Aspects of T cell development contributing to autoimmunity.

Carlsén, Maria

2008

ASPECTS OF T CELL DEVELOPMENT CONTRIBUTING TO AUTOIMMUNITY

Academic dissertation

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With due permission of the Medical Faculty of Lund University, to be presented for public examination at the Clinical Research Centre (CRC) Lecture Hall, Malmö University Hospital on January 18th 2008 at 9.15 a.m.

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Cover: Interaction between a lymphocyte (yellow) and a dendritic cell (blue).
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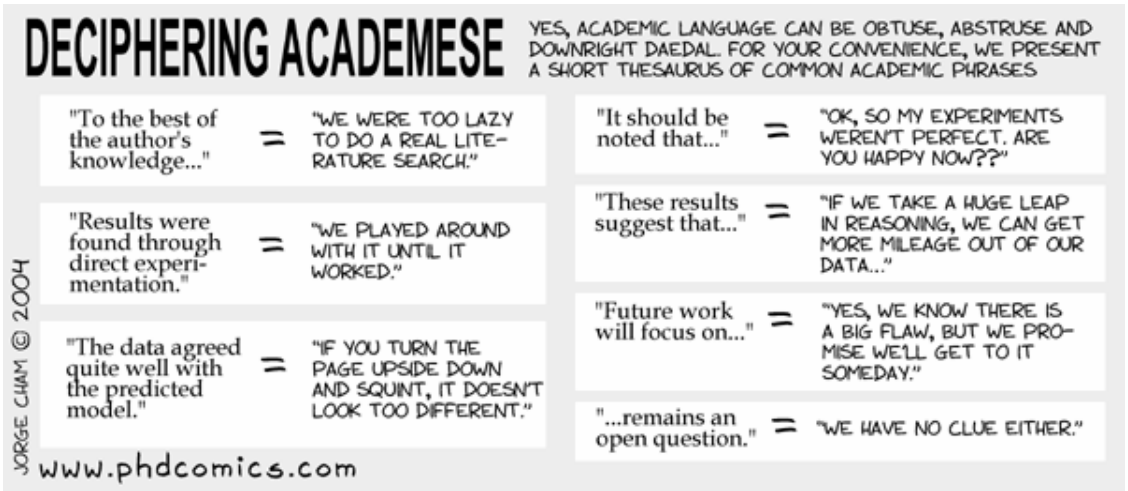
ISSN 1652-8220
ISBN 978-91-85897-56-8
Lund University, Faculty of Medicine Doctoral Dissertation Series 2008:5

Printed by Media-Tryck, Lund University, Lund, Sweden

Basic research is what I'm doing when I don't know what I'm doing

~Wernher Von Braun, German rocket scientist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Blood 2005 106:4234-4240

II. **Maria Carlsén** and Corrado M. Cilio: Normal T cell development in the absence of thymic insulin expression.
Ann. N.Y. Acad. Sci. 2006 1079: 205–212

III. **Maria Carlsén** and Corrado M. Cilio: Evidence for *de novo* expression of thymic insulin by peripheral bone marrow-derived cells.
Submitted for publication

IV. **Maria Carlsén***, Anna Makowska* and Corrado M. Cilio: Apoptosis resistance of non-obese diabetic (NOD) thymocytes is mediated by a defective p53 expression. *Contributed equally to the project
Submitted for publication

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ABBREVIATIONS

ACAD	Activated cell-autonomous death
AICD	Activation-induced cell death
AIRE	Autoimmune regulator
APC	Antigen presenting cell
BM	Bone marrow
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLP	Common lymphoid progenitor
Cre	Cyclisation recombination
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T lymphocyte associated antigen-4
CY	Cyclophosphamide
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
FTOC	Fetal thymic organ culture
GAD	glutamic acid decarboxylase
GC	Glucocorticoid
HLA	Human leukocyte antigen
HSA	Heat stable antigen
ICA	Islet cell autoantibody
IDDM	Insulin dependent diabetes mellitus
IDO	Indoleamine-2, 3-dioxygenase
IEL	Intraepithelial lymphocyte
IFN	Interferon
IL	Interleukin
IS	Immunological synapse
KO	Knock out
LN	Lymph nodes
LoxP	Locus of X-over of P1
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cells
NK	Natural killer cell
NO	Nitric oxide
NOD	Non-obese diabetic mouse
PAE	Peripheral antigen expressing cells
PGE	Promiscuous gene expression
RNA	Ribonucleic acid
SMAC	Supramolecular activation cluster

SP	Single positive
T1D	Type 1 diabetes mellitus
TβRII	TGF-β receptor type II
TcR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper cell
Tr1	Type 1 regulatory T cell
TRA	Tissue-restricted antigen
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
VNTR	Variable number of tandem repeats

ABSTRACT

Organization LUND UNIVERSITY Department of Clinical Sciences Cellular Autoimmunity Unit CRC, Malmö University Hospital Sweden	Document name DOCTORAL DISSERTATION Date of issue January 18th, 2008 Sponsoring organization
Author(s) Maria Carlsén	
Title and subtitle Aspects of T cell development contributing to autoimmunity	

Abstract

Autoimmunity, such as type 1 diabetes (T1D), arises as a result of tolerance breakdown against host tissues, i.e. the β-cells. Central tolerance is established during thymic T cell development and defects in key events during this process could contribute to the pathogenesis of autoimmune diseases. The work presented in this thesis was aiming at unravelling some of those aspects.

We have investigated the impact of thymic expression of TGF-β and insulin on T cell development as well as elucidated the molecular basis for the apoptosis resistance in the non-obese diabetic (NOD) mouse. The role of thymic expression of TGF-β was dissected by using TGF-β type II receptor cre/lox conditional knock out mouse model. By transplanting bone marrow to irradiated recipients we restricted the phenotype to haematopoietic stem cells. These mice develop a lymphoproliferative degenerating disease 6 to 9 weeks after bone marrow transfer. Our analysis revealed no difference in thymocyte distribution or apoptosis induction indicating that TGF-β is dispensable in these aspects. We did however observe increased proliferation of CD8+ thymocytes which might contribute to the immune defect in these animals. The impact of thymic insulin expression on development and selection of thymocytes was investigated by using insulin deficient fetal thymi. Analysis by fetal thymic organ cultures suggested that lack of insulin does not affect thymocyte differentiation. Furthermore, by transplanting insulin deficient fetal thymi under the kidney capsule of nude mice we were able to demonstrate that insulin can be re-expressed in the thymus by influx of bone marrow derived cells. This process might contribute to clonal deletion of insulin-reactive T cells. The NOD mouse displays thymocytes apoptosis resistance, which could also contribute to impaired negative selection and contribute to the development of type 1 diabetes. By analysing the expression of key proteins involved in apoptosis and cell cycle signalling we were able to demonstrate that defective up-regulation of p53 and caspases-1 and 11 are linked to this trait.

In summary, the results presented in this thesis depict factors capable of affecting T cell development and establishment of tolerance, which ultimately might contribute to development of autoimmune disorders such as T1D.

Key words: Autoimmunity, Conditional cre/lox knock out, FTOC, Insulin, Negative selection, NOD, p53, T cell development, TGF-β, Thymus, Tolerance, Type 1 diabetes		
Classification system and/or index termes (if any):		
Supplementary bibliographical information:		Language English
ISSN and key title: 1652-8220		ISBN 978-91-85897-56-8
Recipient's notes	Number of pages 138	Price
	Security classification	

INTRODUCTION

The immune system at a glance

The immune system is a complex machinery capable of fighting a wide range of pathogens. In order to provide the best protection, the immune system has developed several strategies consisting of a primary defence line in the form of the innate immune response and the special elite forces represented by the adaptive immune response. The innate immunity provides a non-specific protection using mechanical and chemical barriers, initialisation of inflammation by recruitment of immune cells through cytokines and chemokines and activation of the alternative complement system. If the invading pathogen is not cleared, the innate immune system paves the way for the adaptive antigen specific immune response through the activation of T and B cells by antigen presenting cells (APC's) in the draining lymph nodes (LN). Once the pathogen has been eliminated the adaptive immune response generates an immunological memory, the ability of certain long-lived memory cells to recognise the pathogen and initiate a rapid immune reaction. However, the prize for this well-organised structure is the possibility of being unable to maintain the immune cells under control and thus risk developing hypersensitivity and autoimmunity. Therefore, establishing and maintaining tolerance is one of the most important features of the immune system. Tolerance describes a state where an antigen that would normally initiate an immune response is either ignored or simply fails to elicit a reaction. Tolerance as a concept was first instigated in 1949 by Burnet's hypothesis of a self and non-self discrimination in the immune system [1]. This idea was proven experimentally by Medawar in 1953 where he and co-workers showed that mice injected with splenocytes or haematopoietic cells *in utero* or just after birth could accept tissue grafts from the donor but not from other mice strains later in life [2]. This discovery awarded Medawar and Burnet the Nobel Price in 1960.

It was not until 1961 that the thymus was recognised as being a crucial organ for lymphocyte development, before then it was more or less considered a redundant organ. After experiments on neonatal thymectomised mice performed by Miller it was determined that the T cells, named after their thymic origin, formed a major part of the white blood cell population together with cells derived from the bone marrow, i.e. B cells [3]. The white blood cell population is divided into several cell types; the granulocytes (neutrophils, eosinophils and basophils), the lymphocytes (T, B and Natural killer (NK) cells) and monocytes/macrophages. Each cell type contributes to fight infections although in diverse ways. An infection, such as the common cold, starts locally where a virus is able to attach and infect the epithelial lining, for instance the throat. The infection of epithelial cells induces secretion of cytokines and chemokines which attracts and allows passage of immune cells from the blood stream to the site of infection. The first cells arriving to the location are part of the innate immune system, such as neutrophils and monocytes. Monocytes

mature into macrophages that are able to phagocyte cells and viruses and secrete additional cytokines to further attract and activate immune cells. These cytokines are also able to raise the body temperature (i.e. induce fever) in order to restrict the ability of viruses to replicate. Professional APC's such as dendritic cells (DC's) constantly patrol the periphery and soon encounters danger signals in the form of cytokines and Toll-like receptor stimulation which activates them. These cells subsequently acquire the ability to home to the draining LN's through up-regulation of CCR7 where they present viral epitopes to B cells and T cells. Naïve T cells circulate the lymphatic vessels and upon entering the LN's start scanning DC's for epitopes corresponding to their T cell receptor (TcR). When a match occurs, they will receive a secondary signal confirming activation through interaction between the T cell co-stimulatory molecule CD28 and the corresponding DC receptors CD80 (B7.1) or CD86 (B7.2). The activated T cells start to multiply thereby expanding the ability of fighting the infection and they also obtain the capacity to leave the LN's and migrate towards the infection. Once in place they are able fight the disease in a more specific manner by recognising infected cells and kill them, thus limiting the infection. The immune system has now adapted their ability to fight the infection. As soon as the infection is resolved, the immune response is silenced by inducing apoptosis of the activated T cells. In order to facilitate the initiation of the adaptive immune system if there were to be another attack by the same virus, some antigen-specific cells are retained and now circulate the lymphatic in the form of resting memory cells. In view of the exponential increase of an immune response, one quickly realises that the ability to dampen activation and establish tolerance is a key factor of the immune system.

T cells

Thymic-derived cells, T cells, compose the major part of the adaptive immune system and they are divided into αβ T cells, γδ T cells and NKT cells based on cell surface markers and the structure of the TcR. NKT cells are distinguished from canonical NK cells through the presence of an αβ TcR and NK lineage markers for instance CD161 (NK1.1). These cells recognise antigens such as lipids and glycoproteins that are presented on the CD1d molecule, the cells are thereby also termed CD1d-restricted T cells. This cell population can be further subdivided into type 1 (invariant, iNKT) or type 2 NKT cells based on the chains making up the TcR [4]. The NKT cells have been implicated in autoimmunity as activation of this cell subset have been shown to reduce inflammation in diabetes, EAE and collagen-induced arthritis [5-8]. Moreover, NKT cells are of particular interest in type 1 diabetes (T1D) as it has been shown that they are defective in function and numbers in both patients and non-obese diabetic (NOD) mice, the major mouse model for T1D [9,10].

γδ T cells are a heterogeneous group of cells that are mostly found in epithelial tissues such as skin, lung and gut lining where they are considered to represent a first line of defence. Although γδ and αβ T cells originate from the same thymic progenitor, they differ from each other in many respects as γδ T cells are much less diverse in antigen recognition and it has been suggested that these cells are mainly involved in immune regulation [11]. Moreover, γδ T cells are the first T cell subset to appear in the periphery implying some importance in protection against pathogens before an adaptive immune system has been fully developed [11]. Given that certain γδ T cells seem to respond to host antigens rather than pathogenic components it is not improbable to consider their role in autoimmunity and studies have indicated both contributing and regulating roles for γδ T cells in this context [12-15]. For instance, NOD mice seem to have increased proportion of γδ T cells in thymus, spleen and blood compared to control mice [16]. Still, studies showing reduction of diabetes development in NOD mice using aerosol insulin treatment have demonstrated a regulatory role for $CD8^+$ γδ T cells and transfer of NOD $CD8^+$ intraepithelial lymphocytes (IEL) into thymectomised NOD mice prevented diabetes further emphasising the regulatory capacity of this particular subset of γδ T cells [17,18]. Thus, γδ T cells have many different functions which probably depend on the cell type as well as the disease model itself.

By far the most frequent T cells in the immune system are the αβ T cells which are comprised of T helper cells (Th) expressing the CD4 co-receptor and cytotoxic T cells expressing CD8. $CD4^+$ T cells are further subdivided to T helper type 1 (Th1) and type 2 cells (Th2), whether they will differentiation into Th1 or Th2 is dependent on the cytokine milieu during activation. Although, this categorisation of T cells is nowadays too rudimentary with the emergence of new subsets such as

Th17 and the various regulatory T cells (Treg). $CD8^+$ cytotoxic T cells participate in immune responses against viruses, intracellular bacteria, parasites and cancer cells. They are activated after encounter of a short (8-10 amino acids) peptide sequence presented by an APC in the context of the MHC class I molecule. The peptides are derived from the cytoplasm where proteosomes constantly degrades proteins [19]. The function of $CD8^+$ T cells mirrors antigen processing in the sense that they are activated by peptides derived from pathogens which can gain access to the cytosol, such as viruses. However, $CD8^+$ T cells can also be activated by exogenous-derived antigens through uptake of apoptotic virus-infected cells or cancer cells [20,21]. This cross-priming can only be mediated by professional APC's [22-24]. Following activation, $CD8^+$ T cells are able to kill any infected cell that present pathogenic peptides on MHC class I, which is expressed by all nucleated cells in the body. Killing occurs either through the release of perforin which perforates the target cell membrane enabling granzyme A and B to enter the cell or by Fas-FasL interaction. Both these pathways initiates programmed cell death through the caspase cascade [25].

In contrast to $CD8^+$ T cells, $CD4^+$ T cells are classically activated by extracellular-derived antigens, although exceptions have been found here as well [26]. Proteins are taken up by APC's through endocytosis or phagocytosis and are subsequently processed in lysosomes into suitable peptides [27,28]. The effector functions of $CD4^+$ T cells are depending on cytokines secreted by the APC. Th1 cells are differentiated by IL-12 induced STAT-1 signalling which triggers the expression of the transcription factor T-bet, the master regulator of Th1 differentiation. With the induction of T-bet follows the effector functions that are characteristic of Th1 cells such as secretion of IFN-γ which in turn maximises the activation of macrophages. Th2 cells on the other hand are differentiated by signalling through the IL-4 receptor via STAT-6 which activates GATA3. This transcription factor drives the expression of the Th2 associated cytokines IL-4, IL-5 and IL-13 which help fuelling the humoral immune response by inducing antibody class switch in B cells [29]. The balance between these two cell subset is a very delicate one, over-activation of Th1 cells is known to cause chronic inflammation and tissue damage whereas Th2 over-stimulation results in allergy and hypersensitivity. For a long time, Th1 cells were considered to be the main contributor to the autoimmune responses in experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). This perception originated from studies performed on disease resistant anti-IL-12p40 treated or IL-12p40$^{-/-}$ mice but with the emergence of the newly discovered cytokine IL-23, which share the same p40 subunit with IL-12, came a new concept [30-32]. The autoimmune responses in EAE and CIA was dependent on IL-23 which promoted the activation of a specific subset of $CD4^+$ cells producing IL-17, i.e. Th17 cells [33-35]. These cells are now thought to represent a distinct lineage of $CD4^+$ T cells and it has been suggested that IL-23 is not the determinant cytokine for Th17 differentiation as IL-6 and TGF-β do promote their development as well [36,37].

The idea of T cells that are able to suppress the activation of other cells was first established in the 70's, but was then disregarded during the 80's mainly due to the difficulty in identifying these cells [38]. The concept was put forward again in 1995 with the experiments performed by Sakaguchi et al. showing that the IL-2 receptor α-chain, CD25, was a marker for regulatory T cells since depletion of $CD4^+CD25^+$ T cells caused autoimmunity when transferred into athymic mice and prevented it when they were reintroduced [39]. It is now widely accepted that these cells are of great importance in maintaining tolerance. It appears that there are a number of distinct subsets of regulatory T cells that mediate suppression in different ways. Type 1 regulatory T cells (Tr1) secrete high amounts of IL-10 which suppresses the secretion of Th1 associated pro-inflammatory cytokines and chemokines from activated macrophages, skews towards a Th2 immune response by stimulating B cells and inhibit lymphocyte proliferation by reducing IL-2 production [40,41]. Tr1 are induced by chronic activation in the presence of IL-10 or immature DC's [42,43]. Furthermore, treatment of NOD mice with the immunogenic peptide of glutamic acid decarboxylase (GAD) 65 induced Th2 and Tr1 cells that were able to suppress diabetogenic T cells [44]. Th3 cells, on the other hand, were identified in tolerance induction against myelin basic protein by oral administration of the antigen [45]. These cells secrete TGF-β which is known for its ability to suppress lymphocyte proliferation and differentiation.

Tr1 and Th3 cells differ from $CD4^+CD25^+$ cells as they are induced in the periphery by DC and cytokine stimulation and do not represent a distinct T cell lineage. Moreover, they mediate suppression via cytokine release rather than direct cell-cell contact. The $CD4^+CD25^+$ regulatory T cells arise in the thymus early in embryogenesis and are selected on different premises than conventional T cells, this topic will be further expanded in coming sections. All of the regulatory subsets described above are $CD4^+$ T cells although several types of $CD8^+$ T cells with suppressive capacities have also been described for example $CD8^+CD28^-$ cells which regulate through cell contact and inhibit up-regulation of co-stimulatory molecules on APC's [46,47].

The thymus

The thymus, which is situated above the heart, is a bi-lobed pyramid-shaped gland which supports T cell development and the first T cell progenitors are detected at embryonic day 11,5 in the mouse [48]. One of the major genes involved in thymic development is *foxn1* which is expressed in thymic epithelial cells (TEC's) and required for their differentiation. Consequently, mutations in this gene causes TEC's to develop abnormally as occurs in the athymic nude mouse were the epithelial cells are unable to support thymocyte differentiation [49,50].

The thymus is very distinctly visible up to adolescence after which it starts to degenerate possibly due to alteration in cytokine milieu thus disenabling thymocyte differentiation [51]. Even so, increase of the peripheral T cell pool through thymic regeneration has been suggested and the loss of thymic mass does not seem to reflect loss of function as thymocytopoiesis do continue and thymic output has been observed later in life [52-54]. Therefore, the thymus might be a more flexible organ than previously believed where thymic output of T cells could at later stages of life reflect peripheral homeostasis.

Thymic anatomy

The thymic structure is composed by two distinct zones namely an outer layer, the cortex, and the inner section, the medulla. T cell progenitors will enter the thymus from the blood stream through high endothelial venules (HEV) located in the perimedullary cortex using selectin-mediated binding and rolling and intergrin-mediated migration across the barrier. The signals which draw these cells from the bloodstream into the thymus are not completely understood, but P-selectin/PSGL-1 has been implicated along with α4β1 or β7/VCAM-1 interaction [55-57]. Moreover, chemokine receptors such as CXCR4, CCR5 and CCR9 and its ligand CCL25 have also been implicated in homing mechanisms, although it is not known whether they function as attractants or if they induce adhesion (figure 1, a)[57-59].

Upon entering the thymus, cells will remain still and expand for about 10 days before start migrating towards the outer cortex (figure 1, b-c) [60]. During development and selection, thymocytes will continuously migrate through the thymus from the outer cortex towards the medulla before exit (figure 1, d-g). The signals that direct these movements are for the most part not completely defined. The outward movements from the perimedullary cortex to the outer cortex seem to be directional and requires cell contact with stromal cells through chemokines such as CXCL12 (receptor CXCR4) and CCL25 (receptor CCR9) and signals involving differentiation, proliferation and survival for example Notch and Kit ligands, IL-7 and Hedgehog (figure 1, b-c). On the other hand, the inward migration from the cortex into the medulla is a more random motion involving transient interaction

with stromal cells during selection and possibly sheer physical force pushing the cells forward as they expand [61]. Although, once cells have been positively selected, the motion once again is polarised with the CCR7 ligands CCL19 an CCL21 as strong candidates (figure 1, d-f) [62,63].

