Institut für Medizinische Mikrobiologie, Immunologie und Hygiene
der Technischen Universität München

Immunological recognition of the SV40 T antigen in a mouse model

Sonja Seewaldt

Vollständiger Abdruck der von der Fakultät für Chemie
der Technischen Universität München zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften
genehmigten Dissertation.

Vorsitzende: Univ.-Prof. Dr. S. Weinkauf

Prüfer der Dissertation:

1. Univ.-Prof. Dr. J. Buchner
2. Univ.-Prof. Dr. I.A. Förster
3. Univ.-Prof. Dr. H. Kessler

Die Dissertation wurde am 30.04.2003 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 20.01.2004 angenommen.

Die Ergebnisse dieser Arbeit sind zum Teil veröffentlicht:

Seewaldt S., Alferink J., Förster I. (2002). Interleukin-10 is crucial for maintenance but not for developmental induction of peripheral T cell tolerance. Eur. J. Immunol. *32*, 3607-3616.

TABLE OF CONTENTS

INDEX OF FIGURES

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AP	Alkaline phospatase
APC	Antigen presenting cell
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD	Cluster of differentiation
CFSE	Carboxy-fluoresceindiacetate succinimidyl ester
CpG	Cytosin-Guanosin
cpm	Counts per minute
CTL	Cytotoxic T cell
CTLA	Cytotoxic T lymphocyte associated antigen
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
IBD	Intestinal bowel disease
Id	Idiotype
IDDM	Insulin-dependent diabetes mellitus
IFN	Interferon
IL	Interleukin
IL-10R	IL-10 receptor
i.p.	Intraperitoneal
LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
NOD	Nonobese diabetic
ODN	Oligodeoxyribonucleotide
PCR	Polymerase chain reaction
POX	Peroxidase
P2	SV40 T antigen-peptide(362-384)
RIP	Rat insulin promoter
RT2	Rip1-Tag2
SEB	Staphylococcal enterotoxin B
SD	Standard deviation
SP	Single positive
Tag	SV40 T antigen
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
wt	Wild type

1 INTRODUCTION

This PhD work was carried out to characterize the immunological recognition of a specific tumor antigen (Ag) - the SV40 T Ag - in a transgenic mouse model. A central question in cancer immunology is whether the recognition of tumor Ags by the immune system leads to activation or tolerance. In the model subject to this study, expression of the SV40 T Ag leads to a profound state of tolerance towards this tumor Ag despite the presence of tumor specific cells of the immune system (Förster et al., 1995). These findings implicate that during tumorigenesis different mechanisms are established that enable the tumor to escape from immunological recognition. What are these mechanisms leading to tumor specific tolerance and how can this specific tolerance be broken?

The first aspect to be addressed here is how the immune system can mediate immunological recognition. The physiological function of the immune system is to protect the individual from pathogens while maintaining tolerance against self. To ensure this function, the immune system has to discriminate between self and non-self. There are different hypotheses, how this discrimination may be achieved. One model proposed by Matzinger (reviewed in Matzinger, 1994) relies on general danger signals, which can be sensed by the immune system. If detectable harm is done to the body, damaged cells may release factors, which are normally found only inside the cells. These factors can be detected by the immune system leading to its activation. Therefore, under normal conditions, the immune system would not be activated to self-determinants, but immunity is generated when pathogens harm the body.

A modification of this model suggested by Janeway focuses on signals derived from the pathogens themselves, which may also act as danger signals (reviewed in Janeway, 1992). Microbes and viruses express characteristic molecular patterns (pathogen-associated molecular patterns (PAMP)), which are not found in mammalian cells. These patterns include cell wall components of gram-negative or gram-positive bacteria (lipopolysaccharides (LPS) or teichoic acid), as well as unmethylated deoxyribonucleic acid (DNA) sequences found in bacteria. Cells of the innate immune system and specialized antigen presenting cells can detect these PAMP by specific pattern recognition receptors such as the Toll-like receptor (TLR) family. This recognition leads to activation of the immune system. By detection of specific molecular patterns not present in the healthy individual the immune system can thus discriminate between self and non-self.

In both models, additional signals representing danger signals or pathogen-derived signals are needed to activate the immune system. Tumors may evade immunological recognition by minimizing additional signals, which could provide activation of the immune system. Generally one would exclude pathogen-derived signals in the context of tumors, with the exception of virally induced tumors. However, invasion of tumors into the surrounding tissue as well as tumor metastases may provide danger signals according to the Matzinger model (Matzinger, 1994) since tissue disruption is involved. During tumor progression, invasive tumors and metastases represent already progressed stages, therefore the tumor already has established mechanisms to evade the immune response.

In order to induce effective tumor immunity, the immune system has to recognize tumor determinants as foreign. Tumor cells, however, derive from normal cells in which growth control has become dysregulated. Therefore, most tumor antigens are also expressed in normal cells and are only weekly immunogenic since they represent self. Consequently, mechanisms leading to self-tolerance may also account for tumor tolerance. Unresponsiveness to self is maintained by several mechanisms preventing the generation of potential harmful cells of the immune system. These tolerance mechanisms involve different anatomical sites and act primarily on antigen-receptor bearing lymphocytes of the adaptive immune system. Generally, one has to distinguish between central and peripheral tolerance induction. Central as well as peripheral tolerance induction can also be demonstrated in tumor animal models (Bogen et al., 1996).

1.1 Central tolerance

Central tolerance is achieved in primary lymphoid organs, namely the thymus for T lymphocytes and bone marrow for B lymphocytes. In these organs, immature self-reactive lymphocytes are eliminated after recognition of self-Ag. T cell precursors enter the thymus and mature while passing through sequential stages. Functional rearrangement of T cell receptor (TCR) genes is accompanied by expression of the coreceptors CD4 and CD8 on the surface. Since the TCR generation is a random process, T cells reactive against self are also generated. The immature T cells have to be able to interact with peptide-MHC complexes on thymic epithelial cells, a process called positive selection (von Boehmer et al., 1994). In general, only self-Ags are presented in the thymus, whereas foreign Ags are captured in peripheral sites.

Therefore, immature T cells recognizing peptide-MHC complexes with high affinity in the thymus have to be eliminated since they represent auto-reactive T cells. This elimination is achieved by induction of apoptosis. This process, termed negative selection, ensures that the repertoire of mature lymphocytes entering the periphery is devoid of T cells recognizing ubiquitously expressed self-Ag (Nossal et al., 1994).

1.2 Peripheral tolerance

Since it appears unlikely that all peripheral Ags can be presented by the thymus, there have to be additional systems to ensure tolerance towards only peripherally expressed Ags. These mechanisms leading to peripheral tolerance are also exploited by cancer cells (Förster et al., 1995; Speiser et al., 1997). Three main mechanisms of peripheral tolerance induction are known so far, which are clonal anergy, clonal deletion and generation of regulatory/suppressor T cells.

1.2.1 Clonal anergy

T cell responses are initiated by T cell activation through recognition of Ag on activated Ag presenting cells (APC). T cells recognize peptides presented by the major histocompatibility complex (MHC) on APCs through their TCR (signal 1) and additionally costimulation is provided by the APC through interaction of accessory molecules (CD80 and CD86) with CD28 on T cells (signal 2). Both signals are required to induce full T cell activation. Anergy is described as unresponsiveness of T cells to antigenic stimulation. *In vitro*, anergic T cells can be induced under conditions where Ag is presented by the APC in the absence of costimulation (reviewed in Schwartz, 2003). Thus, only a weak or incomplete activation of the T cell is provided and these cells are incapable of responding to Ag even if they are restimulated with activated APCs. It was concluded that recognition of organ specific self-Ag on peripheral APCs by autoreactive T cells leads to the induction of anergy, since limited costimulation is provided under normal conditions**,** i.e. in the absence of danger signals. However, anergy induction of naïve cells *in vivo* is always preceded by cell division (reviewed in Schwartz, 2003), suggesting more complex processes in the induction of anergy *in vivo* compared to the *in vitro* situation.

