beta^{+}$ DP and SP cells in a proportion similar to that of controls. The same experiments were carried out with pieces of adult thymus in organ culture (ATOC). Again, we observed typical signs of thymocyte differentiation and despite lethal irradiation, organ-cultured adult thymi contained $TCR\beta^{+}$ DP and SP cells one week after ATOC (data not shown).

Figure 3: Radio-resistant DN1-2 thymocyte precursors reside in the thymus following lethal irradiation.

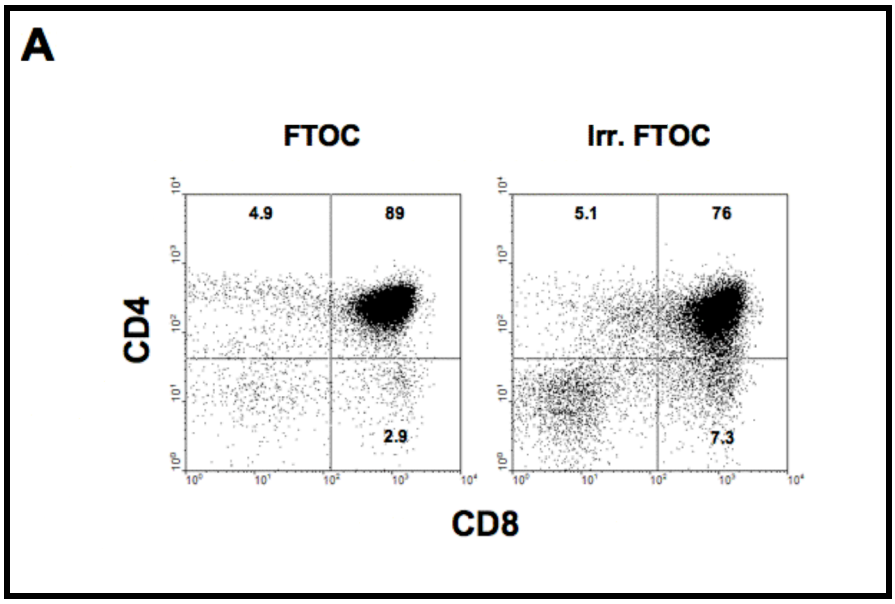


Figure 3A

Shown are the CD4 versus CD8 profiles of day-7 FTOC with (right) or without (left) lethal irradiation (950 cGy) at day 0. Numbers in the quadrants indicate percentages. Shown is one representative experiment out of 5 conducted independently.

Next, C57Bl/6 mice were reconstituted with RAG2$^{-/-}$ BM and treated with anti-IL-7Rα mAb immediately after BMT (figure 3B, right graph). At two weeks post-BMT, following anti-IL-7Rα treatment, the number of host-derived T cells was greatly diminished with thymocyte cellularity reduced from ~50 to 1 x 10^6 cells. This reduction in cellularity was associated with a block of DN to DP stage transition (figure 3B, left panels). We conclude that some T cell precursors survive lethal irradiation, reside within the thymus and that their differentiation is IL-7 dependent.

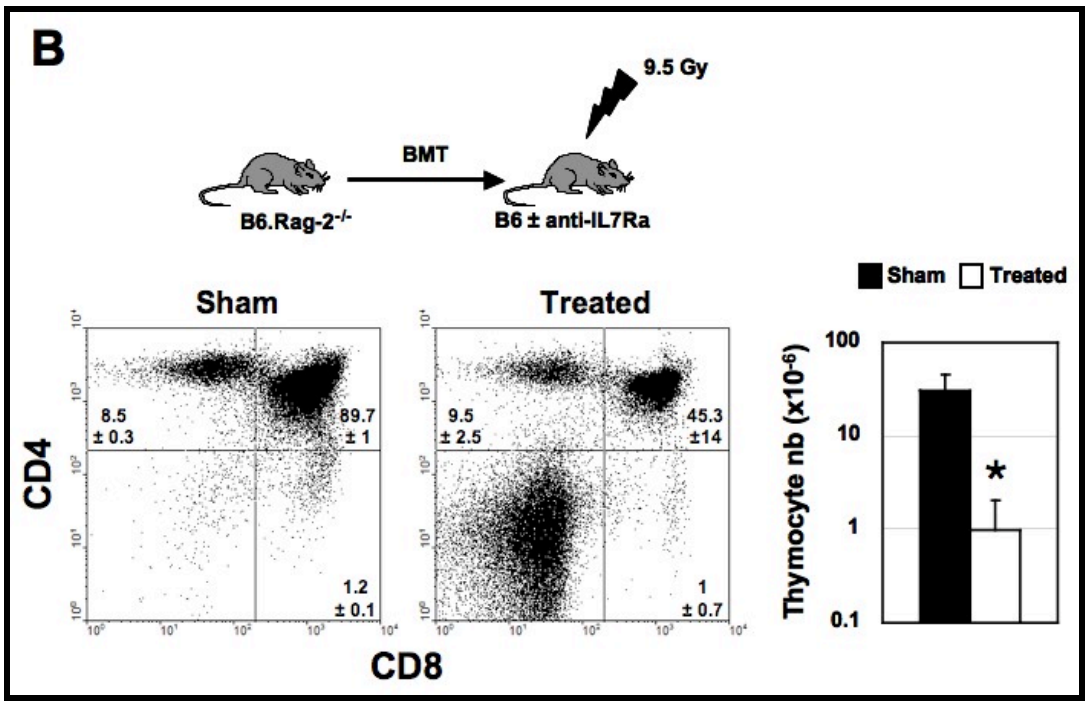


Figure 3A

The same chimeras as in figure 2A were done and following BMT either injected i.p. with 300 µg of anti-IL-7R blocking antibody (A7R34) in 200 µl of PBS once a week (treated) or with PBS only (sham). Two weeks post-BMT, mice were killed and their thymus analysed by FACS. Shown are CD4 versus CD8 profiles (left dot-plots) and mean thymocyte numbers ± SD (n=3) in a logarithmic scale (right histogram bars). ★, P <0.05.

The properties of these thymocyte progenitors were reminiscent of those of typical DN1-2 thymocyte precursors (Balciunaite et al., 2005a; Balciunaite et al., 2005c; Ceredig and Rolink, 2002). We therefore enriched DN cells from C57Bl/6.CD45.1 → C57Bl/6. CD45.2 chimeric mice from 1 to 3 weeks post-BMT and by CD44 and CD117 staining, we were able to reveal the presence of $CD44^{+}CD117^{high}$ cells corresponding to ~0.3 to 0.6% of DN preparations (Figure 4A, left dot plots). As shown in figure 4A, this population contained $CD117^{high}$ DN1 ($CD44^{+}CD25^{-}$) and DN2 ($CD44^{+}CD25^{+}$) T cell precursors.

Figure 4: Radio-resistant DN1-2 thymocytes recapitulate normal thymic ontogeny in vitro.

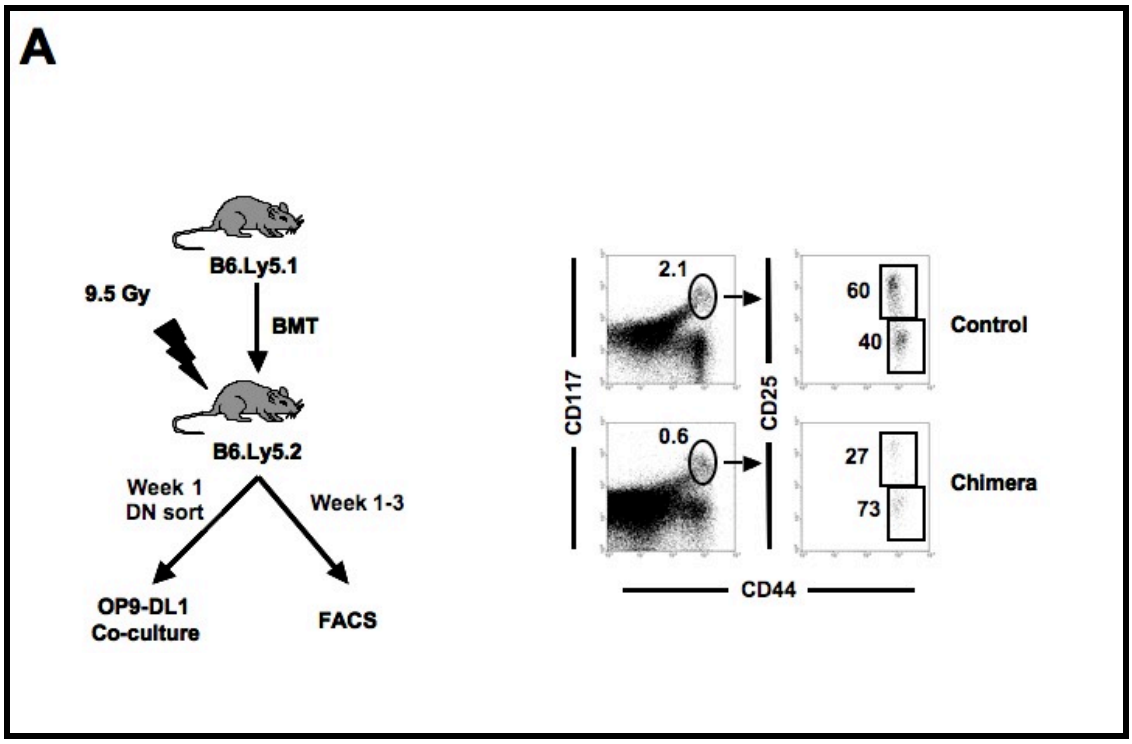


Figure 4A

Shown in the left is the experimental design. Lethally-irradiated C57Bl/6.CD45.2 mice were reconstituted with TCD BM from C57Bl/6.CD45.1 donors. Thymi were analyzed at week 1, 2 and 3 post-BMT. Shown in the right are CD44 versus CD117 on DN preparation and CD44 versus CD25 staining on gated $CD44^{+}CD117^{High}$ cells from an unmanipulated control or a chimeric mouse. $CD44^{+}CD25^{-}$ and $CD44^{+}CD25^{+}$ represent DN1 and DN2 respectively. Numbers in the quadrants indicate representative percentages.

We then quantified the host- and donor-origins of DN1 and DN2 cells from chimeras 1 to 3 weeks post-BMT (Figure 4B, bar graphs). At 1 week post-BMT, DN1-2 cells were detected which were ~99% host-derived and this proportion decreased to ~90% at 2 weeks and finally to

less than 1-5% at 3 weeks post-BMT. Thus, early “classical” T cell progenitors exist in the thymus of chimeric mice even though they are rare cells. Initially, the CD117^{high} progenitor compartment is almost exclusively host-derived but later gets diluted by donor-derived cells.

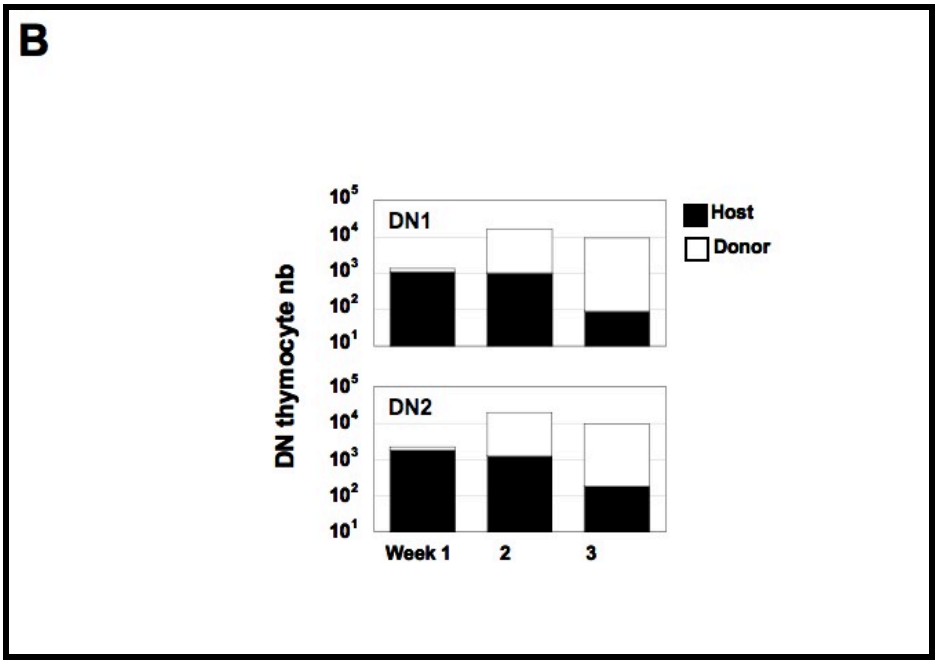


Figure 4B

Bar histograms display the kinetics and mean numbers (n= 3-5 mice for each time point) of host- and donor-derived DN1 and DN2 cells in black and white respectively from 1 to 3 weeks post-BMT.

We next addressed the T-cell development potential *in vitro* of host-derived thymic CD44^{+}CD117^{high} cells found in chimeric mice at week 1 post-BMT. About 2x10^{3} cells were sorted and differentiation potential was assessed by plating them on OP9-DL1 stromal cells (supporting T-lineage development) in the presence of IL-7. Cells expanded rapidly, reaching about 10^{5} cells one week after culture when they were harvested and analyzed by FACS. They underwent typical T-lineage development features by up-regulation of CD25 and down regulation of CD44 expression (figure 4C left dot-plot). One week post-BMT, 95% of the cells were CD45.2^{+} host-derived (figure 4C right histogram).

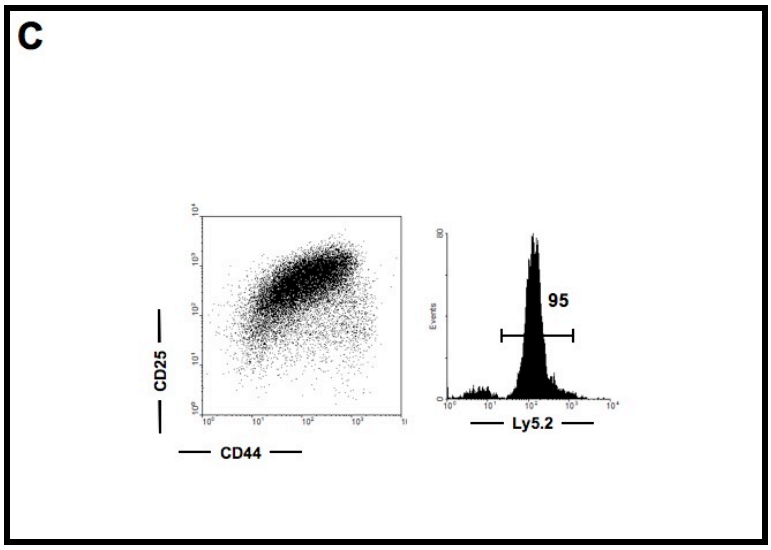


Figure 4C

The same chimeras as described in figure 4A were done, 5 mice were killed pooled and used to sort $CD44^{+}CD117^{High}$ DN1 and DN2 cells one week after BMT. About 2000 cells were plated on OP9-DL1 stroma with IL-7 and analysed by FACS 7-10 days later to assess their in vitro T-cell development potential. Shown are CD44 versus CD25 profiles and CD45.1 (donor-derived) or CD45.2 (host-derived) proportion of recovered thymocytes.

Taken together, our results characterize for the first time the nature of genuine radio-resistant T cell precursors. These cells are (i) intra-thymic in location, (ii) grow in an IL-7-dependent fashion, (iii) display a normal $CD117^{high}$ phenotype and (iv) are functional *bona-fide* DN1-DN2 cells with canonical T cell developmental potential.

Host-derived T cells are functional, but presence of the thymus is required to ensure an unbiased TCR repertoire

A series of chimeras comparing NTX and TX hosts were generated and analysed further to evaluate the functional importance of the thymus in auto-reconstitution. Phenotypically, the most notable difference between the two subpopulations was the increased proportion of naïve ($CD44^{low}CD62L^{+}$) T cells in NTX hosts (17% and 11.8% for $CD4^{+}$ and $CD8^{+}$ T cells respectively) compared with TX hosts (1.8% and 2.3% for $CD4^{+}$ and $CD8^{+}$ T cells respectively) (Figure 5A). In controls (un-manipulated or Bl/6→Bl/6 chimeras), most T cells displayed a naïve phenotype with 66.8% and 46.2% of $CD4^{+}$ and $CD8^{+}$ T cells respectively (Figure 5A left panels) having the $CD44^{low}CD62L^{+}$ phenotype. Thus, NTX chimeras contained a higher proportion of memory phenotype ($CD44^{High}CD62L^{-}$) T cells whereas in TX chimeras almost all T cells were of this phenotype.

Figure 5: Phenotype, repertoire and functionality of host-derived T cells in lethally-irradiated BM chimeras.

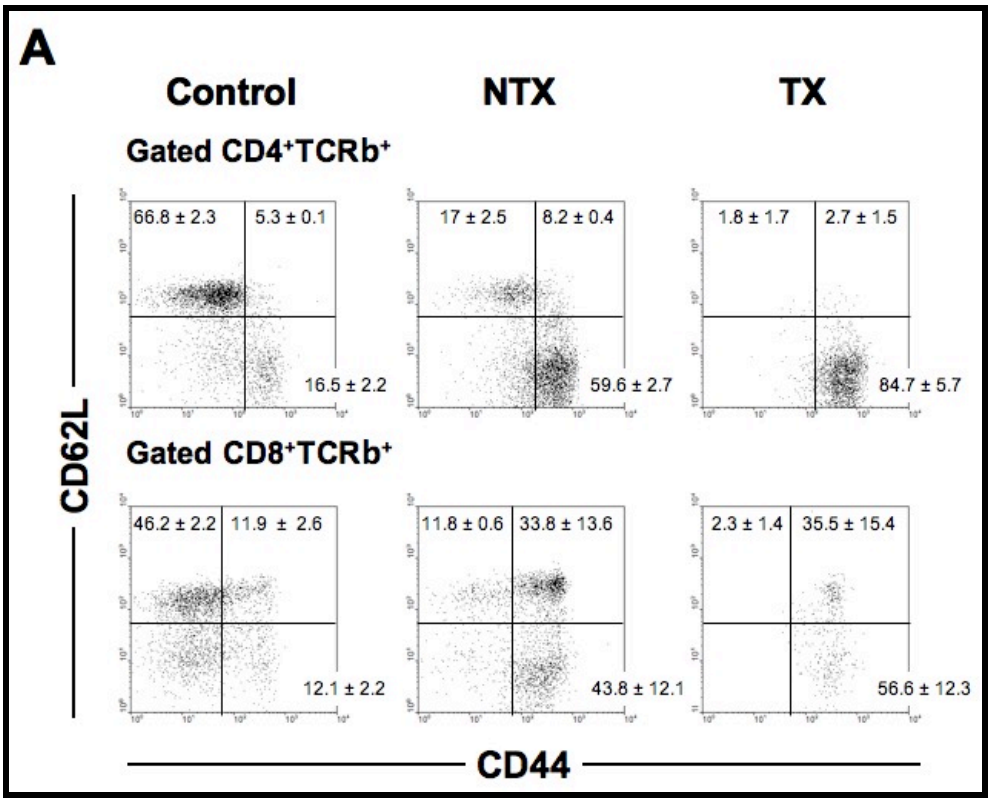


Figure 5A

Shown are CD44 and CD62L expression on gated $CD4^+$ $TCR\beta^+$ (upper dot-plots) or $CD8^+TCR\beta^+$ (lower dot-plots). Numbers in the quadrants indicate mean percentages ± SD from at least 5 mice analysed per group.

The reduced T cell number in TX hosts, where T cells are only extra-thymically derived combined with their severe T cell lymphopenia might result in a repertoire bias and a consequent functional defect. To investigate the repertoire question further, we stained T cells from 10 mice from each group of chimeras with a panel of anti-TCR Vβ-specific mAbs. As shown in Figure 5B, the TCR Vβrepertoire of $CD4^+$ and $CD8^+$ T cells in NTX chimeras consistently resembled that found in controls. This was in clear contrast to that of TX chimeras where in many mice the TCR Vβ repertoire was frequently biased.

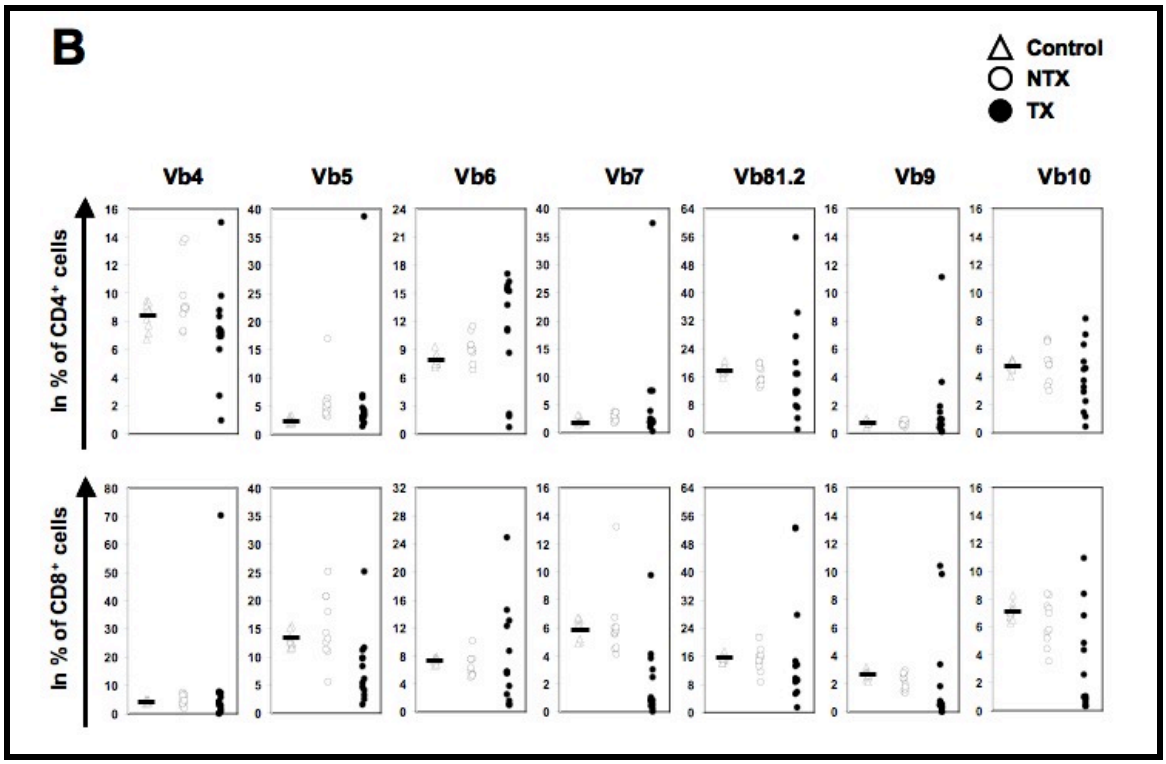


Figure 5B

Vβ repertoire analysis among $CD4^+$ and $CD8^+$ T cells by FACS in chimeras. Each symbol represents a single mouse from control (▲), NTX (O) or TX (●) group. Black bars represent mean value in control group. Ten mice per group were analysed.