Upon entering the medulla, thymocytes now interact with DC's rather than epithelial cells, which is also reflected in their migration (figure 1, f). Negative selection of thymocytes in this area dictates much of the movement and thus the contact between thymocytes and stromal cells consists of co-stimulatory molecules and TcR-MHC interaction. Still, the trafficking of cells within the medulla is probably not one-way as cells most likely go back and forth to increase tolerance induction. TARC (CCL17) has been shown to be expressed by medullary DC's and it has been suggested that this chemokine plays a role in this type of interaction together with CCR7 [64,65].

Once cells are past selection, they remain in the deeper parts of the medulla for further differentiation before exit (figure 1, f). Emigration seem to be dependent on signalling through G-protein-coupled receptors such as the sphingosine-1-phosphate receptor 1 ($S1P_1$) and although the data are contradictory, CD69 signalling has also been implicated in T cell thymic exodus (figure 1, g) [66-71].

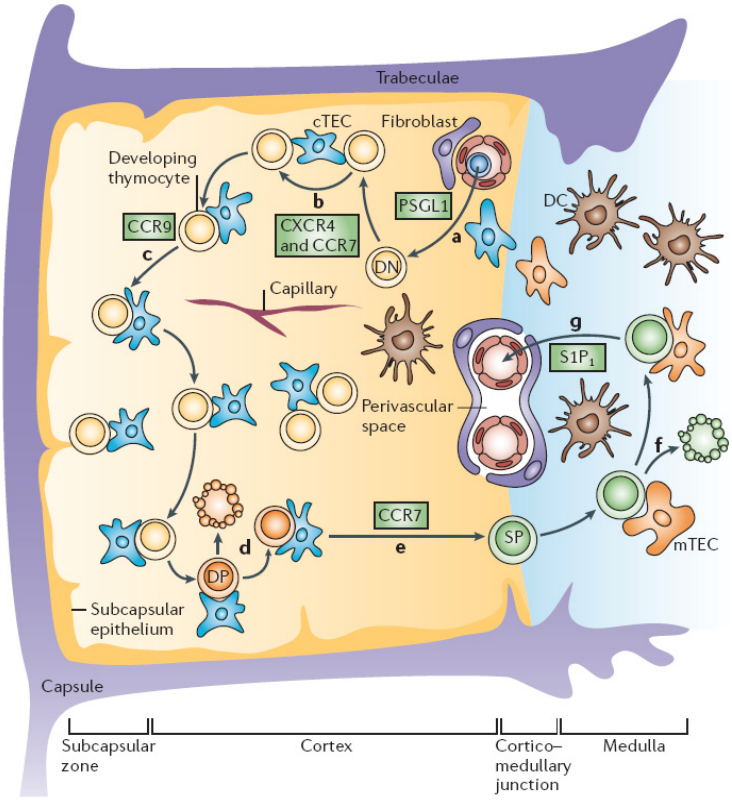


Figure 1 Thymocyte migration during maturation and development. See thymic anatomy section for further details. *Adapted from Nature Reviews Immunology Vol 6 February 2006*

Extrathymic T cell development

Although the thymus constitutes the key organ in T cell development, it is not the only site which can support T cell maturation. For example, chronic exposure to the cytokine oncostatin M has been shown to induce extrathymic T cell development in LN's [72]. The T cells emerging from these sites differ in terms of TcR repertoire and function, but their maturation does seem to be dependent on IL-7 as with conventional T cell development and the distribution of thymocyte subsets were also similar [73,74]. It should be pointed out that this kind of T cell development is lacking in euthymic mice and is a purely synthetic model although it raises interesting suggestions such as the pluripotency and redundancy of the immune system.

The intestine has long been considered to be able to support T cell differentiation as demonstrated from transplantation of small intestine under the kidney capsule of nude mice [75]. Further studies on the ability of the intestine to support T cell development has since then revealed that it is mostly applied to γδ IEL's and the majority of the cells which reside in the intestine in fact are of thymic origin. Still, clusters of lymphoid precursor, so called cryopatches, have been found within the lamina propria which do express c-kit and IL-7R and, upon transfer from nude to SCID mice, were shown to give rise to IEL's [76,77]. By studying TcR rearrangements using green fluorescent protein activity driven by the RAG-2 promoter, it was demonstrated that rearrangement of the TcR did occur in athymic mice in mesenteric LN and Peyer's patches and these cells eventually appeared in the gut mucosa [78]. This phenomenon could however not be detected in euthymic mice once again suggesting that in the presence of a functional thymus, such extrathymic T cell development does not take place. Thus it seems that even though extrathymic development occurs to a very limited extent if any at all, in case for example of severe lymphopenia, there are certain mechanisms which can be induced in order to retrieve some immune function. These mechanisms will most likely not compensate for a loss of thymic function but they probably do represent an evolutionary residual aspect of T cell development.

Thymectomy have since long been a major tool for investigation of T cell development and tolerance induction and recent papers demonstrating the existence of a thymic structure in the neck of Balb/c and C57BL/6 mice have raised concerns regarding the validity of this method [79,80]. Histology sections on the cervical thymus revealed a thymic architecture that resembles the thoracic thymus and functional studies showed that it was able to mediate both negative selection as well as selection of $CD4^+CD25^+FoxP3^+$ regulatory T cells [79,80]. Thus, it appears that conventional T cell development does occur outside the main thymus, the question is to what extent this takes place. Thymectomy of mice did not always result in immunodeficiency which was thought to be due to incomplete removal or presence

of T cells derived from gut or skin. The impact of a cervical thymus on the peripheral T cell pool still remains to be established considering its size being that of a small LN and, as been discussed above, during normal thoracic thymus function the activity of the cervical thymus might be limited.

T cell development and central tolerance

During thymic T cell development, the TcR is created after random rearrangement of the α and β chains. This process is performed in order to achieve maximal diversity of the TcR repertoire thus ensuring recognition of antigens that could be encountered in the periphery. Since it is a random event, there is also a great possibility that the TcR construct will recognise peptides specific for the host, i.e. self-antigens. Therefore, there is a need for a process which eliminates any potential autoreactive cells and this is accomplished by negative selection. As a result, tolerance is established. Thymocyte progenitors migrate from the bone marrow to the thymus where they eventually develop, mature and are exported as naïve $CD4^+$ and $CD8^+$ T cells. It is estimated that over 90% of the progenitors will either never be able to rearrange a functional TcR or will be eliminated due to construction of an autoreactive TcR highlighting the effectiveness of these processes.

Early thymocytes

All immune cells are derived from pluripotent haematopoietic stem cells but at some point these cells give rise to a distinct lineage that will eventually generate T, B, NK cells, macrophages, DC's and so forth. There are differing ideas regarding the origin of these lineages. For example, the concept of T and B cells sharing their ancestor in the common lymphoid progenitor, CLP, has been debated and even though some have shown the existence of such a population in the bone marrow, others have not been able to locate them in the earliest thymocyte population and it has thus been proposed that bone-marrow derived CLP represent an earlier developmental stage and that commitment to the T or B cell line is determined by signals received from their location, i.e. the bone marrow or the thymus [81-83]. Since much of the research of this type is based on the presence or absence of cell surface markers which is used to indicate developmental stages and thus progenitor populations, the obvious difficulty arises when the availability of such markers are limited. Nevertheless, whatever their origin, thymocyte progenitors do upon arrival to the thymus develop into the earliest thymocyte population which lack the expression of CD4 and CD8 and are thereby named double negative cells (DN). These cells differentiate through a series of developmental stages based the absence or presence of the CD25 and CD44 marker; DN1 cells are $CD44^+CD25^-$, DN2 express both CD44 and CD25, DN3 cells have down-regulated CD44 and are thus $CD44^-CD25^+$ whereas DN4 cells express neither CD44 nor CD25. However, it has been proposed that these populations can be further subdivided [84]. For example, the DN1 cell population has been suggested to contain cells with different lineage potential based on heat stable antigen (HSA, CD24) and c-kit (CD117) expression. So called DN1a-DN1e can exhibit diverse differentiation kinetics, proliferative capacities and even lineage potentials. It was thus declared that DN1a and DN1b were precursors for the DN2 cells, whereas DN1c-e did not seem to contribute to continued thymocyte

maturation [85]. Moreover, DN3 cells have also been suggested to include two subtypes that are either able to give rise to γδ T cells (DN3b) or continue into the αβ T cell lineage (figure 2) [86,87].

Events during early thymocyte development include γδ versus αβ lineage commitment, rearrangement of the β-chain and its subsequent functional confirmation during the β-selection and up-regulation of both the CD4 and CD8 co-receptors followed by complete rearrangement of the αβ TcR. Cells that have completed these stages will then be subjected to positive and negative selection which constitutes the later stages of thymocyte development. Lineage commitment of γδ T cells has been difficult to establish due to lack of specific progenitor markers although the consensus is that these cells arise from the DN population as initiation of gene rearrangements of the β, δ and γ loci occur at the DN2-DN3 stage with a subsequent expression of a γδ or pre-TcR at the DN3-DN4 stage. Lineage commitment is suggested to be dependent on signalling strength from the resulting receptor formation, a strong signal through a rearranged γδ TcR results in commitment whereas a weak signal would initiate rearrangement of the α locus resulting in an αβ TcR [88,89]. Signalling through the Notch receptor have also been implicated in thymocyte differentiation as it has been shown to be determinant for T cell lineage commitment and was also implicated in γδ versus αβ lineage commitment where reduced Notch signalling was initially shown to promote γδ T cell development [90-92]. Although, other reports have demonstrated that over-expression of Notch favoured γδ T cell development and Notch signalling has been demonstrated to be present throughout the DN stage [61,93]. A model combining signalling strength from the γδ TcR versus the pre-TcR and the amount of Notch signalling present could explain this discrepancy. Thus, strong γδ TcR signalling would promote γδ T cell commitment whereas a weak pre-TcR signal in combination with presence of moderate Notch signalling would allow transition into double positive (DP) stage. Strong Notch signalling in combination with a γδ TcR would instead promote αβ lineage commitment and so pre-TcR expressing cells are favoured to transfer into DP cells as they are not dependent on availability of Notch ligands [84,89,94].

In order to proceed from DN into the DP stage, cells need to express a functional pre-TcR receptor. DNA rearrangement of the β-locus by the RAG-1 and RAG-2 proteins start at the transition between DN2-DN3, the β-chain is then combined together with the invariant pre-TcRα chain (pTα) and CD3 molecules to form the pre-TcR [95-98]. As already discussed above, signal through the receptor is required for further maturation and induction of α-chain rearrangement and cells that are unable to produce a functional pre-TcR will die by apoptosis which might involve activation of the tumour-suppressor gene p53 (see apoptosis in the immune system). The outcome of a functional pre-TcR induces the cells to proliferate immensely and

undergoing a transient state as immature single positive cells (ISP) with low expression of CD8 after which CD4 and CD8 is up-regulated in the DP stage (figure 2) [99,100]. This proliferative burst expands the bulk of cells that will start to produce an α-chain and thus increases the possibility of a diverse TcR repertoire.

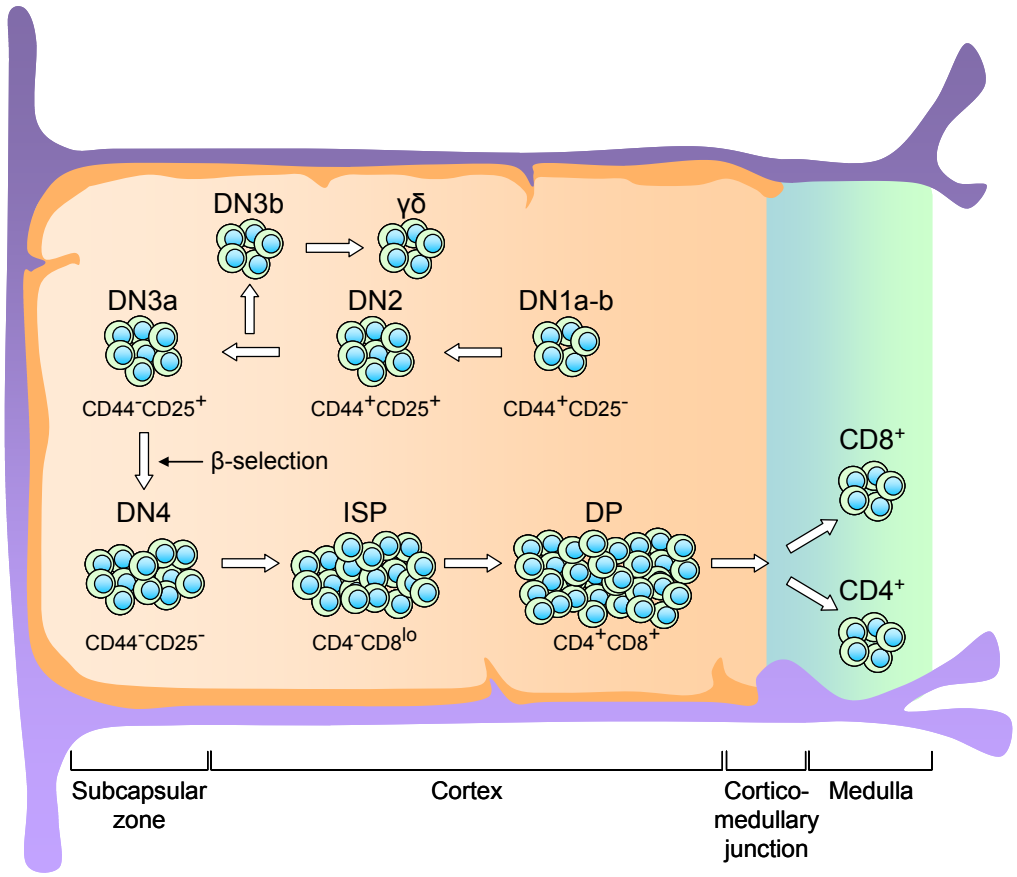


Figure 2 Developmental stages of thymocyte differentiation.

Positive selection

The cells have now changed direction in their migration and are moving towards the medulla. During this process thymocytes will produce a αβ TcR, test its function and commit to either the CD4 or CD8 lineage. DP cells constitute about 80% of total thymocyte due to the proliferative burst at DN4 but approximately 90-95% of the cells will die at this stage through death-by-neglect due to lack of TcR signalling [101,102]. The purpose of positive selection has long been considered to ensure recognition of self-MHC in the periphery. The positive signal received through the TcR-MHC interaction is weak and given that homeostasis in the periphery is suggested to involve regular low TcR stimulation indicates that this process also intends to familiarise the cells to this kind of interaction in order to adjust for any

changes in the peripheral T cell pool [103,104]. Moreover, the low signalling could also imprint a threshold for future activation in the periphery ensuring ignorance of self-peptides in the periphery [105].

The outcome of positive selection is ultimately lineage determination where the cell will be able to either respond to MHC class I in the periphery and thus down-regulate CD4 or recognise MHC class II and down-regulate CD8. Historically, two models have been described to explain how this occurs; the stochastic model which denoted that the down-regulation of one or the other co-receptor was a random process or the instruction model which implied that expression of either co-receptor was repressed depending on the MHC restriction of the TcR [106]. Nevertheless, a third model has surfaced which propagates a strength-of-signal view originating from the idea that co-ligation of CD4-TcR with MHC class II ligand transmits a stronger signal than CD8-TcR/MHC class I interaction[107]. The so called quantitative-instructive model has been based on numerous experiments showing preference of CD4 lineage commitment in the presence of strong stimuli, such as phorbol esther and ionomycin or cross-linking of TcR-CD4 or CD8 [108-110]. The intracellular signalling protein lck has been shown to bind to CD4 with higher avidity than CD8 and is also recruited to a larger extend by CD4 [111-113]. Experimental models have shown that in the presence of low lck activity, CD8 cells were selected whereas CD4 cells depend on high lck signalling [114-116]. These experiments provided a molecular basis for the quantitative-instructive model. Still, the theory dictates a translational signalling process between TcR stimulation and CD4 co-receptor expression. The transcription factor Th-POK has emerged as an interesting candidate. Helper deficient (HD) mice lack $CD4^+$ cells both in thymus and periphery due to a mutation in the Th-POK transcription factor and subsequently MHC class II-restricted cells were diverged to the CD8 lineage[117]. TcR signalling in these animals are intact and any defect in lineage commitment is therefore not depending on difference in TcR stimulation [118] Further studies have shown that Th-POK mRNA is up-regulated in MHC class II-restricted $CD4^+CD8^{lo}$ and CD4 single positive (SP) thymocytes and both HD and wild type transgenic mice over-expressing Th-POK display exclusive commitment to the CD4 lineage [119,120] Additionally, MHC-class I TcR restricted mice transgenic for Th-POK lack $CD8^+$ cells but have a population of $CD4^+$ that express GATA-3 although lack expression of perforin [119,120] Thus, Th-POK seem to be a master of regulation for CD4 lineage commitment.

Even though the quantitative-instructive model is supported by numerous experimental models, an additional hypothesis has recently emerged which is based on some studies showing a temporary $CD4^+CD8^{lo}$ population following co-expression of CD8 and CD4 caused by initial down-regulation of CD8 [121,122]. This theory suggests that during positive selection, cells will down-regulate CD8 and start

to express the IL-7R. If termination of CD8 expression doesn't result in signal loss from the TcR, CD8 receptor expression will remain silent. If however, TcR signal is affected, IL-7R signalling will lead to suppression of CD4 and re-expression of CD8. This model, designated co-receptor reversal, involves indirect and direct participation of stromal cells [123,124]. Still, the theory is so far only based on *in vitro* experiments and need further confirmation. Nonetheless, once positive selection is completed, thymocytes have moved along the cortex, constantly interacting with stromal cells and have now reached the outer medulla where they will encounter DC's and be submitted to negative selection.

Negative selection

While the positive selection ensures functional peripheral T cells, the purpose of negative selection is to guarantee that these cells will not be self-reactive and therefore the process is aiming at purging the T cell repertoire from cells responding to the antigens on which they are tested. This process was proven experimentally with the H-Y TcR transgenic mouse model where the TcR is specific for the male chromosome Y antigen and subsequently male mice did not develop any T cells [125].

Although both positive and negative selection functions on the basis of self-antigen recognition it has been hypothesised that the difference in outcome is dependent on signal intensity, i.e. ligand affinity. Cells bearing a low-affinity TcR will receive a signal rescuing them from death-by-neglect whereas thymocytes with a high-affinity TcR, i.e. autoreactive T cells, will be deleted by apoptosis. These very different outcomes are directed by signals through the same TcR and consequently there ought to exist a distinct divergence in the intracellular signalling pathway leading to either cell survival or apoptosis. Difference in ERK signalling strength has been implicated to differentiate the outcome between positive and negative selection as DP cells in the presence of a low-affinity TcR trigger displayed low levels of ERK activation whereas negative ligands induced high but temporary ERK levels [126,127]. The temporal difference in ERK activation could therefore influence downstream events as low-affinity stimulated ERK activation occurs after p38 and JNK induction which have been shown to be involved in both positive and negative selection. On the other hand, a strong but short-lasting ERK activation from a high-affinity ligand stimulation precedes p38 and JNK induction which might lead activation of different sets of transcription factors [128,129]. For example, the transcription factor E2F1 is implicated in negative selection as E2F1 deficient mice have an increased number of mature T cells as a result of defective thymic apoptosis [130]. In addition, E2F1 induced apoptosis by antigen-specific TcR stimulation has been shown to involve the tumour suppressor gene p53 (see results and discussion, study IV) [131,132].

While successful rearrangement of the β-chain causes allelic exclusion, α-chain rearrangement seem to be an ongoing process even if the cell does express a TcR [133]. This is thought to further increase a diverse repertoire and also has the effect that some cells will ultimately express two different αβ TcR's [134]. This phenomenon has been suggested to cause cells to escape from negative selection and to contribute to autoimmunity based on [135]. Still, these results have been drawn from transgenic mice models and has not been proven to affect severity in autoimmune mouse models [136,137]. Another function of such continued TcR rearrangement could be indicate a second chance by TcR editing meaning that ligand binding induces TcR signalling which might lead to continued receptor rearrangement and a second positive selection. Although several studies have aimed at investigating this aspect, the results have been contradictory and there is no consensus whether or not this theory stands [138,139]. Overall, the contribution of continued TcR rearrangement to the TcR repertoire is therefore uncertain.