1.2.2 Clonal deletion

During an immune response, activation of T cells results in T cell proliferation and expansion of the effector T cell pool. After clearance of the Ag the T cell pool is contracted by programmed cell death (apoptosis) induced by deprivation of survival signals. However, if recently activated T cells are repeatedly stimulated with Ag, a different apoptotic mechanism is induced, termed activation induced cell death (AICD). Persistence of Ag, which would be the case for abundant peripheral self-antigens, leads to repeated stimulation of T cells and these cells upregulate death receptors (Fas and Fas ligand (FasL)) on the membrane. The interaction of Fas with FasL expressed on the same T cell or adjacent T cells activates a cascade of apoptosis inducing molecules (caspases) and results in AICD. T cell tolerance through deletion is thereby established.

Clonal deletion and clonal anergy presumably act in a synergistic manner as illustrated by studies of Kearney et al. in a transgenic T cell transfer model (Kearney et al., 1994). Soluble Ag administered without adjuvant led to proliferation and subsequent contraction of the responding T cell pool. Furthermore, the remaining T cells were unresponsive to restimulation *in vitro*. Thus, clonal deletion as well as clonal anergy induction represent two major processes of tolerance induction occurring during peripheral self-recognition by autoreactive T cells.

1.2.3 Regulatory $CD25^+CD4^+$ T cells

The third principal mechanism of tolerance induction involving regulatory/suppressor T cells has been subject of extensive studies. T cells able to suppress immune responses have already been described in the early 1970s (Gershon et al., 1970; Gershon et al., 1971). However, the lack of efficient molecular markers characterizing these specific T cells as well as the inability to maintain suppressor T cells *in vitro* led to the decline of interest and research in this field. Fundamental work done by Sakaguchi and coworkers in the mid-1990s initiated the discovery of a subset of $CD4^+$ T cells, which is able to suppress proliferation of naïve cells *in vivo* and *in vitro* (Sakaguchi et al., 1995; Asano et al., 1996). These cells constitutively express the interleukin (IL)-2 receptor α-chain (CD25) and constitute 10% of the peripheral $CD4^+$ T cell population in a normal mouse. Simultaneous to the work done by Sakaguchi (Sakaguchi et al., 1995), Mason (Fowell et al., 1993) and Powrie (Powrie et al., 1993) had identified regulatory $CD4^+$ T cells able to suppress autoimmunity in different rat and mouse models. In 2001 several

groups described $CD25^+CD4^+$ T cells also in humans, and these cells displayed the same properties as rodent cells (Jonuleit et al., 2001; Levings et al., 2001; Stephens et al., 2001).

The functional characterization of $CD25^+CD4^+$ T cells was focused on their ability to exert suppressive function and mainly carried out by means of *in vitro* studies. $CD25^+CD4^+$ T cells are anergic to TCR stimulation (Takahashi et al., 1998) and require exogenous IL-2 for survival *in vivo* and *in vitro* (Papiernik et al., 1998). Signaling through the TCR is necessary for suppression of naïve T cells. However, the Ag concentration needed by $CD25^+CD4^+$ T cells to exert suppressive function is much lower than the concentration required for proliferation of naïve T cells (Takahashi et al., 1998). Once activated, the regulatory function of suppressor T cells is Ag unspecific. Consequently, at low Ag concentrations, regulatory T cells raise the threshold for activation of naïve T cells.

Several studies have shown that cell contact between suppressor and naïve T cells is necessary for suppression. In addition, $CD25^+CD4^+$ T cell mediated suppression *in vitro* does not rely on cytokines such as transforming growth factor (TGF)-β, IL-4 or IL-10, as demonstrated by antibody (Ab) blockade of these cytokines during *in vitro* stimulation (Thornton et al., 1998; Takahashi et al., 1998). In contrast, blocking of IL-10 or TGF-β led to abrogation of the suppressive effect of $CD25^+CD4^+$ T cells *in vivo* (Powrie et al., 1996; Asseman et al., 1999). Therefore, although cytokines had not been implicated in the regulatory mechanisms of $CD25^+CD4^+$ T cells *in vitro*, they have been shown to play an essential role in different *in vivo* models of autoimmunity.

So far, no conclusive data on the mechanisms of suppression have been provided, except for the central role of IL-2 during this process. The anergic state and the suppressive capacity of $CD25^+CD4^+$ T cells can be broken by addition of exogenous IL-2. The inhibition of IL-2 production by effector cells (Thornton et al., 1998) was hence suggested as mechanisms of suppression. How this may be achieved remains to be elucidated. Paradoxically, IL-2 has also been shown to be required for the generation of $CD25^+CD4^+$ T cells *in vivo* (Papiernik et al., 1998).

Regulatory $CD25^+CD4^+$ T cells reside mainly in the periphery, but it still remained unclear where these cells were generated. Recently, several laboratories could prove that the thymus continuously produces $CD25^+CD4^+CD8^-$ thymocytes with regulatory capacity (Papiernik et al., 1998; Itoh et al., 1999; Jordan et al., 2001). About 5% of $CD4^+CD8^-$ thymocytes are CD25 positive and these cells have the same functional

characteristics as peripheral regulatory $CD25^+CD4^+$ T cells. The expression of CD25 on thymocytes is probably induced during the transition of the double positive to the single positive stage. So far, however, this could not be demonstrated conclusively.

The thymus representing the organ of central tolerance induction is therefore also implicated in the generation of regulatory T cells. Additionally, the general notion that only ubiquitously expressed and abundant blood-borne self-Ags are presented in the thymus has to be revised. Several studies have demonstrated that many self-Ags formally thought to be tissue-specific are also expressed on thymic epithelial cells (reviewed in Hanahan, 1998; Derbinski et al., 2001). Although the expression of a specific peripheral Ag in the thymus seems to be a rare event, it has been shown in various models to elicit partial self-tolerance (von Herrath et al., 1994; Smith et al., 1997).

Despite generation of regulatory T cells in the thymus, different types of regulatory T cells can also be induced in the periphery. The existence of such peripheral *de novo* development of suppressor T cells has been well documented by studies on oral tolerance induction. Mucosal administration of Ag induces profound T cell tolerance, and the anergized T cells produce immunosuppressive cytokines such as TGF-β. Oral tolerance has a fundamental physiological role since it suppresses immune responses to food Ags and to commensal bacteria residing in the gut. A number of different types of regulatory T cells can further be induced by repeated antigenic stimulation *in vivo* (Santos et al., 1994; Kearney et al., 1994; Homann et al., 2002), as well as through selective culture conditions *in vitro* (Groux et al., 1997; Jonuleit et al., 2000). Taken together, the induction of regulatory T cells is not only achieved in the thymus, but also naïve T cells in the periphery can be educated to become regulatory T cells.

The fundamental interest in regulatory T cells is based on the potential application of these cells in treatment of different human diseases. Understanding the mechanisms leading to regulatory T cell development as well as the *in vivo* function of these cells could help to design new therapies. In general, it could be beneficial to interfere in a positive or negative sense with the control maintained by regulatory T cells. In the case of autoimmunity, an increase of regulatory T cells in order to control auto-aggression would be the goal. The opposite effect would be desired in inducing tumor immunity, where elimination of regulatory T cells has been proven to be effective in animal models (Shimizu et al., 1999). Nevertheless, interventions in either direction are also

linked with the opposite effects. For example, induction of tumor immunity by eliminating regulatory T cells has the caveat of eliciting autoimmunity against healthy tissues (Sakaguchi et al., 2001). Therefore, the beneficial effects have to be validated in the light of potential side effects.