Next, to determine if TX hosts had functional defects, we measured their ability to mount a T-dependent (TD) antibody response. As shown in figure 5C, 14 days following NIP-OVA immunisation, NTX chimeras were able to mount a TD antibody response as efficiently as controls. This was in striking contrast to TX chimeras, where only 4 out of 10 mice were able to mount a TD immune response.

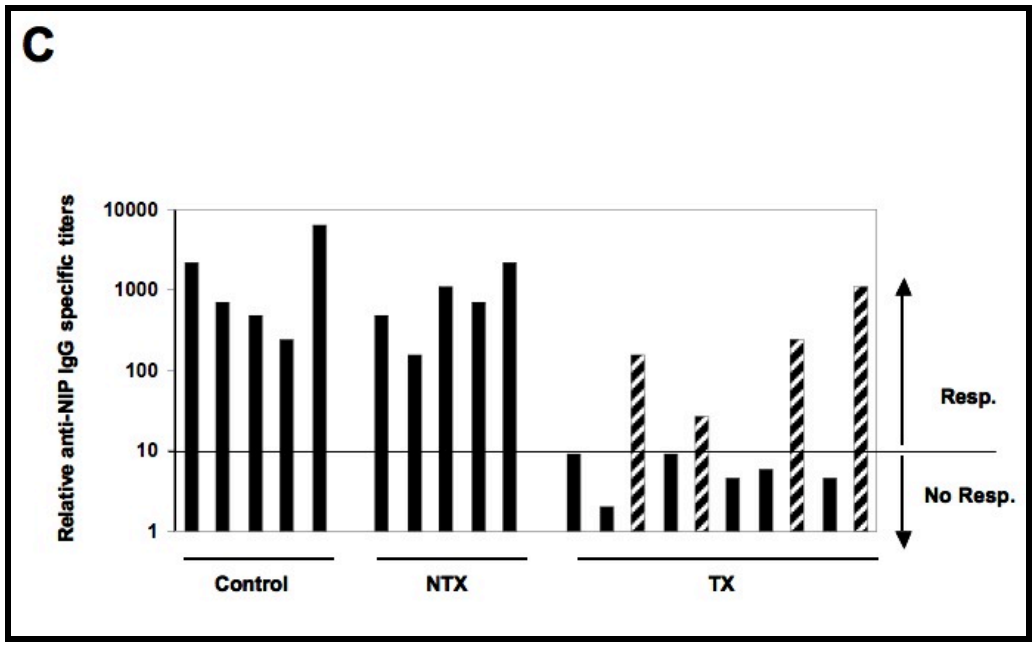


Figure 5C

Depicted is T cell-dependent antibody responses in different group of chimeras day 14 after NIP-OVA immunization. Shown are relative titers of IgG anti-NIP specific antibodies. Each bar represents a single mouse and 5-10 mice were analyzed. When the ratio [anti-NIP IgG titers after immunization / anti-NIP IgG titers before immunization] was below 10 (horizontal line) we considered that the mouse did not mount a T-dependent response. In control and NTX group, 5/5 mice made a specific response with ratio >100. Among TX group, only 4/10 mice made a specific response (hatched bars). Control, NTX or TX chimeras were the same as described in figure 2B.

Taken together, our results show that host-derived T cells in chimeras are functional but in TX hosts, their oligoclonal nature frequently resulted in an inability to mount an efficient antigen-specific TD antibody response.

Discussion

By carefully studying the kinetics of development, distribution, phenotype and function of the host-derived T cells in BM chimeras, we have clarified their previously unappreciated, but important, contribution to the restoration of the immune system. We called this process “auto-reconstitution”. We revealed that following lethal irradiation, host-derived haematopoietic cells in secondary lymphoid organs were almost exclusively composed of T cells and totally devoid of B cells. In addition, host-derived T cells represented a considerable proportion of total T cells (~5% of all T cells six months post-BMT). Kinetic analysis showed that the proportion of host-derived T cells decayed as donor-derived T cell development slowly took place in Bl/6→Bl/6 chimeras. In these competitive settings, where the donor BM generated T cells, it was not possible to study host-derived T cells exclusively. Thus, we generated non-competitive BM chimeras in which BM donor cells (either $RAG2^{-/-}$ or $CD3\varepsilon^{-/-}$) were incapable of generating T cells. Then, we were able for the first time to quantify precisely host-derived T cells in these

non-competitive chimeric settings. Surprisingly, auto-reconstitution could generate a pool of T cells equivalent to 35% that in normal mice. We could also show that T cell auto-reconstitution had two origins: (i) a major, thymic-dependent pathway (TD) and (ii) a minor, thymic-independent pathway (TI). Indeed, in CD3$\varepsilon^{-/-}$→Bl/6 chimeras, by comparing host-derived T cell numbers in euthymic (NTX) versus athymic (TX) hosts, we could quantify TD and TI pathways. Thus, the TD pathway accounted for 70% of host-derived T cells, thereby representing the main source of host T cells in an auto-reconstituted T cell compartment. This estimate is based on the assumption that the size of the TI compartment is not drastically altered by the TD cohort of T cells and *vice versa*.

The TD T cells were composed of the progeny of a single wave of T-cell differentiation which reached a maximum 2 weeks following BMT. As host-derived thymocyte differentiation is only transient and ceases around 3-4 weeks, the thymus does not contain any DP cells later on and its cellularity decreases dramatically. This has been previously characterized by others (Ceredig and MacDonald, 1982). The TD cohort of T cells provides a large number of naïve T cells with a diverse TCR Vβ repertoire. Logically, this observation led us to investigate the nature of radio-resistant thymocyte progenitors.

Recently, when addressing the cellular origin of the host-derived cohort of thymocytes regenerating in BM chimeras, Maillard *et al.* (Maillard et al., 2006) suggested that they arose from an abnormal sequence of differentiation starting from a donor-derived $CD25^{+}$ $CD44^{-}$ $CD117^{-}$ "DN3-like" stage and therefore might involve an atypical thymocyte progenitor. In contrast, we could detect the presence of genuine, or "conventional" $CD25^{-/+}$ $CD44^{+}$ $CD117^{high}$ DN1-2 cells. Such conventional DN1-2 cells were few in number, representing around 2000 cells per mouse one week post-BMT. Further analysis indicated that these T cell progenitors were mainly (~95-99%) host-derived initially and were then replaced by donor derived progenitors around 3 weeks post-BMT.

The toxicity of ionizing radiation to cells is associated with massive apoptosis which is particularly acute in hematopoietic organs, well-known to be highly radiosensitive. Lethal γ-irradiation triggers DNA breaks which in turn activate DNA repair mechanisms and/or p53-dependent apoptosis, unless DNA repair has been carried out correctly (Jeggo and Lobrich, 2006a; O'Driscoll and Jeggo, 2006). There is a body of literature addressing the radio-sensitivity of lymphoid cells, but so far, there are no clear mechanisms demonstrating why only some subpopulations of cells can survive lethal irradiation damage. From in vitro experiments with cell lines, it has been proposed that the cell cycle status at the time of irradiation might determine their radio-sensitivity (Jeggo and Lobrich, 2006b; Lobrich and Jeggo, 2007; Pawlik and Keyomarsi, 2004). Thus, cycling cells harbour much more DNA alterations than their quiescent counterparts and are more prone to die. This dogma by itself would hardly explain what is going

on *in vivo* in lethally irradiated mice. Indeed, it would seem that memory T cells, which are known to be more rapidly cycling than their naïve counterparts, survive whereas B cells, some of which are known to be more cycling than others, are totally deleted. Thus, we believe that certain niches throughout the body might provide lymphocytes with a particular environment with specific radio-protective signals. In our study, the gut contained a higher proportion of radio-resistant T cells and could be an interesting candidate for such a radio-protective niche even though we cannot exclude a preferential relocalisation of surviving T cells following total body irradiation (TBI). Interestingly, a role for the gut in radioprotection was recently proposed because a polypeptide-drug derived from commensal salmonella flagellin, which binds to Toll-like receptor 5 and which activates nuclear factor-κB signalling, was able to prevent mice from gastrointestinal and hematopoietic disorders following lethal TBI (Burdelya et al., 2008). Furthermore, in recipient mice where the anti-apoptotic gene *Bcl-2* was constitutively over-expressed ($H2^K$-BCL-2 transgenic mouse), even host-derived B cells survived following TBI and BMT (data not shown and (Domen et al., 1998)). Thus, the clear contrast in survival properties of B versus T cells might (at least partly) be due to intrinsic differences in their expression of pro- versus anti-apoptotic genes. By intracellular staining, we have already observed a higher baseline level of Bcl-2 expression in T cells versus B cells. Clearly, the issue of the differential survival of T versus B cells following lethal irradiation is warranted and might shed light on important pathways for radioprotection at the cellular level.

As shown in TX recipients, the minor, TI-derived, T cell component of auto-reconstitution was composed of the progeny of mature, radio-resistant peripheral $CD4^+$ and $CD8^+$ T cells. Following their survival and expansion post-irradiation, they expressed a biased TCR Vβ repertoire. Phenotypically, these TI cells had a $CD44^{High}$ $CD62L^{+/-}$ memory-like phenotype which could correspond to their initial phenotype or to a phenotype acquired upon lymphopenia-induced expansion (Bosco et al., 2005; Bosco et al., 2006a). Additionally, as we and others have previously shown, these cells were enriched in FoxP-3^+ regulatory T cells (Anderson et al., 2004; Benard et al., 2006; Komatsu and Hori, 2007; Roord et al., 2008). Following BMT in mice, these TI Treg cells protected the host from GvHD complications in syngeneic conditions (Benard et al., 2006). Others studies have shown that Treg, as well as memory T cells, are more resistant to γ-irradiation, chemotherapy or antibody-mediated depletion *in vitro* and *in vivo* (Anderson et al., 2004; Benard et al., 2006; Bourgeois and Stockinger, 2006; Gladstone et al., 2007; Walzer et al., 2002). The molecular mechanisms behind these interesting observations are still unknown and require further experiments.

Functionally, in NTX $CD3\varepsilon^{-/-}$→Bl/6 thymus-bearing chimeras, host-derived $CD4^+$ T cells alone were sufficient to mount a TD immune response whereas in TX recipients, the immune response was frequently impaired. Clinically, it is recommended that patients be re-vaccinated

following BMT in order to restore a high level of protection against many vaccine-preventable diseases such as measles, tetanus, hepatitis or polio (Avigan et al., 2001; Gangappa et al., 2008; Ljungman et al., 2005; Parkman, 2008; Patel et al., 2007). In this context, mouse BM chimeras might provide a relevant experimental system to study immune response or diseases associated with lymphopenia subsequent to conditioning regimens and BMT in humans. Published observations suggested that host-derived cytomegalovirus-specific (CMV) $CD8^+$ memory T cells could provide efficient anti-viral immunity and help patients survive the severe lymphopenic period following BMT (Chalandon et al., 2006). However, studies in man are difficult to control experimentally and animal models are required to dissect and improve the outcome of BMT.

In conclusion, our results demonstrate a so-far unappreciated, but important, residual host-derived thymic activity following conditioning and BMT. These thymus-derived T cells constitute a significant population of T cells with a polyclonal TCR Vβ repertoire and normal functional properties. By analysing thymectomised recipients, we could demonstrate that in their absence, lymphopenia was more severe, the T cell repertoire was frequently oligoclonal and immune responses were compromised. Therefore, strategies should be developed to improve and expand the TD and/or the TI cohort of T cells which together could serve as a first line of defence in lymphopenic hosts until donor-derived T cell reconstitution takes place. In this regards, improving thymic reconstitution and peripheral T cell survival by, for example, infusing IL-7/anti-IL-7 complexes (Boyman et al., 2006; Boyman et al., 2008) during thymus regeneration would appear to be a promising approach.

Materials and methods

Mice.

Female C57Bl/6, C57Bl/6.RAG2$^{-/-}$, and C57Bl/6.CD3ε$^{-/-}$ mice of either CD45.1 or CD45.2 genotype were maintained in our SPF animal facility. As BM donors, mice were used at 4-8 weeks of age and recipients were killed by CO_2 inhalation prior to analysis. These studies were approved by the institute animal care and user committee.

Thymectomies.

At 4 to 6 weeks of age, mice were anesthetized and thymus was removed by suction through a small upper sternal incision. That thymectomy had been complete was verified in each animal by anatomical inspection at the time of sacrifice.

Cell preparation, immunofluorescence staining and FACS analysis.

Lymphoid organs (thymus, spleen or lymph nodes) were removed from adult unmanipulated or chimeric mice at the indicated times. Single-cell suspensions were generated by disruption of organs through a 40 µm nylon mesh via a syringe plunger and were washed once with 2% FCS-supplemented DMEM. Spleen cell suspensions were depleted of red cells by NH_4Cl treatment prior to staining. Lymphocyte suspensions from liver, lung and skin were prepared by standard procedures and Percoll density gradient centrifugation performed as previously described (Schleussner and Ceredig, 1993) prior to cell recovery. Biotin-, FITC-, PE-, PE.Cy7-, or APC-conjugated mAbs were either home made or purchased from BD Biosciences or eBioscience (San Diego, CA). Cell staining was performed as previously described (Bosco et al., 2005; Bosco et al., 2006a) prior to FACS analysis with a FACScalibur and CELLQuest Pro software (BD Bioscience). The data presented are of live-gated cells based on a combination of forward- and side-scatter signals and when possible propidium iodide exclusion.

Bone marrow transplantation.

Bone marrow cell suspensions from CD45 allogenic donor mice were prepared by flushing femurs and tibias with PBS using a 23g needle. After a red blood cell lysis step, T cells were depleted when necessary by resuspending cells in a mixture of rat IgM anti-CD4 (RL172) and anti-CD8α (31M) mAb hybridoma supernatants and incubated for 20 min at 4°C. Following a washing step, antibody-coated cells were lysed by adding a 1:10 dilution of screened rabbit serum as source of complement in serum free Dulbecco's modified Eagle's medium (DMEM) for 45 min at 37°C. Then, cells were washed, resuspended in serum-free DMEM and counted prior to injection. Host mice were lethally γ-irradiated with a single dose of 950 cGy at a dose of 80 to 90 cGy/min using a Cobalt-source (Gammacell 40, Atomic energy of Canada, Ltd) 4h prior to receiving $5x10^6$ bone marrow cells intravenously. Chimeric mice were analysed at the indicated time points and the host and donor origin of lymphoid cells determined by means of labelled CD45.1- or CD45.2-specific mAbs.

Fetal thymic organ cultures

Fifteen-day pregnant C57Bl/6 mice were lethally irradiated (950 cGy) prior to embryo removal. Thymus lobes from irradiated or control un-irradiated embryos were then used for FTOC. For staining, lobes were dissociated by passing through needles of decreasing size as previously

described (Balciunaite et al., 2005c), washed in DMEM medium and then stained and analyzed by flow cytometry.

Thymus DN preparation, cell sorting and culture on OP9-DL1 stroma.

From total thymocytes, $CD4^-CD8^-$ double negative (DN) cells from control or chimeric mice were prepared by complement-mediated lysis of CD4 and CD8 expressing thymocytes as described above. DN thymocytes were then stained with combinations of fluorescently-labelled CD25, CD44 and CD117 (c-kit) mAbs and pooled $CD25^-$ DN1 and $CD25^+$ DN2 cells sorted as $CD117^{high}CD44^+$ cells using a FACSAria sorter (BD Biosciences). Reanalysis of sorted cells indicated that their purity was routinely ≥ 98%.

OP9 stromal cells expressing the Notch ligand delta-like-1 (OP9-DL1) were kindly provided by Professor Juan-Carlos Zúñiga-Pflücker (University of Toronto, ON, Canada) and maintained in IMDM supplemented with 5 x 10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% (wt/vol) Primatone (Quest, Naarden, The Netherlands), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2% heat-inactivated fetal bovine serum (FBS). Two days prior to coculture, 10^4 stromal cells were seeded per well of a 24-well plate. At day 0, stromal cells had grown to semi-confluency and were then γ-irradiated with 3000 cGy, culture medium removed and replaced by supplemented DMEM plus IL-7 (~10ng/mL). To these cultures were added ~$5x10^3$ sorted DN1 and DN2 thymocytes. At day 10-14 of culture, cells were either analysed by FCM or kept in culture after transfer to fresh OP9-DL1 stromal cells.

T cell-dependent antibody responses.

To induce a T cell-dependent antibody response, reconstituted chimeric mice were injected subcutanously with 200µl complete Freund's adjuvant emulsion containing 50 µg total NIP-ovalbumin. Sera were obtained after bleeding prior to and 14 days after immunization and stored at –20°C. Hapten-specific IgG antibody titres were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Ceredig et al., 2006).

Immunofluoresecent staining of spleen sections.

Spleens were snap frozen with dry ice in Tissue-Tek OCT compound and then 5 to 7-µm sections cut on a cryostat. Sections were then fixed for 10 min in acetone and stored at -80°C. For staining, sections were covered with an antibody solution at saturating concentration diluted in PBS-1% FBS for 1h at room temperature. Anti-$B220^{Biotin}$ and anti-$CD90^{FITC}$ were used to discriminate B and T cell areas respectively, and PNA^{Biotin} (Peanut Agglutinin, Vector

Laboratories) and anti-IgM^{FITC} (M41) used to reveal germinal centres (GC). For the second step, sections were washed in PBS (three times 10 min) and incubated 30 min at room temperature with a Neutralite Avidin-Texas-Red reagent (Southern Biotech) diluted in PBS. Then, sections were washed, coverslipped and analyzed under a fluorescence microscope (Zeiss axioskope) with a 10 to 20x objective.

Statistical analyses.

Data are presented as the mean and SEM. Comparisons between groups were done using a Student's two-tailed *t* test for independent events. *P* values of less than 0.05 were considered significant (★, *p* value <0.05 and ★★, *p* value <0.01).

Part III: TCRβ chains derived from peripheral γδ T cells can take part in αβ T cell development

by Nabil Bosco, Corinne Engdahl, Angèle Bénard, Johanna Rolink, Rhodri Ceredig and Antonius G. Rolink

Introduction

T cell development takes place in the thymus from progenitors of bone marrow origin. By now it is generally believed that a cell called a thymus settling progenitor (TSP), characterized by the expression of CD44, CD117, CD135 and the chemokine receptor CCR9 (Benz and Bleul, 2005; Bhandoola et al., 2007; Schwarz et al., 2007; Zediak et al., 2005) is the bone marrow cell that enters the thymus. However, transplantation experiments in T cell deficient mice have indicated that other progenitors can enter the thymus and may take part in T cell development (Balciunaite et al., 2005b; Bhandoola et al., 2007; Martin et al., 2003).

Early T cell progenitors in the thymus lack CD4 and CD8 expression and are therefore called double negative (DN) cells. Based on the differential expression of CD44, CD25 and CD117, DN cells can be subdivided into DN1-4 subpopulations. Thus DN1 cells are $CD44^{+}$, $CD25^{-}$ and $CD117^{High}$ and within this population, TSPs are included as a $CD135^{+}$ subpopulation (Bhandoola et al., 2007; Ceredig et al., 2007; Ceredig and Rolink, 2002; Zediak et al., 2005). DN1 cells differentiate through a $CD44^{+}$, $CD25^{+}$, $CD117^{high}$ DN2 stage to become $CD44^{-}$, $CD25^{+}$, $CD117^{-/low}$ DN3 cells (Ceredig and Rolink, 2002). Both DN1 and 2 cells still possess multi-lineage developmental potential, including that for NK, dendritic and myeloid cells (Balciunaite et al., 2005c; Bell and Bhandoola, 2008; Wada et al., 2008). However, B cell lineage potential seems to be restricted to the TSP population within the DN1 subset (Benz and Bleul, 2005; Bhandoola et al., 2007; Ceredig et al., 2007; Zediak et al., 2005). By largely unknown mechanisms, commitment to the T cell lineage is acquired at the DN2 to DN3 cell transition (Balciunaite et al., 2005a; Balciunaite et al., 2005c). However, cells at the DN3 stage still have the option to develop into either αβ or γδ T cell lineage cells (Hayday and Pennington, 2007; Kreslavsky et al., 2008; von Boehmer et al., 1998). A relatively large number of studies have indicated that the expression of the pre-TCR plays a crucial role in the development of αβ T cells (Fehling et al., 1995; Kreslavsky et al., 2008; von Boehmer et al., 1998). Recent, elegant, single cell studies have indicated that γδ TCR expressing cells still have the option to develop into either γδ or αβ TCR expressing T cells (Haks et al., 2005; Hayes et al., 2005; Kreslavsky et al., 2008). The strength of the TCR-mediated signal that a γδ T cell receives seems to determine the outcome of this option. Thus, a strong signal (positive selection) drives these cells into the γδ T cell lineage whereas no, or a weak, signal leads to the differentiation

into αβ T cell lineage cells (Haks et al., 2005; Hayes et al., 2005; Kreslavsky et al., 2008). The finding that at least some peripheral γδ T cells are positively selected in the thymus strongly supports this model (Hayday and Pennington, 2007; Lewis et al., 2006).