The anatomical location for negative selection is somewhat disputed. Some results have concluded that the cortex is able to support negative selection based on experiments showing depletion of DP cells and cortical atrophy after treatment of a high-affinity antigen in mice with a transgenic TcR [140,141]. Since DP cells reside in the cortex it was assumed that negative selection originated here. On the other hand, some elegant experiments using transgenic mice only expressing MHC class II or MHC class I on cortical stromal cells and not in the medulla demonstrated that in these mice, negative selection was absent [142,143]. Moreover, some of the signals and conditions, such as expression of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) which seem to be required for optimal negative selection can only be found in the medulla [144-146]. In addition, the medulla is rich in thymic DC's and medullary thymic epithelial cells (mTEC's) which are central for induction of negative selection [147,148].

Overall, it seems that negative selection in the cortex cannot be excluded, although the requirements for clonal deletion are more advantageous in the medulla. Positive selection in the cortex does indeed involve ligation to peptide with the purpose of transmitting a rescue signal in the case of proper TcR rearrangement. If strong signalling is obtained, the cell will be deleted as it is self-reacting. This is, by definition, clonal deletion. Nevertheless, the ligands on which thymocytes are selected might differ between the cortex and medulla. Consequently, purging of self-reactive TcR in the cortex could be more rudimentary and reflect TcR function, whereas negative selection in the medulla represents fine-tuning of the repertoire. The expression of self-antigens in the thymus has become the focus of many recent studies as it was shown that TEC's are able to express endogenous tissue-restricted proteins that contribute to clonal deletion.

The immunological homunculus; ligands involved in tolerance induction

In order to establish tolerance to self tissues, the emerging T cells must at some point learn to discriminate between self and non-self. Clonal deletion in the thymus involves recognition of self-antigens expressed by APC's. These antigens can either be derived through peripheral influx of proteins which are taken up and processed by thymic DC's or they could already be expressed in the thymus. The concept of the immunological homunculus is an immunological counterpart of the brain homunculus, that is representation of the body but within the thymus [149].

It is now widely recognised that there is such an ectopic expression of tissue-restricted antigens (TRA) in the thymus, also known as promiscuous gene expression (PGE). The expression of these genes, which seem to represent basically every organ of the body, have been traced to both cortical epithelial cells (cTEC's) as well as mTEC's, even though the majority is expressed in mTEC's [150]. Further investigation showed that gene expression in mTEC's also differed with level of maturity indicating the possibility of acquired speciality in antigen presentation [151]. Considering the expression pattern of TRA's in thymic epithelial cells where more complex gene expression is observed with higher level of maturation, i.e. CD80 and MHC class II expression, it has been postulated that acquisition of PGE requires two steps; mTEC lineage commitment followed by maturation (up-regulation of CD80 and MHC class II). Consequently, mature mTEC's are more adept to mediate negative selection and thus express the highest levels of TRA's. It has been proposed, based on the observation that mTEC's are very short-lived, that as mTEC's are differentiated and matured, they are pushed along the medulla toward the cortex thus creating a highly specialised area of negative selection in the cortico-medullary junction [152]. The difficulty in proving this theory has been the lack of mTEC progenitor identification. Although, a recent study identified early mTEC markers which persisted into adult mTEC's and were co-expressed with the autoimmune regulator (Aire) and high levels of CD80 [153]. On the other hand, it has also been argued that expression of TRA's occurs at a pluripotent stage and following differentiation, gene expression would be more restricted creating a random expression pattern of TRA's. This mechanism has been seen in haematopoietic stem cells [154]. However, it remains to be seen if this is also applicable to thymic epithelial cells.

The condition autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is an uncommon disease manifested by massive lymphocyte infiltration and presence of circulating autoantibodies. The disease was shown to be caused by a mutation in the *AIRE* gene which was highly expressed in both human and murine thymus [155-158]. The expression of *Aire* in the thymus is restricted to DC's and mature mTEC's expressing higher levels of MHC class II and B7.1 and thus it has been suggested that *Aire* controls PGE in the thymus as *Aire*-deficient mice lacked

expression of TRA's subsets [151,159]. Nevertheless, there also seem to be an *Aire*-independent expression of TRA's as some are still expressed in the absence of *Aire* and so far only one study has been able to directly link reduced *Aire*-dependent thymic expression of a single self-antigen (interphotoreceptor retinoid-binding protein, IRBP) and subsequent development of autoimmunity (autoimmune eye disease) [151,160].

Insulin expression has been shown to be greatly reduced in the absence of Aire in a dose-dependent manner. Levels of thymic insulin expression has earlier been ascribed to the variable number of tandem repeat (VNTR) region in the T1D IDDM2 susceptibility locus where carriers of long class III repeats, which is shown to correlate to disease protection, had higher levels of thymic insulin expression. In contrast, carriers of short class I repeats, which is predisposing to diabetes development, demonstrated lower levels of thymic insulin [161,162]. This indicates that there could be another level of regulation for TRA's in general and insulin in particular. Studies trying to elucidate the correlation between *AIRE* and TRA's in human thymic samples have shown that such correlation also seem to apply in human samples [163,164]. Moreover, in an effort to correlate *AIRE* expression and VNTR haplotype (class1/III and class I/I carriers) it was suggested that AIRE interacted with separate effectiveness to the VNTR haplotypes as difference in expression were only correlated to the different VNTR haplotypes when AIRE were correlated to antigen levels suggesting additional molecular regulation of AIRE [164].

Even though ectopic gene expression in mTEC is a widely recognised concept, expression of self-antigens in the thymus was actually first ascribed to peripheral cells immigrating to the thymus termed peripheral antigen expressing cells (PAE) which were subsequently indentified as being of DC and macrophage phenotype [165-167]. Later studies revealed that these cells, which expressed the T1D pancreatic autoantigens insulin, GAD and IA-2, showed DC and macrophage phenotype and did not express epithelial markers or *Aire* [168]. Moreover, they also seem to contribute to clonal deletion as immunohistochemistry stainings have located this cell subset in the thymic medulla surrounded by apoptotic cells [168].

Selection of regulatory T cells

The regulatory T cells can be divided into several subsets as previously discussed and there are a number of evidences indicating that the so called natural $CD4^+CD25^+$ regulatory T cells (hereafter referred to as Treg) arise in the thymus and are selected on a different premises than conventional T cells. For example, experiments using transplantation of thymic epithelia to athymic mice demonstrated that transfer of tolerance indeed was possible as these mice accepted skin and heart grafts from the donor strain which was due to a dominant tolerance mechanism via

the $CD4^+$ population [169]. Thymectomised three day old neonatal mice develop multi-organ autoimmunity and in one study using nude mice grafted with neonatal thymi, which were then either removed after four days or left intact, showed that in the case of intact thymi, the autoimmune phenotype was reduced [170]. Moreover, a later study showed that thymectomy caused reduction of $CD25^+$ cells which, upon reconstitution, prevented development of autoimmunity [171].

Eventually it was shown that Treg indeed emanated from the thymus as transfer of DN cells into athymic mice was shown to give rise to $CD25^+CD4^+CD8^-$ thymocytes and elimination of the same population resulted in autoimmunity [172]. As it has become evident that Treg are self-reactive and that they do arise in the thymus it was postulated that they were selected based on medium to high-affinity recognition of self-ligands thus rendering them anergic (figure 3). This was based on experiments showing an increase in Treg when hemagglutinin (HA) TcR transgenic cells were selected on HA ligands [173,174].

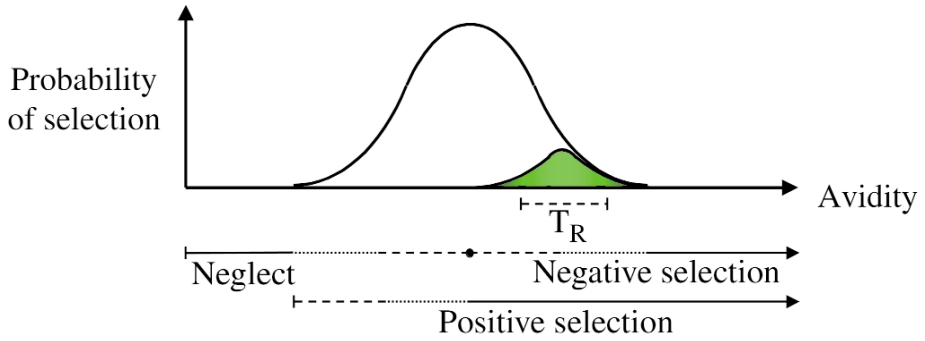


Figure 3 Selection of Treg based on high-TcR affinity model. *Adapted from Nature Immunology Vol 2 September 2001*

The transcription factor *foxp3* is a marker for Treg as it is predominantly expressed in these cells and also seems to be necessary for their function and development [175-177]. The role of Foxp3 during thymic generation of Treg was uncertain and it was not known whether expression of *foxp3* resulted in Treg lineage commitment or if it was expressed as a consequence of it. Recent studies have demonstrated that although Treg can develop in the absence of Foxp3, they do not function without it, thus Foxp3 is indispensable for their effector function [178].

Given that Treg are selected based on affinity to self-ligands, the role of Aire in generation of thymic Treg is of great interest. Nevertheless, studies on the *Aire*$^{-/-}$ mice showed that Treg indeed could be generated in the absence of Aire [179]. Still, a recent study using HA-TcR double transgenic mice expressing HA under the aire

promoter in mTEC and DC's were able to show that large numbers of HA-specific Foxp3$^+$ Treg did emit from the thymus and that their selection was independent of antigen cross-presentation by DC's but instead relied on antigen presentation by mTEC's [180]. Thus it seems that thymic generation of Treg is independent of Foxp3 and Aire, although presence of aire-driven expression in mature mTEC probably provides optimal conditions for Treg development. Nevertheless, there can be other factors than TcR affinity and antigen presentation which can drive the Treg lineage commitment. The epithelial-derived chemokine thymic stromal lymphopoietin (TSLP) has been shown to be a potent activator of DC's and the connection between TSLP, DC's and thymic generation of Treg became clear with a study focusing on Hassall's corpuscle. These bodies consisting of epithelial cells are distributed in the medulla and although their exact function is largely unknown it was speculated that these formations were involved in thymocyte apoptosis or mTEC differentiation. In a study showing the differential recruitment of CD8^{lo}CD4$^+$ cells expressing CD30 but not CD45RA to Hassall's corpuscle by secretion of MDC (macrophage-derived chemokine) suggested that these cells had already gone through positive selection and were possibly undergoing negative selection mediated by the epithelial cells in Hassall's corpuscle [181]. The conclusions was based on the observation that the epithelial cells expressed CD30L which has been suggested to mediate apoptotic signalling during negative selection [182,183]. Moreover, CCR4, which is the receptor for MDC, has been shown to be highly expressed by peripheral Treg and the medulla did contain CCR4$^+$ cells that were co-localised with the CD8^{lo}CD4$^+$ [184]. Therefore, it can be speculated that this region is specialised in the induction of thymic Treg. Indeed, the study showing TSLP secretion by epithelial cells in Hassall's corpuscle proposed that secretion of TSLP results in maturation of surrounding DC's which then are able to induce high-affinity thymocytes into Treg. The results were derived from experiments demonstrating that culture of CD4$^+$CD8$^-$CD25$^-$ thymocytes in the presence of TSLP-matured DC's induced CD4$^+$CD25$^+$Foxp3$^+$ cells that were able to mediate suppression [185]. Hence, secretion of MDC by epithelial cells in Hassall's corpuscle might preferentially recruit positively selected thymocyte with high-affinity TcR's into a region containing highly mature DC's induced by TSLP that are able to mediate Treg lineage commitment. Even though the study performed by Watanabe et al [185] was based on *in vitro* observations and more data are needed to confirm their results, they did co-localise CD4$^+$CD25$^+$ thymocytes in the medulla together with mature DC's and Hassall's corpuscle.

In summary, the information on thymic Treg generation is somewhat paradoxical but the consensus nevertheless seem to indicate that Treg induction requires specific conditioning whether it is provided through TcR stimulation, antigen presentation or cytokine milieu.

TGF-ß and T cell development

Transforming growth factor β (TGF-β) is a cytokine with pleiotropic functions affecting cell differentiation, proliferation, migration and survival but is also involved in development and wound healing [186]. There are three different isoforms, TGF-β 1-3, that are differently expressed in various organs and where TGF-β1 is the prevalent form expressed in the immune system. Hence, the discussion of TGF-β in this text will thus refer to TGF-β1.

TGF-β binds to the TGF-β type II receptor (TβRII) as a dimer which causes recruitment of the type I receptor (TβRI) and the two sub-units subsequently form a heterotetrameric complex. TβRII then phosphorylates and activates TβRI, which in turn phosphorylates regulatory Smads (R-Smad 2 or 3). The phosphorylated R-Smads subsequently form a complex with so called Co-Smads (Smad 4) which is translocated to the nuclei to exert gene regulation (figure 4) [187].

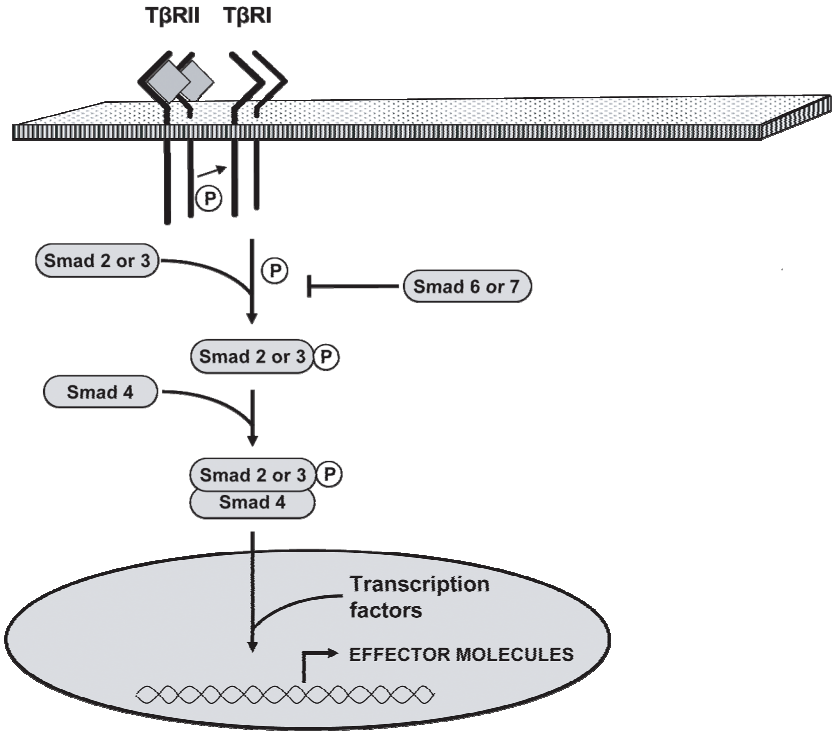


Figure 4 TGF-β signalling pathway.

The use of TGF-β null mice have emphasised the immunosuppressive effects of TGF-β as these mice develop a wasting disease characterised by multi-organ infiltration of activated lymphocytes [188]. These mice also display thymic reduction, although hyperplasia in medulla was seen as well as a reduced cortex [189]. However, these characteristics are probably secondary to the inflammation and since the null

mutation affect all cells it cannot be excluded that the developmental role of TGF-β during embryogenesis is influencing the results. Studies using exogenous administration of TGF-β in fetal thymic organ culture (FTOC) confirmed the suppressive effects of TGF-β as cell proliferation was decreased and T cell differentiation was initially blocked at the DN1 stage and $CD4^+$ as well as DP cells were affected [190]. Differentiation of $CD8^+$ thymocytes seemed to be unaltered which was contradictory to earlier reports which suggested a TGF-β mediated induction of CD8 expression in DN cells, although these experiments were performed in the presence of IL-7 [191]. Furthermore, another *in vitro* study suggested that TGF-β regulates the differentiation rate of $CD4^-CD8^{lo}$ into $CD4^+CD8^+$ [192].

In an attempt to restrict the effect of TGF-β deficiency to T cells by introducing a dominant negative TβRII it was shown that thymocyte development was not significantly affected [193,194]. Still, this approach cannot be used to study the effect of early thymocytes as the construct was placed under the CD4 or CD2 promoter. Moreover, as these mice developed a less severe autoimmune phenotype it has been suggested that the block in receptor signalling was incomplete. There are obvious difficulties in obtaining reliable results from the impact of TGF-β on thymocyte development due to its numerous functions and therefore the available information is limited. We attempted to shed some light in the field using a unique transplantation system which will be further discussed in following sections (see experimental strategy and study I).

Peripheral tolerance

Despite the seemingly rigorous control over T cell development, autoreactive T cells do escape thymic clonal deletion and circulate the periphery. Therefore, a secondary peripheral system is needed to restrain the possible activation of these cells and also to hinder a potential over-activation of the immune system during an infection. Mechanisms contributing to the peripheral tolerance include suppression mediated by the regulatory T cells, activation-induced cell death (AICD), activated cell-autonomous death (ACAD) and anergy. AICD induces apoptosis of activated T cells through Fas-FasL and occurs in the presence of a TcR re-stimulation without confirmation of activation by a second signal. ACAD results in the absence of TcR signalling during for example cytokine deprivation and is the consequence of altered ratio between pro- and anti-apoptotic gene expression. Anergy, which is a permanent non-responsive state, is induced via TcR signalling without proper co-stimulation [195,196].

T cell activation and the IS

The immunological synapse (IS) is the formation between the T cell and the APC which constitutes the basis for T cell activation. Within the LN, T cells constantly scan APC's for ligands fitting their cognate TcR and if match occurs, the cells stay in contact. The formation, which has been shown to persist for hours, can be described as a bull's eye structure consisting of so called central and peripheral supramolecular activation clusters (c- and pSMAC). The pSMAC are made up by CD2 and the adhesion molecules LFA-1 and ICAM-1 whereas the cSMAC is occupied by the TcR-MHC complex together with CD28, CTLA-4 and the co-receptors CD4 or CD8 [197] (figure 5). The first event following TcR binding is the intracellular lck-mediated phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAM) of the TcR-associated CD3 chains. This is followed by recruitment and activation of ZAP-70 which phosphorylates a number of adaptor proteins necessary for continued downstream signalling such as LAT leading to recruitment and activation of phospholipase C (PLC)-γ. The activation of PLC-γ generates diacylglycerol (DAG) and inositol-tri-phosphate (IP3) which in turn activates protein kinase C and intracellular calcium influx ultimately leading to the activation of the transcription factors NFκB and NF-AT [196]. All this occurs within minutes during which the IS is formed and the cytoskeleton is aligned. In order to achieve full T cell activation, additional stimulation is required which is achieved via CD28/B7.1 or 2 interaction, although other co-stimulatory molecules can most certainly replace CD28 [195]. As T cells have reached full activation, they start extensive proliferation and obtain final effector functions.

Upon T cell activation, the inhibitory molecule cytotoxic T lymphocyte associated antigen (CTLA)-4 is up-regulated. It is a negative regulator of T cell activation and binds to B7.1/2 with 20-100 fold higher affinity than CD28 compensating for the lower surface expression of CTLA-4 in resting state compared to CD28 [198-201]. In quiescent T cells, CTLA-4 is stored in intracellular vesicles and following TcR and CD28 ligation, CTLA-4 is accumulated to the IS and retained there as a result of tyrosine phosphorylation of the cytoplasmic tail mediated by TcR signalling [202-205]. Ligation of CTLA-4 causes reduced IL-2 secretion, inhibition of cyclin D3 and cyclin dependent kinases as well as degradation of the cell cycle inhibitor $p27^{kip1}$ all resulting in cell cycle arrest [206,207]. The role of CTLA-4 in inhibition of cell activation has been demonstrated in CTLA-4 deficient mice which die after 3-4 weeks from massive lymphoproliferation and multiple organ infiltration [208,209]. Furthermore, CTLA-4 is constitutively expressed on $CD4^+CD25^+$ regulatory T cells indicating a suppressive mechanism via CTLA-4 mediated inhibition of activation [210,211].