1.3 Role of cytokines in self-tolerance

Cytokines are the principal mediators of communication between cells of the immune system. Depending on the secretion of distinct patterns of cytokines T helper (Th) cells are further subdivided into Th1 and Th2 cells. Th1 cells produce IL-2, interferon (IFN)-γ, and lymphotoxin (LT), and they stimulate macrophages and cytotoxic T cells (CTL), which is essential for defense against infections. Th2 cells secrete IL-4, IL-5 and IL-10 and provide help for B cell activation. Furthermore, cytokine production by Th2 cells downregulates Th1 responses and vice versa. $CD4^+$ effector T cells implicated in various autoimmune diseases tend to be biased towards the Th1 or Th2 type depending on the type of autoimmunity. Th1 cells promote organ specific autoimmune diseases (insulin dependent diabetes mellitus (IDDM), thyroiditis), while Th2 cells mainly mediate systemic autoimmune diseases (lupus) (reviewed in Sakaguchi, 2000). Consequently, interfering with a given cytokine profile during an autoimmune response can be beneficial. For instance, in the nonobese diabetic (NOD) model of IDDM a cytokine shift from Th1 to Th2 induced by administration of IL-4 or anti-IFN-γ treatment has been demonstrated to prevent development of disease (Liblau et al., 1995).

In vivo experiments with regulatory T cells also stressed the important function of cytokines especially TGF-β and IL-10, in mediating suppression induced by these cells (reviewed in Maloy et al., 2001). Both cytokines have been shown to possess immunosuppressive activity in different systems. TGF-β inhibits proliferation and differentiation of T cells, B cells, as well as the activation of macrophages (reviewed in Letterio and Roberts, 1998). Furthermore, TGF-β blocks cell-cycle progression and presumably also has a direct effect on the expression of IL-2 (Brabletz et al., 1993). Like TGF-β, IL-10 also regulates growth and differentiation of various cell types of the immune system (reviewed in Moore et al., 2001). IL-10 is an essential regulatory factor for macrophages (Takeda et al., 1999) and downregulates MHC II as well as costimulatory molecules on APCs. Repeated antigenic-stimulation of naïve $CD4^+$ T

cells in the presence of IL-10 gives rise to regulatory T cells (Tr1) producing high levels of IL-10 and having immunosuppressive function *in vivo* (Groux et al., 1997).

The importance of TGF-β and IL-10 in induction and maintenance of tolerance is demonstrated by the respective knock-out animals. TGF-β-deficient mice develop severe, spontaneous autoimmune disease leading to death at 3-4 weeks of age (Shull et al., 1992; Kulkarni et al., 1993). This autoimmune phenotype is induced by sustained activation of $CD4^+$ T cells (Christ et al., 1994; Diebold et al., 1995; Letterio et al., 1996). Furthermore, selective unresponsiveness of T cells to TGF-β leads to the development of autoimmune disease, albeit the disease development is less severe compared to TGF-β-deficient mice (Gorelik et al., 2000). IL-10 deficient mice also develop spontaneous inflammatory reactions, but in contrast to TGF-β deficient mice, which show multi-organ autoimmunity, IL-10 deficiency leads to chronic enterocolitis (Kuhn et al., 1993). The intestinal inflammation in IL-10 deficient mice originates from uncontrolled reactivity to commensal bacteria (Berg et al., 1996). These findings illustrate that TGF-β is essential to maintain self-tolerance under normal homeostatic conditions, whereas IL-10 has a more specific role in the control of the intestinal immune system.

Strikingly, the immunosuppressive function of IL-10 and TGF-β is not only essential for the maintenance of self-tolerance, but it is also exploited by tumors. Several reports have described elevated expression levels of IL-10 and TGF-β associated with various tumors (Smith et al., 1994; Pisa et al., 1992). Secretion of IL-10 and TGF-β by tumors can actively inhibit the effector function of cells of the adaptive and innate immune system. This may also explain the finding that many tumors show infiltrates of lymphocytes as well as dendritic cells (DCs) and macrophages, which remain inactive. The tumor may thus have created a microenvironment, which paralyses the immune system.

1.4 Tumor antigens

The majority of Ags expressed by tumors can also be found in normal cells and are therefore not recognized by the immune system. However, several types of tumor Ags have been characterized, which can be detected by the immune system. These tumor Ags are considered as main targets for antitumor immunity established by T cells. Tumor Ags are closely associated with the alterations found in cancer cells. Mutants of

cellular genes as well as products of oncogenes involved in the transformation of normal cells to cancer cells are described as tumor Ags. In addition, overexpression or aberrant expression of normal cellular proteins found in tumor cells can provide a source of tumor Ags. The third main class of tumor Ags are products of oncogenic viruses.

The majority of tumor Ags characterized so far are related to recognition by CTLs, since activated CTLs can directly kill tumor cells after recognition of Ag expressed as peptide-MHC I complexes by tumor cells. Tumor Ags recognized by Th cells may also be a critical component in tumor recognition. Th cells are needed for sustained activation of CTLs, production of cytokines and recruitment of macrophages.

Several studies could demonstrate that DCs in the lymph nodes (LN) draining the tumor site present tumor Ags (Huang et al., 1994; Mayordomo et al., 1995; Nguyen et al., 2002). Strikingly, DCs not only present endocytosed Ag via the classical MHC II pathway but also via MHC I. This specific type of presentation has been termed cross-priming or cross-presentation (reviewed in Heath and Carbone, 2001), since the DC can prime CTLs specific for Ags of another cell. Through cross-presentation, the same APC can activate CTLs as well as Th cells specific for tumor Ags.

The immune system not only has to recognize tumor Ags, but the tumor Ag has to be presented in a context allowing activation of the immune system. Therefore, one strategy to induce effective tumor immunity is to provide additional stimuli like adjuvants for DC maturation *in vivo* in combination with the specific tumor Ag. Another approach is to isolate DC from cancer patients, propagate and activate them *in vitro* in the presence of tumor antigens and adoptively retransfer them.

1.5 The model: RT2/TCR1 mice

RT2/TCR1 mice represent an animal model of peripheral tolerance induction towards an endogenously expressed tumor Ag (Förster et al., 1995). This model consists of two different transgenic lines:

RIP1-Tag2 (RT2) mice express the SV40 T Ag under control of the rat insulin promoter (RIP) in their pancreatic β-cells (Hanahan, 1985). Since the transgene is already expressed early during embryonic development (embryonic d10) it essentially becomes a self-Ag and the animals develop profound tolerance against it. Ectopic expression of the SV40 T Ag could be detected in the thymus, implicating the possibility of central tolerance induction towards the transgene. The SV40 T Ag is a potent oncoprotein

causing transformation of cells by inhibiting tumor suppressor gene products p53 and Rb (Ludlow, 1993). The tumor development in these animals proceeds through defined stages. At an age of 4-5 weeks, first signs of the transforming action of the SV40 T Ag can be observed as proliferation of β-cells is detected resulting in hyperplasia of islets (Teitelman et al., 1988). The next stage is defined by growth of new blood vessels (neovascularization) leading to angiogenic islets at an age of 7-9 weeks (Folkman et al., 1989). Finally, encapsulated tumors arise from angiogenic islets (10-12 weeks), which invade into the exocrine pancreas at a low frequency (invasive carcinomas) (Perl et al., 1998). With increasing tumor mass, RT2 animals succumb to hypoglycemia.