It is well known that 10-20% of the peripheral γδT cells express a TCRβ protein in their cytoplasm (Wilson and MacDonald, 1998). However, it still unknown why these cells became γδ and not αβ T cells and why they maintain TCRβ gene transcription and protein expression. Here we tested whether TCRβchains isolated from peripheral γδ T cells could take part in the development αβ T cells. To this end, cDNAs encoding expressed TCRβ genes were cloned from sorted lymph node γδ T cells and retrovirally introduced into in vitro-propagated Pax-5/TCRβ double deficient pro-B cells. The potential of these transduced cells to give rise to αβ T cells was analyzed in vivo in sublethally-irradiated CD3ε-deficient recipient mice. Results obtained unambiguously demonstrated that with respect to the reconstitution of the thymus and the peripheral αβ T cell compartment in this experimental system, TCRβ chains from γδ T cells were as efficient as those derived from αβ T cells at reconstituting the αβ T cell compartment. Thus the TCRβ chains found in γδ T cells can potentially mediate αβ T cell development. The potential implications of these findings for γδ – αβ T cell development will be discussed.

Results

Thymus reconstitution by Pax5/TCRβ double deficient proB cells

Previously, we have shown that upon transplantation into immune-deficient hosts, in vitro-propagated Pax5 deficient pro-B cells could readily reconstitute all thymocyte subpopulations as well as peripheral lymphoid organs with mature αβ T cells (Rolink et al., 1999). Now, we have generated a Pax5 x TCRβ double-deficient pro-B cell line in order to test the ability of TCRβ chains of different origins to take part in αβ T cell development. As a first control, Pax5/TCRβ double deficient pro-B cells were transduced with an empty pMIG retroviral vector and 5 – 7 days later sorted for GFP expression. After a further two weeks of in vitro propagation, cells were injected intra-venously into sub-lethally irradiated CD3ε-deficient recipient mice. Three weeks after transfer, the thymi of these mice contained 10 – 15 x 10^6 cells, 85 – 95% of which were GFP positive (Figure 1A). Of the GFP^+ cells, 15 – 20% were DN, 65 – 75 % DP and 5 – 10% single $CD4^+$ (Figure 1B). Analysis of TCR expression on these various subpopulations revealed that 15 – 25% DN cells were γδ TCR^+ whereas the other subpopulations of CD4/8-

defined cells were not (Figure1 C-E). Obviously, none of the cells expressed αβ TCR (Figure 1 C-E). Thus, Pax5/TCRβ double deficient pro-B cells can modestly reconstitute the thymus of CD3ε-deficient mice and some of the resulting DN cells express a γδ TCR.

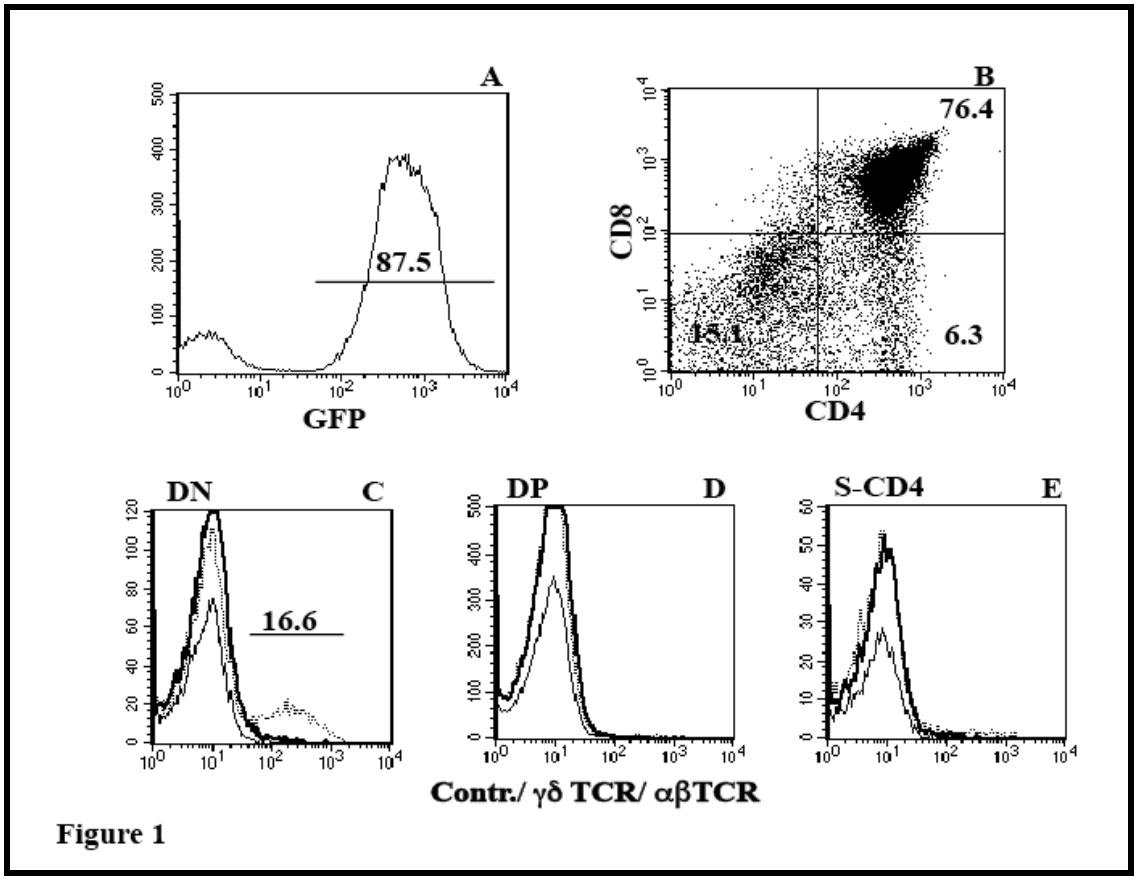


Figure1: Phenotype of thymocytes in CD3ε-deficient mice three weeks after the injection of PAX5/TCRβ double deficient proB cells transduced with empty pMIG vector.

(A) Shown is the GFP expression. (B) Shown are the CD4 and CD8 expression on the GFP positive cells. (C-E) gd TCR (dotted histograms), ab TCR (bold histograms) and controls (thin line histograms) on gated DN (C), DP (D) and CD4 single positive (E) GFP positive thymocytes. Numbers represent the percentage of cells in one representative mouse, more than 10 were analyzed.

Thymus reconstitution by Pax5/TCRβ double deficient pro-B cells using TCRβ chains from γδ or αβ T cells

Previously it was shown that about 10-15% of the peripheral γδ T cells are cytoplasmically TCRβ$^+$ (Wilson and MacDonald, 1998), results confirmed in Figure 2.

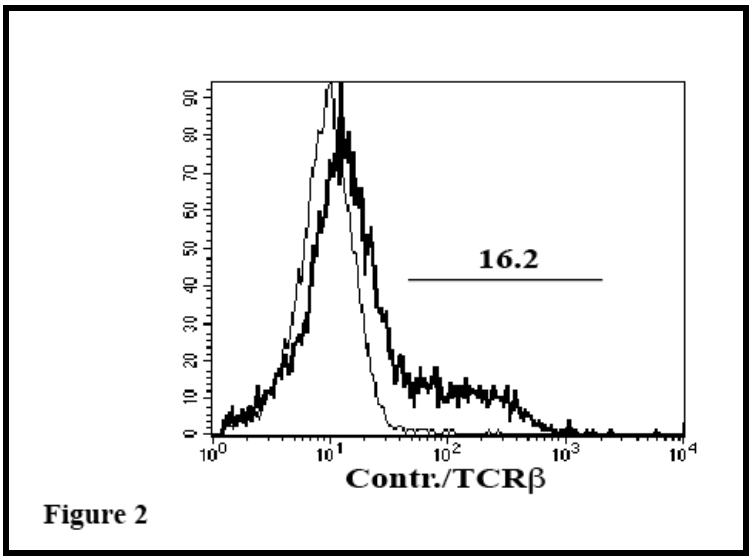


Figure 2: Cytoplasmic TCRβ expression by γδ T cells from WT Bl/6 lymph nodes.

Lymph node cells were stained for γδ TCR cell surface expression and subsequently for intracellular TCRβ. Histograms represent γδ TCR positive gated cells. Thin line histogram represents the control and the bold line histogram represents the TCRβ stained sample. The number indicates the percentage of positive cells in one representative mouse.

To test whether TCRβ chains derived from γδ T cells could take part in αβ T cell development, we firstly amplified by RT-PCR cDNA from sorted LN γδ T cells (as shown in supplementary Fig. 1) using oligonucleotides specific for Vβ4, Vβ6 or a Vβ8 family members. Amplicons were sequenced prior to cloning into a pMIG retroviral vector and expressed in the Pax5/TCRβ double deficient pro-B cells. (The TCR Vβ sequences used in this study are shown in Supplementary Table I). Subsequently, TCRβ-expressing, GFP^+ cells were injected i.v. into sublethally-irradiated CD3ε deficient mice. Pax5/TCRβ double deficient pro-B cells transduced with vectors containing TCRβ chains amplified from sorted LN αβ T cells (as shown supplementary Fig. 1) were used as controls. Three weeks later, the thymus of mice that had received Pax5/TCRβ double deficient pro-B cells transduced with γδ T cell-derived TCRβ chains contained between 80 and 130 x 10^6 cells (Figure 3A). Similar thymocyte numbers were found in recipients of Pax5/TCRβ double deficient pro-B cells transduced with TCRβ chains derived from sorted LN αβ T cells (Figure 3A). Thus, thymus cellularity in both groups were close to those of wild type mice and moreover, were 5 -10 fold greater than in mice that had received cells transduced with the empty vector (Figure 3A).

Figure 3: Numbers and phenotype of thymocytes derived from CD3ε deficient mice at 3 weeks after the injection of Pax5/TCRβ double deficient pro B cells transduced with TCRβ derived from lymph node αβ or γδ T cells.

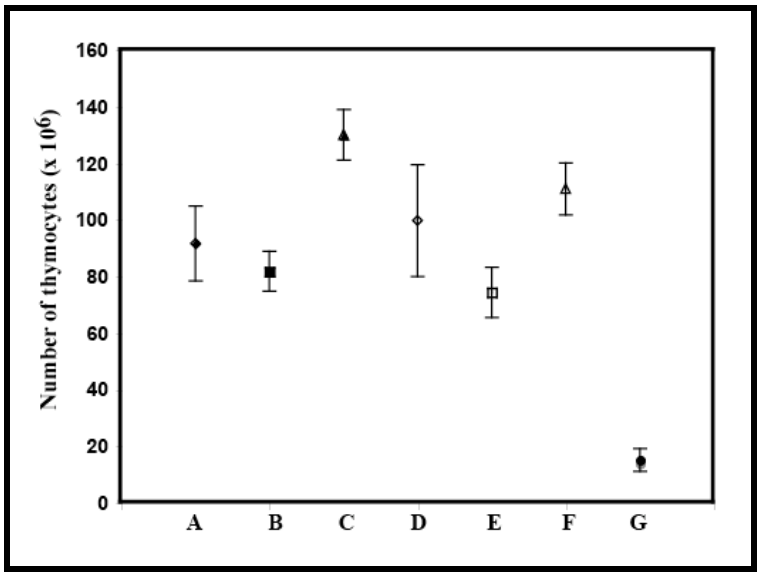


Figure 3A

Numbers of thymocytes recovered are shown. Each symbol represents the mean ± SD of 5 mice. Groups A, B and C were mice injected with different γδ T cell derived TCRβ chain transduced cells. Groups D, E and F were mice injected with different αβ T cell derived TCRβ chain transduced cells. Group G represents the mice that were injected with empty vector transduced cells.

With respect to CD4, CD8 and TCR expression, thymocytes from recipient mice reconstituted with cells expressing TCRβ chains from either TCRγδ or TCRαβ-derived cells were very similar to those of wild type mice. Figure 3B and 3C show a typical example of thymocytes from mice reconstituted with either γδ or αβ-derived TCRβ chains. For comparative purposes, both of these transduced TCRβ's used a Vβ8 family member. In both recipients, over 95% of thymocytes were GFP^+ with 2-5% DN, 75-85% DP, 5-10% CD8 single positive and 10-20% CD4 single positive cells. Moreover, as in wild type mice, αβ TCR expression was low on DP cells and relatively high on single positive cells. Surprisingly, and in marked contrast to the mice that had received the Pax5/TCRβ double deficient pro B cells transduced with the empty vector (see Figure 1), no γδ T cells were detected in the thymus of these mice (see supplementary Fig. 2).

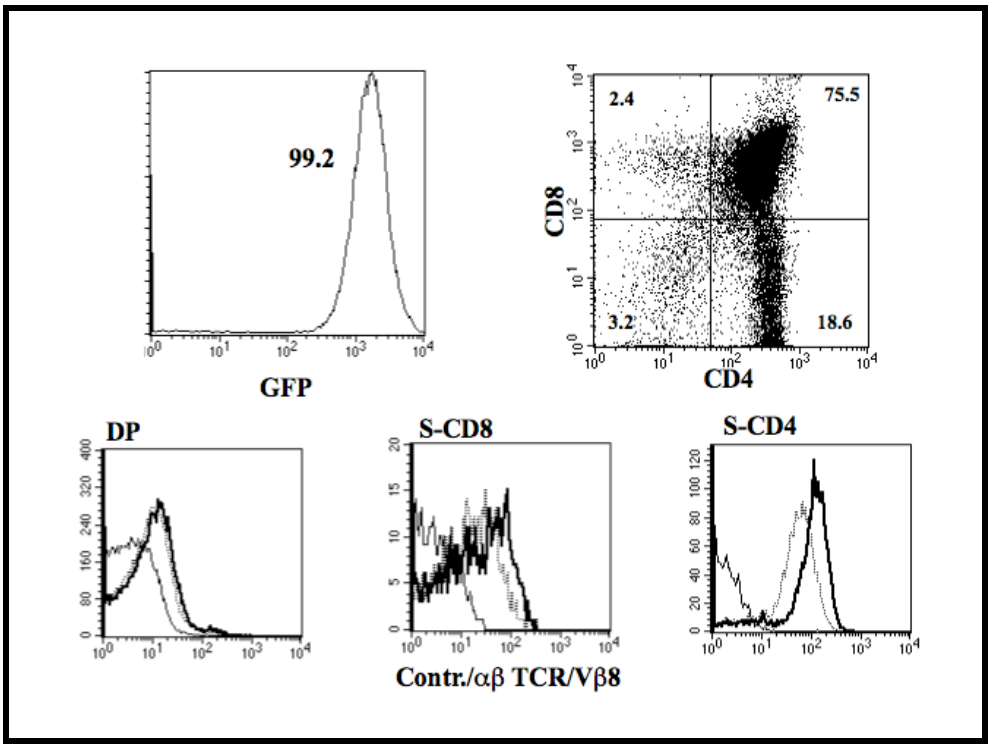


Figure 3B

GFP, CD4, CD8, αβ TCR and Vβ8 expression by thymocytes derived from a mouse injected with cells transduced with a Vβ8 using TCRβ chain derived αβ T cells. Three lower histograms represent αβ TCR (bold line histogram) and Vβ8 (dotted line histogram) expression by double positive (DP) and single positive CD8 and CD4 cells. Thin line histograms represent the negative controls. Numbers in quadrants represent the percentage of one representative mouse among 5 analyzed.

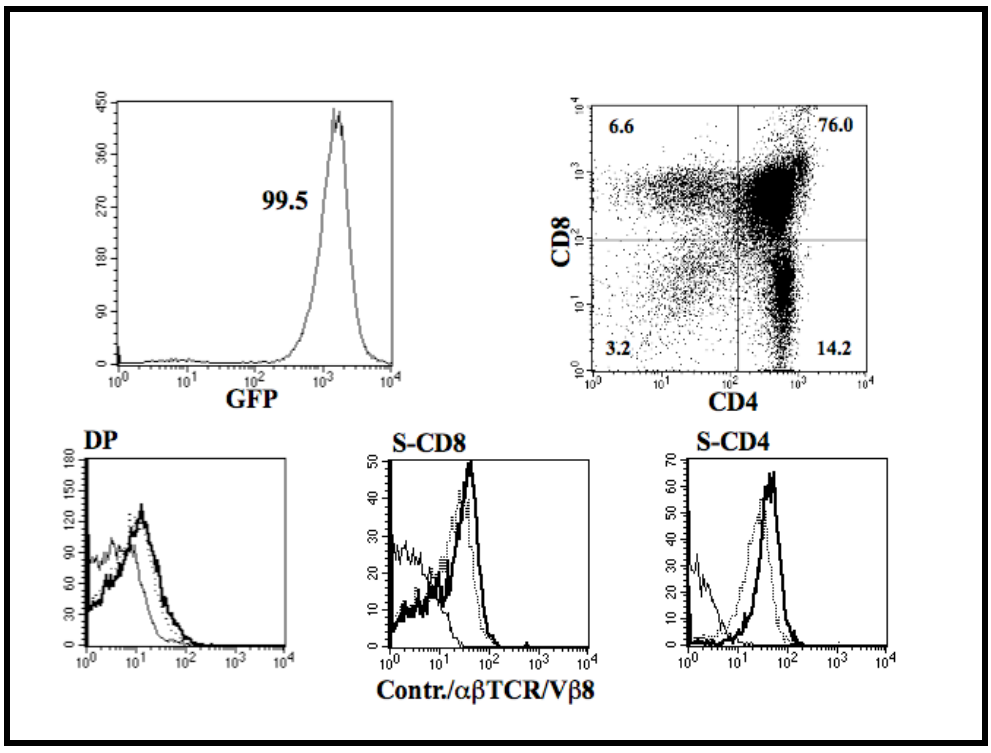


Figure 3C

GFP, CD4, CD8, αβ TCR and Vβ8 expression by thymocytes derived from a mouse injected with cells transduced with a Vβ8 using TCRβ chain derived γδT cells. Three lower histograms represent αβ TCR (bold line histogram) and Vβ8 (dotted line histogram) expression by double positive (DP) and single positive CD8 and CD4 cells. Thin line histograms represent the negative controls. Numbers indicate the percentage of cells. (>5 mice were analyzed independently).

Taken together, these results indicate that TCRβ chains from γδ cells can guide αβ T cell development as efficiently as those from αβ T cells. This conclusion is based on the combined results obtained with 6 TCRβ chains derived from γδ T cells and 6 from αβ T cells.

Reconstituted mice develop lethal autoimmune disease

In a next set of experiments, we wanted to test the functional capacity of αβ T cells generated from TCRβ transduced Pax5/TCRβ double deficient pro-B cells. However, these studies were hampered by the fact that independently of the origin of the transduced TCRβ chains, from four weeks after injection, all recipient mice showed weight loss and had diarrhea and practically all died within 5 to 15 weeks after transplantation (Figure 4A).

Figure 4: CD3ε mice injected with TCRβ transduced Pax5/TCRβ double deficient pro B cells develop a fatal autoimmune disease.

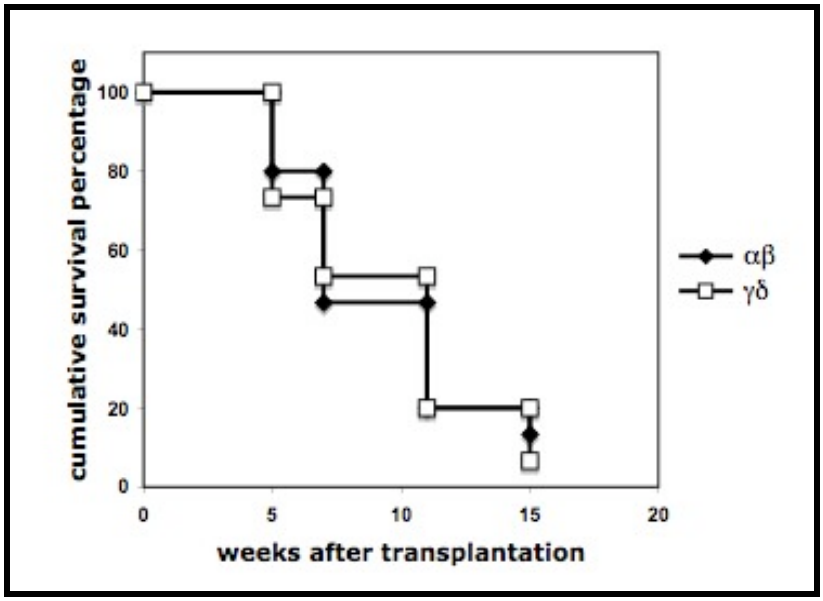


Figure 4A

Survival curve of CD3ε mice injected with empty vector transduced cells (▲, 12 mice), γδ T cell derived TCRβ transduced cells (◆, 15 mice) and αβ T cell derived TCRβ transduced cells (■, 15 mice) are shown.

Moreover, all mice had relatively high titers of IgG anti-nuclear autoantibodies in their serum (Figure 4B).

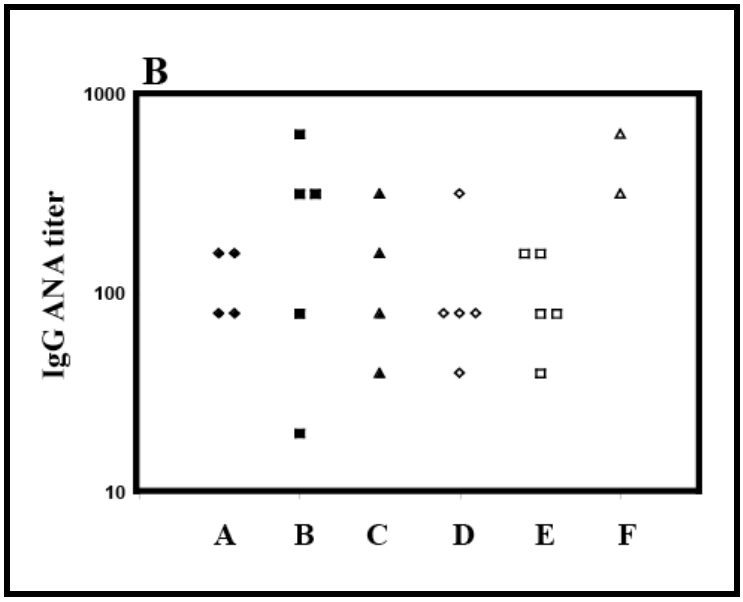


Figure 4B

IgG anti-nuclear autoantibody titer in CD3ε mice injected with γδ T cell derived TCRβ transduced cells (groups A-C,◆,■,_) and αβ T cell derived TCRβ transduced cells (groups D-F,◇, □,△) are depicted.