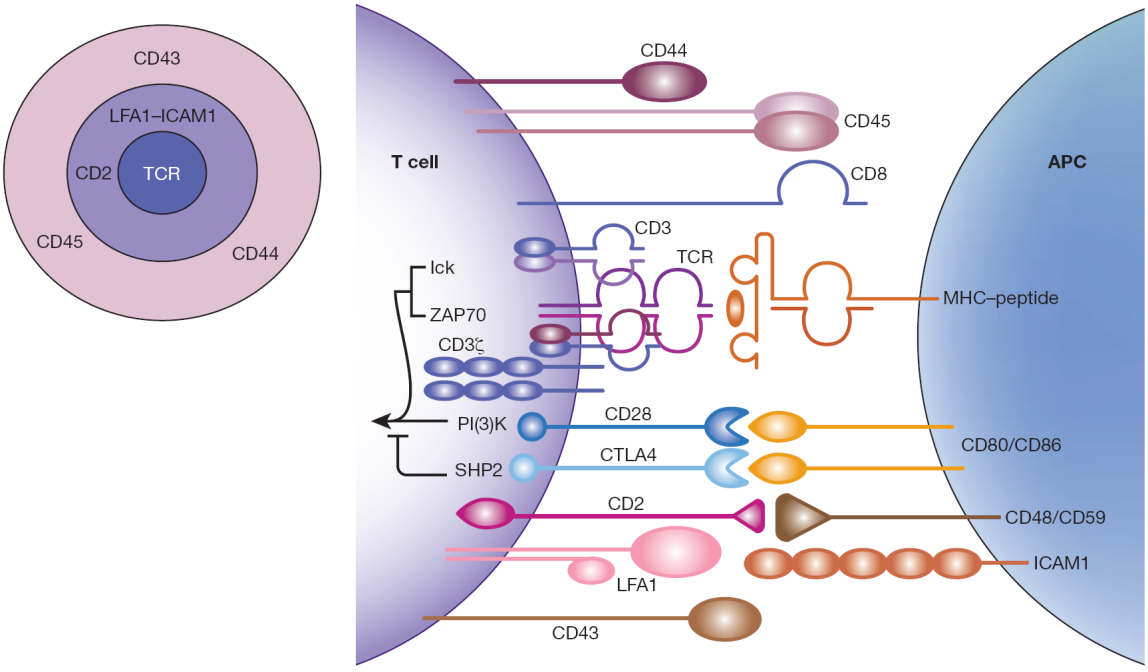


Figure 5 Schematic overview of the immunological synapse. *Adapted from Nature Vol 430 July 2004.*

Regulatory T cells

Peripheral tolerance is also maintained through the active suppression mediated by the $CD4^+CD25^+$ Treg. There are a number of T cell subsets capable of suppressing the immune response as discussed before (see T cells) and this section will focus on the thymic derived so called natural Treg which can be phenotyped by the presence of CD4, CD25, Foxp3, and CTLA-4.

The impact of Foxp3 in the immune system is illuminated by the mutation in this gene which causes the lymphoproliferative disorder in Scurfy mice [212]. The human counterpart, the X-linked syndrome IPEX, causes severe over-activation of the immune system with organ-specific autoimmunity and allergies [213,214]. It later became clear that Foxp3 was not just a marker for Treg but was also required for their function [175-177].

Treg suppress activation by cell-cell contact, either directly on the responder cell or via DC's. For example, interaction with DC's via TcR, CTLA-4 and B7.1/2 might induce suppressor effector function in Treg or ligation of CTLA-4 could also mediate suppression in a more indirect manner. For instance, the enzyme indoleamine-2,3-dioxygenase (IDO) is expressed in DC's under the transcriptional control of INF-γ and catalyses the conversion of tryptophan into its metabolites. Tryptophan is essential for T cell function and viability and by restricting the supply through modulation of IDO expression DC's can down-regulate T cell proliferation [215]. Moreover, it has been suggested that Treg are able to condition DC by inducing IDO activity and this is achieved via CTLA-4 [216]. Furthermore, CTLA-4 can also induce down-regulation of B7.1/2 on the corresponding DC thereby rendering them less able to induce proliferation [217]. Treg depend on IL-2 production for their preservation which is probably supplied by $CD25^{low}CD4^+$ as neutralisation of IL-2 have been shown to reduce $CD4^+CD25^+FoxP3^+$ cells in mice [218]. Moreover, they can also mediate suppression of proliferation by competing for IL-2 [219].

Elucidating the role of Treg in autoimmunity is not easy due to the polygenic nature of autoimmune diseases and the impact of environmental factors. Although findings such as decreased number of peripheral Treg and reduced function have been found in many patient groups suggesting a possible defect, the question remains whether these results are causative or indicative of disease.

Autoimmunity and loss of tolerance

It is apparent that the immune system operates by balancing activation and suppression of an immune response. What then, is the factor which causes the balance to shift? Autoimmune diseases are very complex as they often develop as a result of genetic predisposition, environmental factors and immune dysregulation, therefore to identify a single cause is futile (figure 6). Nevertheless, whatever the cause or triggering factor, autoimmunity always arises due to loss of tolerance against self-tissues.

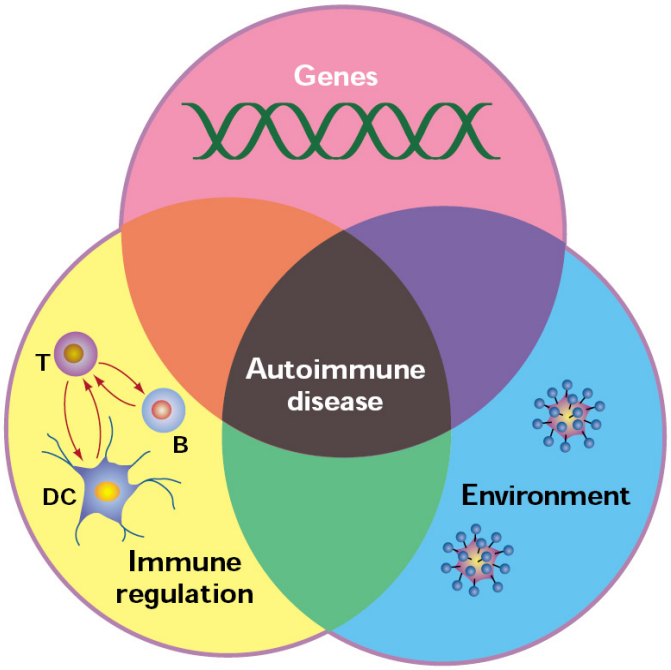


Figure 6 Factors contributing to development of autoimmunity. *Adapted from Nature Immunology Vol 2 September 2001*

Type 1 diabetes

T1D is the outcome of T cell mediated destruction of the insulin-producing β-cells in the islets of Langerhans located within the pancreas. It is the result of genetic predisposition, environmental triggering factors and failings in immune regulation. It is most often manifested in young adolescence and clinical symptoms are not noticeable until almost 80% of the β-cells are already destroyed making it hard to intervene with preventive therapies. Although, high-risk individuals can now be identified using risk markers that are shown to predispose to diabetes development. The most important of any risk markers so far identified is the IDDM1 locus containing the HLA region where DR3/DQ2-DR4/DQ8 alleles are highly

predisposing whereas DR2/DQ6 carry protection. Polymorphisms in these HLA-alleles are thought to affect peptide-MHC binding thus influencing self-antigen presentation. Presence of antibodies against pancreatic derived proteins, so called islet cell autoantibodies (ICA), such as GAD65, insulin and IA-2 can be used as marker of disease prediction although they are not causative [220]. Other genetic risk factors include the VNTR region (IDDM2) which has already been mentioned, the PTPN22 gene encoding a tyrosine phosphatase involved in TcR signalling and polymorphisms in the CTLA-4 gene [221-224]. All in all, about 18 IDDM loci have so far been identified.

Whatever the genetic risk involved, there is also an environmental impact as not all individuals carrying risk genes develop T1D. It has been suggested that certain triggering factors are able to ignite the immune response which is then cross-reacting against the β-cells. For example, infection of Coxsackie virus was indicated to induce autoimmunity through molecular mimicry as a viral amino acid sequence resembles GAD65. However, the correlation between viral infection and diabetes development is not restricted to Coxsackie virus as echovirus, Epstein-Barr virus, cytomegalovirus and more have been connected to T1D. Other suggested triggers involve dietary factors and it has been indicated that intake of cow milk protein in an early age would predispose to T1D whereas vitamin D has been proposed to be protective [225]. Vitamin D treatment has been shown to prevent diabetes development in the NOD mouse [226]. It is thought to modulate the immune response by binding to Vitamin D receptor on DC's which renders them tolerogenic through decreased IL-12 production thus skewing the immune response away from a Th1 towards a Th2 pathway. Moreover, treatment of NOD mice with vitamin D has also been shown to increase the frequency of Treg in pancreatic LN's. [227]

The destruction of the β-cells is initiated by infiltration of mononuclear cells, a process known as insulitis. Both $CD4^+$ and $CD8^+$ cells participate in the damaging process which probably occurs via Fas-FasL induced apoptosis. The T cells are primed in the adjacent pancreatic LN's by APC's carrying β-cell derived antigens. Once initiated the destruction is most likely augmented by the release of oxygen free radicals and exposure to nitric oxide (NO) and inflammatory cytokines such as IL-1β, IFN-γ and TNF-α [228,229]. Furthermore, the release of reactive oxygen species and NO could also modify antigens by structural changes thus creating new antigen epitopes which can be presented to the immune system. In this way tolerance can be broken as the tolerated antigen has been modified. This process occurs in artherosclerosis where oxidised low-density lipoprotein (LDL) is more easily absorbed by macrophages than unmodified LDL and autoantibodies against oxidised LDL are produced [230]. In addition, a study using modified myelin oligodendrocyte glycoprotein (MOG), which is a target antigen in MS, demonstrated increased immunogenic properties of the modified protein and increased APC

uptake [231]. Thus, the same process might occur in the pancreatic islets. Indeed, oxidised modification of GAD was shown to bind stronger to sera from T1D patients than the native form [232].

Animal models for T1DM; the NOD mouse

The non-obese diabetic mouse was developed over 20 years ago in Japan [233]. It spontaneously develops diabetes which closely resembles the human T1D and is therefore a widely used model. However, the disease is gender biased as 60-80% of the females develop diabetes but only 20-30% of the males which might be due to an oestrogen effect as one study discovered increased IFN-γ production from IL-12 and oestrogen stimulated NOD $CD4^+$ T cells that had been previously activated [234]. Moreover, as prolactin has been shown to promote DC differentiation and considering that females are more prone to autoimmunity, this could also contribute to the gender effect seen in NOD mice [235,236]. Peri-insulitis first appears at 3-4 weeks of age and insulitis at 10 weeks which progresses to diabetes at around 12-14 weeks in females and somewhat later in males. Furthermore, insulitis occurs to the same extend in males and females highlighting some form of immune regulation in the later stages of disease development in male mice [237]. Destruction of β-cells is, as in human T1D, mediated by $CD4^+$ and $CD8^+$ T cells and genetic predisposition also resembles the human condition with nearly 20 *Idd* loci and the highest contribution from the MHC haplotype $H2^{g7}$ [238]. Even though there is no murine counterpart to the VNTR-region, CTLA-4 is a contributing factor in this disease model and it is the candidate gene for the *Idd5* locus [239].

There are many reports suggesting immune defects contributing to the pathogenesis in NOD mice such as impaired macrophage maturation and function, and defects in NK, NKT cells and Treg even though $CD4^+$ and $CD8^+$ T cells are considered to be the most important driving factors [238]. The antigens recognised by the T cells are all derived from the pancreas and include insulin, GAD, IA-2 and heat shock protein (hsp) 60 [240]. Much focus has been placed on thymic selection and establishment of central tolerance in the NOD mouse model, although no conclusive findings have been revealed. Some studies have proposed defects in establishment of central tolerance whereas others could not find such an impairment [241,242]. It has been suggested that binding of low-affinity peptides is a property of the MHC haplotype which might lead to less efficient negative selection [243]. No deficiency in *Aire* expression or function has been found although a morphological abnormality has been seen in *Aire*-expressing cells [244]. Altogether, there are many studies suggesting both central and peripheral deficiencies in the NOD mouse model. Additionally, several reports have indicated that an underlying resistance in apoptosis could contribute to either over-stimulation or less efficient clonal deletion of the immune system in the NOD mouse.

Apoptosis in the immune system

Apoptosis is the mechanism of programmed cell death which cells undergo in the presence of proper induction signalling. It is an essential component of the immune system as it is the method by which activated cells are terminated after an immune response thereby maintaining peripheral homeostasis. Apoptosis as opposed to necrosis is a controlled way of inducing cell death which occurs through a series of organised events. The reason for this being that it enables cell death without any damaging processes such as cellular leakage leading to inflammation and macrophage and DC activation. The earliest sign of apoptosis is exposure on the outside of the membrane of phosphatidylserine (PS) motifs, which normally reside on the inner side of the membrane. These motifs serve as recognition signals for macrophage-mediated phagocytosis. Next, the cell starts to shrink and exhibits chromatin condensation followed by fragmentation of DNA and nuclei. Finally apoptotic bodies are formed by blebbing of the cellular membrane. Programmed cell death is very different from necrosis where cells swell up, membrane integrity is lost early in the process and cells finally burst spreading cellular content [245].

As mentioned before, the apoptotic pathways involved in down-regulating the immune response include AICD and ACAD. AICD is a TcR-mediated system where activated T cells mainly undergo a Fas-FasL (CD95/CD95L) induced cell death in the presence of TcR re-stimulation but in the absence of proper co-stimulation, this is also referred to the extrinsic apoptosis pathway. Triggering of the death receptors transmits via so called death-inducing signalling complexes (DISC) at the cell membrane leading to assembly and eventually activation of caspases 8 and 10 by cleaving their pro-structures. The intracellular signalling can then be transmitted either by a caspase cascade involving caspases-3, 6 and 7 or in the case of low CD95L signalling via an amplification loop involving the Bcl-family proteins Bax and Bak which brings about mitochondrial cytochrome c release (intrinsic pathway). This will result in formation of so called apoptosomes which recruits and cleaves pro-caspase 9 thereby inducing a caspase cascade involving the caspases mentioned above (figure 7) The activation of caspases leads to cleavage of cell-death substrates such as actin and nuclear lamins resulting in chromatin condensation, DNA and nuclear fragmentation and formation of apoptotic bodies [195].

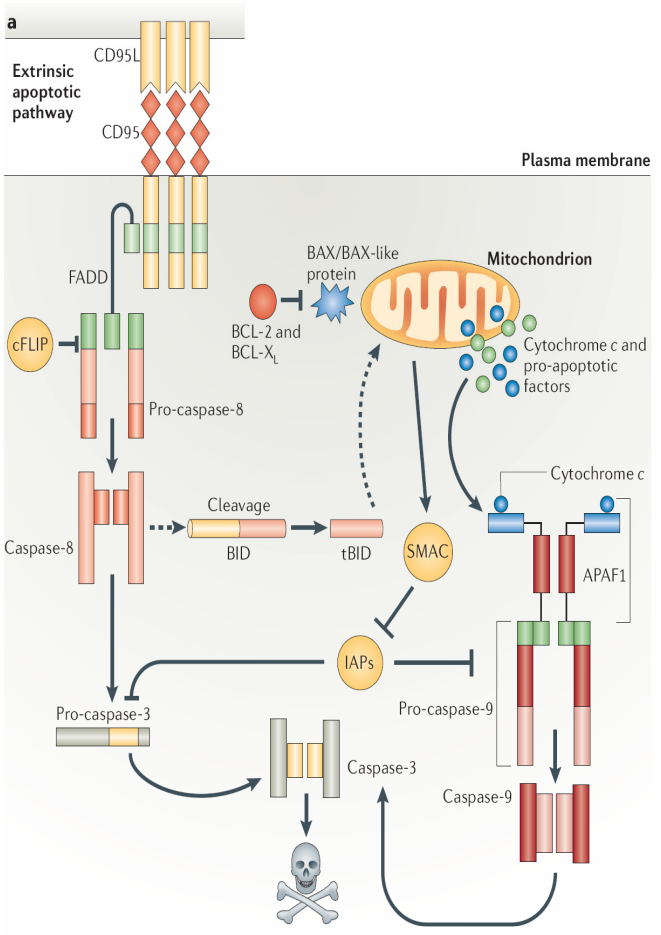


Figure 7 Overview of extrinsic and intrinsic apoptosis signalling pathway. *Adapted from Nature Reviews Immunology Vol 6 November 2006*

ACAD results from absence of survival signals such as lack of stimulating cytokines. It involves the Bcl-family proteins such as Bim and PUMA which are up-regulated and are able to withdraw the inhibition on apoptosis induction exerted on Bax and Bak by Bcl-2 and Bcl-x_L thus initiating cytochrome c release from the mitochrondria (figure 7). The ACAD pathway is therefore a result of altered balance between an anti-apoptotic (Bcl-2 and Bcl-x_L) and a pro-apoptotic state (Bim and PUMA) [195].

The transcription factor p53, which has been extensively studied in cancer, is a protein able to generate cell cycle arrest or apoptosis. Expression of p53 is induced in presence of stress stimuli such as DNA damage or UV light. In absence of stimuli, the p53 protein is negatively regulated by Mdm2 which interferes with the transcriptional activity of p53 as well as labels it for degradation. The bi-functional role of p53 is carried out by different signalling molecules where cell cycle arrest is

mediated mostly by the activation of the cyclin-dependent kinase inhibitor $p21^{WAF-1}$. Induction of apoptosis is initiated by activation of genes resulting in programmed cell death by different pathways. For instance, p53 activates gene expression of the death receptors DR4, DR5 and CD95 leading to apoptosis by caspase activation. In addition, p53 also induces expression of Bax, Noxa and Puma leading to mitochondrial cytochrome c release. Finally, apoptosis through loss of mitochondrial integrity is mediated by p53-induction of so called redox genes. Whether p53 induces cell cycle arrest or apoptosis is thought to be dependent on additional cellular proteins signalling for survival or not and differential phosphorylation of p53 is suggested to result in gene target discrepancy [246,247].

The mechanisms to induce apoptosis during T cell development seem to differ between thymocyte developmental stages. For example, early DN thymocytes rely on IL-7R signalling for continued survival which induces Bcl-2 expression thus rescuing cells from apoptosis [248,249]. However, DN cells express death receptors such as DR3 and 5 signifying a role for the extrinsic apoptotic pathway during β-selection [250]. Whereas immature DP thymocytes have been suggested to undergo Fas-independent apoptosis but require Bim expression, Fas-dependency for semi-mature $HSA^+CD4^+CD8^-$ varies with TcR stimulation where cell death following high amount of TcR cross-linking were Fas-driven and low levels of stimulation was not [251,252]. However, later studies have indicated that semimature thymocytes also require Bim for apoptosis induction and are not dependent on Fas regardless of stimuli strength [253]. In addition, several studies have implicated p53 as a key protein involved in apoptosis during both β- as well as negative selection although this most likely occurs through different mechanisms [131,132,254-257].

Apoptosis resistance in the NOD mouse

As apoptosis clearly is a mechanism which contributes to clonal thymic deletion as well as peripheral homeostasis, the NOD mouse has been extensively studied in these aspects. For example, NOD DP thymocytes are resistant to dexamethasone-induced apoptosis which has been mapped to the *Idd6* susceptibility loci [258,259]. Dexamethasone is a glucocorticoid (GC) which induces apoptosis by cytochrome c release and subsequent activation of the caspase pathway [260]. The role of GC's during thymocyte development and selection is controversial where some have observed a definite impact and others haven't depending on the mouse model. However, it has been suggested that signalling through either the TcR or glucocorticoid receptor (GR) would lead to apoptosis whereas combined signalling would counteract each other thus letting cells with moderate TcR signalling be positively selected whereas high- or low-affinity TcR's would generate apoptosis either via TcR (high-affinity) or GR (low-affinity) induced pathways [261].

Cyclophosphamide (CY) is a compound which accelerates diabetes in NOD mice and induces apoptosis in the periphery. NOD mice are more resistant to CY-induced apoptosis than control mice, a trait was later mapped to the *Idd5* region containing the CTLA-4 gene [239,262,263]. Further analysis revealed an impaired up-regulation of CD28 and defective expression of CTLA-4 implicating these co-stimulatory receptors in mechanisms contributing to apoptosis resistance [239].

DNA damage is induced by γ-irradiation and the result of such destruction depends on the signalling outcome between systems involved in DNA repair, cell cycle arrest and apoptosis induction. NOD mice exposed to γ-irradiation display decreased apoptosis among peripheral lymphocytes and thymocytes which was once again mapped to the *Idd5* susceptibility loci [264]. Moreover, the contribution of CTLA-4 was further reinforced as *Ctla-4*$^{-/-}$ mice demonstrated the same feature of apoptosis resistance as NOD mice [264].

By re-stimulating T cells *in vitro* it was shown that NOD lymphocytes display AICD resistance [265,266]. This has been implicated with diminished caspase-3 and 8 up-regulation which in turn could be the result of persistent expression of c-FLIP which blocks caspase-8 signalling (figure 7). Furthermore, c-FLIP also maps to the *Idd5* loci further implicating its importance [266]. Improper ACAD by IL-2 withdrawal has also been demonstrated in NOD CD4^{+} cells which was suggested to be a consequence of inadequate Bcl-x down-regulation [267].

The studies mentioned above emphasise that possible defects in the NOD mouse model affecting an important tolerance mechanism could indeed contribute to the pathogenesis in these animals. This issue is also the basis for one of the studies included in this thesis (see study IV).

AIMS OF THIS THESIS

Tolerance is a key feature of the immune system and is induced centrally in the thymus and maintained by peripheral mechanisms. Development of autoimmunity arises when tolerance against self-tissues has been broken. Therefore, investigating T cell development may highlight pathogenetic mechanisms contributing to development of autoimmune diseases. The purpose of this thesis was to identify factors important for thymocyte development and the establishment of T cell tolerance.