The second line (TCR1 mice) expresses a transgenic TCR specific for the SV40 T Ag epitope (362-384) restricted to I-A^K. The transgenic TCR has been generated based on TCR genes found in SV40 T Ag reactive $CD4^+$ T cells isolated from infiltrates of a different SV40 T Ag expressing line (RIP-Tag5). RIP-Tag5 animals show a late onset of transgene expression in the pancreas (starting from 8-10 weeks) and develop an autoimmune response manifested by lymphocytic infiltrates in the pancreas. In the periphery of TCR1 mice, about 5 % of the total $CD4^+$ T cell population expresses the transgenic TCR. These cells can be detected with an anti-idiotypic monoclonal Ab.

By crossing RT2 mice with TCR1 animals, autoreactive $CD4^+$ T cells specific for a defined tumor neo-self Ag are introduced. The question was whether these self-reactive T cells would lead to auto-aggression, or if they could be rendered tolerant. Strikingly, RT2/TCR1 mice develop peripheral tolerance towards SV40 T Ag (Förster et al., 1995). The majority of peripheral autoreactive T cells get deleted during ontogeny. However, the percentages of transgenic T cells in the thymus of RT2/TCR1 mice are comparable to TCR1 mice, thereby excluding deletion of transgenic T cells by negative selection in the thymus. Additionally, the remaining peripheral transgenic T cells are functionally impaired in their responsiveness towards SV40 T Ag. Taken together, peripheral tolerance induction in the RT2/TCR1 model is characterized by clonal deletion and induction of anergy.

Tolerance against the SV40 T Ag is established during the first six weeks of live in RT2/TCR1 mice. At three weeks of age, RT2/TCR1 mice are normally responsive to the SV40 T Ag and they have the same percentages of transgenic T cells as TCR1 mice (Förster and Lieberam, 1996). However, already at the age of one week activated transgenic T cells can be found in the local environment of the pancreas in RT2/TCR1 mice. Additionally, young RT2/TCR1 mice show a transient insulitis with maximal

infiltration at 2-3 weeks of age. Therefore, tolerance induction in RT2/TCR1 mice is preceded by a phase of activation of autoreactive T cells (Förster and Lieberam, 1996). The timing of tolerance induction in RT2/TCR1 mice is presumably linked to the release and presentation of pancreatic Ags. Several studies have demonstrated that the presentation of islet β cell Ags is developmentally regulated (Höglund et al., 1999; Morgan et al., 1999). Juvenile animals have compromised presentation of pancreatic Ags, and activation through cross-presentation is inefficient during the perinatal period (Rafii-Tabar et al., 1986). The following sequence of events could occur in RT2/TCR1 animals. During an initial phase, transgenic T cells in RT2/TCR1 mice can see their Ag and get activated in LNs draining the site of Ag expression (pancreatic draining LNs, mesenteric LNs (MLNs)). However, this activation does not lead to tolerance induction. The activated T cells can infiltrate into the pancreas and cause limited inflammation, thus resulting in an increased Ag release. The repeated antigenic stimulation in conjunction with developmental changes in Ag presentation occurring around the age of 3 weeks (Scaglia et al., 1997; Trudeau et al., 2000) then leads to tolerance induction. Tolerant RT2/TCR1 mice still possess activated transgenic T cells in the MLN, indicating that maintenance of tolerance is an active process. Furthermore, tolerance induction in RT2/TCR1 mice correlates with the systemic appearance of $CD25^+$ transgenic T cells. At an age of 8 weeks more than 50% of the remaining transgenic T cells also express CD25. The increasing percentage of $CD25^+$ transgenic T cells during tolerance induction could be due to specific induction of CD25 expression. Another explanation would be the preferential deletion of $CD25^-$ transgenic T cells (Papiernik et al., 1998). The remaining population would consequently be enriched for $CD25^+$ transgenic T cells. In any case, the existence of $CD25^+$ transgenic T cells indicates that, besides tolerance induction by clonal deletion and anergy, active regulation by suppressor T cells may also be induced.

The RT2/TCR1 model has several advantages compared to other TCR transgenic models. First of all, only a small percentage of peripheral T cells express the transgenic TCR. This represents a rather physiological situation compared to TCR transgenic mice, where the majority of peripheral T cells are transgenic. Furthermore, the RT2/TCR1 model is also very attractive to study tumor immunology since tolerance is established towards an endogenously growing tumor in contrast to models of transplanted tumors.

1.6 Aim of this PhD work

Peripheral tolerance induction in the RT2/TCR1 model has been described to proceed through different stages. The end-stage is characterized by profound tolerance towards the tumor Ag (Förster et al., 1995; Förster and Lieberam, 1996). The central question is to understand how tolerance is established and maintained in RT2/TCR1 mice. What are the factors and conditions necessary to induce tolerance? Are regulatory T cells involved in this process?

As outlined above, cytokines like IL-10 and TGF-β have a crucial regulatory role in the induction and maintenance of tolerance. A regulatory role for IL-10 in limiting inflammation and immunopathology has been suggested in autoimmune models of arthritis and thyroiditis (Katsikis et al., 1994; Mignon-Godefroy et al., 1995). Furthermore, IL-10 has also been implicated in the regulatory function exerted by $CD45RB^{low}CD4^{+}$ T cells in transfer models of intestinal bowel disease (IBD) (Assemann et al., 1999). The central role of IL-10 in controlling immune responses was further strengthened by the general intestinal pathology seen in IL-10 deficient animals. However, no systemic signs of autoimmunity can be found in $IL\text{-}10^{KO}$ mice. It remains therefore to be determined whether IL-10 has any impact on the development or maintenance of peripheral tolerance towards self-Ag.

The first aim of this PhD thesis was to further elucidate the role of IL-10 in peripheral tolerance induction in the RT2/TCR1 model. For this purpose, RT2/TCR1 mice were crossed into an IL-10 deficient background. The tolerance status of $RT2/TCR1/IL\text{-}10^{KO}$ mice was analyzed by different immunological methods in comparison to RT2/TCR1 mice.

The second part of this work was focused on regulatory T cells. In RT2/TCR1 mice tolerance induction correlates with the appearance of $CD25^{+}$ transgenic T cells. The constitutive expression of CD25 has been attributed to a specific population of regulatory T cells. In the RT2/TCR1 model, it remained to be determined whether these $CD25^{+}$ transgenic T cells had indeed regulatory function and this question was an additional aspect of this PhD work.

The third part of this work was addressing the role of peripheral LNs in the generation of regulatory T cells and tolerance induction. In RT2/TCR1 mice initial activation of transgenic T cells takes place in lymphoid structures draining the site of Ag expression, whereas $CD25^{+}$ transgenic T cells can be found systemically in tolerant RT2/TCR1 mice. In a model of IDDM it has been demonstrated that induction of autoreactive T

cells requires the draining LNs (Gagnerault et al., 2002). Consequently, we hypothesized that these sites may also be needed for tolerance induction. Lymphotoxin beta receptor $(LT\beta R)^{KO}$ mice lack all peripheral LNs and therefore represent an ideal tool to investigate the dependency of tolerance induction on the presence of peripheral LNs. Hence, RT2/TCR1 mice were crossed into a LTβR deficient background and analyzed for the development of peripheral tolerance.

The results of this PhD work have been published in part (section 3.1.1-3.1.3):
Seewaldt S., Alferink J., Förster I. (2002). Interleukin-10 is crucial for maintenance but not for developmental induction of peripheral T cell tolerance. Eur. J. Immunol. *32*, 3607-3616.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals, radioactive substances, media

Chemical substances were generally purchased from Sigma, Taufkirchen. [Methyl-^{3}H]-thymidine (specific activity 5.0 Ci/mMol) was bought from Amersham, Braunschweig. Cell culture media and cell culture additives were obtained from GibcoBRL, Eggenstein unless otherwise stated.