Histological analysis revealed pathological signs resembling inflammatory bowel disease of the large intestines (Figure 4C) and marked leukocyte infiltrations in the lungs (Figure 4D).

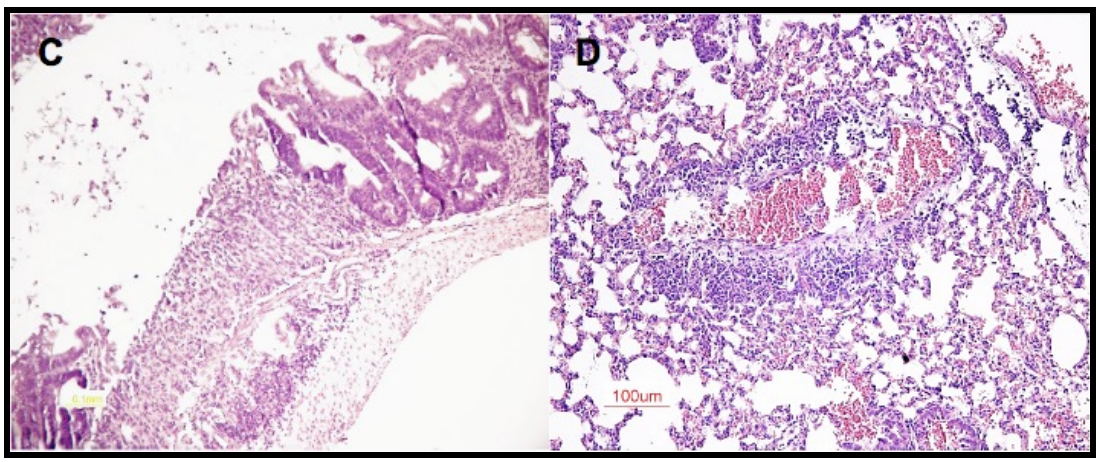


Figure 4 C-D

C and D. Shown are representative pictures of widespread lymphocytic infiltration in non-lymphoid organs of CD3ε mice injected with TCRβ transduced Pax5/TCRβ double deficient pro B cells. Picture in C is a photograph of a H+E stained section of the lower colon (magnification x240). Picture D is a photograph of a H+E stained section of the lung (magnification x240).

No such signs of disease were observed among mice that had received Pax5/TCRβ double deficient pro-B cells transduced with the empty vector. Thus, the presence of αβ T cells developing from the TCRβ-transduced Pax5/TCRβ double deficient pro-B cells seemed to be the cause of this disease. This was despite the fact that T cells in mice reconstituted with pro-B cells expressing TCRβ chains from either γδ or αβ cells contained FoxP3$^+$ cells (not shown).

Recently, we showed that sublethally-irradiated C57Bl/6 RAG-2 deficient mice reconstituted with T cell-depleted wild type syngeneic bone marrow developed a similar type of disease (Benard et al., 2006). In that study, we also demonstrated that the disease induction could be prevented by the co-transplantation of naturally-arising regulatory T (Treg) cells (Benard et al., 2006). To test whether naturally-arising regulatory T cells could also prevent the development of the disease in CD3ε-deficient recipients, mice were injected with a mixture of 2 x10^5 sorted GFP$^+$ Treg cells from FoxP3 knock-in mice and 5 x 10^6 TCRβ-transduced Pax5/TCRβ double deficient pro-B cells. As in the previous experimental system, co-injection of Treg cells protected the mice from developing lethal autoimmune disease. Thus, mice containing co-injected Treg did not show weight loss, had no diarrhea and did not have elevated IgG anti nuclear autoantibody titers in their serum. At 8 weeks after injection, these mice were analyzed for the presence of T cells in the periphery. In the spleen of all injected mice, 10 – 20 % cells were GFP positive and expressed TCRVβ, in this case Vβ8, introduced into injected proB cells and the vast majority expressed an αβ TCR. In corollary, GFP$^+$ Vβ8$^-$, i. e. cells derived from the injected Treg, were undetectable (not shown). The lymph nodes contained 50-60% GFP$^+$, αβ TCR$^+$ T cells. An example of such CD3ε-deficient mice injected with Pax5/TCRβ double deficient pro-B cells expressing a Vβ8 TCR chain from either a αβ or γδ T cell are shown in Figures 5A and 5B respectively. In the first mouse (Figure 5A) 53% of lymph node cells were GFP positive and 67.5% GFP$^+$ cells expressed CD4 and 12% CD8. In all recipients, a low, but significant, number of GFP$^+$, CD4$^-$ CD8$^-$ cells were found, none of which expressed a TCR. Phenotypically, these cells resembled the injected Pax5/TCRβ double deficient pro-B cells and thus most likely were cells that had not homed to the thymus and did not differentiate into T cells. Both GFP$^+$ CD4 and CD8 cells expressed an αβ TCR that used in this case the Vβ8 TCR chain (Figure 5A). Therefore, these peripheral GFP$^+$ T cells were derived from the transduced cells and not from a contamination in the co-injected Treg population.

Figure 5. Phenotype and function of peripheral T cells derived from TCRβ transduced PAX5/TCRβ double deficient proB cells when mice were co-injected with 2x10^5 polyclonal Treg cells.

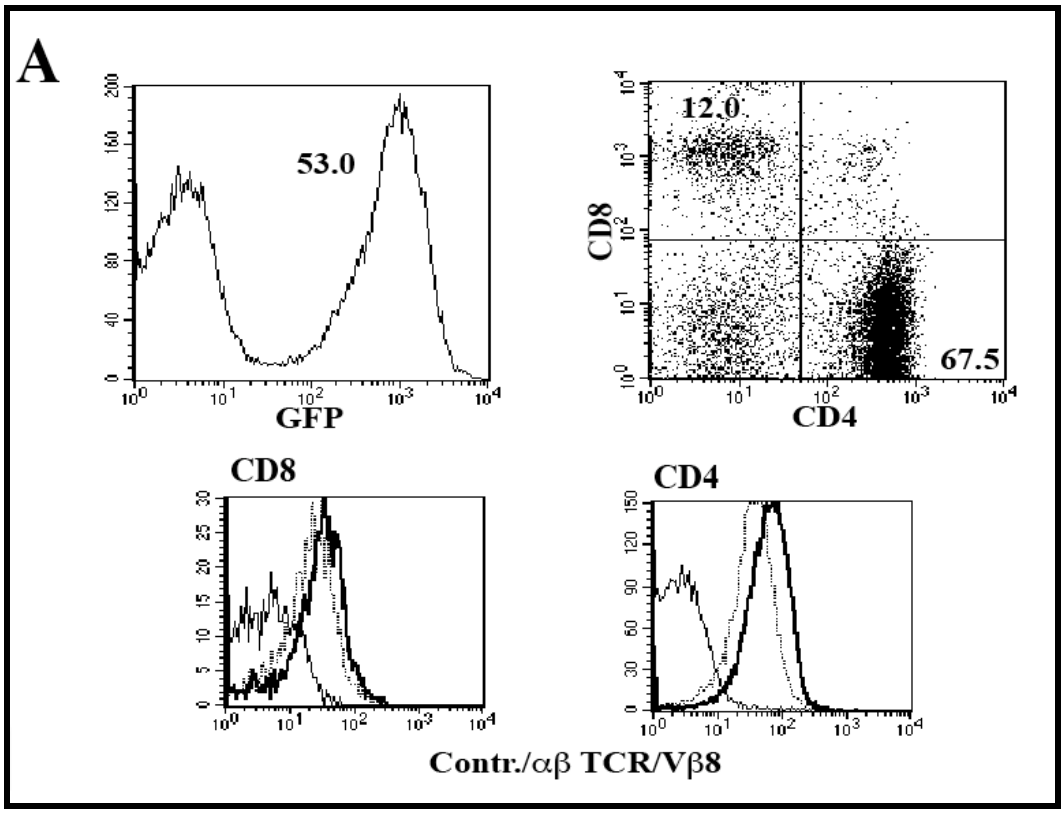


Figure 5A

GFP, CD4, CD8 and TCR expression by lymph-node cells of a CD3ε-deficient mouse at 8 weeks after injection of PAX5/TCRβ double deficient pro B cells transduced with a Vβ8 using TCRβ chains derived from αβT cells. The CD4-CD8 dot plot represents GFP positive cells. The histograms show αβ TCR (bold line) and Vβ8 (dotted line) expression on gated CD8 and CD4 cells. Thin line histograms represent the negative controls. Numbers represent the percentage of one representative mouse among 5-10 analyzed.

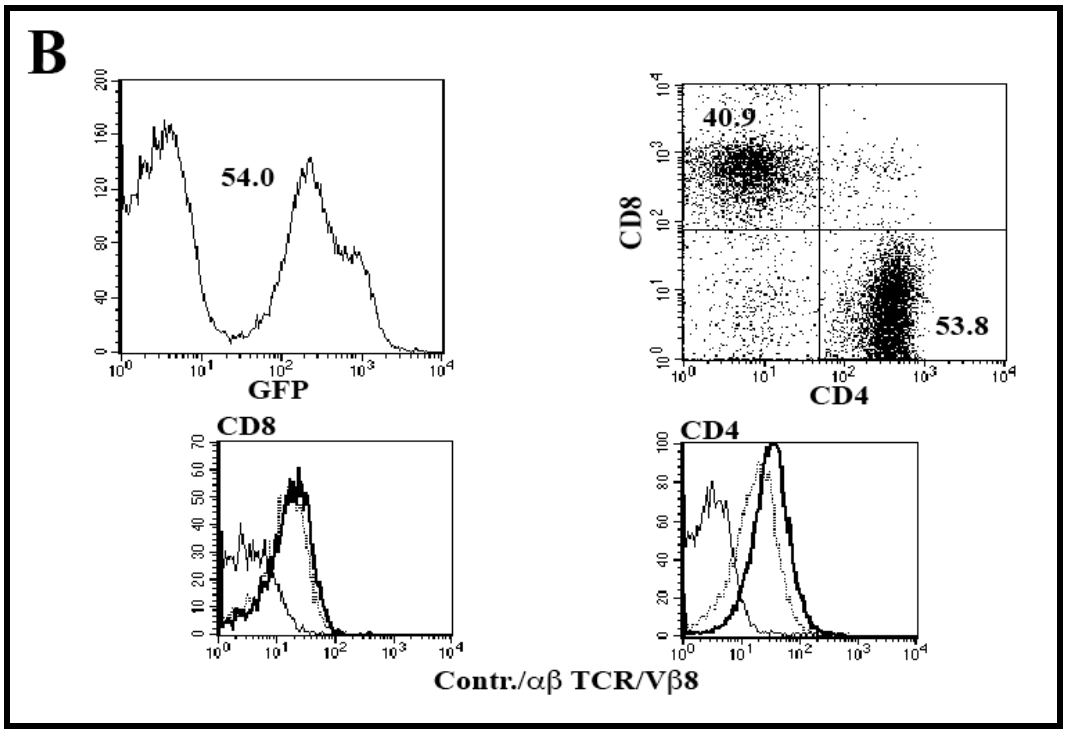


Figure 5B

GFP, CD4, CD8 and TCR expression by lymph node cells of a CD3ε-deficient mouse at 8 weeks after injection of PAX5/TCRβ double deficient proB cells transduced with a Vβ8 using TCRβ chains derived from γδ T cells. The CD4-CD8 dot plot represents GFP positive cells. The histograms show αβ TCR (bold line) and Vβ8 (dotted line) expression on gated CD8 and CD4 cells. Thin line histograms represent the negative controls. Numbers in quadrants represent the percentage of one representative mouse among 5-10 analyzed.

Figure 5B shows the equivalent staining from a CD3ε-deficient mouse reconstituted with cells transduced with a γδ T cell-derived Vβ8 TCR chain. In this mouse, 54% of cells were GFP^+ with 53.8% GFP^+ cells expressing CD4 and 40.9% expressing CD8. Again, all CD4 and CD8 cells expressed a Vβ8 αβTCR (Figure 5B).

αβ T cells in reconstituted mice are functional

To address the functionality of these αβ T cells, their responsiveness to anti-CD3ε antibody activation was measured. Figure 5C shows the results of such an experiment. Thus T cells obtained from mice reconstituted with cells transduced with αβ or γδ-derived TCRβ chains responded equally well and similar to T cells derived from wild type Bl/6 mice, thereby indicating that these T cells were fully functional.

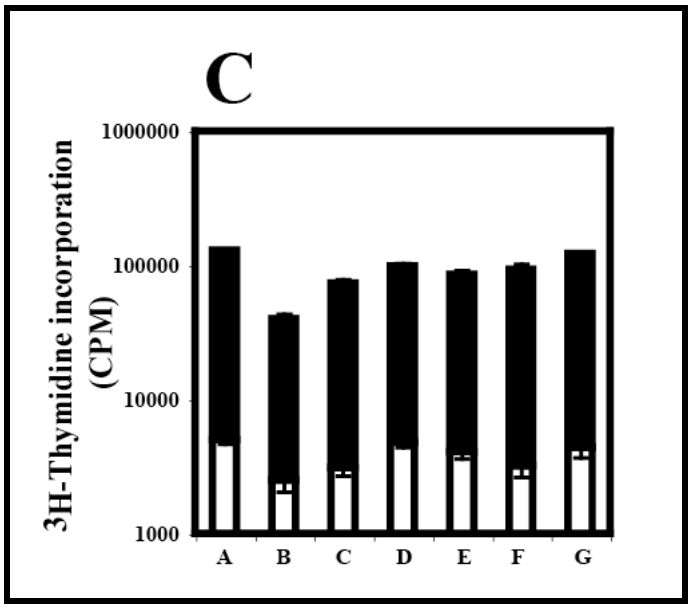


Figure 5C

^{3}H-thymidine incorporation upon anti-CD3 stimulation. Groups A-C are MACS purified T cells from CD3ε-deficient mice injected with PAX5/TCRβ double deficient proB cells transduced with different TCRβ chains derived from γδ T cells. Groups D-F are MACS purified T cells from CD3ε-deficient mice injected with PAX5/TCRβ double deficient proB cells transduced with different TCRβ chains derived from αβ T cells. Group G represents T cells derived from wild type B6 mice. White columns show the amount of ^{3}H-thymidine incorporated in absence anti-CD3 stimulation.

In vitro activation of ex vivo isolated T cells derived from P5TB cells transduced with different TCRβ's

In order to test whether these T cells were also able to induce T cell dependent humoral immune response, various groups of mice were injected with NIP-Ova in alum and 14 days later, the specific IgG anti-NIP titers determined. Serum taken at day 7 before immunization was used as a negative control and serum from identically immunized Bl/6 mice as a positive control. CD3ε deficient mice in which the T cell compartment was reconstituted with cells expressing TCRβ chains from either γδ or αβ T cells had IgG anti-NIP titers 3 -10 fold above control levels (Figure 5D). In fact, in every individual mouse, the titer was higher after immunization, indicating that these T cells could provide help for a humoral response. However, the anti-NIP titers in the reconstituted mice were 5-10-fold lower than in wild type Bl/6 mice (Figure 5D). This latter result most likely reflects the oligoclonality of the T cell repertoire generated in these reconstituted mice restricted as it is by the expression of a single TCRβ chain. Importantly, the anti-NIP titers of CD3ε-deficient mice reconstituted with Treg alone were not significantly different from non-reconstituted CD3ε-deficient mice (data not shown).

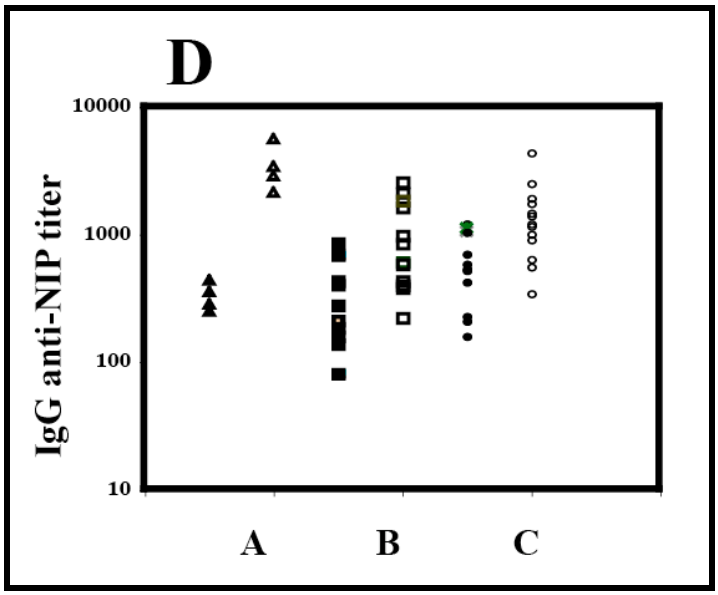


Figure 5D

Depicted are IgG anti-NIP titers at 7 days before (filled symbols) and 14 days after NIP-Ova immunization (non-filled symbols). A (▲, △) shows wild type B6 mice (n=4), B (■, □) shows titers found in mice injected with PAX5/TCRβ double deficient proB cells transduced with different TCRβ chains derived from γδ T cells (n=12) and C (●, ○) shows titers found in mice injected with PAX5/TCRβ double deficient proB cells transduced with different TCRβ chains derived from αβ T cells (n=13).

Discussion

Although it has been known for many years that γδ T cells can express a TCRβ chain (Wilson and MacDonald, 1998), the reason why these cells did not end up in the αβ T cell lineage is still obscure. Using a novel strategy for reconstituting the T cell compartment of recipient mice with cells expressing individual TCRβ chains, the data presented here clearly and formally demonstrate that TCRβ chains isolated from γδ T cells can take part in αβ T cell lineage development and that the resulting mature T cells are fully functional.

Previously, we have shown that for developing B cells, not all expressed IgH chains could pair with the surrogate light chain molecules λ5 and VpreB to form a pre BCR (ten Boekel et al., 1997). In an analogous fashion, one could have imagined that for T cell development, not all the TCRβ chains would be able to pair with preTα and be able to form a pre TCR. This might have been particularly the case for TCRβ chains expressed by γδ T cells. However, the data presented herein strongly argue against this. All the TCRβ chains derived from γδ T cells used in this study were as efficient in the generation of αβ T cells as those derived from αβ T cells. The fact that thymus cellularity in reconstituted mice was close to that of normal wild type mice and much higher than that in preTα deficient mice (Fehling et al., 1995) strongly indicates that T cell development in these mice went via a preTCR-driven proliferation stage. Previously it has been shown that allelic exclusion of the TCRβ locus is mediated by the preTCR (Aifantis et al.,

1997; Krotkova et al., 1997). Recently, using αβ T cell-derived transgenic TCRβ chains, Ferrero et al (Ferrero et al., 2007) have analyzed TCRβ allelic exclusion specifically in γδ T cells of both wild type and preTα deficient mice. They clearly demonstrated that in wild type γδ T cells, expression of a TCRβ transgene resulted in allelic exclusion of the endogenous TCRβ locus, whereas on a preTα deficient genetic background, they did not. This result also indicates indirectly that TCRβ chains expressed by γδ T cells can form a preTCR.

At least 3 different scenarios could be imagined to explain why TCRβ-expressing γδ T cells can nevertheless develop. First, γδ T cells that have been generated and selected and therefore committed to the γδ lineage could still rearrange and express a TCRβ chain, which in this case might be inert. Whether such a scenario is feasible is rather questionable since these cells would have to still express the various enzymes required for the rearrangement after selection and commitment. That the TCRβ chains isolated from γδ cells seem to be fully functional also makes this scenario unlikely.

Second, γδ T cells expressing a TCRβ chain might have developed from a precursor that did not express preTα and thus, despite expressing TCRβ protein, were unable to form a pre TCR. The preTα "knock-in" Cre-reporter mouse recently described by the Fehling group (Luche et al., 2007) would be an ideal tool with which to test whether γδ T cells are derived from precursors in which the preTα gene was never expressed. Third, and to us the most appealing scenario, TCRβ expressing γδ T cells are derived from cells that co-express the γδ TCR and the pre TCR. The strength of TCR signal that these cells would receive via their γδ TCR would determine their fate. Thus, and as recently shown (Haks et al., 2005; Hayes et al., 2005; Kreslavsky et al., 2008), a strong signal would drive these cells into γδ T cell lineage in these cells would TCRβ$^+$ while a weak or no signal would drive these cells into the αβ T cell lineage. When true, the expression of a pre TCR would not automatically be an absolute sign for αβ T cell commitment as previously has been suggested (Kreslavsky et al., 2008).

Expression of and signaling via the TCRβ/preTCR complex at the DN3 stage leads to a very strong proliferative expansion and differentiation of cells to the next stage of development, namely DN4 cells. This proliferation accounts for the fact that about 75% of the VDJ TCR β rearrangements found in DN4 cells are in frame (Dudley et al., 1994; Kisielow and von Boehmer, 1995; von Boehmer, 2005). This process is also referred to as preTCR mediated β selection (Dudley et al., 1994; Kisielow and von Boehmer, 1995; von Boehmer, 2005). Mertsching et al. (Mertsching et al., 1997) determined the in frame/out of frame ratios of VDJ TCRβ rearrangements in fetal thymic γδ T cells and found 42% of them to be in frame. Since this is about the double of the percentage one would expect from a non-selected repertoire (i.e. 22.2% in frame) these authors concluded that some γδ T cells in the thymus were β selected. A

similar conclusion was reached by Wilson et al. (Wilson and MacDonald, 1998) by showing that about twice as many thymic γδ T expressing a TCRβ were in cell cycle compared to those that did not. However, it should be noted that pre TCR expression is not a requirement for γδ T cell generation since TCRβ (Mombaerts et al., 1992) as well as preTa (Fehling et al., 1995) deficient mice have normal, or even elevated, numbers of γδ T cells.