The specific aims were to:

- investigate the impact of TGF-β during T cell development
- clarify the role of thymic insulin expression on the maturation of thymocytes.
- study the peripheral contribution of ectopic thymic insulin expression and its possible implication during clonal deletion
- elucidate the molecular pathway contributing to thymocyte cell death resistance after γ-irradiation-induced apoptosis observed in the NOD mouse model

EXPERIMENTAL STRATEGY

Conditional knock out animal model

The conditional knock out model is a very useful system when removal of a particular gene might cause embryonic lethality. Moreover, this biotechnology also allows for a tissue-specific gene deletion. The inducible cre/lox system is based on the bacterially derived cyclisation recombinase enzyme (cre), which recognises a specific site in the genome, loxP (locus of X-over of P1), and consequently the enzyme catalyzes DNA recombination between two loxP sites. The loxP sites consist of two 13 base pair long repeats which can be placed on each side of the DNA region that is to be deleted. Subsequently, a conditional knock out mouse model can be obtained by crossing mice transgenic for cre under a desired promoter with mice genetically modified to contain two loxP site hanging over a specific DNA target, [268]. Different methods have been used to study the role of TGF-β during T cell development by either introducing a TGF-β null mutation or by using a TβRII dominant negative approach. However, none of these strategies have really been successful in limiting the phenotype to the immune system or to be able to include even the earliest T cell progenitors. To address this issue, we took advantage of TGF-β receptor II conditional knock out mice.

TGF-β signalling is transmitted through the TβRI/II heterotetrameric complex by phosphorylation of the TβRI cytoplasmic tail by TβRII (figure 4). Therefore, in order to eliminate receptor signalling of TGF-β, the cytoplasmic tail of TβRII encoded by exon 4 was targeted with loxP sites. The cre enzyme expression was controlled by the interferon inducible promoter Mx1 and thus cre activity was generated by injection of the interferon inducer polyinosinic-polycytidylic (polyI:polyC) acid to directly delete the exon 4 region (figure 8).

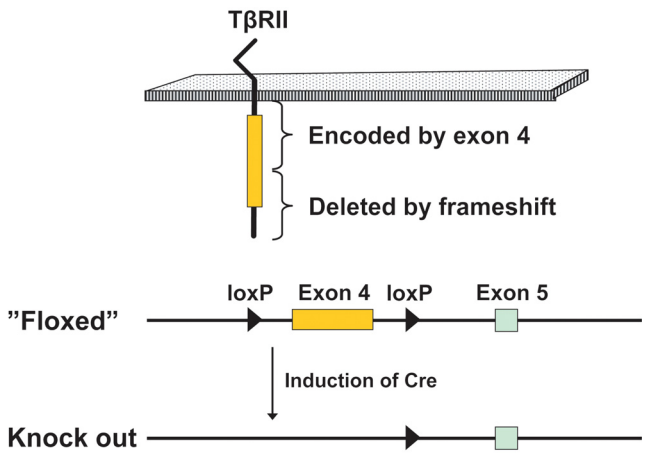


Figure 8 Conditional knock out of TβRII.

TβRII$^{-/-}$ mice develop a lethal inflammatory disease 8 to 10 weeks after induction. However, as we wanted to restrict the phenotype to the immune system, bone marrow cells were isolated from TβRII cre/lox mice one week after induction and transferred to irradiated recipients. The recipients develop the same kind of inflammatory disorder as donors but at 6 to 9 weeks after transfer [269]. Nevertheless, they show no clinical signs after 3 weeks, which then constituted our starting point for investigation of T cell development in order to avoid the effect of systemic inflammation on thymocyte development and differentiation (figure 9). Control mice used in the experiment did not show any clinical symptoms and were transplanted with bone marrow from donors that were heterozygous for the loxP site (TβRII$^{+/-}$) and therefore gene deletion could not be induced on both alleles. We also performed competitive experiments by transplanting recipient mice with mixed bone marrow cells from TβRII$^{+/-}$ and TβRII$^{-/-}$ at a 1:1 ratio in order to confirm that our read-out was not affected by inflammation. Donor cells and any radioresistant remaining immune cells in the recipient mice were distinguished by the Ly5.1 and Ly5.2 markers. Recipients carried either the Ly5.1 (CD45.1) cell surface marker (non-competitive transplantations) or were Ly5.1^{+}/5.2^{+} double positive (competitive transplantations), donor cells were always Ly5.2^{+}.

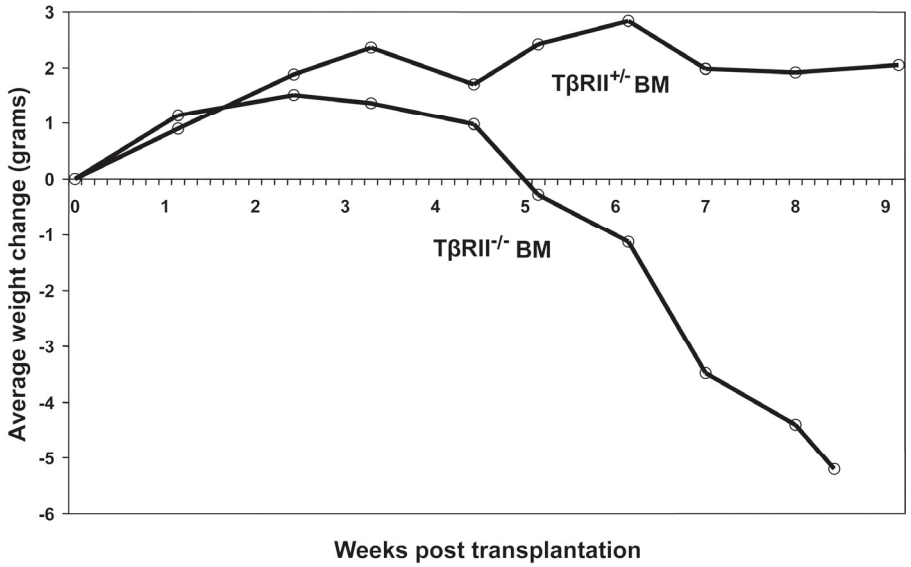


Figure 9 Curve following weight change after transplantation of TβRII$^{-/-}$ bone marrow (BM) compared to control TβRII$^{+/-}$ BM recipient. Clinical symptoms are not noticeable at 3 weeks post transplantation which was the starting point of experiments.

Proliferation assays

Proliferation assays are fundamental for investigation of antigen specific responses. Proliferation can be followed *in vitro* and *in vivo* by using bromodeoxyuridine (BrdU) and carboxyfluoroscein succinimidyl ester (CFSE). BrdU is a synthetic thymidine nucleoside analogue which is incorporated into the DNA during cell division. BrdU incorporation is detected by using anti-BrdU fluorescent-conjugated antibodies, in this manner cell proliferation can be tracked by flow cytometry. CFSE is a fluorescent dye, which passively diffuse into the cell and once modified by intracellular esterases will be unable to exit the intracellular compartment. Consequently, at each round of cell division the amount of dye will be equally divided into each daughter cell resulting in a corresponding decrease of fluorescence intensity. Using flow cytometry it is then possible to accurately measure the number of cell divisions of a given cell population during *in vivo* or *in vitro* stimulation (figure 10). In contrast, BrdU incorporation measures the frequency of cells that are proliferating during a given time. The advantage of these methods is the ability to study proliferation in different cell populations by flow cytometry whereas for example [methyl-^{3}H]-thymidine incorporation only measures proliferation from the entire cultured cell population. For study I we decided to track proliferation rate *in vivo* by using i.p. injection of BrdU. After 8 hours, thymi were collected and stained for the cell surface markers CD4, CD8, Ly5.1 and Ly5.2 and intracellularly for BrdU (study I page 3).

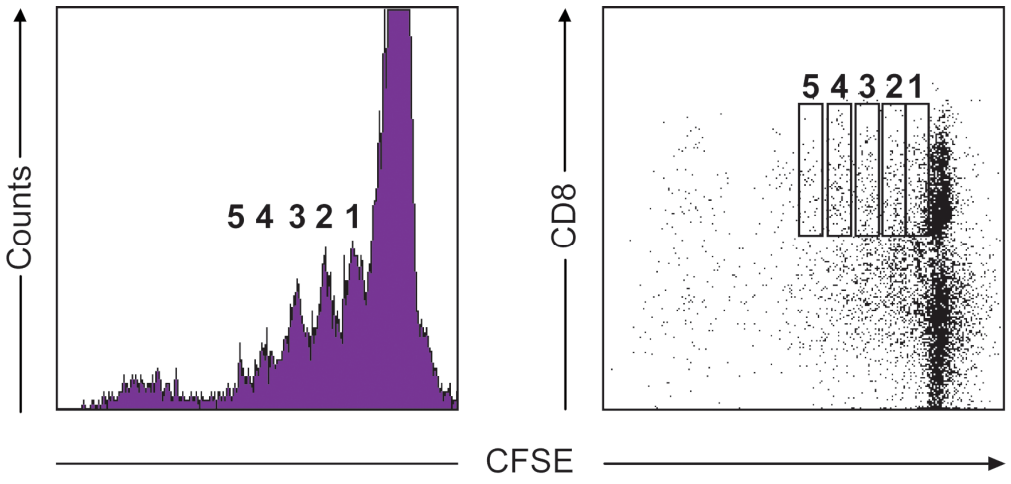


Figure 10 Histogram plot (right) and dot plot (left) from flow cytometry proliferation analysis of CFSE dilution. Number of cell divisions can be distinguished and is indicated in the plots.

In study III we were interested in measuring T cell antigen-specific responses towards insulin and therefore we investigated T cell proliferation *in vitro* by using insulin-pulsed CD11c-purified DC's as APC's. Proliferation was analysed using CFSE staining and therefore B-cell depleted splenocytes were stained with CFSE preceding co-culture with insulin-pulsed DC's (study III page 4).

Proliferation can also be induced in a non-antigen specific manner using mitogenic stimulation. In study I we wanted to verify deletion of TβRII by using an *in vitro* proliferation assay where cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the presence or absence of TGF-β. By using this approach APC's are not needed as the plate-bound antibody will cross-link the TcR and activation is optimised by the presence of anti-CD28 which provides co-stimulatory signalling. Proliferation read out was performed using [methyl-^{3}H]-thymidine incorporation where cells were culture for 72 hours, pulsed with ^{3}H-thymidine for 4 hours, harvested with a Tomtec cell harvester (Hamden, CT) and subsequently analyzed using a microbeta liquid scintillation β-counter (study I page 2).

Induction of apoptosis

In order to study any intrinsic defects in apoptosis signalling pathways it might be necessary to induce apoptosis in a non-physiologic manner. For example, in study I thymocyte apoptosis was induced *in vivo* by i.p. injection of 100 μg anti-CD3, which simulates TcR signalling and therefore cause deletion among DP thymocytes (study 1 page 3). Apoptosis susceptibility can then be measured on the basis of DP depletion. A more specific analysis for apoptosis induction can be achieved using Annexin V and 7-AAD staining. As cells proceed through apoptosis the inner layer of the cell membrane will be exposed thereby uncovering phosphatidylserine motifs which Annexin V binds to in the presence of calcium ions. Apoptosis also increases the permeability of cellular membranes allowing the dye 7-AAD to enter and stain cells that are in later stages of apoptosis. Thus, it is possible to discriminate between early and late phase of apoptosis using Annexin V together with 7-AAD (figure 11).

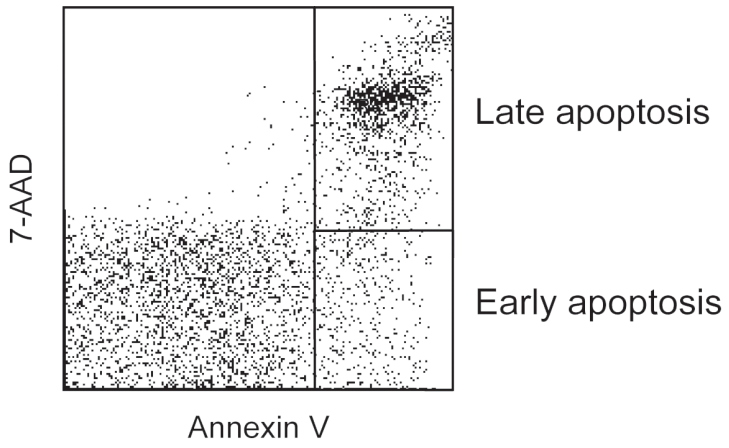


Figure 11 Flow cytometry dot plot of apoptosis analysis using Annexin V and 7-AAD staining.

Sensitivity in apoptosis induction can furthermore be investigated using γ-irradiation, which might also be considered to be a non-physiological method. However, γ-irradiation causes DNA damage which is a crucial apoptosis-inducing signal in cells and hence it is a widely used method for studying underlying defects in apoptotic pathways. In study IV we wanted to investigate signalling pathways involved in apoptosis and therefore we used whole-body γ-irradiation at a non-lethal dose of 6 Gray. Thymi were collected at 6 or 12 hours after irradiation and analysed by Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling (TUNEL) and propidium iodide (PI) for apoptotic cells (study IV page 3). During apoptosis, DNA is fragmented by endonucleases. The TUNEL assay is based on the activity of a terminal-deoxynucleotidyl transferase (TdT) which catalyses the incorporation of fluorescent-conjugated dUTP into DNA single strand breaks thus labelling the nuclei of apoptotic cells. The amount of DNA fragmentation is then analysed by flow cytometry. DNA content can also be analysed using PI staining whereby cells are fixed, permeabilised and stained with PI which intercalates the major groove of double-stranded DNA. Analysis by histogram plots reveals frequency of apoptotic cells with hypodiploid DNA content and enables cell cycle analysis for cells in G0/G1, S and G2/M phase (figure 12).

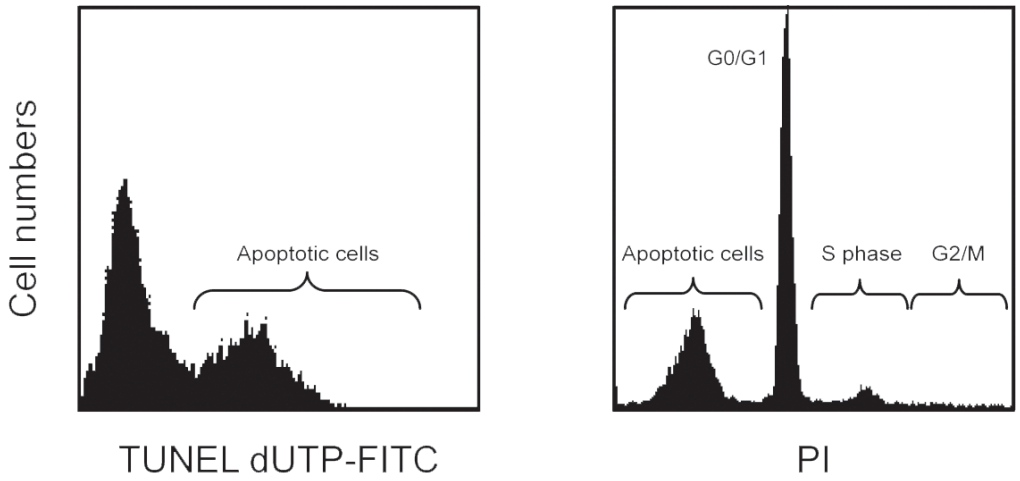


Figure 12 Analysis of apoptosis by flow cytometry. Right panel shows frequency of apoptotic cells using TUNEL. PI staining (left panel) reflects cell DNA content and enables analysis of apoptotic cells as well as cell cycle progression.

Fetal Thymic Organ Culture (FTOC)

Fetal thymic organ culture (FTOC) is a highly useful technique for studying and manipulating T cell development as the intact lobes are able to support T cell maturation as well as negative and positive selection [270]. Embryonic thymic lobes are preferably dissected at 14-16 days post coitum. At embryonic day 14, the lobes are still separated and are situated on either side of the oesophagus whereas they migrate and fuse at later stages of embryonic development. Following dissection, the separate lobes are placed on membranes floating on media in a 6-well plate (figure 13). A modification of the FTOC procedure, reaggregation thymic organ culture (RTOC), can be used to keep stromal cells intact and reconstitute the thymi with given thymocyte precursors. In study I we wanted to study T cell development in thymocytes lacking TβRII signalling using the same immunological thymic environment as for control thymocyte precursors. Thus we used irradiated fetal thymi from same donor embryos to deplete endogenous precursors. Thereafter TβRII$^{+/-}$ or TβRII$^{-/-}$ DN thymocytes cells were added to the irradiated thymi in hanging drop cultures using Terasaki plates. This procedure allows the DN cells population to reconstitute the lobes. Reaggregated thymi were then transferred to floating membranes and cultured in the same way as FTOC to induce T cell development (study I page 2)(figure 13).

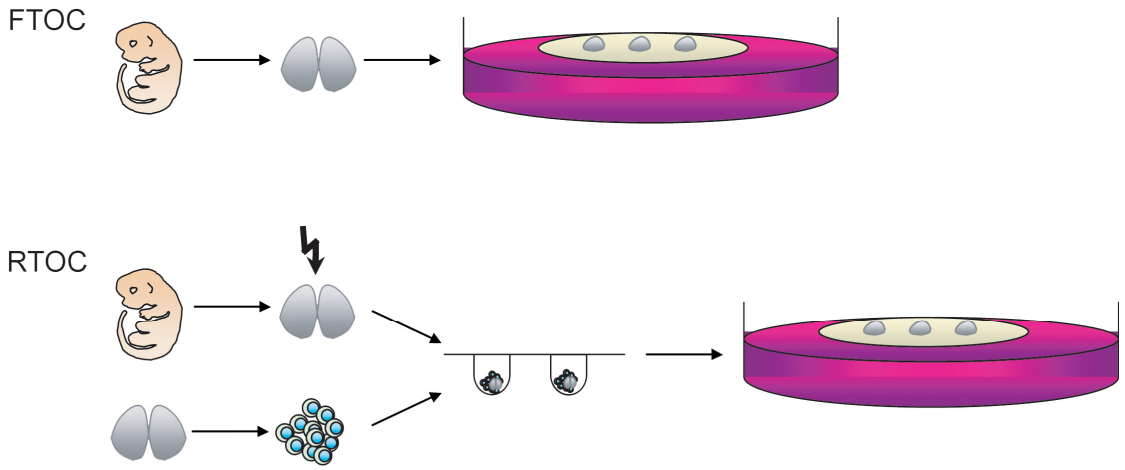


Figure 13 Fetal thymic organ culture (FTOC) versus reaggregated thymic organ culture (RTOC).

After 7 (study I page 2 and study II page 3) or 14 days (study I page 2), thymocyte suspensions were prepared and stained for cell surface markers identifying different stages of thymic development, e.g. DN cells (CD25 and CD44), DP cells (CD4 and CD8), SP cells (CD4 or CD8) and thymic Treg (CD25, study II). In order to study the expression pattern of true lineage negative thymocytes and restrict the analysis to early T cell progenitors, a dump channel was applied during flow cytometry acquisition to exclude cells expressing CD3, CD4 and CD8 (figure 14).

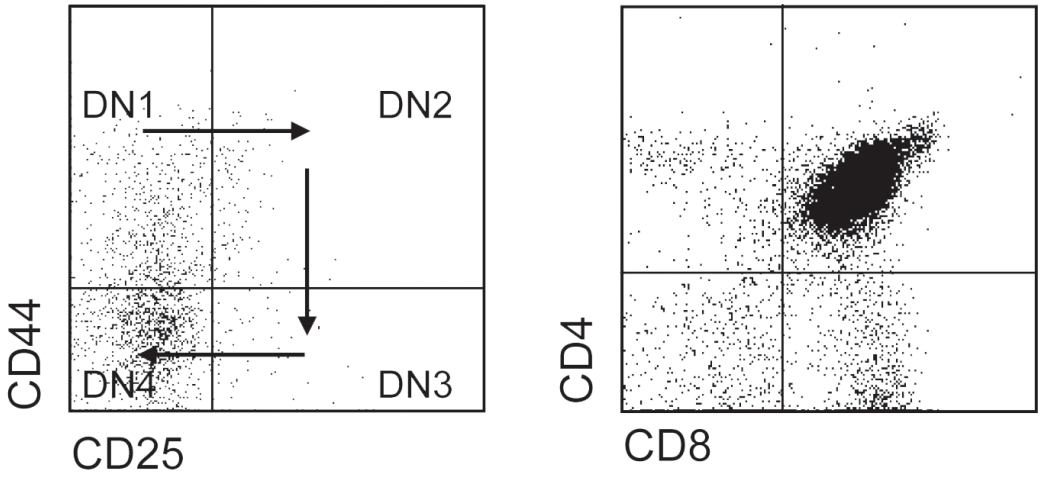


Figure 14 Flow cytometry analysis of DN and DP cells, true DN cells are obtained by gating on CD3, CD4 and CD8 lineage negative cells.