Chemicals and Enzyms	Source of Supply
Acetic acid	Merck, Darmstadt
Aceton	Pharmacy, Klinikum Rechts der Isar, München
Agarose	GibcoBRL, Karlsruhe
Ammonium sulfat	Merck, Darmstadt
β-mercaptoethanol	Sigma, Taufkirchen
Bovine serum albumin (BSA)	Roche Diagnostics GmbH, Mannheim Biomol, Hamburg
CFSE	Sigma, Taufkirchen
Deoxynucleotides (dNTPs) (dATP, dGTP, dCTP, dTTP)	GibcoBRL, Karlsruhe
EDTA	Sigma, Taufkirchen
Eosin Y	Sigma, Taufkirchen
Ethanol	Pharmacy, Klinikum Rechts der Isar, München
Ethidiumbromid	Roth, Karlsruhe
Fetal calf serum (FCS)	PAN Biotech, Aidenbach
Formaldehyd (37%)	Sigma, Taufkirchen
Glycerol	Sigma, Taufkirchen
Hematoxylin	Sigma, Taufkirchen
Hydrochlorid acid	Roth, Karlsruhe
Levamisole solution	Vector Labs, USA
Marker 1kb DNA-ladder	GibcoBRL, Karlsruhe
Mineral oil	Sigma, Taufkirchen
2-methylbutan	Sigma, Taufkirchen
Lipopolysaccharide (LPS)	Sigma, Taufkirchen
NGS (Normal goat serum)	Dianova, Hamburg
Orange G	Sigma, Taufkirchen
Saponin	Sigma, Taufkirchen
SDS (natriumdodecylsulfat)	Roth, Karlsruhe
Tris-(hydroxymethyl)-aminomethan	Roth, Karlsruhe
Tween20	Sigma, Taufkirchen
10 x Phosphate buffered salt solution (PBS)	Biochrom, Berlin
Staphylococcus enterotoxin B (SEB)	Sigma, Taufkirchen/ Toxin Technology, USA
10 x Tris-Acetat-EDTA (TAE)	GibcoBRL, Karlsruhe
1668 Thioat (CpG-ODN)	TIB Molbiol, Berlin

Collagenase D	Sigma, Taufkirchen
DNAse I	Roche Diagnostics GmbH, Mannheim
Proteinase K	Roche Diagnostics GmbH, Mannheim
Taq-DNA-Polymerase	GibcoBRL, Karlsruhe

2.1.2 Reagents and laboratory supplies

Reagents and laboratory supply	Source of supply
AP substrate-kit III	Vector labs, USA
Cell strainer	NUNC, Wiesbaden
Cryomold® tissue molds	Miles Inc, USA
Cytometric Bead Array (CBA) kit	BD PharMingen, Heidelberg
DAKO Pen	DAKO Diagnostika, Hamburg
Entellan	Merck, Darmstadt
MACS columns	Myltenyi, Bergisch-Gladbach
NovaRed substrate-kit	Vector labs, USA
OPTEIA^{TM} mouse TNF-α -set	BD PharMingen
Plastics	NUNC, Falcon, Corning USA
ProteinG sepharose column	Amersham Pharmacia, USA
Tissue freezing media	Leica, Heidelberg
VectaMount	Vector labs, USA
Vectastain® ABC-AP-kit	Vector labs, USA

2.1.3 Buffers

Buffer	Composition	
Binding buffer (Ab purification)	20 mM	sodium phosphate pH 7.0
Eosin staining solution	15% (v/v) 85 % (v/v) 0.5% (v/v)	eosin solution H_20_{dd} acetic acid
Elution buffer (Ab purification)	0.1 M	glycin-HCl pH 2.7
FACS staining buffer	1x 0.5% (w/v) 0.01% (w/v)	PBS BSA NaN_3
MACS buffer	1x 0.5% (w/v) 0.01% (w/v) 2 mM	PBS BSA NaN_3 EDTA
6x Orange G loading buffer	1 mg/ml 20 mM 30 % (v/v)	orange G Tris glycerol

Red blood cell lysis buffer	0.15 mM	NH_4Cl
	10 mM	$KHCO_3$
	0.1 mM	Na_2EDTA
		pH 7.3
Tail buffer	100 mM	Tris-HCl pH 8.5
	5 mM	EDTA
	0.2% (w/w)	SDS
	200 mM	NaCl
TE buffer	10 mM	Tris, pH 8.0
	1 mM	EDTA, pH 8.0
10x PCR buffer	670 mM	Tris
	260 mM	HCl
	33 mM	$MgCl_2$
	166 mM	$(NH_4)_2SO_4$
	100 mM	β-mercaptoethanol

2.1.4 Peptides and primers

The 23 AA SV40 T Ag-peptide(362-384) P2 was synthesized in immunograde quality by Neosystem, France. AA-sequence: TNRFNDLLDRMDIMFGSTGSADI

All listed primers were purchased from TIB Molbiol (Berlin).

Strain	Primer name	Sequence (5' → 3')
RT2/TCR1	SV1	GGA CAA ACC ACA ACT AGA ATG CAG
	SV5	CAG AGC AGA ATT GTG GAG TGG
	IF19K	CTG AAC TGC AGT TAT GAG GAC AGC
	IF21K	TAT AAT TAG CTT GGT CCC AGA GC
IL-10	IL-10 T1	GTG GGT GCA GTT ATT GTC TTC CCG
	IL-10 T2	GCC TTC AGT ATA AAA GGG GGA CC
	IL-10 T4	AGA ACC TGC GTG CAA TCC ATC TTG
LTβR	NR7	TGT CAG CCG GGG ATG TCC TG
	NR4	CTG GTA TGG GGT TGA CAG CG
	HSV-TK	ATT CGC CAA TGA CAA GAC GCT GG

2.1.5 Antibodies and second step reagents

Unless otherwise stated, all antibodies are directed against mouse antigens.

Antibody	Clon/Species	Application/dilution	Source of supply
Id-FITC	9H5.6/mouse	FACS/ 1:50	(Förster et al., 1995)
Id-PE	9H5.6/mouse	FACS/ 1:100	(Förster et al., 1995)
Id-biotin	9H5.6/mouse	FACS/ 1:50	(Förster et al., 1995)
B220-PE	RA3-6B2/rat	FACS/ 1:50	BD PharMingen
B220-biotin	RA3-6B2/rat	Histochem. /FACS 1/100	BD PharMingen
B7.1-PE	16-10A1/ armenian hamster	FACS/ 1:50	BD PharMingen
B7.2-FITC	GL-1/rat	FACS/ 1:50	BD PharMingen
B7.2-PE	GL-1/rat	FACS/ 1:50	BD PharMingen
CD3ε	145-2C11/ armenian hamster	*in vitro* stimulation 1µg/ml	BD PharMingen
CD3ε-biotin	145-2C11/ armenian hamster	Histochemistry/ 1:100	BD PharMingen
CD4	GK1.5/rat	Histochemistry/ 1:100	BD PharMingen
CD4-FITC	GK1.5/rat	FACS/ 1:50	BD PharMingen
CD4-PE	GK1.5/rat	FACS/ 1:100	BD PharMingen
CD4-bio	GK1.5/rat	FACS/ 1:50	BD PharMingen
CD4-APC	RM4-5/rat	FACS/ 1:100	BD PharMingen
CD4-PE.Cy5.5	RM4-5/rat	FACS/ 1:200	CALTAG
CD8-PE	53-6.7/rat	FACS/ 1:100	BD PharMingen
CD8-bio	53-6.7/rat	FACS/ 1:50	BD PharMingen
CD8-APC	53-6.7/rat	FACS/ 1:100	BD PharMingen
CD11b-FITC	M1/70/rat	FACS/ 1:50	BD PharMingen
CD11b-biotin	M1/70/rat	FACS/ 1:50	BD PharMingen
CD11c-FITC	HL3/arm. hamster	FACS/ 1:100	BD PharMingen
CD11c-PE	HL3/arm. hamster	FACS/ 1:100	BD PharMingen
CD11c-biotin	HL3/arm. hamster	FACS/ 1:50	BD PharMingen