Another point worth mentioning is the finding that CD3ε-deficient mice transplanted with the Pax5/TCRβdouble deficient pro-B cells transduced with the empty vector generated decent numbers of γδ T cells in the thymus whereas those injected with TCRβ chains derived from either γδ or αβ T cells did not. These findings suggest that early expression of TCRβ chains prevents γδ T cell development or makes it very inefficient (Gerber et al., 2004; von Boehmer et al., 1988).

Recently, we showed that sublethally-irradiated RAG-2 and CD3ε deficient mice reconstituted with wild type syngeneic bone marrow cells develop a fatal autoimmune disease whose induction could be prevented by the co-transplantation of Treg cells (Benard et al., 2006). The development of a similar type of disease is also observed here in mice injected with TCRβ transduced cells and not with those transduced with the empty vector. Thus, the presence of αβ T cells expressing the rather limited (only 1 TCRβ chain) T cell repertoire derived from these transduced cells might not be responsible for causing this disease. Also in this experimental system the disease could be prevented by the co-injection of Treg cells. Despite reconstituted mice containing FoxP3$^+$ cells in both thymus and periphery, by themselves, they were incapable of preventing the onset of autoimmunity. The crucial questions arising from these observations are why in these chimeric settings are insufficient endogenous TReg generated and why should there be a requirement for co-transfer of TReg to prevent autoimmunity? Thus far, we have not been able to answer these questions. Nevertheless, by preventing the onset of autoimmune disease, we have been able to address the functional activity of the T cells derived from the TCRβ transduced cells. With respect to anti-CD3 stimulation, our findings demonstrate that they are practically identical to T cells from wild type mice. Moreover, the T cells derived from TCRβ transduced cells were also able to provide help for a T cell-dependent B cell response. However, in these mice, antibody titers were 5 -10 fold lower than those observed in wild type mice. This latter is most likely due to the fact that in the chimeric mice, TCR receptor diversity is limited by the expression of a single TCRβ chain.

Previously the Pax5 deficient pro B cell system has successfully been used to analyze mutant forms of the LAT adaptor in vivo (Ardouin et al., 2005). Herein, we have demonstrated that TCRβ chains isolated from γδ T cells can very efficiently participate in αβ T cell development and can give rise to functional T cells. Thus the reconstitution of mice with Pax5 deficient pro B

cells expressing various genes is an easy, rapid and very powerful way to analyze the function of such genes in T cell development in vivo.

Materials and Methods

Mice

C57Bl/6, CD3ε deficient (Malissen et al., 1995) and Foxp3/EGFP reporter (Wang et al., 2008) mice were maintained in our own animal SPF facilities. Female mice were used at 6 to 12 weeks of age and all experiments were carried out according to Institutional guidelines. $PAX5^{+/-}$ (Urbanek et al., 1994) and $TCR\beta^{-/-}$ (Mombaerts et al., 1992) mice were bred at the former Basel Institute for Immunology to generate $PAX5^{-/-}$ $TCRb^{-/-}$ double deficient mice.

TCRβ chain cloning and vector construction

Full-length Vβ4, Vβ6 and Vβ8 using TCRβ cDNA's were amplified from cDNA of sorted lympnode γδ or αβ T cells using the following primers:

TCRβ-Vβ4-Fwd: Atagctgcagcctgtgtgacactgctatg

TCRβ-Vβ6-Fwd: atagctgcaggaaactccctccaaactatg

TCRβ-Vβ8-Fwd: Atcaagcttcatatcctagaggaagcatg

TCRβ-Cβ1-Rev: ataggatccatcttcacatctgacttcatga

TCRβ-Cβ2-Rev: Ataggatccataaaagtttgtctcaggaattt

Products were cloned into the pGem Teasy vector (Promega) after specific digestion with restriction enzymes (bold characters above are introduced restriction sites digested with PstI for Vβ4 and Vβ6 or HindIII for Vβ8 and BamHI for Cβ1 or Cβ2) and sequenced. In frame products were subsequently cloned into the pMIG plasmid (Addgene plasmid 9044), allowing the bi-cistronic expression of TCRβ together with green fluorescent protein (GFP). The retroviral vectors were transfected into the Phoenix retroviral packaging line (ATCC Number: SD 3443).

Cell culture and retroviral infection

Pro B cells were derived from $PAX5^{-/-}$ $TCR\beta^{-/-}$ double deficient mice and were grown on a monolayer of γ-irradiated ST-2 stromal cells in IMDM supplemented with $5x10^{-5}$M β-mercaptoethanol, 1mM glutamine, 0.03‰ (w/v) Primatone (Quest, Naarden, NL), 100U/ml penicillin, 100 μg/ml streptomycin and 2% FCS and in the presence of recombinant IL-7. Cells

were transferred every 3 days on a new layer of irradiated stromal cells. Pro-B cells were retroviral transduced by spin-infection using standard procedures. Infections resulted in 10-20% GFP positive cells. High GFP expressing cells were sorted using a FACS ARIA (BD Biosciences) and expanded.

For anti-CD3 stimulation 96-well plates were coated with 50 µl PBS containing 5µg/ml anti-CD3ε (clone: 2C11) overnight at 4 degree. After extensive washing 10^5 purified T cells were added per well. T cells were purified by MACS according to the manufacturer's instruction. After 3 days 1 µCi/well ^{3}H-thymidine was added for the last 18 h prior to harvesting.

Reconstitution of CD3ε$^{-/-}$ mice

CD3ε$^{-/-}$ mice at 6-12 weeks of age were γ-irradiated (450 Gray) and 3-5 h thereafter i.v. injected with $5x10^6$ pro B cells and in some experiments with $2x10^5$ Treg cells. Treg cells were obtained from Foxp3/EGFP reporter mice by sorting.

Antibodies and flow cytometry

The following mAb's labeled with various fluorochromes were purchased from BD Biosciences: anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-TCRβ(H57-597), anti-γδTCR (GL3) and anti-Vβ8 (F23.1). Cell staining was performed as previously described (Rolink et al., 1996). Samples were analyzed using a FACSCalibur (Becton Dickinson). For cell sorting a FACSAria was used (Becton Dickinson).

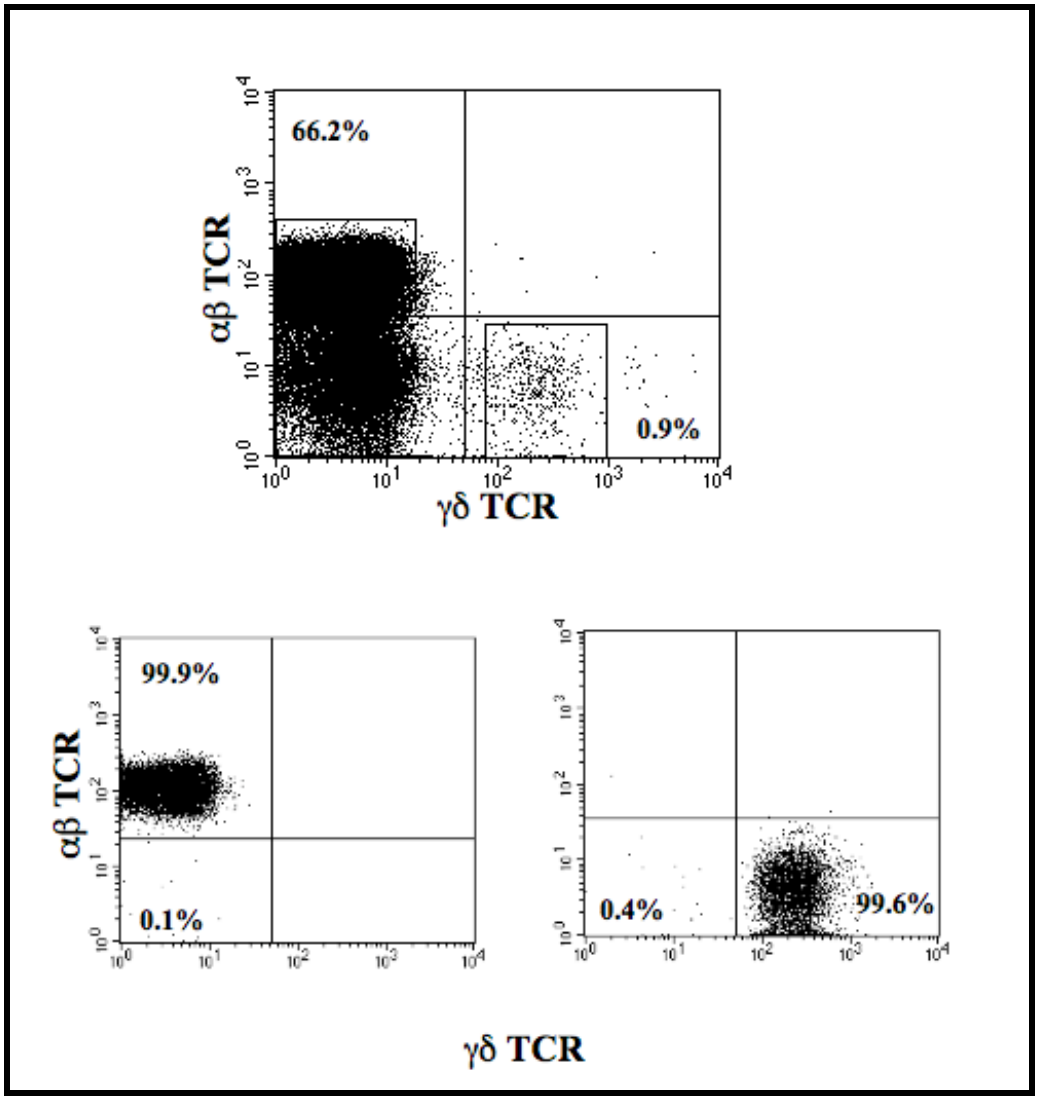
Immune responses

Mice were immunized i.p. with 100µg NIP-Ova (Biosearch Technologies Inc., Novato, CA) in alum. At day 14 after immunization the mice were bled and the serum IgG anti-NIP titer was determined by ELISA as previously described (Schubart et al., 1996).

Histology

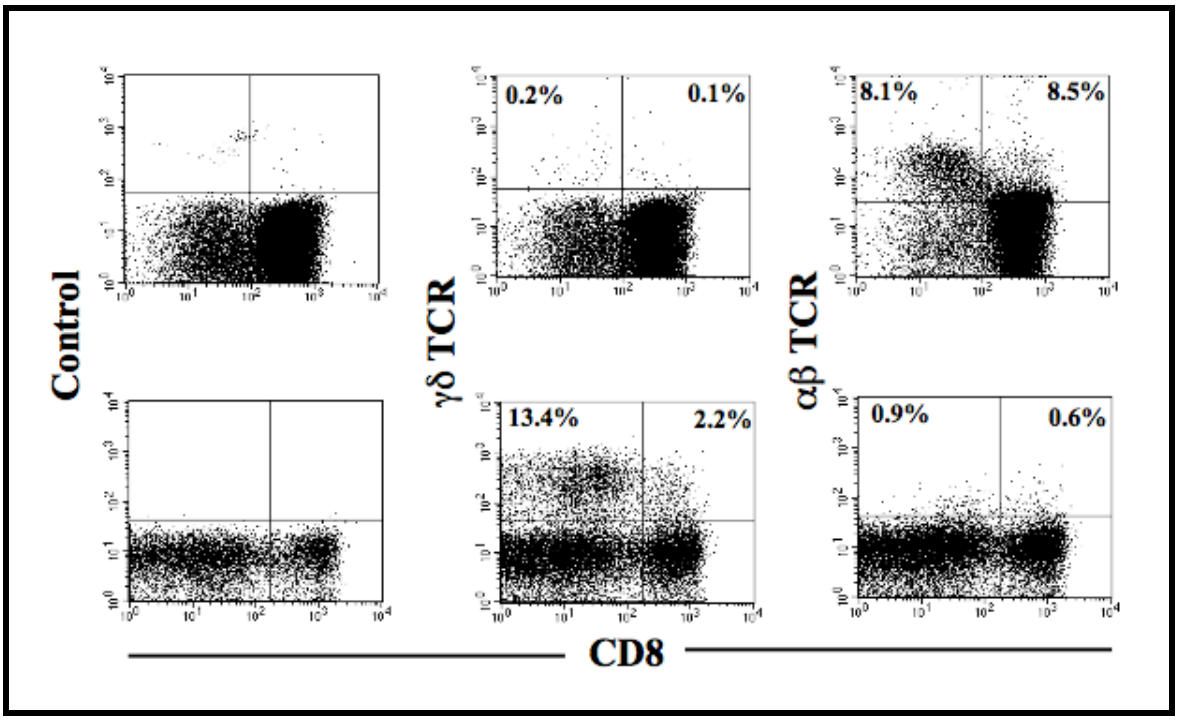
Organs were fixed in 4% paraformaldehyde and embedded in parafin. Sections of 3µm were prepared and stained with hematoxylin/eosin.

Supplementary data



Supplementary Figure 1. Gating strategy and post-sort analysis of LN derived αβ and γδ T cells.

Inguinal, axillary and brachial LN from 4-week old C57Bl/6 mice were pooled and cell suspensions stained with APC-labeled anti-TCRαβ (H57) and PE-labeled anti-TCRγδ (GL3) prior to being sorted with a FACS Aria (BD Biosciences). The upper panel shows a cytogram display of stained cells and the gates used for sorting. Figures in % show the proportion of gated cells. Below are shown post-sort cytograms indicating that the purity of sorted TCRαβ cells was 99.9% (left cytogram) and of TCRγδ cells 99.6% (right cytogram) in both cases, contaminating cells were not of the opposite TCR specificity.



Supplementary Figure 2. Expression of TCRβ chains by PAX5/TCRβ double deficient proB cells abolishes TCRγδ expression.

Shown are cytogram displays of gated GFP+ thymocytes stained with the indicated markers from mice reconstituted with PAX5/TCRβ double deficient proB cells transduced with either TCRβ-expressing (upper panels), or empty-vector (lower panels). As shown in the middle panel, expression of a TCRβ chain abolished TCRγδ expression. Results are from a representative mouse. Numbers in quadrants represent the percent positive cells.

Supplementary Table I. Shown are the sequences and Vβ family names of six TCRβ chains from either αβ or γδ sorted LN T cells used in this study for transduction of PAX5/TCRβ double deficient proB cells. Above each sequence, is from the left to the right, the name of the given clone, its αβ or γδ origin and Vβ family.

12.3	alpha-beta	V beta 4	

ATGGGCTCCATTTTCCTCAGTTGCCTGGCCGTTTGTCTCCTGGTGGCAGGTCCAGTCGACCCGAAAATTATCCAGAAACC
AAAATATCTGGTGGCAGTCACAGGGAGCGAAAAAATCCTGATATGCGAACAGTATCTAGGCCACAATGCTATGTATTGGT
ATAGACAAAGTGCTAAGAAGCCTCTAGAGTTCATGTTTTCCTACAGCTATCAAAAACTTATGGACAATCAGACTGCCTCAA
GTCGCTTCCAACCTCAAAGTTCAAAGAAAAACCATTTAGACCTTCAGATCACAGCTCTAAAGCCTGATGACTCGGCCACAT
ACTTCTGTGCCAGCAGCCAGGAGGGGGGGCCGGTGCAGAAACGCTGTATTTTGGCTCAGGAACCAGACTGACTGTTC
TTGAGGATCTGAGAAATGTGACTCCACCCAAGGTCTCCTTGTTTGAGCCATCAAAAGCAGAGATTGCAAACAAACAAAAG
GCTACCCTCGTGTGCTTGGCCAGGGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGTGGGTGAATGGCAAGGAGGTC
CACAGTGGGGTCAGCACGGACCCTCAGGCCTACAAGGAGAGCAATTATAGCTACTGCCTGAGCAGCCGCCTGAGGGTC
TCTGCTACCTTCTGGCACAATCCTCGAAACCACTTCCGCTGCCAAGTGCAGTTCCATGGGCTTTCAGAGGAGGACAAGTG
GCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGGCCGAGCAGACTGTGGAATCACTTC
AGCATCCTATCATCAGGGGGTTCTGTCTGCAACCATCCTCTATGAGATCCTACTGGGGAAGGCCACCCTATATGCTGTGC
TGGTCAGTGGCCTGGTGCTGATGGCCATGGTCAAGAAAAAAAATTCCTGA

137.6	gamma-delta	V beta 6	

ATGAACAAGTGGGTTTTCTGCTGGGTAACCCTTTGTCTCCTTACTGTAGAGACCACACATGGTGATGGTGGCATCATTACT
CAGACACCCAAATTCCTGATTGGTCAGGAAGGGCAAAAACTGACCTTGAAATGTCAACAGAATTTCAATCATGATACAATG
TACTGGTACCGACAGGATTCAGGGAAAGGATTGAGACTGATCTACTATTCAATAACTGAAAACGATCTTCAAAAAGGCGAT
CTATCTGAAGGCTATGATGCGTCTCGAGAGAAGAAGTCATCTTTTTCTCTCACTGTGACATCTGCCCAGAAGAACGAGAT
GGCCGTTTTTCTCTGTGCCAGCAGGACTGGGGGGCAAGACACCCAGTACTTTGGGCCAGGCACTCGGCTCCTCGTGTTA
GAGGATCTGAGAAATGTGACTCCACCCAAGGTCTCCTTGTTTGAGCCATCAAAAGCAGAGATTGCAAACAAACAAAAGGC
TACCCTCGTGTGCTTGGCCAGGGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGTGGGTGAATGGCAAGGAGGTCCA
CAGTGGGGTCAGCACGGACCCTCAGGCCTACAAGGAGAGCAATTATAGCTACTGCCTGAGCAGCCGCCTGAGGGTCTC
TGCTACCTTCTGGCACAATCCTCGAAACCACTTCCGCTGCCAAGTGCAGTTCCATGGGCTTTCAGAGGAGGACAAGTGG
CCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGGCCGAGCAGACTGTGGAATCACTTCA
GCATCCTATCATCAGGGGGTTCTGTCTGCAACCATCCTCTATGAGATCCTACTGGGGAAGGCCACCCTATATGCTGTGCT
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31.1	alpha-beta	V beta 6	

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GTACTGGTACCGACAGGATTCAGGGAAAGGATTGAGACTGATCTACTATTCAATAACTGAAAACGATCTTCAAAAAGGCG
ATCTATCTGAAGGCTATGATGCGTCTCGAGAGAAGAAGTCATCTTTTTCTCTCACTGTGACATCTGCCCAGAAGAACGAG
ATGGCCGTTTTTCTCTGTGCCAGCAGTGGGGACACCTTGTACTTTGGTGCGGGCACCCGACTATCGGTGCTAGAGGATC
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CATCAGGGGGTTCTGTCTGCAACCATCCTCTATGAGATCCTACTGGGGAAGGCCACCCTATATGCTGTGCTGGTCAGTG
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146.1	gamma-delta	V beta 8	

ATGTCTAACACTGCCTTCCCTGACCCCGCCTGGAACACCACCCTGCTATCTTGGGTTGCTCTCTTTCTCCTGGGAACAAA
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TGCTGGCAGCACTGAGAAAGGAGATATCCCTGATGGATACAAGGCCTCCAGACCAAGCCAAGAGAACTTCTCCCTCATT
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GGGCTTTCAGAGGAGGACAAGTGGCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGGC
CGAGCAGACTGTGGGATTACCTCAGCATCCTATCAACAAGGGGTCTTGTCTGCCACCATCCTCTATGAGATCCTGCTAGG
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174.10	gamma-delta	V beta 8	

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AATGGAGAGAGATAAAGGAAACCTGCCCAGCAGATTCTCAGTCCAACAGTTTGATGACTATCACTCTGAGATGAACATGA
GTGCCTTGGAGCTAGAGGACTCTGCCGTGTACTTCTGTGCCAGCTCTCTCTTAAGCTCCTATGAACAGTACTTCGGTCCC
GGCACCAGGCTCACGGTTTTAGAGGATCTGAGAAATGTGACTCCACCCAAGGTCTCCTTGTTTGAGCCATCAAAAGCAGA
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GCCACCCTATATGCTGTGCTGGTCAGTGGCCTGGTGCTGATGGCCATGGTCAAGAAAAAAAATTCCTGA

38.7	alpha-beta	V beta 6	

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TACTGGTACCGACAGGATTCAGGGAAAGGATTGAGACTGATCTACTATTCAATAACTGAAAACGATCTTCAAAAAGGCGAT
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CGTGTTAGAGGATCTGAGAAATGTGACTCCACCCAAGGTCTCCTTGTTTGAGCCATCAAAAGCAGAGATTGCAAACAAAC
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CAAGTGGCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGGCCGAGCAGACTGTGGAAT
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CTGTGCTGGTCAGTGGCCTGGTGCTGATGGCCATGGTCAAGAAAAAAAATTCCTGA

22.2	alpha-beta	V beta 6	

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GTGACAGAGGATCTGAGAAATGTGACTCCACCCAAGGTCCCCTTGTTTGAGCCATCAAAAGCAGAGATTGCAAACAAACA
AAAGGCTACCCTCGTGTGCTTGGCCAGGGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGTGGGTGAATGGCAAGGA
GGTCCACAGTGGGGTCAGCACGGACCCTCAGGCCTACAAGGAGAGCAATTATAGCTACTGCCTGAGCAGCCGCCTGAG
GGTCTCTGCTACCTTCTGGCACAATCCTCGCAACCACTTCCGCTGCCAAGTGCAGTTCCATGGGCTTTCAGAGGAGGAC
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46.4	gamma-delta	V beta 4	

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TCCTATGCAAGCCACCACAGCTCTCTTACATCTCAGTGTAGGAGTGAATGTGGAACATCAGAGGATCTGAGAAATGTGAC
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TGTCTGCCACCATCCTCTATGAGATCCTGCTAGGGAAAGCCACCCTGTATGCTGTGCTTGTCAGTACACTGGTGGTGATG
GCTATGGTCAAAAGAAAGAATTCATGA