RNA analysis

RNA can be either detected or quantified using a variety of methods. For the studies included in this thesis, RNA was analysed using reverse transcriptase polymerase chain reaction (RT-PCR), real-time quantitative PCR (QT-PCR), semi-quantitative PCR (SQ-PCR) and ribonuclease protection assay (RPA).

RT-PCR is a non-quantitative method, which detects the presence or absence of mRNA. It is, however, a highly sensitive and specific method which can detect rare transcripts in small amounts of sample. An appropriate choice of thoroughly designed primers is desired as contamination of genomic DNA during RNA preparation might interfere and give false positive results. The optimal primers are those which span across the exon-intron border ensuring that genomic DNA will not be detected. However, it is not always possible to design such primers as many aspects need to be taken into account. Therefore, the second approach is to design primers which will flank one intron making the potentially contaminating DNA product larger than the complementary DNA (cDNA) product. Still, there is also the possibility of eliminating genomic DNA following RNA preparation. The most common approach is to treat the RNA sample with RNase-free DNase, which will digest any residual DNA. However, if the RNA sample is very small and transcripts are rare, this treatment might jeopardise RNA integrity unless all products are certified RNase-free and RNase inhibitors are added. Another method for cDNA preparation, which was used in study III, is the Qiagen Quantitect Reverse Transcription Kit where cDNA was prepared using a two-step system. The first step eliminated genomic DNA in a 2 minute PCR step. The sample was then directly used for cDNA preparation (study III page 3). For study II, cDNA was prepared using random hexamers primers and Superscript Rnase H^- Reverse Transcriptase (Life Technologies, MD) (study II pages 2-3).

SQ-PCR is a relative measurement of RNA expression where the cDNA template is diluted before PCR and subsequently compared on agarose gel. It is a simple method that will only provide an estimation of RNA levels. In study I RNA levels of TβRII in DN cells from BM recipients was measured and quantified using SQ-PCR to ensure complete gene deletion. The PCR included two primer pairs for detection of floxed and null allele (study I page 2).

Quantification of RNA is most accurately performed using real-time QT-PCR which can detect as little as a 2-fold change in expression level. The PCR reaction can be divided into three phases: the exponential phase where theoretically each cycle doubles the amount of product, the linear phase during which reaction is slowing down due to exhaustion of reaction components and the plateau where no more product is formed. Therefore, in each given cycle there is a quantitative

relationship between starting material and product. Quantification is typically performed either using a fluorescence-based probe assay or by using SYBR (Synergy Brands) Green dye. SYBR Green is a dye which binds to the minor grove of the DNA molecule and by doing so the fluorescence that is emitted is decreased. The quantification is thus based on decreased signal from SYBR Green when product is accumulated and only reflects the amount of double stranded DNA. Fluorescence-based probes have two different dyes attached, one quencher consisting of a high-energy dye like TAMRA (tetramethyl-6-carboxyrhodamine) and a reporter, which is a low-energy dye for example FAM (6-carboxy-flourescein). When probes are intact and in near proximity of another the fluorescence from the reporter dye will be lower as it is reduced by the quencher. The probe will anneal to a specific template sequence between the forward and reverse primer. As the Taq DNA polymerase removes any obstacles downstream during extension through its 5' exo-nuclease activity, it will cleave the probe and remove the reporter dye and thus the fluorescence is increased as the quencher no longer is able to suppress light emission (figure 15). Hence, the more product, the higher fluorescence emission, which is displayed by the software as a plot showing fluorescence intensity on a logarithmic scale on the X-axis and number of PCR cycles on the Y-axis. The cycle threshold (Ct) value is then extrapolated which is the cycle at which the sample reaches the threshold of detection where the fluorescence intensity is above background. Therefore, the higher levels of RNA (i.e. more cDNA), the lower Ct value (figure 16). The quantification can be either absolute with an exact number of gene copies present or relative which is a quantified difference in gene copies related to a control group after both have been normalised against an endogenous control. By constructing a standard curve for each primer set, quantification of both sample and endogenous control is obtained from the standard curve and sample value is then normalised against the endogenous control. The standard curve method is preferred when using primer sets of unequal or less than 100% amplification efficiency. Although, it is reagent consuming as a standard curve must be performed every time the assay is run. Therefore, when using primers with 100% efficiency, the ΔΔCt method is the method of choice. This method will provide a fold-change in expression and is based on comparison between two sample sets, for instance knock out (KO) group and controls. It first normalizes expression against endogenous control for both sample sets according to $\Delta Ct = \text{avg } \Delta Ct_{GOI} - \text{avg } \Delta Ct_{ref}$ where avg is average, GOI is gene of interest and ref is reference. The ΔΔCt is then obtained by subtracting the so called calibrator ΔCt value from the sample ΔCt, the calibrator being the group which the rest of the samples will be compared against. Fold-change in expression is then acquired according to $2^{-(\Delta\Delta Ct)}$. This method was applied in study III where fold-change in insulin expression was compared in grafted thymi against FTOC thymi by QT-PCR on the ABI Prism 7900 Sequence Detection System (study III pages 3-4) (Applied Biosystems, Forster City USA).

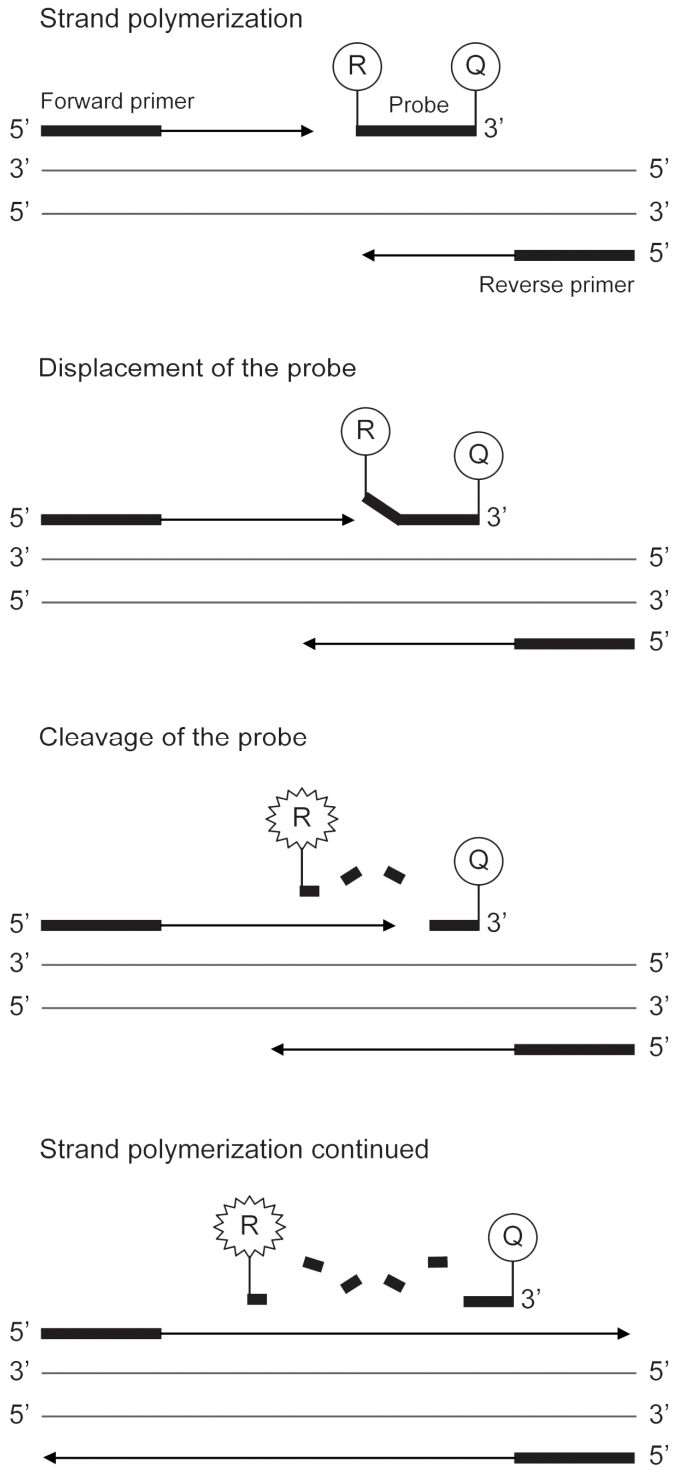


Figure 15 Principle of RT-PCR *Adapted and modified from Applied Biosystems online support real-time PCR tutorial.*

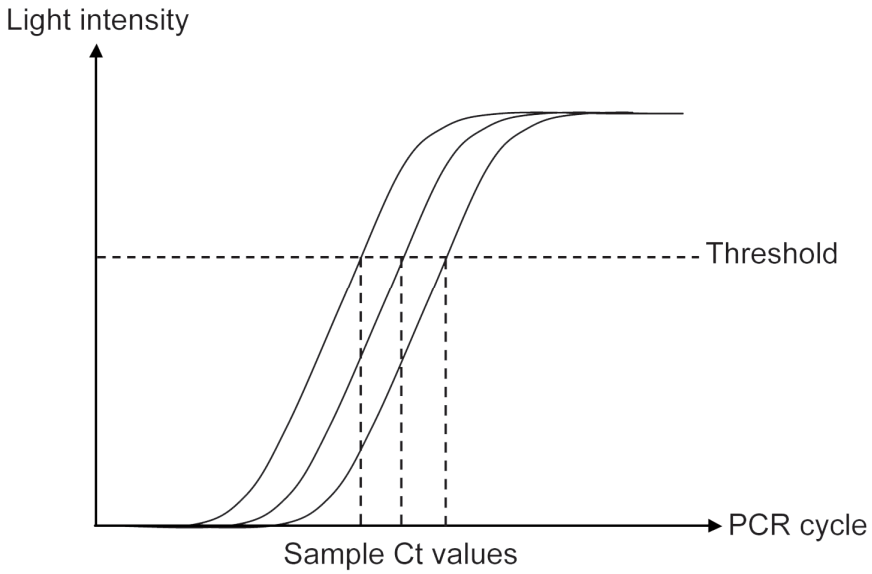


Figure 16 Extracting Ct value based on fluorescence above the threshold of detection.

RNase protection assay (RPA) is a sensitive method which allows a quantitative expression analysis of multiple genes in a single sample. It is based on the principle that double-stranded RNA is protected from RNase digestion. Protected RNA-RNA hybrids are created by synthesising RNA probes complementary to the GOI. The sample is then treated with RNase which digests all other remaining RNA. The probe is labelled with different form of detection agent, such as a radioactive isotope or biotin. The protected samples are separated on a SDS-PAGE gel and bands are subsequently detected and quantified using the appropriate instrument (Densitometer or ECL detection). Included on the gel are unprotected probes which serves as markers, they are however longer than the protected probes and thus migrate slower on the gel (figure 17). In study IV, the apoptosis-1 multi-probe template (BD Pharmingen, San Diego, CA) was used for the synthesis of [α^{32}P] labelled anti-sense RNA to detect difference in RNA expression coding for multiple caspases involved in apoptosis signalling (study IV pages 3-4).

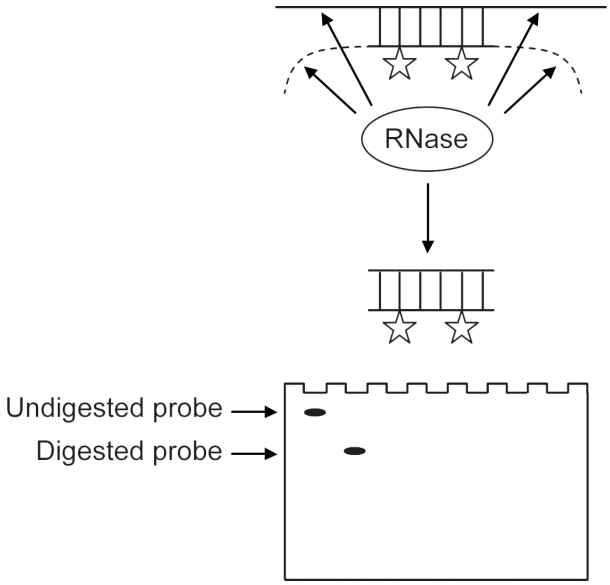


Figure 17 RPA principle. A labelled RNA probe hybridises with target RNA after which RNase digests single stranded RNA. The RNA-RNA hybrid is separated on a gel together with undigested longer probes which serves as markers.

RESULTS AND DISCUSSION

Thymic TGF-ß and T cell development (study I)

The immunosuppressive effect of TGF-β on T cell proliferation in the periphery is a major contribution to maintain homeostasis and tolerance as revealed by the lymphoproliferative degenerative disease caused by elimination of TGF-β or TGF-β signalling in various animal models [189,193,194]. Thymic T cell development is dominated by proliferative and apoptotic events, therefore thymic expression of TGF-β might indeed contribute to tolerance induction by, for example, restricting proliferation. However, in the last years several investigators have obtained conflicting results using different experimental approaches. In this project we intended to further investigate the role of TGF-β signalling during thymocytes development in an attempt to conclusively clarify this issue. To this end we generated mice with conditional TβRII deficiency restricted to the haematopoietic system by transplanting induced TβRII$^{-/-}$ bone marrow cells into irradiated recipient mice (see experimental strategy). In this model thymocytes could develop in an environment expressing TGF-β, but would remain unresponsive to TGF-β signalling whereas all non-haematopoietic cells would still express a functional TβRII. These mice did develop a lymphoproliferative wasting disease in accordance with previous studies. The disease was characterised by massive lymphocyte and granulocyte infiltration in the stomach, pancreas and lung and activated T cell phenotype in the periphery. Moreover, the mice exhibited reduced thymic cellularity due to cortex atrophy and extensive cellular depletion [269]. In order to further dissect the impact of TGF-β on the development of T cells, thymocyte differentiation was investigated by flow cytometry both *ex vivo* 3 weeks after induction and by FTOC's in order to avoid the impact of circulating pro-inflammatory factors.

The results surprisingly revealed no difference in thymocyte subset distribution either *ex vivo* or after 7 and 14 days of culture suggesting that differentiation and/or lineage determination is not dependent on TGF-β at any point during thymocyte development. This was an unexpected finding as it has been previously proposed by Plum et al. [190] that TGF-β inhibited progression from DN1 as well as reduced CD4 SP and DP cells. However, these experiments were performed in FTOC's with exogenous addition of TGF-β, which might not reflect the actual physiological secretion of TGF-β by the local environment or the circulating levels of this cytokine. Since thymocyte proliferation does mostly occur at the DN1, DN4-DP and SP stages [84], the results obtained by Plum et al. [190] could be interpreted as inhibition of proliferation by addition of TGF-β and not through the inability of thymocytes to proceed in differentiation. In line with this hypothesis, a study using a cortical epithelial-like cell line demonstrated that these cells inhibit cell cycle progression and differentiation from ISP to DP [192]. This effect was indeed dependent on proliferation as addition of TGF-β arrested cell cycle progression at

the G1 stage in ISP once again suggesting that the effect of TGF-β is mostly exerted by inhibition of proliferation and does not affect differentiation *per se*. However, in our model we could not detect a skewed thymocyte distribution, suggesting that there might be other factors, probably provided by stromal cells, which could explain the conflicting results. Alternatively, exogenous addition of TGF-β in the experiments described above might have affected thymocyte proliferation in a different manner than *in vivo* conditions.

Even though we could not detect differences in subset distribution of TβRII$^{-/-}$ thymocytes in our experiments, a defect in the induction of apoptosis and/or in their proliferative potential could not be excluded. These defects could still contribute to the uncontrolled lymphoproliferative disease observed in the periphery. Therefore, we set out to investigate the frequency of thymocyte apoptosis after i.p. injection of anti-CD3 in TβRII$^{-/-}$ or TβRII$^{+/-}$ mice. Cross-talk between TcR and TGF-β signalling was previously suggested in a study showing Smad2 phophorylation following TcR ligation in peripheral human lymphocytes [271]. Moreover, TGF-β null mice did display increased thymic apoptosis although both in the presence and absence of stimuli suggesting that this phenotype could be secondary to inflammation [272]. We did not observe any increased susceptibility to apoptosis in the presence or absence of anti-CD3 as studied by DP thymocytes depletion and Annexin V/7-AAD staining (see experimental strategy) suggesting that in our model TGF-β is dispensable for the induction of apoptosis in the thymus induced by TcR stimulation.

As no difference in apoptosis could be observed, we next focused on the proliferative capacity of thymocytes lacking TGF-β signalling. We tested this hypothesis by measuring thymocyte proliferation *in vivo*. Mice were injected with BrdU i.p and thymocytes were stained for detection of BrdU incorporation after 8 hours (see experimental strategy). The experiments revealed an increase in proliferation of TβRII$^{-/-}$ CD8 SP thymocytes (figure 18). To confirm our results and to avoid the influence of peripheral inflammation, we repeated the experiment in a competitive manner by transplanting mice with a 1:1 ratio bone marrow mix of TβRII$^{-/-}$ or TβRII$^{+/-}$ and B6SJL. The thymocyte population origin were distinguished by flow cytometry using the Ly5.1 and Ly5.2 markers where donors cells were Ly5.2^{+} (TβRII$^{-/-}$ or TβRII$^{+/-}$) and Ly5.1^{+} (B6SJL) while the remaining radio-resistant host cells were Ly5.1^{+}/ Ly5.2^{+} and were excluded from the analysis (see experimental strategy). The results confirmed our previous finding that CD8^{+} thymocytes proliferated to a greater extent than other thymocyte subsets (figure 18). Therefore, we concluded that this is an intrinsic effect due to defective TGF-β signalling and not secondary to inflammation. Several studies have implicated a role for TGF-β in CD8 expression. Although we observed an increased proliferation rate of CD8^{+} thymocytes, this was not reflected by an increased proportion of these

cells. One explanation might be that the augmented proliferation of CD8+ TβRII-/- thymocytes was paralleled by an increase thymic output thereby compensating for the increased frequency.

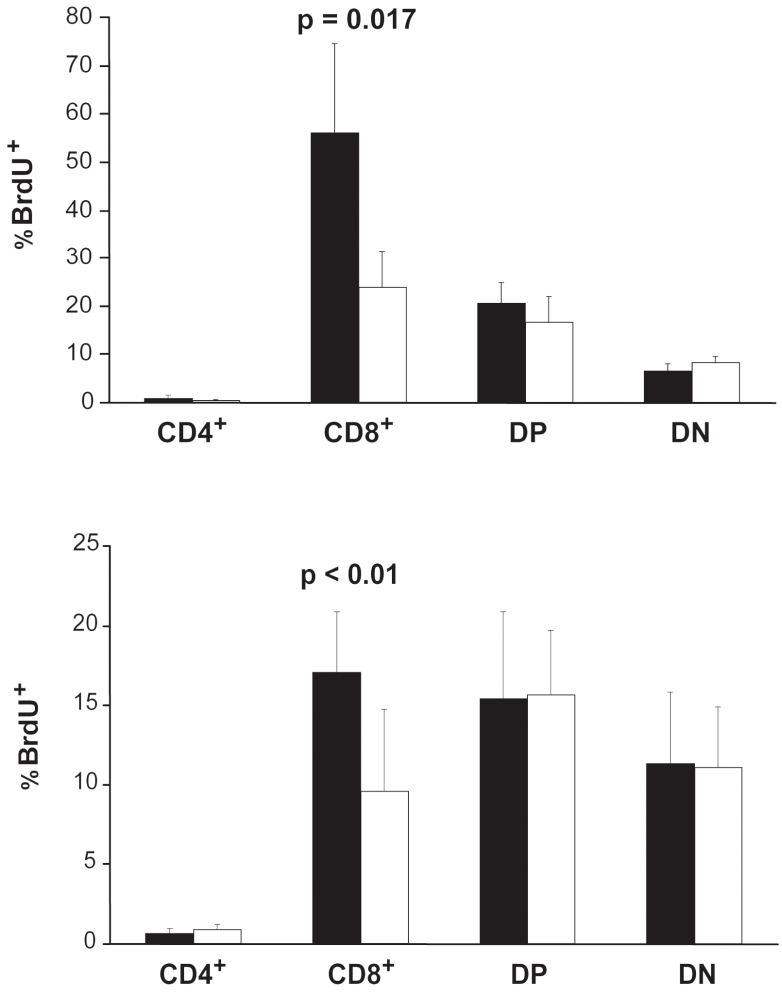


Figure 18 Increased proliferation of TβRII-/- CD8 SP thymocytes after non-competitive (upper) and competitive (lower) BM transplantation.