CD11c-APC	HL3/arm. hamster	FACS/ 1:100	BD PharMingen
CD25-FITC	7D4/rat	FACS/ 1:50	BD PharMingen
CD25-PE	PC61/rat	FACS/ 1:50	BD PharMingen
CD44-biotin	IM7/rat	FACS/ 1:150	BD PharMingen
CD45RB-PE	16-A/rat	FACS/ 1:200	BD PharMingen
CD62L-biotin	MEL-14/rat	FACS/ 1:50	BD PharMingen
CD69-PE	H1.2F3/arm. hamster	FACS/ 1:30	BD PharMingen
CTLA-4-PE	UC10-4F10-11/ armenian hamster	FACS/ 1:50	BD PharMingen
F4/80-biotin	CI:A3-1/rat	FACS/ 1:50	Serotech
I-A^K-FITC	10-3.6/mouse (CWB)	FACS/ 1:50	BD PharMingen
Vβ8 TCR	F23-1/ mouse(C57LJ)	FACS/ 1:50	BD PharMingen
Fc-block (CD16/32)	2.46G2/rat	FACS/ 1:50	BD PharMingen
anti-IL10R	1B1.3a/rat IgG1	*in vivo* studies	DNAX
anti-SV40 T Ag serum	rabbit	Histochemistry/ 1:1000	Eurogentec

Second step reagents

Reagent	Application/ dilution	Source of supply
Extravidin-POX	Histochemistry/ 1:300	Sigma
anti-rat -POX	Histochemistry/ 1:100	Dianova
anti-rabbit-AP	Histochemistry/ 1:1000	Dianova
anti-armenian hamster-HRP	Histochemistry/ 1:100	Linaris
Streptavidin-FITC	FACS/ 1:100	BD PharMingen
Streptavidin-PE	FACS/ 1:200	BD PharMingen
Streptavidin-PerCP	FACS/ 1:30	BD PharMingen
Streptavidin-APC	FACS/ 1:100	BD PharMingen

Isotype controls

Isotype	Clon/Species	Application /dilution	Source of supply
IgG2a-FITC	G155-178/mouse	FACS/ 1:50	BD PharMingen
IgG2b-PE	A95-1/rat	FACS/ 1:50	BD PharMingen
IgG2aκ	R35-95/rat	FACS/ 1:50	BD PharMingen
IgG2κ	B81-3/hamster	FACS/ 1:50	BD PharMingen

Antibodies coupled to MACS beads

Antibody	Application	Source of supply
B220	MACS	Myltenyi
CD4	MACS	Myltenyi
CD11c	MACS	Myltenyi

2.1.6 Mice

Different transgenic and KO mouse lines were analyzed during this work. RIP1-Tag2 (RT2) mice express the SV40 T Ag under control of the rat insulin promoter (RIP) (Hanahan, 1985). TCR1 mice bear a transgenic TCR specific for the SV40 T Ag (Förster et al., 1995). The transgenic TCR is restricted to I-A^k and originated from the C3HeB/FeJ background. To generate IL-10-deficient mice in the same background, IL-10^{KO} mice of the mixed C57BL/6/129Ola background (Kuhn et al., 1993) were backcrossed to C3HeB/FeJ for 7 generations. Subsequently, mice were intercrossed with RT2/TCR1 transgenic mice. We noticed that IL-10^{KO} (C3HeB/FeJ) mice show first signs of IBD (diarrhea) at the age of 5-7 weeks and subsequently develop chronic enterocolitis. Mice suffering from severe symptoms of IBD were excluded from the experiments. Both, IL-$10^{+/+}$ and IL-$10^{KO/+}$ mice, were used as wt controls.

To generate RT2/TCR1 mice deficient for LTβR, LTβR^{KO} mice of the mixed C57BL/6/129Ola background (Fütterer et al., 1998) were backcrossed to C3HeB/FeJ for 4 or 9 generations and then intercrossed with RT2/TCR1 mice. The initial analysis of RT2/TCR1/LTβR^{KO} mice was done with animals backcrossed for 4 generations. The results were later on verified with animals of the N9 generation.

All mice were kept under specific pathogen-free conditions at the animal facility of the Institute for Med. Microbiology, Immunology, and Hygiene (Technical University Munich). Animal experiments were approved and authorized by local government under the permission number 211-2531-14/2001. Experiments were performed with 6- to 12-week-old mice, unless otherwise stated.

2.2 Methods

2.2.1 Genotyping

Isolation of chromosomal DNA

Tail biopsies were digested o/n with 0.2 mg/ml Proteinase K in tail buffer at 55°C. Samples were vortexed and cellular debris were spun down (13,000 rpm/10 min). The supernatant was mixed with one volume isopropanol, leading to precipitation of DNA. The DNA cloud was fished out and transferred into 250 µl TE buffer.

PCR-Amplification of DNA

The polymerase chain reaction (PCR) is used to amplify a particular DNA sequence. It relies on the ability of DNA polymerases to synthesize a complementary strand starting from a single stranded DNA matrix. Short oligonucleotides flanking the 5' and 3' ends of the DNA segment of interest are used as primers for DNA synthesis. The PCR consists of three different steps. First the complementary strands are separated by a brief heat treatment (denaturation step). Secondly specific primers hybridize with their complementary sequence (annealing step) and as last step a temperature-resistant DNA polymerase synthesizes the complementary strand (elongation step). Repeated cycles of synthesis, melting and annealing lead to exponential amplification of the sequence of interest.

Reaction mix	1.5 µl	DNA
	1 µl	Primer 1-3 (10 µM)
	1 µl	dNTPs (10 mM each)
	2 µl	BSA (4 mg/ml)
	5 µl	10x PCR buffer
	1 µl	Taq-Polymerase (2.5U)
	ad 50 µl	H_20_{bidest}

Standard PCR program

Step	time	temperature
I) denaturation	20 sec	94°C
II) annealing	60 sec	63°C
III) elongation	120 sec	72°C
Go to step I; 35 cycles		
IV) finishing up	10 min	72°C

Analytical agarose gel electrophoresis

Agarose gel electrophoresis is a standard method to separate DNA fragments. Under the influence of an applied electric field the negatively charged DNA segments migrate towards the anode. This migration leads to separation of DNA fragments according to their size. To visualize the DNA fragments an intercalating substance (ethidiumbromid) is added to the agarose gel, thereby allowing the detection of fluorescent DNA bands by UV-irradiation at 280 nm.

2.2.2 Ab production, purification and *in vitro* testing

Hybridoma cells producing an α-IL10R antibody (1B1.3a, rat IgG1) were cultured in complete RPMI medium containing 5% very low IgG FCS. The supernatants were collected and the antibody was purified by affinity chromatography using a ProteinG sepharose column. ProteinG is a protein from *Staphylococcus aureus* and it binds with high affinity to the Fc region of IgG molecules. The column was loaded with supernatant at neutral pH, washed and elution of the antibody was accomplished by using a low pH buffer (pH 2.7). To diminish the risk of denaturation of the antibody at low pH, the eluted antibody was collected in 1M Tris buffer (pH 9.0) to reestablish a neutral pH. The eluted antibody was dialyzed over night against PBS. The concentration was determined by measuring the absorption at 280 nm and corrected by the specific extinction coefficient for IgG (OD280nm /1.4 = Ab concentration mg/ml). For *in vitro* testing, $1.5x10^6$ Raw macrophages were stimulated for 4 hours with 100 ng/ml LPS, 10 ng/ml IL-10 and/ or 10 ng/ml α-IL10R antibody. TNF-α was quantified in the supernatants by means of a sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA was carried out as recommended by the manufacturer (BD PharMingen) who also provided matched pairs of capture and detection antibodies.