68.5	gamma-delta	V beta 6	

ATGAACAAGTGGGTTTTCTGCTGGGTAACCCTTTGTCTCCTTACTGTAGAGACCACACATGGTGATGGTGGCATCATTACT
CAGACACCCAAATTCCTGATTGGTCAGGAAGGGCAAAAACTGACCTTGAAATGTCAACAGAATTTCAATCATGATACAATG
TACTGGTACCGACAGGATTCAGGGAAAGGATTGAGACTGATCTACTATTCAATAACTGAAAACGATCTTCAAAAAGGCGAT
CTATCTGAAGGCTATGATGCGTCTCGAGAGAAGAAGTCATCTTTTTCTCTCACTGTGACATCTGCCCAGAAGAACGAGAT
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AAAGGCTACCCTCGTGTGCTTGGCCAGGGGCTTCTTCCCTGACCACGTGGAGCTGAGCTGGTGGGTGAATGGCAAGGA
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TGTGCTTGTCAGTACACTGGTGGTGATGGCTATGGTCAAAAGAAAGAATTCATGA

80.2	gamma-delta	V beta 6	

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TACTGGTACCGACAGGATTCAGGGAAAGGATTGAGACTGATCTACTATTCAATAACTGAAAACGATCTTCAAAAAGGCGAT
CTATCTGAAGGCTATGATGCGTCTCGAGAGAAGAAGTCATCTTTTTCTCTCACTGTGACATCTGCCCAGAAGAACGAGAT
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CAAGTGGCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGGCCGAGCAGACTGTGGAAT
CACTTCAGCATCCTATCATCAGGGGGTTCTGTCTGCAACCATCCTCTATGAGATCCTACTGGGGAAGGCCACCCTATATG
CTGTGCTGGTCAGTGGCCTGGTGCTGATGGCCATGGTCAAGAAAAAAAATTCCTGA

86.4	alpha-beta	V beta 8	

ATGTCTAACACTGCCTTCCCTGACCCCGCCTGGAACACCACCCTGCTATCTTGGGTTGCTCTCTTTCTCCTGGGAACAAA
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CAGACTAATAACCACAACAACATGTACTGGTATCGGCAGGACACGGGGCATGGGCTGAGGCTGATCCATTATTCATATGG
TGCTGGCAGCACTGAGAAAGGAGATATCCCTGATGGATACAAGGCCTCCAGACCAAGCCAAGAGAACTCCTCCCTCATT
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GCCTGAGCAGCCGCCTGAGGGTCTCTGCTACCTTCTGGCACAATCCTCGAAACCACTTCCGCTGCCAAGTGCAGTTCCA
TGGGCTTTCAGAGGAGGACAAGTGGCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGGG
CCGAGCAGACTGTGGAATCACTTCAGCATCCTATCATCAGGGGGTTCTGTCTGCAACCATCCTCTATGAGATCCTACTGG
GGAAGGCCACCCTATATGCTGTGCTGGTCAGTGGCCTGGTGCTGATGGCCATGGTCAAGAAAAAAAATTCCTGA

99.1	alpha-beta	V beta 8	

ATGTCTAACACTGCCTTCCCTGACCCCGCCTGGAACACCACCCTGCTATCTTGGGTTGCTCTCTTTCTCCTGGGAACAAA
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CAGACTAATAACCACAACAACATGTACTGGTATCGGCAGGACACGGGGCATGGGCTGAGGCTGATCCATTATTCATATGG
TGCTGGCAGCACTGAGAAAGGAGATATCCCTGATGGATACAAGGCCTCCAGACCAAGCCAAGAGAACTTCTCCCTCATT
CTGGAGTTGGCTACCCCCTCTCAGACATCAGTGTACTTCTGTGCCAGCGGTGATGCTTATTCTGGAAATACGCTCTATTTT
GGAGAAGGAAGCCGGCTCATTGTTGTAGAGGATCTGAGAAATGTGACTCCACCCAAGGTCTCCTTGTTTGAGCCATCAAA
AGCAGAGATTGCAAACAAACAAAAGGCTACCCTCGTGTGCTTGGCCAGGGGCTTCTTCCCTGACCACGTGGAGCTGAGC
TGGTGGGTGAATGGCAAGGAGGTCCACAGTGGGGTCAGCACGGACCCTCAGGCCTACAAGGAGAGCAATTATAGCTAC
TGCCTGAGCAGCCGCCTGAGGGTCTCTGCTACCTTCTGGCACAATCCTCGCAACCACTTCCGCTGCCAAGTGCAGTTCC
ATGGGCTTTCAGAGGAGGACAAGTGGCCAGAGGGCTCACCCAAACCTGTCACACAGAACATCAGTGCAGAGGCCTGGG
GCCGAGCAGACTGTGGGATTACCTCAGCATCCTATCAACAAGGGGTCTTGTCTGCCACCATCCTCTATGAGATCCTGCTA
GGGAAAGCCACCCTGTATGCTGTGCTTGTCAGTACACTGGTGGTGATGGCTATGGTCAAAAGAAAGAATTCATGA

Part IV: T cell mediated tolerance in double transgenic mice co-expressing OVA specific TCR and OVA as agonistic antigen.

Introduction

As reported earlier, T cell tolerance is mediated through diverse mechanisms at different levels. In the thymus, central tolerance is achieved through negative selection and regulatory T cell development. In the periphery it is the result of a combination of effects, namely induction of apoptosis, activation induced cell death, regulatory T cell mediated suppression and anergy. Models of choice for studying T tolerance are double transgenic mice expressing a given antigen and a TCR specific for this same antigen. The choice of the promoter used in transgenic constructs allows for the restriction of antigen expression in specific cell subsets and therefore for the study of different mechanisms of tolerance. These models enable to follow Ag specific T cells fate, tolerance or autoimmunity in the periphery being the direct read out in these double transgenic mice.

Despite strong negative selection, CD11c-HA x TCR-HA mice suffer severe systemic auto-immunity and hyper-inflammation symptoms start at 3 weeks of age (Figure 1A). Most of them consequently died about 5 weeks after birth (Figure 1B). On the opposite KLC-HAxTCR-HA double transgenic mice display no detectable antinuclear auto-antibodies (ANA).

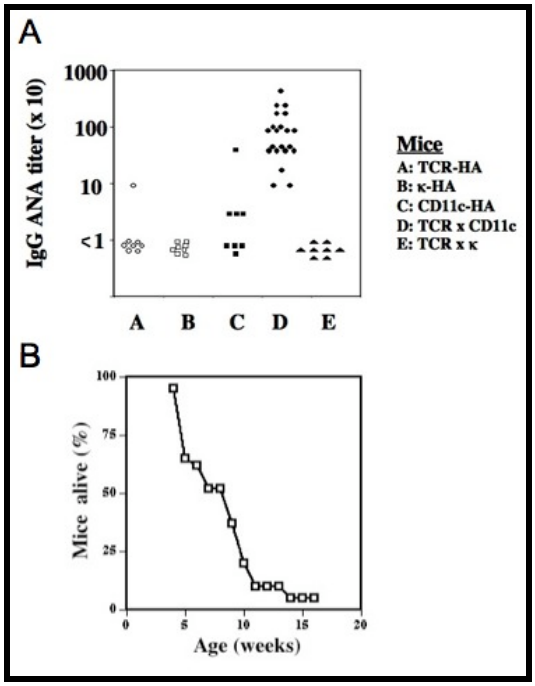

Figure 27: CD11c-HAxTCR-HA mice suffer from systemic autoimmunity.

A. IgG ANA titers in serum of TCR-HA and HA double transgenic mice. When comparing different groups of double transgenic mice expressing specifically HA in various cell types, only the mice expressing HA in the DCs population and expressing HA specific TCR, suffer from autoimmune

disease. They display very high titer of ANA. In contrast the level of ANA in KLC-HAxHA-TCR mice is in the normal range of a wild type mouse. Those mice are protected from autoimmunity.

B. CD11c-HAxTCR-HA mice succumb to systemic autoimmunity starting at 5 weeks of age. The presence of antinuclear auto-antiodies, extensive lymphocytic infiltration and arthritis leads to rapid death of these double transgenic mice.

When CD11c-HA mice were crossed with TCR-HA mice, DCs express HA in the thymus and induce negative selection of the T cells expressing HA specific TCR.

However a specific population of T cells was able to escape negative selection thanks to TCR editing, by expressing on their surface a second TCR α chain while down regulating autoreactive alpha chain.

After being positively selected, these dual TCR expressing T cells reach the periphery where they express on their surface two different TCRs, one of the two being autoreactive.

The encounter with self-antigen, namely HA presented by DCs in the periphery, leads to their proliferation and triggers effector functions. As a consequence, mice die of systemic autoimmunity (Figure 2).

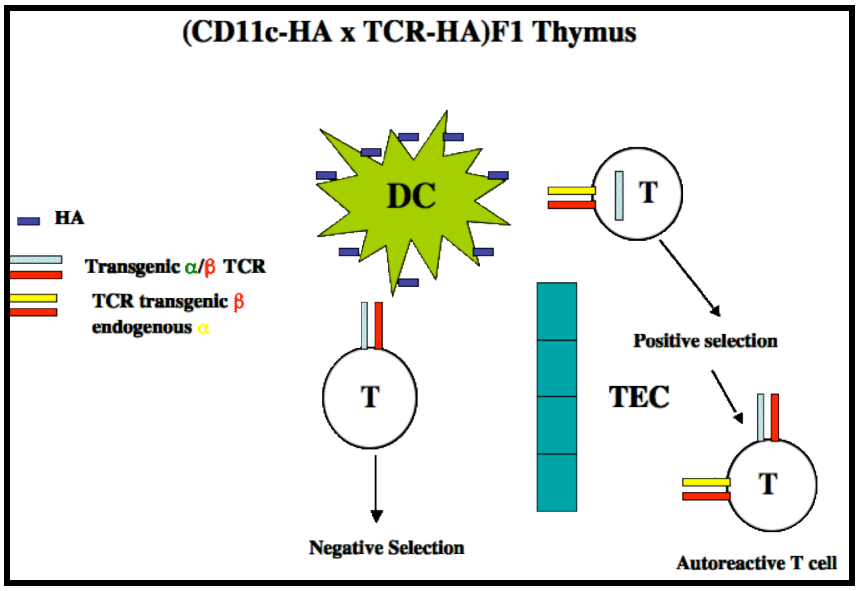

Figure 2: Theoretical model of T cell mediated autoimmunity development in CD11c-HAxTCR-HA mice.

When HA is presented by thymic DCs, the majority of HA specific T cells are eliminated by negative selection. However the few T cells undergoing receptor editing, down regulate transgenic α chain. Hence they are able to escape negative selection and are responsible for autoimmunity in the periphery.

On the contrary, double transgenic mouse models, where agonist antigen is presented by TECs, have been reported to have an increase in Treg population (Jordan et al., 2001). This led to the conclusion that interaction of a T cell with its agonist antigen presented by radio-resistant TECs leads to Treg development. As an example, KLC-HA x TCR-HA showed sharp increase of regulatory T cells, mice stayed healthy and did not show any sign of autoimmunity (Figure 3).

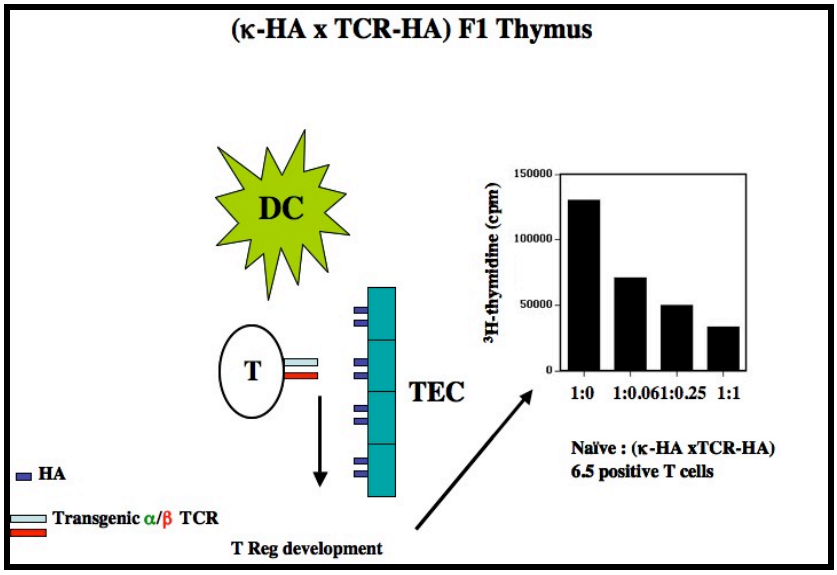

Figure 3: Theoretical model of Regulatroy T cell development in KLC-HAxTCR-HA mice.

In the alternative model where HA is expressed under the control of KLC promoter, thymic epithelial cells present the neo self-antigen to developing T cells thanks to promiscuous gene expression. This is supposed to drive HA specific TCR cells towards regulatory T cells development as reported in several other similar studies. In fact HA specific T cells can be found in higher proportion in the periphery where they have been shown to exert their suppressive function on effector HA specific T cells as shown on the graph. Autoimmunity in these mice is thought to be prevented by the higher proportion of regulatory T cells.

Following these observations two working hypotheses were proposed, the first one being that expression of HA by DCs promotes development of dual TCR expressing T cells responsible for autoimmunity, whereas when HA is presented by TECs, Tregs development is favoured and autoimmunity is prevented.

However the question still remains whether this phenomenon is specific to the HA system or if it can be generalized to any other neo-self antigen expression system. To resolve this issue, we generated double transgenic mice according to the same principle but using a different antigen namely chicken ovalbumin (OVA) as an intracellular protein antigen.

We generated for this purpose two different transgenic mouse strains expressing chicken OVA cDNA under the control of CD11c and KLC promoter, restricting expression to DCs and B cells respectively. We crossed these mice with OT1 and OT2 mice, carrying a transgenic TCR specific for OVA, respectively MHC-I and MHC-II restricted.

The generation of transgenic mice CD11c-OVA and KLC-OVA

We proposed to study tolerance under different conditions where OVA antigen expression is restricted to particular cell types. For this purpose we used two different DNA plasmid constructs

generated to direct *Ova* cDNA transcription by cell specific promoters, namely CD11c promoter to target expression of antigen to DCs and KLC promoter to target OVA expression to B cells.

Cloning of chicken ovalbumin cDNA

The chicken ovalbumin cDNA used for all constructs is a 1.8Kb digested out with EcoRI restriction enzyme from the *pAc-Neo-Ova* vector, a gift from Dr. M.J. Bevan (Moore et al., 1988). The cDNA encode for a soluble cytosolic protein of 385 amino acids (AA) with a molecular weight of 43kDa, OVA protein contains one disulfide bond and carries two major T cell epitopes encoded by $AA_{258-265}$ recognised by MHC-I restricted $CD8^+$ T cells of OT1 mice and $AA_{323-339}$ recognised by MHC-II restricted T cells of OT2 mice (Figure 2).

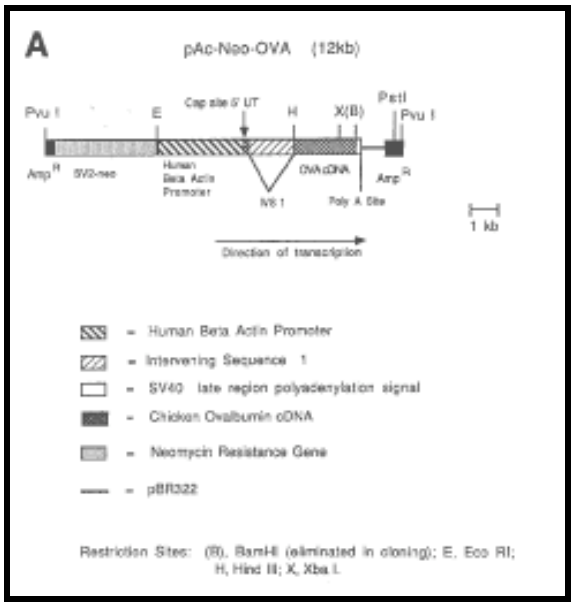

Figure 28: Restriction map of pAc-neo-*Ova*.

The complete *Ova* cDNA was subcloned into the mammalian expression vector pHI3APr-I-neo at the BamHI and HindIII sites, under control of the human 6-actin promoter. This plasmid also contains the neomycin resistance gene, under control of the *SV40* promoter, to provide a selectable marker for transfection. (Adapted from (Moore et al., 1988).

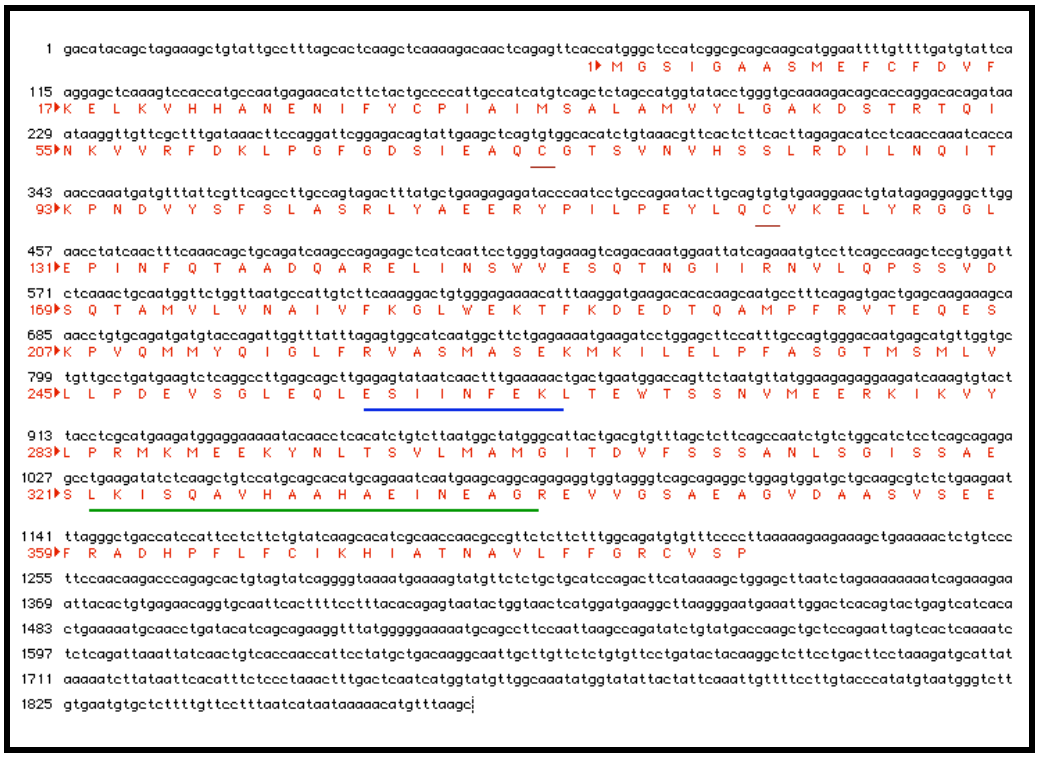

Figure 29: *Ova* sequence

In red is shown the open reading frame amino acid (AA) sequence corresponding to the OVA protein. Two cysteins engaged in a disulfide bond are shown in brown and OT1 and OT2 epitope AA sequence is displayed respectively in blue and green.

Cloning of the CD11c promoter-Ova DNA construct

Detection of the CD11c β2 integrin by mAb N418 has pointed out that its expression was restricted to mouse dendritic cells (Agger et al., 1990; Metlay et al., 1990). It is thought to be the most faithful molecule for dendritic cells and its corresponding promoter is considered to drive expression with a high specificity to dendritic cells of various tissues including spleen and thymus.

We used the pBSK(-)-CD11c promoter-HA construct originally obtained from Dr T. Brocker from which we cloned out HA sequence by using EcoRI sites. *Ova* cDNA was removed from the *pAC-Neo-Ova* vector from Michael Bevan by EcoRI mediated restriction digest and then inserted into pBSK(-)-CD11c promoter vector.

The pBSK(-)-CD11c promoter vector contains a long sequence containing 5900bp located upstream the CD11c start codon providing all the promoter elements (Brocker et al., 1997). It includes also a rabbit β-globin cDNA expression cassette containing splicing sites and polyadenylation signal generally used to stabilize and prevent the degradation of the cDNA of interest (Cavener and Ray, 1991). The *Ova* cDNA sequence was inserted into this cassette at the EcoRI site as it was reported to support productive DNA expression (Kouskoff et al., 1993).

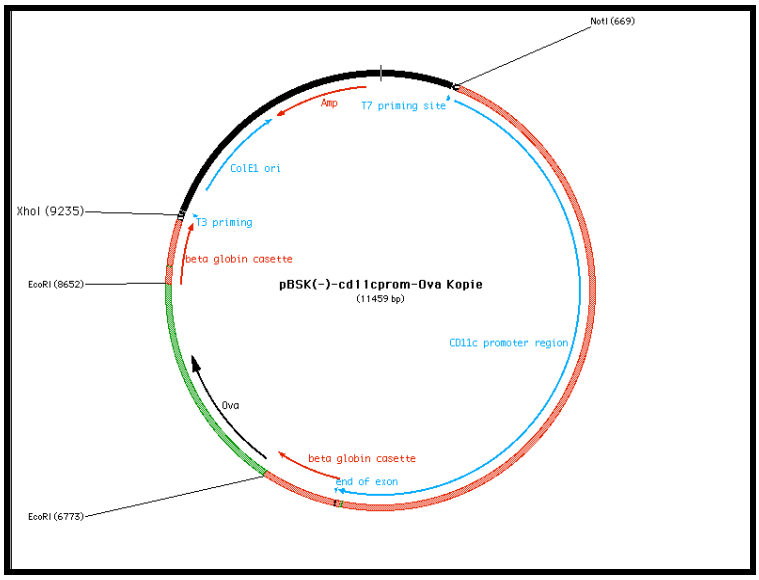

Figure 30: pBSK(-)-CD11c promoter-OVA

In green is shown OVA cDNA sequence surrounded by β-globin cassette in red. Enzyme restriction sites are indicated in black.

The linear DNA fragment used to generate transgenic mice was obtained by XhoI/NotI restriction digest and used to transform ES cells injected into blastocyst of Bl/6 mice.