Since TGF-β has been shown to induce expression of CD8 *in vitro,* the effect imposed by TGF-β on CD8+ thymocytes could be two-sided [191]. TGF-β might augment CD8 expression in concert with other cytokines such as IL-7 and TNF-α as been proposed before, but could also exert inhibitory effect specifically on CD8+ thymocyte proliferation. In our model, therefore, it is possible that the induction of CD8 expression is maintained by the redundant effect of other cytokines. Dominant negative inactivation of TGF-β signalling in T cells using the CD2 promoter has indeed demonstrated an increased proliferation of peripheral CD8+ T cells in line

with our finding [194]. However, a recent study using the same approach of dominant negative disruption of TβRII but under the CD4 promoter showed that CD8 maturation in the thymus was inhibited [273]. This study did not, however, investigate the proliferation of CD8 expressing thymocytes and it was pointed out by the authors that in our experiments we did not investigated the ISP thymocyte compartment. Future investigation of the ISP frequency in our model could reveal if this cell subset contributed to our original observation. In summary, in this project we investigated thymocyte development in the absence of TGF-β signalling. The results revealed no significant impact on thymocyte subset distribution or apoptosis induction. However, we observed an increased proliferative capacity of $CD8^+$ thymocytes which might have an impact in the periphery and thus contribute to disease development in this mouse model.

The role of insulin in T cell development (study II)

Thymic insulin expression has been attributed to take part in the induction of negative selection of autoreactive thymocytes thereby contributing to the establishment of tolerance. However, insulin is also a growth hormone which might influence thymocyte expansion. Studies supporting this hypothesis have demonstrated, for example, that somatostatin, another pancreatic hormone, is expressed in the thymus by TEC's and might reduce as well as enhance thymocyte numbers and maturation [274,275]. Moreover, growth hormone (GH) have been shown to affect T cell development by inducing proliferation, cytokine secretion and migration possibly mediated by the insulin-like growth factor-1 (IGF-1) which is expressed in TEC [276]. Furthermore, IGF-2, which is highly expressed by mTEC's, have likewise been suggested to influence early thymocyte differentiation as well as development of thymic epithelia [277,278]. Therefore, since we were interested in studying the role of thymic insulin expression on the induction of thymocytes selection, it was important to exclude any effects of insulin as a growth factor for the development and differentiation of thymocyte subsets. In order to investigate these aspects we used mice deficient in the two insulin genes, *Ins1* and *Ins2* double KO mice. These mice die within 48 hours after birth due to insulin deficiency and fatal neonatal diabetes. Therefore we approached this problem by using embryonic thymi. T cell development in the absence of thymic insulin was studied by FTOC from 14.5 day old embryos which were cultured for 7 days and thereafter stained and analysed by flow cytometry (see experimental strategy). As a consequence of the intercrossing, three different genotypes were obtained and were all used in the analysis; $Ins1^{-/-}Ins2^{-/-}$, $Ins1^{-/-}Ins2^{+/-}$ and $Ins1^{-/-}Ins2^{+/+}$.

Our results revealed no difference in thymic subset distribution including early DN1-4 thymocytes as well as later thymocytes, i.e. CD4 SP, CD8 SP, DP and thymic $CD4^{+}CD25^{+}$ Treg. The experiments were also performed in serum free Aim V media to guarantee that possible presence of bovine insulin in the fetal calf serum would not influence the experiment (figure 19). Our results are in line with a previous experiment showing that treatment of FTOC's with antibodies against proinsulin did not affect thymocyte development [277]. However, another report did observe increase in DN3 thymocyte frequency after insulin treatment [279]. These conflicting data could nevertheless be explained by the fact that insulin was exogenously added to FTOC's in this experiment. Therefore, it seems likely that thymic insulin expression does not contribute to thymocyte maturation and differentiation whereas it is conceivable that circulating insulin indeed could affect thymocytes that are in proliferative stages. However, whether this effect is by direct action on thymocytes themselves or a secondary effect mediated by additional cytokine release from epithelial cells is unknown. Indeed, both thymocytes and TEC do express insulin receptors suggesting that both alternatives are possible [280]. Freitas

et al [279] did report that insulin could not increase thymocyte expansion in cell suspension cultures in the absence of stromal cells, further indicating that exogenous insulin administration affect thymocytes through TEC rather than direct binding to their receptors. In conclusion, our data suggest that insulin expression in thymus indeed most likely contributes to tolerance induction rather than promoting expansion and maturation of thymocytes.

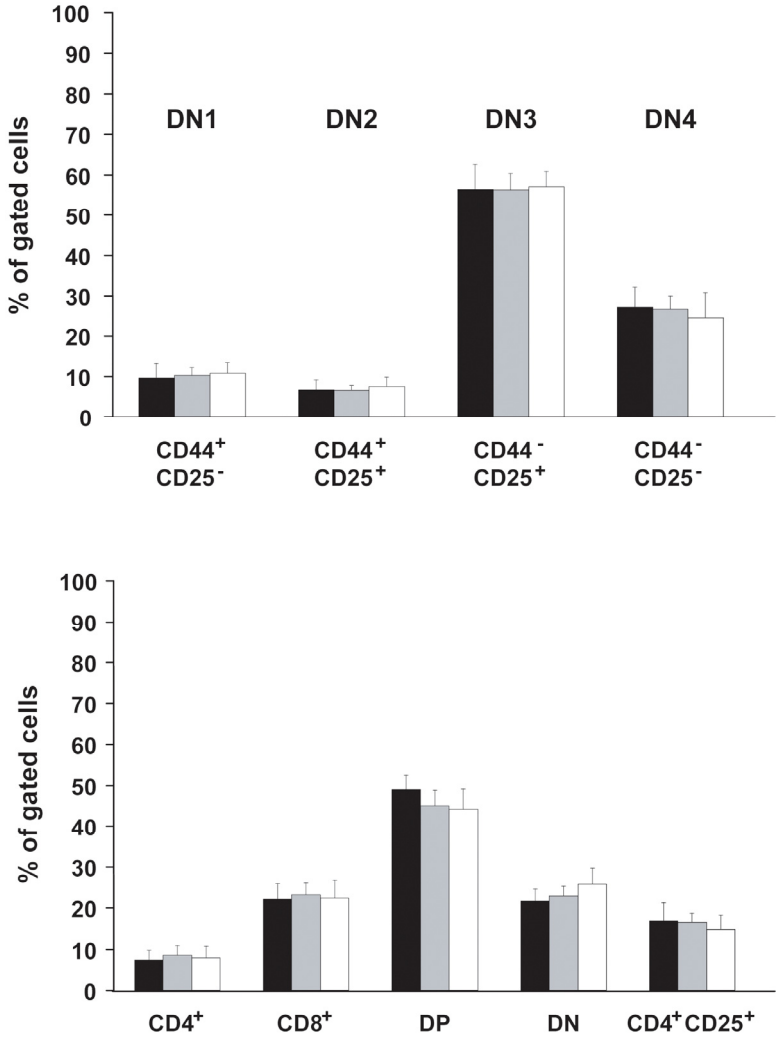


Figure 19 Frequency of early DN1-DN4 thymocytes (upper panel) and late thymocytes (lower panel) after 7 day FTOC in serum free Aim V media. Filled bars represent *Ins1*$^{-/-}$ *Ins2*$^{-/-}$, shaded bars *Ins1*$^{-/-}$*Ins2*$^{+/-}$ and open bars *Ins1*$^{-/-}$*Ins2*$^{+/+}$. DN cells were gated on CD3,4,8 lineage negative cells. CD4^{+}CD25^{+} were gated on CD4 SP thymocytes.

Thymic insulin expression and negative selection (study III)

Our previous study suggested that thymic insulin expression was dispensable for thymocyte expansion and maturation, therefore we decided to further investigate the impact of thymic derived insulin on negative selection of thymocytes using this valuable mouse model. In order to study these aspects *in vivo*, we transplanted *Ins1*$^{-/-}$*Ins2*$^{-/-}$ or *Ins1*$^{-/-}$*Ins2*$^{+/+}$ fetal thymi under the kidney capsule of athymic nude mice. Grafting of fetal thymi under the kidney capsule is an efficient method to reconstitute the immune system and study tolerance induction. Nude mice lack functional thymi due to mutation of the *foxn1* gene causing abnormal development of epithelial cells unable to support thymocyte development [49,50]. However, thymic bone marrow progenitors are intact and engraftment of a fetal thymus enables these cells to migrate and develop physiologically *in vivo*. Using this system also allowed us to investigate if thymic insulin expression is an intrinsic and independent property (immunological homunculus) or whether migration of APC's to the thymus could contribute to ectopic insulin expression. Therefore, we first analysed grafted thymi for insulin re-expression by RT- and QT-PCR (see experimental strategy). The results indeed revealed a re-expression of *Ins2* in previously insulin deficient grafted thymi (figure 20). Moreover, these results were additionally confirmed by grafting insulin knock out thymi under the kidney capsule of *Ins1*$^{-/-}$*Ins2*$^{+/+}$ mice (figure 21, unpublished results). Therefore, thymic insulin expression does not exclusively depend on mTEC expression and seem to, in part, arise from peripheral sources.

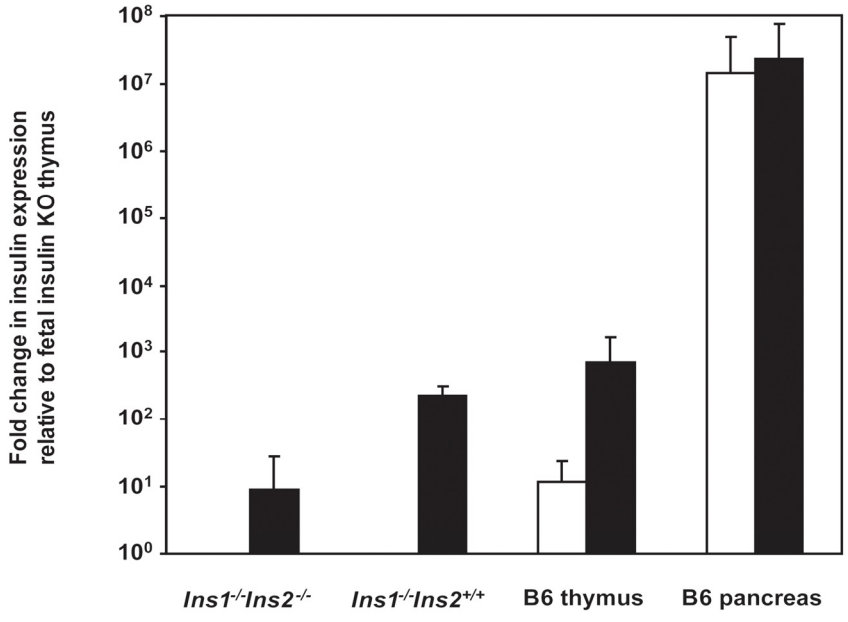


Figure 20 Re-expression of *Ins2* (black bars) in *Ins1*$^{-/-}$*Ins2*$^{-/-}$ transplanted nude mice. White bars indicate *Ins1* expression in control samples.

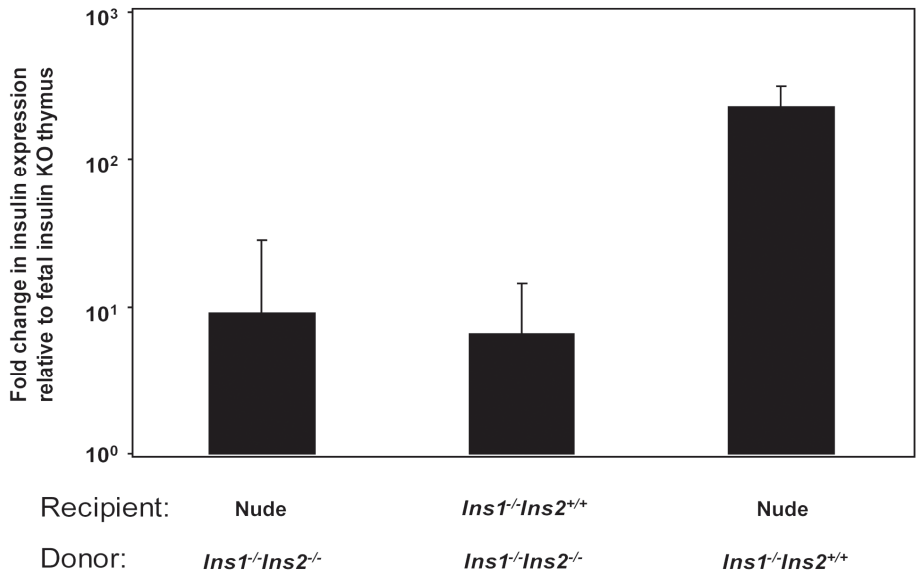


Figure 21 Re-expression of *Ins2* in *Ins1*$^{-/-}$*Ins2*$^{-/-}$ transplanted *Ins1*$^{-/-}$*Ins2*$^{+/+}$ mice (middle bar) compared to *Ins1*$^{-/-}$*Ins2*$^{-/-}$ and *Ins1*$^{-/-}$*Ins2*$^{+/+}$ transplanted nude mice.

As insulin deficient thymi were reconstituted by insulin-expressing cells, we wanted to investigate if these cells also contributed to clonal deletion. To study peripheral T cell reactivity to insulin, CFSE-stained splenocytes, B cell depleted, were cultured with insulin-pulsed CD11c^{+} DC's (see experimental strategy). Proliferation was therafter analysed by flow cytometry. The results revealed no difference in CFSE dilution between splenocytes from *Ins1*$^{-/-}$*Ins2*$^{-/-}$ or *Ins1*$^{-/-}$*Ins2*$^{+/+}$ thymi recipients suggesting that re-expression of insulin might have re-established negative selection of insulin-reactive thymocytes. In fact, a subset of bone marrow derived CD11^{+} PEA cells have been demonstrated to transcribe insulin which is subsequently processed and presented in an immunogenic form [281]. Moreover, these cells do migrate to the thymus and most likely contributes to clonal deletion. Indeed immunohistochemistry stainings have localised this DC subset in the cortico-medullary junction positive for insulin encircled by apoptotic thymocytes [168]. Our results are also supported by these studies and demonstrate that insulin expression in thymus takes places at many levels.

Given that thymic Treg are suggested to arise as a result of high-affinity interaction with self-antigens, we wanted to explore Treg in grafted thymi and in the periphery of transplanted nude mice. No difference in the frequency of CD4^{+}CD25^{+}FoxP3^{+}

cells could be observed neither in the thymus nor in the periphery of mice transplanted with *Ins1*$^{-/-}$*Ins2*$^{-/-}$ or *Ins1*$^{-/-}$*Ins2*$^{+/+}$ thymi. Together with the previous results, these findings reinforce the idea that development of Treg is not affected by thymic insulin deficiency or by the re-expression of insulin after transplantation. However, since we did not test the suppression ability of these cells it is still possible that the generation of insulin-specific Treg could have been affected. Interestingly, the thymic region of Hassall's corpuscle, which is suggested to provide an unique environment for Treg development via TSLP-activated DC's, have indeed been shown to express proinsulin [185,282]. These fascinating data suggest that Treg specific for insulin might be generated in this area. If that were the case, insulin-specific Treg in our model would indeed be affected as the re-expression of insulin probably is the result of immigrating PAE's and not present in Hassall's corpuscle. However, we did not have the opportunity to study these aspects, which will be the focus of future investigations.

The impact of thymic insulin expression on negative selection of thymocytes has been supported by many studies. For example, NOD mice have been shown to express lower levels of insulin expression in the thymus which is in accordance with T1D patients where lower thymic insulin levels seem to be under the control of the diabetes susceptibility short VNTR loci [161,162,283]. Insulin is not the only T1D autoantigen which is ectopically expressed in the thymus as GAD65, ICA69 and IA-2 are also present [284-286]. Therefore, loss of tolerance arises not only for insulin, but also for other proteins. For example, a study investigating ICA69 thymic expression in NOD mice revealed lower levels of ICA69 compared to wild type strains [287]. The suggestion that insulin is the dominant autoantigen in T1D is supported by studies showing that full insulin knock out NOD mice combined with a mutated proinsulin transgene completely arbrogated diabetes development whereas deletion of ICA69, IA-2 or GAD65 did not [288-291]. NOD mice deficient for *Ins2* develop an accelerated form of diabetes further emphasising the role for insulin in tolerance breakdown [292,293]. In these mice thymic expression of the highly immunogenic insulin epitope B:9-23 encoded by the *Ins2* gene, which has been shown to activate autoreactive diabetogenic T cells, is deleted but the peptide still managed to induce a T cell response after immunisation to NOD *Ins2*$^{-/-}$ mice [292]. These data might indicate that thymic *Ins2* expression in NOD mice fails to eliminate B:9-23 reactive thymocytes and deletion of *Ins2* will not alter that process. However, it is also possible that cells specific for the *Ins1* variant of this epitope, which only differs with one amino acid, cross-reacted to the peptide in this study. Furthermore, the authors observed activation from an additional peptide sharing the same amino acid sequence for both *Ins1* and *Ins2* located in the A-chain. The peptide activated CD4^{+} NOD *Ins2*$^{-/-}$ cells but not wild type cells suggesting that these cells were indeed generated in the absence of *Ins2*. Therefore, there could be separated mechanisms giving rise to B:9-23 or A-chain specific T cells mediated for example by differences in protein

processing. In addition, post-translational modification of a peptide activating human A-chain reactive T cells has been reported indicating that additional changes might contribute to this complex scenario [294]. In fact modification of autoantigens through processes such as oxidation could eventually change epitope reactivity during the course of the disease. Once β-cell death is initiated novel epitopes are either exposed to the immune system or created as a result of destructive conditions during necrosis. Therefore, such epitope spreading complicates investigations aiming at understanding the impact of thymic insulin expression on the generation of autoreactive T cells and/or Treg.

Thymic expression of genes involved in negative selection has been attributed to the Aire protein as *Aire* deficient mice lack several tissue-restricted antigens. However, it seems that this protein cannot account for all ectopic expression since some TRA's are Aire independent and insulin-expressing PAE do not express Aire [151,159,168]. Consequently, thymocyte selection could have opposite outcomes depending on the type of APC presenting the antigen. It has been suggested that optimal negative selection requires high APC maturation status thereby implicating the necessity for co-stimulation. Peptide presentation by different APC's during negative selection might indeed contribute to altered peripheral T cell repertoires. This is highlighted by selection of Treg from TSLP-activated DC's in the Hassall's corpuscle [185]. Therefore, in T1D, defective clonal deletion of insulin-reactive thymocytes might not only arise from reduced insulin expression in mTEC's but could also reflect either a lack of co-stimulatory abilities of immigrating PAE's or altered ability of insulin transcription in these cells. In line with this reasoning, studies have indicated that bone-marrow derived NOD DC's have lower co-stimulatory abilities after activation although the results from these studies were later challenged by others which were unable to reproduce the findings [295-298]. The controversy was suggested to depend on the use of GM-CSF alone instead of GM-CSF/IL-4 in cultures to induce immature DC's but additional studies did however confirm lower up-regulation of co-stimulation in NOD DC's even in the presence of IL-4 [297-299]. Furthermore, children with T1D have shown decreased expression of B7.1 after maturation and activation of PBMC-derived DC's [300]. However, reports concerning the frequency of different DC subsets in peripheral blood of type 1 diabetic patients have been contradictory [300-302]. Nevertheless, even though there is a discrepancy regarding *in vitro*-obtained DC's or naturally occurring circulating DC's, observations of defects obtained from studying *in vitro*-generated DC's might be of importance for understanding immune activation and/or regulation of immune responses. Consequently, decreased ability to provide co-stimulation and antigen presentation might ultimately affect negative selection mediated by PAE in the thymus.

In summary, the results from our study suggest that self-antigen expression in the thymus is not only an intrinsic independent process as we clearly show that thymic

influx of peripheral bone marrow derived cells contribute to the ectopic expression of insulin in the thymus. Further investigations aimed at identifying the key cellular subsets that are involved in this process and their possible contribution to the establishment of central tolerance will definitely be of importance to understand the pathogenesis of autoimmune diseases.

Apoptosis resistance in NOD mice; the role of p53 (study IV)

Apoptosis is a major mechanism to maintain cellular homeostasis in the periphery as well as inducing tolerance in the thymus. Previous studies have shown that NOD mice are resistant against various apoptosis-inducing agents including γ-irradiation, therefore in this project we wanted to further dissect the molecular basis for γ-irradiation induced apoptosis resistance in NOD thymus [258,263,267,303]. Cell death in response to γ-irradiation is generated as a result of DNA damage which cannot be rescued by DNA repair mechanisms. Thus, before the cell enters the path of programmed cell death, a series of checkpoints are carried out. Any defects in these mechanisms might lead to either premature or late apoptosis initiation consequently rendering cells sensitive or resistant to apoptosis induction. We choose to investigate apoptosis induction in NOD thymocytes using γ-irradiation not only because NOD thymocytes are intrinsically resistant to it but also because this phenotype could reveal defective intracellular pathways relevant for diabetes pathogenesis. In support of this hypothesis, resistance to γ-irradiation induced apoptosis in NOD thymocytes has been previously genetically mapped to the *Idd5* susceptibility locus [239]. Apoptosis induction through γ-irradiation could also reveal defects in thymic apoptosis induced by non-functional pre-TcR or high-affinity TcR signalling since they share several intracellular signalling pathways. We investigated these aspects in NOD mouse using whole-body sublethal γ-irradiation. Our results confirmed previously publications showing apoptosis resistance in thymocytes compared to wild type using TUNEL and PI stainings (figure 22).