2.2.3 Peptide/SEB treatment

Adult mice were injected intraperitoneally (i.p.). with 100 µg SV40 T Ag-peptide(362-384) (P2) in PBS. Three days later, cells were harvested from MLN and analyzed. For tolerance induction in adult mice, 8- to 10-week-old animals were injected i.p. with 100 µg P2 in PBS and received a second injection 15 days later. The analysis took place three weeks after the first injection. During the course of the experiment, blood was taken at different time points from the tail vein.

To induce tolerance by SEB, animals were injected i.p. with different concentrations of SEB followed by a second SEB injection within one week. Analysis took place two weeks after the first injection.

To induce tumor immunity, animals received different amounts of CpG-ODN (1668 Thioat) together with 100 µg P2 in PBS. Systemic blockade of IL-10 was achieved by i.p. injection of 500 µg α-IL10R one day prior to peptide treatment.

2.2.4 Organ removal

Animals were sacrificed by cervical dislocation. The peritoneal region was desinfected with 70% ethanol and the organs were removed under aseptic conditions.

2.2.5 APC isolation

Peritoneal exudate cells (PEC) were isolated by washing the peritoneal cavity with cold RPMI 1640 medium containing 10% FCS. DCs were isolated by digesting whole spleens with 1 mg/ml collagenase D and 300 U/ml DNase I for 15 min at 37°C. To obtain a highly enriched DC population, spleen cell suspensions were labeled with CD11c-specific magnetic beads and separated on MACS columns (see description below).

2.2.6 Magnetic activated cell sorting (MACS)

MACS is used to separate cell populations characterized by a specific cell surface antigen out of a complex cell mixture. The cells of interest are labeled with MACS Microbeads, which are magnetic beads coupled to a specific antibody. The separation takes place in a high gradient magnetic separation column placed in a strong magnetic field. The magnetically labeled cells are retained in the column, while non-labeled cells pass through. By removing the column from the magnetic field, the magnetically

retained cells can be eluted. Applying this method, both, labeled and non-labeled cells can be recovered.

MACS was performed as recommended by the manufacturer. In brief, cells were incubated with MACS Microbeads 10x diluted in degassed MACS staining buffer for 15 min at 4°C. The separation column was placed in the MACS magnet and equilibrated with MACS buffer. The cells were washed and pipetted onto the separation column, which was subsequently washed 3 times with one column volume MACS buffer. After removal of the column from the separator, MACS buffer was applied and the retained cells were flushed out by means of a plunger.

2.2.7 Flow cytometry

Flow cytometry allows measurements of various phenotypic, biochemical and molecular characteristics of individual cells (or particles). It is often referred to as Fluorescence Activated Cell Sorting (FACS- a trademark of BD) and by means of specialized flow cytometers it is possible to sort and collect cells with defined properties.

Prior to analysis cells are labeled with antibodies conjugated to fluorescent dyes. During analysis the cells are made to flow rapidly through the flow cell, or stream in air, where they are illuminated by a focused laser beam at a certain wavelength (488 nm wavelength light from an argon laser). The dyes fluoresce and the emitted light is detected by very sensitive photomultipliers. As each cell intercepts the laser beam it additionally scatters some of the light. The intensity of the scattered laser light gives information about the diameter, shape, and granularity of the cell. The intensity of the fluorescent emission gives information about the expression level of the analyzed cellular marker. It is also possible to measure intracellular proteins for example cytokines by flow cytometry. For this analysis, the cells have to be fixed and permeabilized to permit entry of the cytokine-specific antibody into the cytoplasm.

Different fluorescent dyes have been used to enable analysis of multiple markers. Commonly used dyes are FITC (Max emission 530 nm), PE (Max emission 585 nm), Cy5 (Max emission 674 nm), APC (Max emission 660 nm).

Cell surface staining

$2x10^6$ cells were incubated with the indicated antibodies in 30 µl FACS buffer and 2.4G2 mAb was added to block Fcγ receptors in 96-well V-bottom microtiter plates for

20 min on ice. Cells were washed twice with FACS buffer, incubated with second step reagents (streptavidin conjugates) and washed again twice.

Intracellular staining

For intracellular staining cell surface stained cells were fixed with 2% formaldehyde in PBS for 20 min at RT, washed in PBS and permeabilized with 0.5% Saponin in FACS buffer for 10 min. Intracellular staining was performed by incubating the cells with the respective antibody in 0.5% Saponin/ FACS buffer for 20 min at RT. Cells were washed with 0.5% Saponin/ FACS buffer followed by a second wash with FACS buffer prior to analysis.

Analysis

Analysis was done on a FACScan cytofluorograph (Becton Dickinson) and 10,000 cells were collected per sample. For analysis of Id^+CD4^+ T cells 3,000 Id^+CD4^+ cells were recorded per sample. To analyze the frequency of $Id^+CD4^+CD8^-$ cells in the thymus, 200,000 $CD4^+CD8^-$ thymocytes were collected. In cell sorting experiments Id^+CD4^+ T cells were isolated using a MoFlow Cytometer (Cytomation) and purity was >80%.

2.2.8 Proliferation assays

Proliferation assays were carried out to determine the responsiveness of lymphocytes to different stimuli. To activate T cells, two different types of stimuli were used. Transgenic T cells were stimulated Ag-specifically by P2 peptide, whereas polyclonal stimulation was achieved by using α-CD3 antibodies. In some experiments *staphylococcus aureus* enterotoxin B (SEB) was used to restimulate T cells *in vitro*. Two different methods were applied to measure the proliferative response:

[^{3}H]-Thymidine proliferation assay

Thymidine is a pyrimidine base, which constitutes one of the four building bases of DNA. By adding radioactively labeled [^{3}H]-thymidine to cultures of stimulated lymphocytes it is incorporated into newly synthesized DNA, thereby allowing detection of proliferation by measuring β irradiation.

Single cell suspensions of MLN cells or splenocytes ($4x10^5$ cells/well) or sorted $4x10^3$ Id^+CD4^+ T cells and $2x10^5$ MLN cells as APCs were stimulated with titrated amounts of P2 in complete RPMI 1640 medium. Cultures were maintained in 96-well flat-bottom

(round-bottom for sorted cells) tissue culture plates for 64h and [^{3}H]-thymidine was added to each well for the last 16h. Cultures were performed in triplicates. Cells were harvested on glass fiber filters and incorporated [^{3}H]-thymidine was quantified using a Matrix 9600 direct β counter (Canberra Packard, Frankfurt, Germany). The counting efficiency of the Matrix 9600 direct β counter is 6- to 8-fold lower compared to a liquid scintillation counter, explaining the lower counts per minute (cpm) values obtained. The rate of proliferation is indicated as a stimulation index (cpm response/cpm background).

Suppression assay of sorted cells

$1x10^4$ $CD25^+$ or $CD25^-Id^+CD4^+$ T cells were cultured alone or mixed at a ratio of 1:1. $1x10^4$ irradiated (30 Gray) splenocytes were used as APCs and the cells were stimulated with 20 nM P2 in complete RPMI 1640 medium. For analysis of suppression by $CD25^+CD4^+$ T cells $2.5x10^4$ FACS sorted cells were cultured in the presence of $2.5x10^4$ irradiated (30 Gray) splenocytes and stimulated with 1 μg/ml α-CD3. Cultures were maintained in 96-well round-bottom tissue culture plates and proliferation detected as [^{3}H]-thymidine incorporation (see above).