Cloning of the KLC promoter-Ova DNA construct

The β-globin cassette was cloned out of the CD11c promoter construct, a pBSK(-) backbone by PstI/ClaI restriction digest. This 1.2kb fragment was inserted into a PstI/ClaI digested pBSK(+) backbone vector resulting in *pBSK-β-globin* cassette vector. This vector was digested with EcoRI and into that site the OVA fragment, isolated also by EcoRI from *pAC-Neo-Ova*, was inserted.

The KLC promoter and H.C enhancer representing a fragment of 2kb was cloned out of the vector *pCD4-Hg1 CE1*, kindly provided by Klaus Karjalainen, by a EcoRI/XbaI restriction digest. This fragment was inserted into a pBSK(+) copy digested by the same enzymes, EcoRI and XbaI. This insertion provided the *pBSK(+)-KLC-HC* vector with new restriction enzyme sites for EcoR5 and NotI, which were used to clone out the KLC promoter and H.C. enhancer fragment. This fragment was then inserted into the *pBSK-β-globin-Ova* beforehand digested with NotI and SmaI. EcoRV and SmaI are restriction enzymes generating blunt end DNA that allowed the ligation of KLC promoter and H.C. enhancer fragment to *pBSK-β-globin-OVA*.

Once again the linear DNA fragment used to generate transgenic mice was obtained by XhoI/NotI restriction digest. Transgenic constructs were transfected into packaging cells and tested for OVA expression in vitro.

Screening for the presence of Ova transgene in transgenic mice

Offsprings of foster mothers were analyzed for the presence of the OVA transgene. Hence, *Ova* specific PCR on tail DNA revealed the mice that had integrated the transgenic construct into their genome. The following primers specific for *Ova* were used: *Ova* upstream primer: 5'-CAATCCTGCCAGAATACTTGC-3' and *Ova* dowstream primer: 5'-TCCATCTTCATGCGAGGTAA-3' resulting in a PCR product of 554bp. As positive controls, primers specific for *β-actin* were used. After 39 cycles (20s 94°C, 30s 55°C, 80s 72°C) amplified DNA was detected on a 1.5% agarose gel containing ethidium bromide.

OT1 mice

OT1 mice were a kind gift of Ed Palmer. These mice were originally developed by Hogquist et Al (Barnden et al., 1998; Hogquist et al., 1994). OT1 mice express a Vα2/Vβ5 TCR derived from the K^b-restricted $OVA_{257-264}$ specific T cell clone 149.2. This TCR recognizes the OT1 peptide SIINFEKL in the context of H-2Kb.

OT1 mice display normal numbers of lymphocytes in the thymus and in the periphery. FACS analysis in the thymus showed a bigger CD8 SP T cell population than WT mice; with a parallel increase in CD4 SP T cell population (Figure 8). The expression of the specific Vα2/Vβ5 TCR starts early at the DN stage for 95% of the DN cells and stays high throughout all stages of thymic development. Surprisingly CD4 SP T cell subpopulation show also high expression of both transgenic TCR chains and $Va2^+Vb5^+$ cell population represents up to 90% of CD4 SP T cell population. In fact MHC-I restricted CD8SP T cells in OT1 mice are thought to engage toward CD4SP development pathway (Barnden et al., 1998). DP T cells showed decreased percentage of cells positive for Vα2/Vβ5, about 55% as well as decreased mean fluorescence intensity of Vα2 and Vβ5. It has been previously reported that TCR expression level is lower in DP and reaches its maximum expression level at SP stages. A quarter of this DP population does not express Vβ5 but is reduced by half at the following developmental stage of SP T cells. It is noteworthy that among the CD4 SP T cell population a significant percentage of cells, about 7%, undergo TCR editing and downregulate Vα2 chain. This phenomenon is not observed in the CD8 SP population. Eventually the highest expression level of Vα2/Vβ5 is found at the CD8 SP stage with 86% of cells displaying high mean fluorescence intensity.

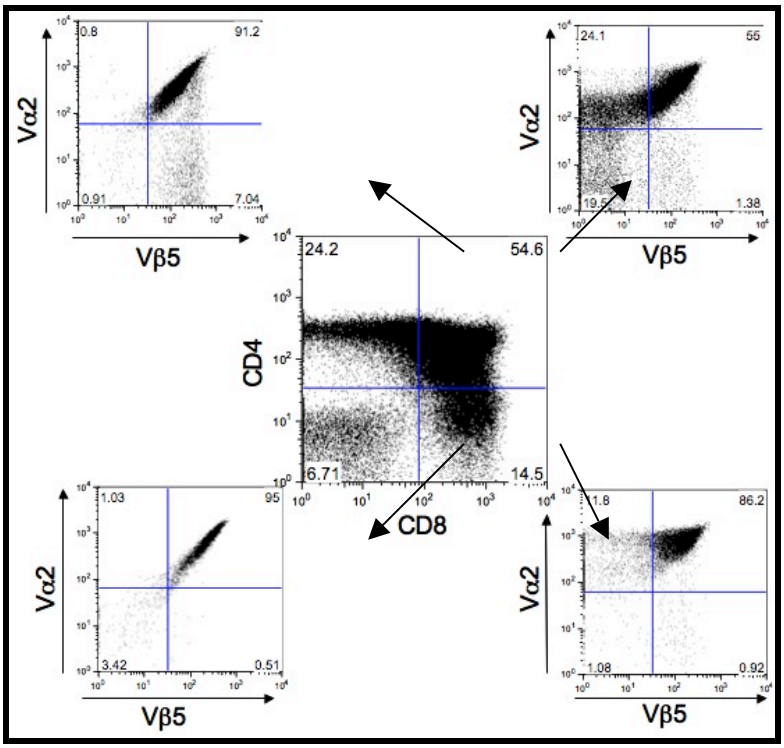

Figure 31: FACS analysis of thymic subpopulations from a Bl/6 OT1 mouse

The middle panel shows CD4 and CD8 expression on total thymocytes and Vβ5 and Vα2 expression on gated CD4 SP T cells (upper left), gated DP (upper right), gated DN (lower left) and gated CD8 SP (lower right). Numbers shown in each corner of the dot plots represents percentage of cells for each quadrant.

Eventually spleen and LN also showed normal numbers of lymphocytes compared to WT mice but with considerable skewing towards CD8 T cell population unlike in the thymus (Figure 6). The CD8 T cell population is more than 10 fold higher than the CD4 T cell population with almost 95% of cells being OVA specific. In contrast the CD4 T cell population is very small and the 20% of $V\alpha2^{+}V\beta5^{+}$ within $CD4^{+}$ T cell population might be considered as MHC-I restricted although this has not been checked. Again a minority of T cells showed TCR editing, being $V\beta5^{+}$ but $V\alpha2^{-}$.

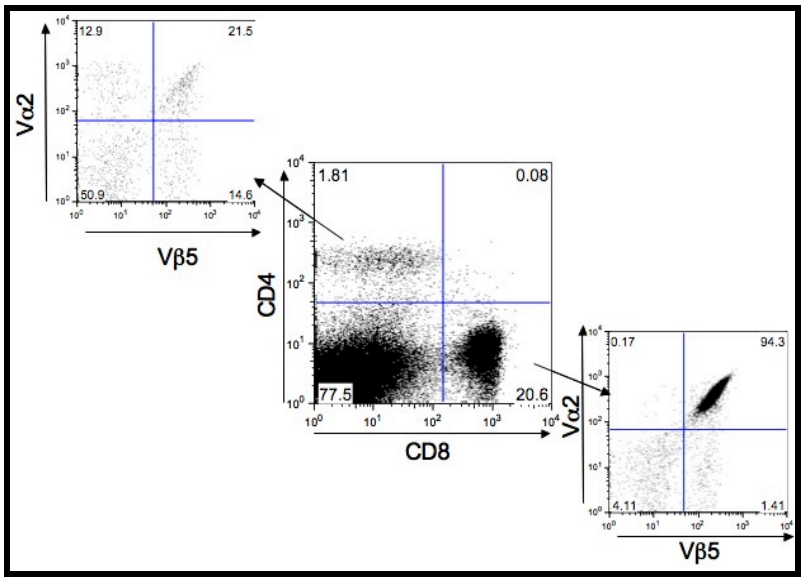

Figure 32: FACS analysis of a spleen of a Bl/6 OT1 mouse

The middle panel shows CD4 and CD8 expression on total splenocytes. Each CD4 and CD8 subpopulations are stained for Vβ5 and Vα2 on gated $CD4^+$T cells (upper left) and gated $CD8^+$ T cells (lower right).

OT2 mice

OT2 mice were a kind gift of Hans Acha-Orbea. These mice were originally developed by Barnden et Al (Barnden et al., 1998; Hogquist et al., 1994). OT2 mice on a Bl/6 background express also Vα2 and Vβ5 TCR that pairs with the CD4 co-receptor. This TCR is specific for $OVA_{323-339}$ peptide: ISQAVHAAHAEINEAGR in the context of $I\text{-}A^b$ MHC molecule.

These mice possess lower numbers of thymocytes, but lymphocyte numbers in the periphery are comparable to WT mice. FACS analysis of the thymus show forced TCR transgene expression already at the DN stage albeit at low level, this concerns only 8% of DN cells (Figure 7). The DP thymocyte population of OT2 mice display a higher percentage of cells expressing the transgenic TCR, about 20%, but again with lower mean fluorescence intensity. Eventually transgenic TCR expression reaches its strongest level in the SP $CD4^+$ T cell population. $V\alpha2^+V\beta5^+$ OVA specific T cells stand for 80% of the $CD4^+$ SP cells in the thymus. There is a significant skewing of T cell development towards $CD4^+$ T cell population since the transgenic TCR expressed by the majority of the T cells is MHC-II restricted, hence $CD8^+$ T cell compartment is very small and represents only about 1% of the whole thymic population. Moreover about 50% of the $CD8^+$ SP cells in these mice are $V\alpha2^+V\beta5^+$ suggesting that they are MHC-II restricted as well.

It is noteworthy that almost all SP CD4 T cell that are Vβ5 are also Vα2 positive whereas about 2% of the Vα2$^+$ CD4$^+$ SP are not Vβ5 positive. Since allelic exclusion process is almost perfect in the TCRβ locus it seems unlikely that these cells would have edited their TCRβ chain. It is probable that those cells represent γ/δ T cells since Vα2 mAb is known to cross-react with Vγ8 TCR chain. It has to be noted that the percentage of Vα2$^+$ T cell in CD4 SP of a WT thymus is about 12%.

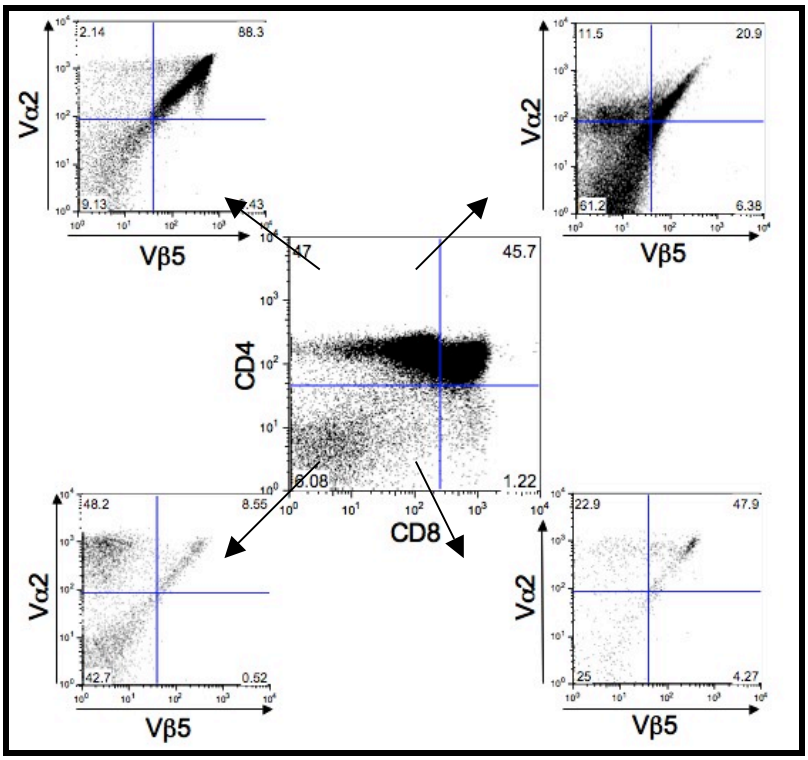

Figure 33: FACS analysis of thymic subpopulations from a Bl/6 OT2 mouse

The middle panel shows CD4 and CD8 expression on total thymocytes and Vβ5 and Vα2 expression on gated CD4 SP T cells (upper left), gated DP (upper right), gated DN (lower left) and gated CD8 SP (lower right). Numbers shown in each corner of the dot plots represents the percentage of cells for each quadrant.

In the spleen, the pronounced skewing toward CD4 T cell population is again particularly evident (Figure 8). CD4 T cell population is nearly four times bigger than the CD8 T cell population. The majority of CD4 T cells, namely more than 80%, are OVA specific TCR bearing T cells that are Vα2$^+$ and Vβ5$^+$, whereas it is not the case for the CD8 population where less than 20% are Vα2$^+$ and Vβ5$^+$. LN of OT2 mice show a very similar pattern of expression in the same populations.

It is noticeable that very few T cells in these mice undergo spontaneous TCR editing since nearly all Vβ5$^+$ are also Vα2$^+$.

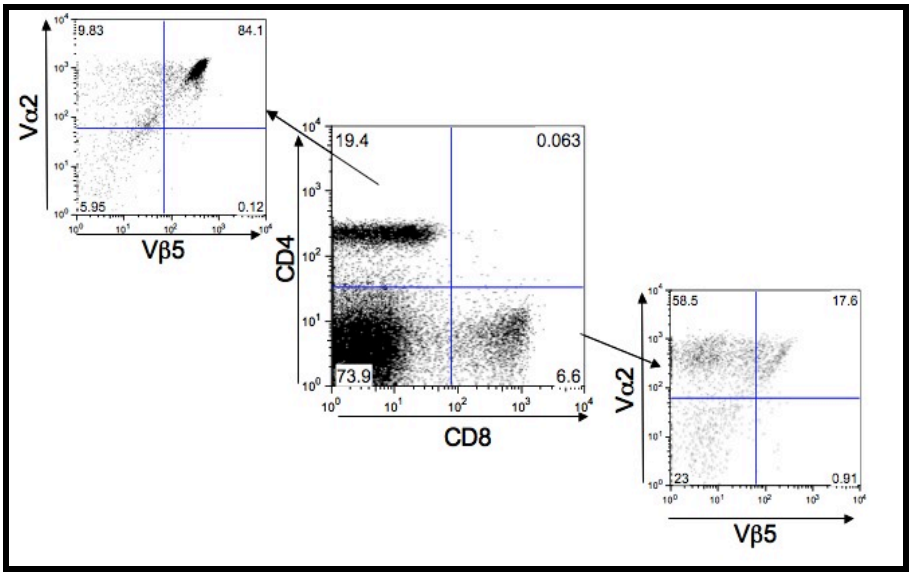

Figure 34: FACS analysis of a spleen of a Bl/6 OT2 mouse

The middle panel shows CD4 and CD8 expression on total splenocytes. Each CD4 and CD8 subpopulations are stained for Vβ5 and Vα2 on gated CD4$^+$T cells (upper left), and gated CD8$^+$ T cells (lower right). Numbers shown in each corner of the dot plots represents the percentage of cells for each quadrant.

Results

CD11c-OVAxOT1 and KLC-OVAxOT1

Single *Ova* transgenic mice were crossed with OT1 transgenic animals. Hence F1 mice were screened by PCR for OVA expression (as described earlier) and by FACS analysis for OT1 phenotype by using Vα2 and Vβ5 antibody.

Thymus, spleen and LN from these double transgenic animals were analyzed by flow cytometry in order to monitor OVA specific T cell fate. Two founders from each mouse line CD11c-OVA (designed as F3 and F4) or KLC-OVA (designed as F1 and F2) were selected for good negative selection. The following figures show data from one or the other founder, as representative for each mouse line since their phenotype were very similar. Cell numbers of thymus and spleen are shown in Figure 9. Mice were used between 6 to 11 weeks of age.

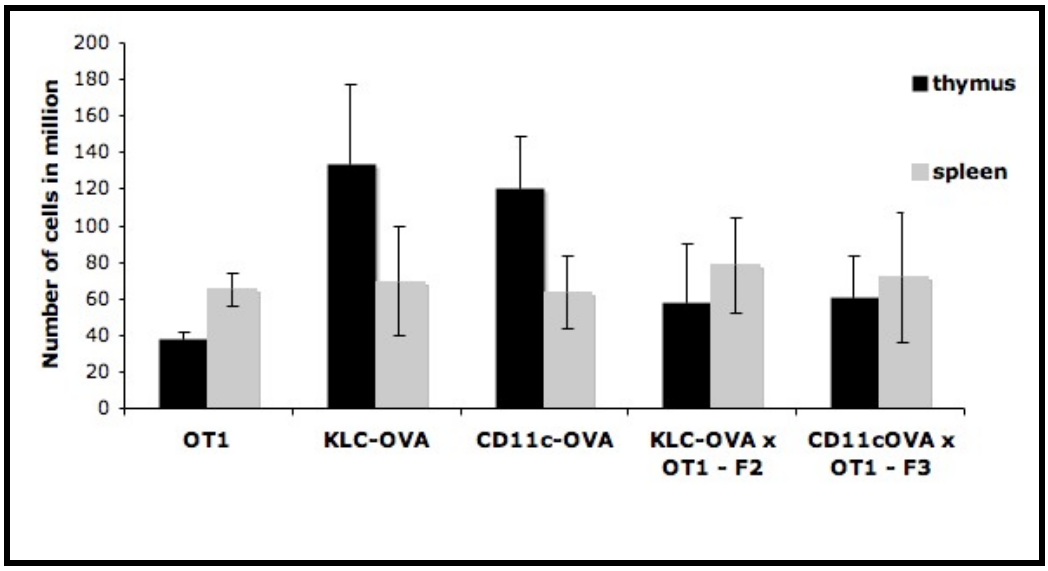

Figure 35: Thymocyte and splenocyte numbers of double transgenic mice KLC-OVAxOT1 and CD11c-OVAxOT1 compared with OVA single transegnic mice.

Here is shown mean and standard deviation of thymocyte and splenocyte numbers from 7 to 11 week old mice, either single or double transgenic: OT1 (n=2); KLC-OVA (n=6); CD11c-OVAxOT1 (n=6); KLC-OVAxOT1 (n=6); CD11c-OVAxOT1 (n=11).

Double transgenic mice displayed numbers of thymocytes that are similar to single transgenic OT1 mice. However, thymocyte numbers of double transgenic mice are lower than those of WT or single transgenic CD11c-OVA or KLC-OVA. This can be explained by the relative immuno-deficiency of OT1 mice, a feature that is common to several TCR transgenic mouse models (Hogquist et al., 1994). Spleen and LN cell numbers did not differ between single and double transgenic mice (Figure 9 and data not shown).

FACS analysis of the different thymic and peripheral T cell subpopulations showed that strong negative selection is going on in these double transgenic mice (Figure 10). Thus the CD8 SP compartment in the thymus dropped from about 23% in OT1 mice to 2% in double transgenic mice Moreover a 4 - 6 fold reduction of peripheral CD8 T cells was observed.

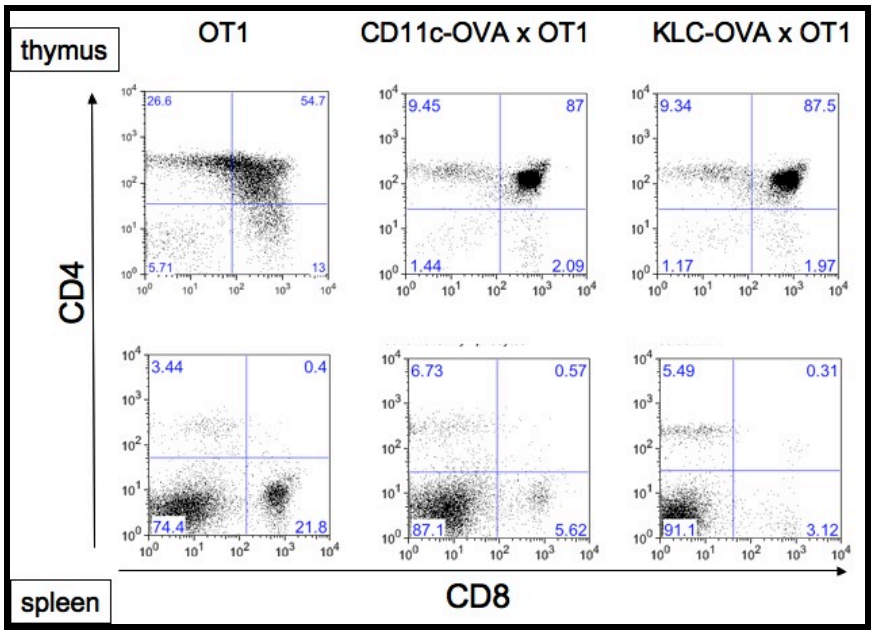

Figure 36: FACS analysis of thymic and spleen T cell subpopulations of single and double transgenic mice.

Organs of OT1, CD11c-OVAxOT1 and KLC-OVAxOT1 were removed, washed, stained with fluorescent anti-CD4 and anti-CD8 mAb and analysed by FACS. The upper row shows thymus cells and the lower row represents spleen cells. All graphs are gated on lymphocyte population. Numbers shown in each corner of the dot plots represents the percentage of cells for each quadrant.

The CD4 T cell compartment was affected as well, albeit to a lesser extent.

Along with percentages, cell numbers of CD8 SP population of double trangenic mice are also greatly decreased compared to OT1 single transgenic mice, this illustrates faithfully the negative selection mediated by OVA presentation in the thymus

CD11c-OVAxOT2 and KLC-OVAxOT2

The cellularity of the thymus in both double transgenic CD11c-OVAxOT2 and KLC-OVAxOT2 mice did not strongly differ from the single OT2 transgenic mice and was only slightly reduced compared to single transgenics CD11c-OVA and KLC-OVA of the same age. However CD4 SP population was absent in CD11c-OVAxOT2 and KLC-OVAxOT2 indicating strong negative selection in double transgenic mice. FACS analysis of thymocytes showed that the CD4 SP population dropped about 7 to 9 fold in double transgenic mice. Moreover, in the periphery the number of $CD4^+$ T cells was found to be 5 to 8 fold lower in double transgenic mice than in single OT2 mice and practically none of them expressed the OVA specific OT2 TCR (data not shown).