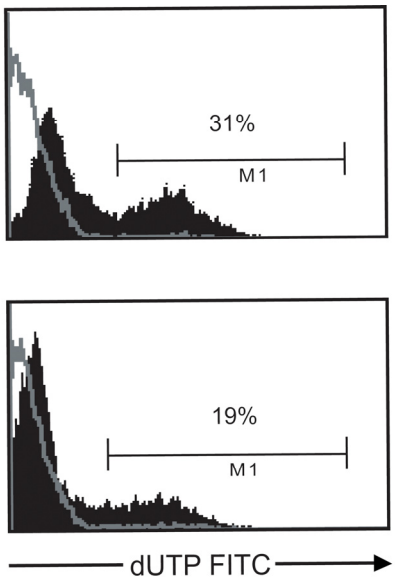


Figure 22 TUNEL staining showing less apoptosis induction in NOD thymocytes (lower panel) compared to C57BL/6 control (upper panel).

In order to further elucidate the signalling pathways contributing to this phenotype, we decided to investigate the expression of several caspase proteins using RPA (see experimental strategy). Caspases are highly involved in apoptosis signalling as activation of the caspase cascade leads to condensation of chromatin and cleavage of actin and lamins which brings about cellular decomposition [195]. Moreover, several defects in caspase expression have been reported in the NOD mice [265,266]. However, in contrast to previous reports we could not detect any changes in caspase-3 or 8 which nevertheless can be explained by the different experimental approaches as TcR stimulation were used to study AICD in these investigations. We did nonetheless observe impaired up-regulation of the caspases-1 and 11. Moreover, as we further investigated proteins involved in apoptotic and cell cycle signalling pathways, we observed that the tumour suppressor gene p53 was not up-regulated in response to γ-irradiation in the thymus of NOD mice. Based on our observations, we propose two possible scenarios where defective up-regulation of p53 and caspases-1 and 11 might contribute to apoptosis resistance in the NOD mouse model. The first model implies p53 activation and accumulation in response to double DNA strand breaks (DSB) created at the V(D)J recombination during pre-TcR rearrangement. This leads to DNA repair mechanisms mediated by DNA-protein kinases (DNA-PK). DNA-PK and the p38 MAP kinase, which is activated during V(D)J recombination, have both been shown to phosphorylate p53 [256,304-306]. Accumulation of p53 leads to induced cell cycle arrest via $p21^{WAF-1}$ for β-selection checkpoint. If DNA rearrangement has been successful, pre-TcR signalling will inhibit p53 possibly via the negative p53 regulator Mdm2 [307]. In the absence of a rescue signal, accumulation of p53 will eventually lead to apoptosis (figure 23). However, as p53 up-regulation seems to be defective in NOD mice, less accumulation of p53 will result into a higher threshold for induction of apoptosis in NOD thymocytes.

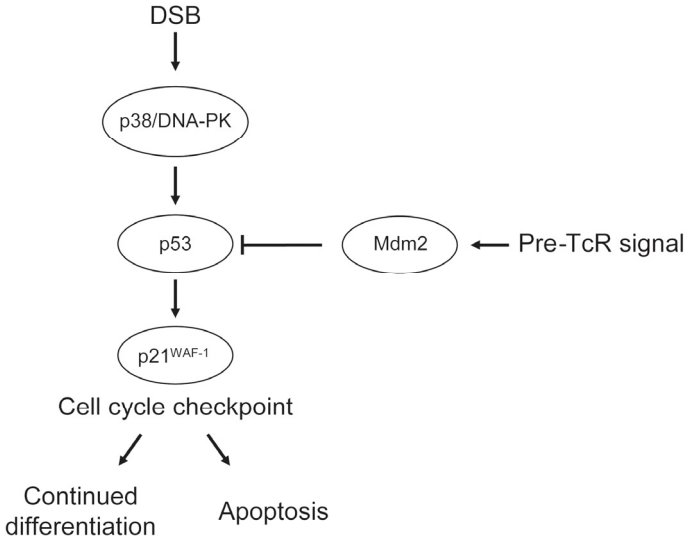


Figure 23 Proposed model of p53-induced apoptosis during β-selection.

The second scenario involves the engagement of p53 and caspases-1 and 11 during negative selection induced apoptosis. High-affinity TcR signalling leads to activation of the transcription factor E2F1 which via ARF leads to inhibition of Mdm2 thus enabling p53 accumulation and subsequent induction of apoptosis [131,132]. The contribution of nitric oxide (NO) to thymocyte apoptosis is uncertain as it has been demonstrated to either induce or protect thymocytes from cell death depending on the experimental setting [308]. However, NO promotes apoptosis by a p53 and caspase-1 dependent mechanism which indeed might also involve caspase-11 as it induces caspase-1 activity [309-311]. As mentioned above, apoptosis resistance of NOD thymocytes has been linked to the *Idd5* locus containing the CTLA-4 gene and *Ctla-4*$^{-/-}$ mice display similar apoptosis resistance as NOD thymocytes indicating that this co-stimulatory molecule could be involved in the signalling pathways contributing to the NOD phenotype [239,264]. Interestingly, the CTLA-4 signalling protein SHP-2 promote cell cycle arrest and could possibly act in concert with p53-induced p21^{WAF-1} [312](figure 24). NOD thymocytes have been also shown to be defective in up-regulation of Bim and caspases-3 and 8 during apoptosis induction [265,266,313]. Therefore, insufficient up-regulation of p53, Bim, CTLA-4, caspases-1, 3, 8 and 11 in response to high-affinity TcR signalling and NO production might contribute to resistance in apoptosis induction and/or inadequate cell cycle arrest in NOD thymocytes. The combination of these two scenarios could ultimately lead to a peripheral T cell repertoire with T cells carrying high-affinity TcR's and altered apoptosis thresholds thus contributing to the T1D pathogenesis in NOD mice by protecting autoreactive T cells from clonal deletion.

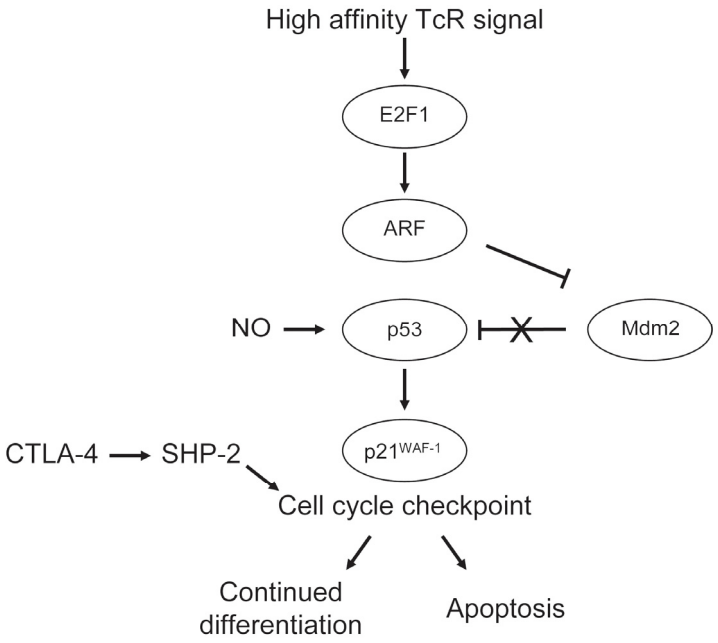


Figure 24 Proposed model of p53-induced apoptosis during negative selection.

SUMMARY OF RESULTS AND CONCLUSIONS

The main findings of this thesis demonstrate that:

- Thymic TGF-β is not essential for thymocyte differentiation.
- Induction of apoptosis in thymocytes is not affected by the lack of thymic TGF-β signalling.
- Proliferation of $CD8^+$ thymocytes is increased in the absence of thymic TGF-β signalling which might contribute to the lymphoproliferative disorder observed in mice lacking TGF-β signalling.
- Insulin expression in thymus is dispensable for thymocyte differentiation.
- Expression of insulin in the thymus is not only intrinsically regulated but could also be derived from thymic influx of peripheral bone-marrow cells probably contributing to the establishment of central tolerance.
- NOD mice are resistant against γ-irradiation induced apoptosis.
- Apoptosis resistance in NOD mice is in part caused by a defective up-regulation of p53, caspases-1 and 11.

In conclusion, aspects such as defective thymic expression of TGF-β and insulin as well as resistance in apoptosis induction through impaired up-regulation of p53 and caspase proteins can affect T cell development which ultimately might contribute to the development of autoimmune disorders such as T1D.

SWEDISH SUMMARY (populärvetenskaplig sammanfattning)

Autoimmuna sjukdomar, såsom typ 1 diabetes, uppstår till följd av en obalans i immunsystemet där immunceller ser kroppens egna vävnader som ett hot och angriper dem. Vid typ 1 diabetes är det de insulin-producerande β-cellerna i bukspottskörteln som förstörs, vilket leder till att man resten av livet får tillföra kroppen insulin genom injektioner. Anledningen till varför β-cellerna angrips är fortfarande till stor del okänd. Defekter i vissa mekanismer, som i normala fall hjälper till att hålla immunsystemet under kontroll, kan bidra till uppkomst av autoimmunitet. Dessa mekanismer upprätthåller ett tillstånd av tolerans mot kroppen och verkar på två olika nivåer. Så kallad central tolerans skapas under mognad och utveckling av en viss typ av immunceller, T-cellerna. Perifer tolerans bibehålls ute i vävnaderna genom olika processer, t.ex. via en speciell grupp T-celler vilka dämpar aktivering av andra celler.

T-celler tillhör, tillsammans med B-celler, den adaptiva grenen av vårt immunsystem. Det adaptiva immunsystemet formas utefter infektionen och aktiveras vid senare faser av sjukdomen eftersom cellerna behöver tid att mobilisera sig och angripa infekterade celler mer specifikt än vad som sker i början av sjukdomsförloppet. Föregångare till T- och B-celler, s.k. hematopoetiska stamceller, finns i benmärgen. Medan B-celler utvecklas och mognas i benmärgen (därav B-celler), vandrar de blivande T-cellerna till ett organ kallat tymus (därav T-celler) som är lokaliserat ovanför hjärtat. Här genomgår de olika mognadsprocesser som garanterar att de celler som så småningom kommer ut i cirkulationen är kapabla att aktiveras vid infektion, men också att de inte aktiveras av kroppens egna ämnen. Dessa egenskaper formas när T-cellerna genomgår positiv och negativ selektion under utvecklingen. Medan den positiva selektionen ser till att cellerna kan aktiveras korrekt, säkerställer den negativa selektionen att de celler som överaktiveras sållas bort. Därmed skapas central tolerans. Den här avhandlingen fokuserar på hur defekter vid etablering av tolerans under T-cellsutvecklingen kan bidra till uppkomst av autoimmuna sjukdomar såsom typ 1 diabetes.

I den första studien undersöker vi hur uttrycket i tymus av ett protein, TGF-β, kan påverka T-cellsutveckling. Vi använde oss av en s.k. konditionell knockout modell som vid induktion avlägsnar den kodande genen för TGF-β-receptorn. Därefter transplanterade vi benmärgen till strålade möss och på så sätt är det endast celler som kommer från benmärgen som inte kan svara på TGF-β. Dessa möss utvecklar autoimmunitet där immunsystemet är överaktiverat. Genom att undersöka hur tymocyterna, d.v.s. omogna T-celler, ser ut och fungerar kan vi avgöra om avsaknad av TGF-β under T-cellsutveckling kan leda till autoimmunitet. Vi såg att tymocyter utvecklades normalt men att en grupp av $CD8^+$ tymocyter uppvisade en ökad

förmåga till celldelning, proliferation. Denna egenskap kan leda till en okontrollerbar överaktivitet och därigenom till autoimmunitet.

Nästa studie fokuserar på hur insulin påverkar T-cellsutveckling. Insulin är ett hormon som utsöndras av β-cellerna i bukspottskörteln vid förhöjda glukoshalter i blodet. Dessutom är insulin ett av de proteiner som aktiverar autoreaktiva T-celler hos diabetespatienter. Men, insulin uttrycks inte enbart av β-celler. Det har på senare tid visats att många proteiner som igenkänns av autoreaktiva T-celler i sjukdomar såsom MS, diabetes och tyroidea, uttrycks i tymus. Det har därför spekulerats om dessa proteiner bidrar till etablering av tolerans under den negativa selektionen på så sätt att de tymocyter som aktiveras av dem under T-cellsutveckling sållas bort. Men, insulin fungerar även som ett tillväxthormon. Därför ville vi undersöka hur tymocyter utvecklas vid avsaknad av insulinuttryck i tymus. Detta gjordes med hjälp av en musmodell som saknar insulin. Möss som inte har insulin överlever dock inte mer än 48 timmar efter födseln så därför studerade vi T-cellsutvecklingen med hjälp av fetaltymus. Vi kunde inte påvisa någon förändring i fördelningen av tymocyter vid frånvaro av insulin och slutsatsen var därför att insulin i tymus sannolikt påverkar den negativa selektionen och uttrycks inte i syfte som tillväxthormon.

Den tredje studien var en fortsättning från förra då vi vidare studerade hur insulin påverkade den negativa selektionen av tymocyter och på så sätt hjälpte till att forma central tolerans. Vi transplanterade fetaltymus, som saknade insulin, till möss som inte har egen tymus men som däremot har T-cellsföregångare i benmärgen vilka kommer att utvecklas i den transplanterade tymusen. På så sätt kan vi se hur T-celler som mognats i en tymus utan insulin uppför sig ute i kroppen. Studien visade dock att insulin verkar återuttryckas i tymus genom en viss typ av celler som vandrar in utifrån periferin och presenterar insulin för tymocyter och därigenom bidrar till att etablera central tolerans.

Den fjärde och sista studien fokuserade på en av de mekanismer som framkallar negativ selektion i tymus och som i periferin hjälper till att nedreglera ett immunsvar när infektionen är över. Detta sker genom en speciell typ av programmerad celldöd som kallas apoptos. En musmodell för typ 1 diabetes, NOD-musen, har uppvisat defekter i apoptos och vi ville i detta projekt undersöka de bakomliggande orsakerna. Därför framkallade vi apoptos hos tymocyter genom γ-strålning. NOD-mössen påvisade mindre apoptos än kontrollmössen och efter att ha jämfört vissa proteiner som är inblandade i apoptos-signalering såg vi att NOD-mössen inte uttryckte p53 och vissa kaspas-proteiner i lika hög grad som kontrollmössen. Slutsatsen var alltså att defekt i apoptos hos NOD-musen under negativ selektion av tymocyter kan bero på felaktig uppreglering av dessa proteiner och detta kan således leda till att autoreaktiva tymocyter undkommer negativ selektion vilka så småningom bidrar till uppkomst av diabetes.

ACKNOWLEDGEMENTS

The completion of this thesis would not be possible without the help and support from so many people; family, friends and co-workers.

First, I would like to thank my supervisor, Corrado, for introducing me to the field of immunology in the first place. Under your guidance I have grown as a scientist and learnt the importance of asking questions. Your enthusiasm for science is what drew me to embark on this adventure and it's what kept me going even when things were tough. Well, that and the good times spent outside of work testing grappa and discussing unpublished results.

I would also like to thank Åke Lernmark, who was kind enough to let me be his student and for helping out with the bureaucracy when needed…

Since I started this project, our "islet of immunology" in Malmö has really expanded. Thank you Jeanette, one can always rely on you to bring cakes to meetings, Elisabeth for spreading joy in the lab, Tina for superb crayfish party, Mārtiņš the paparazzo and my companion in the animal house, Carro for getting me started with my jogging (good luck with the running!), Stina for always knowing what is needed and when you need it, Cissi for being an excellent room mate accompanying me in the lab during long experiments, thank you Stina and Cissi also for critically reading the thesis. Thank you present and former lab members for the support and for helping out when needed!

My practical supervisor (from hell….) Anna, who taught me the techniques and who also became a close friend. I miss you and hope to be able to visit you soon!

Per, for the excellent and fruitful collaboration and for providing with whiskey when discussing unpublished results late nights in the lab. Looking forward to the next goose dinner!

During the course of this thesis, I got to know a group of people who initially were co-workers in the lab but as the after work-sessions accumulated became close friends; Jenny, sneaky, since you already know what I'm about to say there's not really any point in me saying it (great minds…) but here goes anyway: thank you for being the best spinning and shopping friend who always knows how to enjoy life and how to cheer up the day. Johan, for outstanding game nights (I'm counting on another one very soon no matter how many sambuca it takes!), hilarious msn-chats and for always following me home after nights out in the city, Marketa for arranging the best PhD-parties and for always being one to count on for a good night out, Fredrik for being a first-rate master of schlager, Emma and Camilla for always brightening up the party. Looking forward to the next AW-session!

All the PhD's or PhD's-to-be who helped create a great work place: Lovisa (thank you for all the help on all RNA-related issues!), Tina, Charlotte G, Hemang, Nael, Eka, Christiano, Avinash, Eero, Charlotta and Anna.

Thank you to all members from the former Wallenberg lab and the new CRC labs: Leif, Marju, Olle, Martin, Charlotte L, Barbro L, Elisabeth, Carl-David, Holger, Valeriya, Johan, June, Pernilla, Tereza, Anna, Esa, Maggan, Lena, Malin, Lisbeth, Mona, Bodil, Marie, Maj-Lis, Agneta, Charlotte B, Britt, Joanna, Barbro G, Anita, Kristian, Anastasia, Sabina, Thea, Lina, Rasmus and Ida.

A friend is someone who knows all about you and still likes you…I don't think anyone knows more about me than the "circle of trust"; Sandra, Wenche, Erika and Tessan. Thank you all for always listening and understanding, for all the good times in the past and to come. I really look forward to join the circle again after my absence and hopefully the circle can be complete with Tessan moving back here someday!

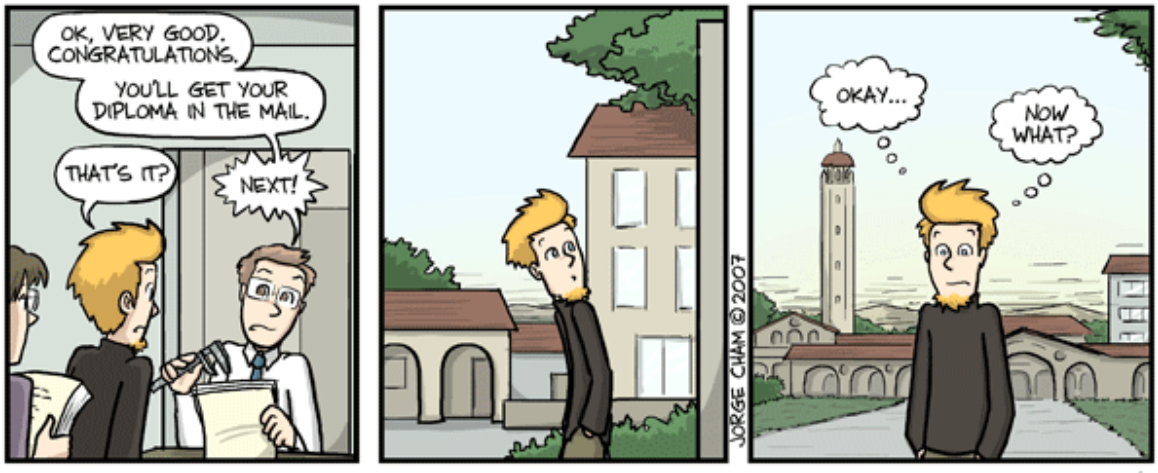
If there where someone who would know more about me it would probably be you, guys, Erik and Hanna, since you have been there from day one in Lund. Thank you, Erik, for all the talks we've had during the university years, for outstanding parties in Malmö with Tor and for coffee breaks whenever you're in town, I hope you will be able to move back here so we can get them more often. Thank you Hanna, for being an excellent lab-mate and "co-worker" at the nation, I hope you decide to come back to the south.

To my oldest friend from Nässjö, Dille, thanks for late birthday calls and company to "Hotet" even though the trips have been scarce these past years. I can always count on you to bring me the latest update on the Nässjö people.

My cousin and friend Jenny, thank you for dinners, "fika" and celebrations. Funny that none of us ended up working in a bank…..

Finally but most important my family: Mamma och pappa, for endless love and support, for providing the foundation from which I grew and for always reminding me to believe in myself. My brothers: Stefan, who really knows what doing a PhD is like (thanks for the pep talks), and Tomas for being the best room-mate ever, I miss our Tuesday movie nights.

"In friendship and in love, the two side by side raise hands together to find what one cannot reach alone." Anders, no words can express what I feel and I cannot imagine what life would be like without you by my side.



"What we call the beginning is often the end. And to make an end is to make a beginning. The end is where we start from." ~T.S. Eliot

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