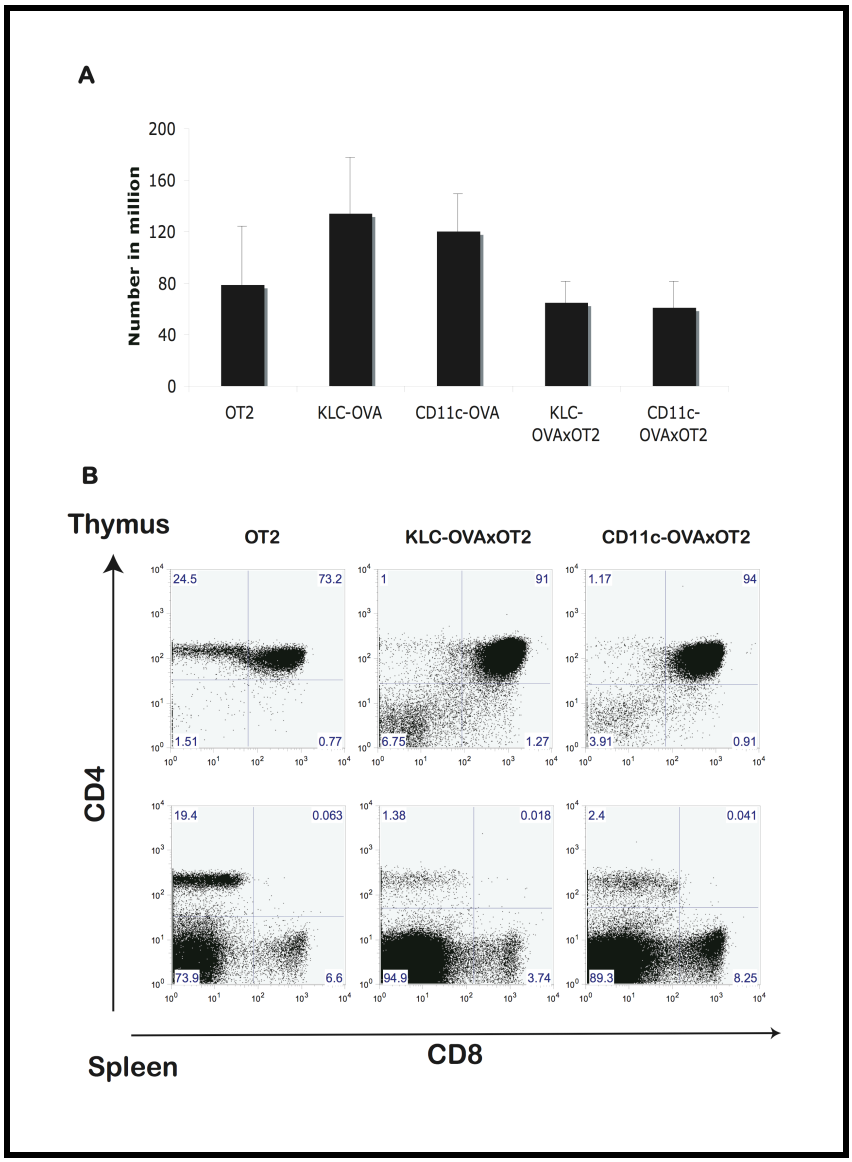

Figure 37: double transgenic mice CD11c-OVAxOT2 and KLC-OVAxOT2 showed strong negative selection of CD4 T cells.

A. Thymocyte numbers of double transgenic mice KLC-OVAxOT2 and CD11c-OVAxOT2. Here is shown mean and standard deviation of thymocyte numbers from 6 to 14 week old mice, either single or double transgenic: OT2 (n=2); CD11c-OVA (n=6), KLC-OVA (n=6); CD11c-OVAxOT2 (n=16); KLC-OVAxOT2 (n=8).

B. FACS analysis of thymic and spleen subpopulations of single and double transgenic mice. Organs of OT2, CD11c-OVAxOT2 and KLC-OVAxOT2 were removed, washed, stained with fluorescent anti-CD4 and anti-CD8 mAb and analysed by FACS. The upper row shows thymus cells and the lower row represents spleen cells. All graphs are gated on lymphocyte population. Numbers shown in each corner of the dot plots represents the percentage of cells for each quadrant.

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OT2 cells proliferate in vivo when encountering CD11c-OVA APCs and KLC-OVA B cells.

Given the results of the *in vitro* mixed lymphocyte reaction, we hypothesized that OT2 T cells adoptively transferred in double transgenic host would show an anergic phenotype as well. Interestingly OT2 cells, stained beforehand with CFSE to follow their division, show a high degree of proliferation in the periphery of the double transgenic hosts as soon as day 3 after adoptive transfer. In CD11c-OVA founder 3 and 4 after 3 days, only about 5% and less than 1% respectively were still CFSE high whereas in Bl/6 hosts the same population represented 75 to 80% in spleen and LN. Similar results were obtained when KLC-OVA transgenic animal were used as host mice. There is no difference between the proliferative capacity in the spleen or in the LN and very little differences found between founders. When Bl/6 hosts were adoptively transferred with OT2 cells and immunized 24 hours later subcutaneously by footpad injection with OVA in CFA as a positive control, no proliferation could be recorded as shown by the high MFI of the CFSE profile of OT2 cells in these mice. It might be due to early time point of analysis since high dilution of CFSE staining could be seen a day 6 in the same mice (data not shown). When markers for activation were analysed by FACS and despite extensive proliferation of OT2 cells in the periphery of double transgenic mice, we could only characterise naïve status of these cells. As a conclusion OVA specific T cells in the periphery of double transgenic mice are not anergic in vivo and even able to proliferate in the periphery. It might be interesting to examine further these adoptively transferred mice to follow development of any pathology on a long-term basis.

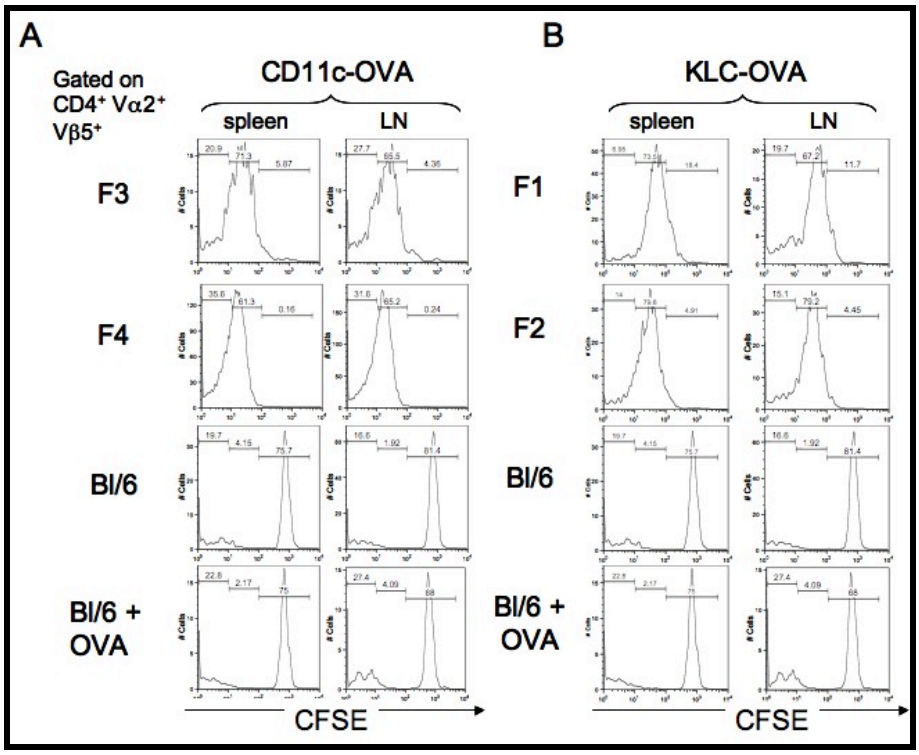

Figure 38: OT2 adoptively transferred into CD11c-OVA and KLC-OVA hosts proliferate in the periphery.

OT2 lymph node cells were stained with CFSE and injected intraveinously of CD11c-OVA (F3 and F4) or KLC-OVA (F1 and F2) or Bl/6 hosts (n=2-3 for each group). 24hrs later half of the Bl/6 mice were immunized subcutaneously with OVA/CFA and on day 3 after adoptive transfer spleen and lymph node cells were isolated, stained with fluorescent labelled mAb to perform FACS analysis and examined for CFSE dilution. Here is shown lymphocytes gated on $CD4^+V\alpha2^+V\beta5^+$ T cells OT2 cells.

Discussion

We have generated four different mouse strains expressing the ovalbumin antigen in specific cell subsets along with MHC I or MHC II restricted TCR specific for ovalbumin, i.e CD11c-OVAxOT1, KLC-OVAxOT1 and CD11c-OVAxOT2, KLC-OVAxOT2. These mice represent models to study central as well as peripheral T cell tolerance. We have shown that all these mice are tolerant and do not display any sign of autoimmunity unlike previously observed in a similar HA model using the same promoters (Curti et Al.). Sharp reductions of OVA specific CD8 SP or OVA specific CD4 SP T cells respectively for OT1 and OT2 systems in the thymus of double transgenic mice show that these populations were negatively selected and that negative selection is almost complete in these mice. However a few autoreactive T cells escape negative selection. In the periphery these OVA specific $V\beta5^+$ $V\alpha2^+$ T cells represent 0.1 to $1x10^6$ cells (data not shown). This number can be considered relatively low, however in a WT mouse the frequency of peripheral T cells specific for a given peptide epitope has been evaluated as 0.5 to $1x10^{-5}$ which is 150 to 1500 times less than the number of OVA specific T cells in double transgenic mouse. Despite the presence of a few OVA specific T cells in the periphery, their numbers do not seem to be sufficient for triggering autoimmunity, since no antinuclear

autoantibodies, splenomegaly or sign of arthritis could be detected. Similar to double transgenic OVA mice CD11c-HAxTCR-HA mice previously developed in our laboratory show practically complete negative selection. However, in contrast to CD11c-OVAxOT2 mice, significant numbers of phenotypically activated T cells expressing the TCR clonotype can be found in the periphery of CD11c-HAxTCR-HA. In addition the number of antigen specific T cells varies between the HA and the OVA model, whereas 6.5^+ TCR cells represent about 10% of the $CD4^+$ T cell peripheral population in CD11c-HAxTCR-HA, only 3% $CD4^+$ T peripheral T cells are $Va2^+Vb5^+$ cells. The difference between the two models may lie in the efficiency of antigen presentation. Despite clear evidence of thymus presentation of OVA shown by nearly complete negative selection, it might be hypothesized that OVA presentation in the periphery is qualitatively or quantitatively different from HA and is by consequence not able to activate and trigger proliferation of OVA specific T cells in the periphery. Our last experiment showing proliferation of naïve OT2 cells after adoptive transfer into CD11c-OVA as well as KLC-OVA mice disproves this last hypothesis. OVA in CD11c-OVA or KLC-OVA mice is efficiently presented and is able to trigger proliferation of OVA specific T cells. However double transgenic mice show limited proliferation of OVA specific T cells, one of the probable explanations for this lack of response despite efficient OVA presentation is that OVA specific T cells in double transgenic mice may display anergic properties.

The characterization of the anergic state of OVA specific T cells in double transgenic cells could be further analysed by measuring the response of these cells to ex vivo restimulation with OVA or in vivo immunization with antigen pulsed DCs. Because of the very low numbers of OVA specific T cells in the periphery of double transgenic mice, this experiment is technically challenging and could not be performed.

To account for the difference observed between the two systems, it is noteworthy that already single transgenic mice, namely TCR-HA and OT2 mice are different. Whereas the first strain display only 30 to 40% of clonotype positive $CD4^+$ T cells in the periphery, the second possess about 90% of OVA specific T cells in the periphery. The low frequency of clonotype positive T cells in TCR-HA mice could be interpreted as a result of a low probability of pairing between the α and the β chain of the HA-TCR. Indeed T cells from TCR-HA single transgenic show a high occurrence of TCR editing which is more pronounced in double transgenic mice. Conversely TCR editing seems to be a minor event in OVA double transgenic mice (data not shown). In fact TCR editing is supposed to be responsible for the development of autoimmunity in CD11c-OVAxTCR-HA mice, it allows HA-TCR T cells to escape thymic negative selection but give rise to dual TCR expressing cells which get activated in the periphery. Due to stronger negative selection and minimum TCR editing, a lower number of OVA specific T cells are found in the periphery, in addition these cells display anergic properties.

We have shown that Ova presentation in the periphery of CD11c-OVA as well as KLC-OVA mice is sufficient for triggering proliferation of naïve OT2 cells. However this proliferation does not lead to hyperinflammation in double transgenic mice. What could account for the anergy of OVA specific T cells compared to activation of HA specific T cells?

The nature of the antigen might influence the degree of activation in the periphery and could explain the discrepancies observed between the two models. Indeed OVA is a large protein of about 45kDa expressed intracellularly in CD11c-OVA as well as in KLC-OVA double transgenic mice. On the opposite HA is a highly immunogenic protein isolated from a virus and attached to the membrane. Because of its intracellular location, OVA would be expected to be processed and presented through MHC class I mediated presentation pathway to CD8 T cells. In addition it has been shown that professional APCs can capture antigen as debris from apoptotic antigen expressing cells by either receptor mediated endocytosis, pinocytosis or phagocytosis. Eventually if OVA is released extracellulary, APCs could load surface MHC-II molecules with OVA peptide. It is known that OVA specific T cells become anergic upon encounter with a soluble form of OVA (Lohr et al 2004). That is why OVA specific ELISA test was developed to detect any possible presence of OVA in the serum of those mice, but OVA concentration was below detection threshold of the ELISA (data not shown).

The relevance of the use of these two systems could also be discussed, in fact all double transgenic systems mentioned previously used MHC-II restricted TCR and MHC-II ligand as agonist antigen. In our case, the expression of the entire OVA protein displaying two different epitopes, each of them being the ligand for either MHC-I or MHC-II allowed to study tolerance of both CD4 and CD8 T cells by using OT1 and OT2 mice. We could show that negative selection happens in both CD8 and CD4 population.

Considering these elements it is assumed that OVA peptide presentation in the thymus would be relatively low especially in KLC-OVAxOT2 mice. In fact it is known that MHC products and MHC-peptide complexes densities are 10 to 100 times higher on DC than on other APCs like TECs through promiscuous gene expression (Inaba). This could explain the slight difference observed between negative selection in CD11c-OVAxOT2 and KLC-OVAxOT2 mice. However both levels of expression either on DCs or on TECs are sufficient to induce strong degree of negative selection.

The mechanisms of tolerance in the periphery have been well described for OVA as an agonist antigen by Kawahata et Al. (ref). They showed that OVA specific T cells in OVA expressing mice are able to proliferate in the periphery to a limited extent and get activated without triggering inflammation. The presence of negative selection in both double transgenic systems is a direct proof for OVA expression in the thymus, however it is possible that OVA expression

in the periphery of those mice would be weaker, this has been suggested by in vitro mixed lymphocyte reaction in single transgenic mice (data not shown). Although the level of Ova presentation is sufficient to provoke proliferation in vivo as shown by CFSE dilution of OVA specific T cells after adoptive transfer experiment, it might be too low to break tolerance and instead would induce anergy.

Furthermore the discrepancy between HA and OVA systems might lie in the affinity of the TCR to its specific antigen or in the antigen dose available. Indeed $CD4^+$ T cell stimulation with high antigen concentration or strong agonist peptides can bypass the need for costimulation, more specifically for CD28 signal (Teh hs 1997). This might well be the case in the HA double transgenic mice since HA is considered to bind to HA-specific TCR with very high affinity (jordan 2001). Although the affinity of OT1 T cells for $OVA_{257-264}$ peptide/K^b is relatively high with a K_d of about 6.5µM and the one of OT2 T cells for $OVA_{323-339}$ peptide/K^b is thought to be lower, this difference of affinity between the two models could account for the difference in their capacity of activating T cells.

Eventually the HA system developed previously in our lab and the OVA system described here present many dissimilarities and might not be fully comparable.

Current experiments are ongoing to breed our double transgenic mice on the RAG deficient background; this would lead to the production of only OVA specific T cells. Our prediction is that this would not however trigger autoimmunity in these mice. Another way to induce hyper inflammation would be to inject mice with CPG or LPS and see if activation of APCs would be able to induce response of OVA specific T cells. Finally long term adoptive transfer of naïve OT1 and OT2 cells into CD11c-OVA and KLC-OVA will allow us to check whether autoimmunity develops or not in OVA expressing host.

Materials and methods

Mice

CD11c-OVA and KLC-OVA mice were generated as indicated above. CD11c-OVA and KLC-OVA mice as well as C57Bl/6 mice were maintained in our own animal SPF facilities. Female mice were used at 6 to 12 weeks of age and all experiments were carried out according to Institutional guidelines.

Reagents and antibodies.

The following mAb were purchased from PharMingen (San Diego, USA): anti-CD25^{FITC} (7D4), FITC or biotin-conjugated anti-CD8α (53-6.7). Anti-CD4^{PE} (RM4-5).

Flow cytometric analysis

Single-cell suspensions of thymus, spleen and lymph nodes were prepared in PBS supplemented with 2% FBS and 0.2% sodium azide as described previously (Balciunaite et al., 2005b). Cells were adjusted to 20–10 _ 10^6 cells/ml and 0.5–1 _ 10^6 cells incubated for 30 min at 4°C with the indicated reagents at saturating concentrations as previously described. Stained cells resuspended in PBS 2% FBS 0.2% azide were analyzed using a FACSCalibur (Becton Dickinson) and data analyzed using FlowJo (Tree Star). Viable lymphoid cells were defined by a combination of FSC, SSC.

Adoptive transfer

LN cell suspensions from 3 to 5 OT2 donor mice were prepared. After red blood cell lysis, LN cells were washed, stained with 10μM CFSE for 5 min at room temperature, washed again and resuspended in DMEM prior to i.v. injection. CD11c-OVA and KLC-OVA or Bl/6 recipient mice received about 10x10^6 cells LN OT2 cells. Twenty-four hours later Bl/6 mice were immunized by footpad injection with OVA/CFA. Three days later mice were killed and spleen and LN were analyzed by FACS.

Abbreviations

3D	Three-dimensional
AA	Amino acids
AIRE	Autoimmune regulator
ANA	Antinuclear autoantibody
APECED	Autoimmune Polyendocrine Syndrome type 1
APC	Antigen presenting cells
ATOC	Adult thymic organ culture
BCL-2	B-cell lymphoma 2 anti-apoptotic protein
BCR	B cell receptor
BIM	BCL-2 interacting mediator
Bl/6	C57 BL6 mice
BM	Bone marrow
BMT	Bone marrow transplantation
BMdDCs	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMLP	Common myeloid lymphoid progenitor
CMV	Cytomegalovirus
CRP	C reactive protein
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte antigen 4

DC	Dendritic cell
DETC	Dendritic epidermal T cell
DMEM	Dulbecco's modified Eagle's medium
DN	Double negative
DP	Double positive
DsRNA	Double strand ribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EBF	Early B cell factor
ELISA	Enzyme-linked immunosorbent assay
ELP	Early lymphoid progenitor
ERK	Extracellular signal-regulated kinase
ETP	Early thymic progenitor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcεR1γ	High-affinity IgE receptor
FGF	Fibroblast growth factor
FLT3	Fms-related tyrosine kinase 3
FoxP3	Forkhead box P3
FTOC	Fetal thymic organ culture
GAD67	Glutamic acid decarboxylase
GC	Germinal center
GDP/GTP	Guanosine di/tri-phosphate
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GMP	Granulocyte/macrophage progenitor
GRB2	Growth factor receptor-bound protein 2
GvHD	Graft versus host disease
HA	Hemagglutinin

HSC	Haematopoietic stem cell
IBD	Inflammatory bowel disease
ID2	Inhibitor of DNA binding 2
IEL	Intraepithelial lymphocyte
IFN-g	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IRF	IFN regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	Jun-N terminal kinase
KD	Kilodalton
KLC	Kappa light chain
KI	Knock in
KO	Knock out
LAT	Linker for activation of T cells
LEF1	Lymphoid enhancer binding factor 1
Lck	Leukocyte specific tyrosine kinase
Lin^-	Lineage negative
LN	Lymph node
LPS	Lipopolysaccharide
LSL	Lineage negative, stem-cell antigen 1 positive, cKIT high
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEP	Megakaryocyte/erythroid progenitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MPP	Multipotential progenitor
MyD88	Myeloid differentiation primary response 88
NF-AT	Nuclear factor of activated T cell
NFκB	Nuclear factor kappa B
NK	Natural killer cell
NTreg	Natural regulatory T cell
Nurr-77	Nuclear orphan steroid receptor
OBF1	Octamer binding transcription factor- binding factor 1
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
Pax-5	Paired box protein 5
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE	Promicuous gene expression
PHSC	Pluripotent haematopeitic stem cell
PI	Propidium iodide
PLCγ	Phospholipase C gamma 2
PNA	Peanut agglutinin
PRR	Pathogen recognition receptor
PTEN	Phosphatase and tensin homologue
RAG	Recombination activating gene
Sca1	Stem-cell antigen 1
SCF	Stem cell factor
SOX-4	Sex determining region Y box 4
SP	Single positive
TBI	Total body irradiation
TCD	T cell depleted

TCR	T cell receptor
TD	Thymus dependant
TdT	Terminal deoxynucleotidyl transferase
M/cTEC	Medullary/cortical thymic epithelial cell
Teff	Effector T cell
TGFβ	Tramforming growth factor beta
Th	Helper T cell
TI	Thymus indepedant
Treg	Regulatory T cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor
TSA	Tissue-restricted self-antigen
TSLP	Thymic stromal lymphopoietin
TSP	Thymus settling progenitor
TX	Thymectomised
NTX	Non-thymectomised
WT	Wild type

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