CFSE Assay

Carboxy-fluoresceindiacetate succinimidyl ester (CFDA SE) is used as fluorescent vital dye for proliferation analysis of cells. CFDA SE is a nonpolar molecule that spontaneously penetrates cell membranes and is converted to anionic CFSE by intracellular esterases. CFSE irreversibly couples to proteins by reaction with lysine side-chains and other available amine-groups, resulting in stable long-term intracellular retention. During cell division, CFSE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as their parents. As a result, each successive generation in a population of proliferating cells is marked by a halving of cellular fluorescence intensity that is readily followed by flow cytometry (CFSE excitation/ emissionmax: 488nm/ 525nm).

For analysis of cell division rates, single cell suspensions of lymphoid organs were prepared and $1x10^7$ cells/ml were incubated with 5 μM CFSE in pre-warmed proteinfree RPMI 1640 medium for 10 min at 37° C. The labeling reaction was stopped by adding one volume FCS, followed by two washes with complete RPMI. $3x10^6$ labeled cells were cultured with or without 1 μM P2 in 24 well plates at $3x10^6$ cells/well in cell

culture medium. After 2-3 days of culture, supernatants were collected and cells were harvested and analyzed by flow cytometry.

2.2.9 Cytokine detection

Cytokines were determined in supernatants after two days of culture with 1 μM SV40 T Ag-peptide P2. The amounts of IL-2, IFN-γ and TNF-α were evaluated using the mouse Th1/Th2 Cytometric Bead Array (CBA). The CBA allows simultaneous detection of five different cytokines (IL-2, IL-4, IL-5, IFN-γ, TNF-α) in one sample. The test consists of five bead populations coated with antibodies specific for the respective cytokine. Each of these bead populations has a distinct fluorescent intensity, which can be detected in the FL3 channel of a flow cytometer. The five different bead populations are mixed together, incubated with the sample and PE-conjugated detection antibodies. The various levels of cytokines can be seen during analysis as shifts of the respective bead population in the FL2 channel. The CBA provides standards and the concentrations of the cytokines are calculated along with the standard curves. The array was performed according to manufacturers instructions.

2.2.10 Histology and immunohistology

Classical histological methods are applied to examine the morphology of a given tissue or organ. Two different reagents are used: hematoxylin is a basic reagent, which stains the nucleus blue, eosin is a acidic reagent and stains the cytoplasm in a red color. A more sophisticated method is the immunohistological analysis of organs where certain cell types can be detected by specific antibodies. These antibodies are coupled in a second step to an enzyme and finally by adding the specific substrate a colored product is formed allowing the detection of the antigen by microscopy.

For histological analysis organs were snap frozen in N_2-cooled 2-methylbutan and cut into 7 μm cryosections. Sections were fixed in ice-cold aceton for 10 min and stored at -80°C.

Hematoxylin/Eosin staining

The sections were stained 15-30 min in 4 fold diluted hematoxylin, washed 3 times 5 min in PBS and incubated for 10 min in eosin staining solution. Finally the sections were washed once for 1 min, dried and embedded with Entellan.

Immunohistology

After bringing sections to room temperature they were encircled using a DAKO Pen (water repellant wax) to create a boundary between adjacent tissue sections. The whole staining procedure was performed in a humid staining chamber. Sections were rehydrated in PBS and unspecific Ab-binding was blocked for 20 min with a 1% BSA/ 5% NGS containing PBS solution. Sections were incubated for 45 minutes with diluted detection antibodies (see description of antibodies above) in PBS/ 1% BSA (100 µl per section). Subsequently the tissue sections were washed 3 times for 3 min in PBS. As second step reagents were used either enzyme-conjugated antibodies or enzyme-conjugated streptavidin. The enzymes applied for detection were alkaline phosphatase or peroxidase. Second step reagents were incubated for 45 minutes (used in titrated concentrations (see description of second step reagents above) in PBS/ 1% BSA; 100 µl per section). Thereafter the sections were washed 3 times for 3 min in PBS. As substrates NovaRED substrate kit for peroxidase detection and AP substrate-kit III for alkaline phosphatase detection were used as recommended by the manufacturer. Finally the sections were dried and embedded with Vectamount.

3 RESULTS

3.1 Role of IL-10 in peripheral tolerance induction

3.1.1 Developmental induction of tolerance in the absence of IL-10

Peripheral tolerance in the RT2/TCR1 model is characterized by deletion of the majority of autoreactive T cells as well as unresponsiveness of the remaining transgenic T cells to peptide stimulation *in vitro* (Förster et al., 1995). To assess the influence of IL-10 on peripheral tolerance induction, RT2/TCR1 mice were crossed into an IL-10 deficient background. Animals were analyzed at an age of 6-8 weeks, since tolerance in RT2/TCR1 mice is established during the first 6 weeks of life. As functional readout for tolerance induction, the numbers of transgenic T cells in the periphery were determined as well as the ability of these cells to proliferate in response to Ag stimulation *in vitro*.

The percentages of transgenic T cells (idiotype $(Id)^+$ $CD4^+$ T cells) of TCR1, TCR1/IL-10^{KO}, RT2/TCR1 and RT2/TCR1/IL-10^{K0} mice in the thymus versus periphery were compared by FACS analysis. In the thymus no significant differences in the levels of Id^+ cells among $CD4^+CD8^-$ thymocytes could be detected (Fig. 1).

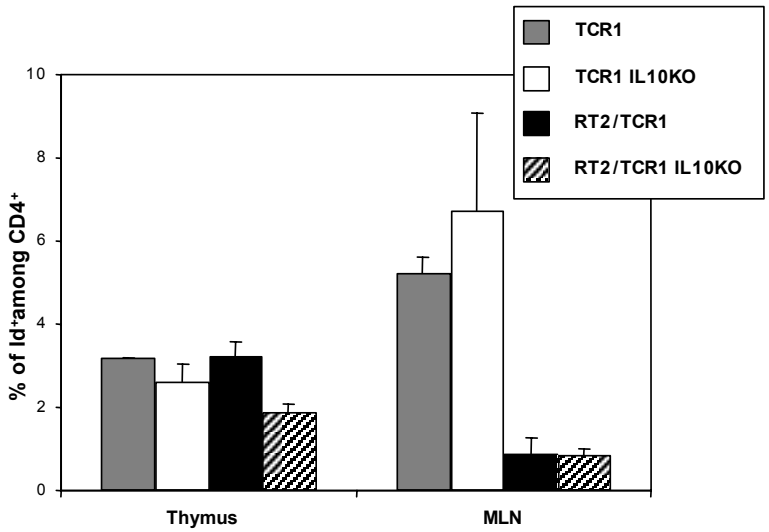

Figure 1: Frequency of Id^+CD4^+ in thymus and periphery of wt and IL-10^{KO} mice.
Given are mean values ± SD obtained from two (TCR1; TCR1/IL-10^{KO}) or three (RT2/TCR1; RT2/TCR1/IL-10^{KO}) individual mice. Frequencies of Id^+CD4^+ cells in MLN of the 4 different groups of mice given in the text represent mean values ± SD from data obtained in several independent experiments.

In contrast, in MLN the frequency of Id^+CD4^+ T cells was strongly reduced in RT2/TCR1 mice (1.4±0.6% (n=28)) compared to TCR1 animals (5.3±1.3% (n=39)). A similar reduction of transgenic T cells in RT2/TCR1 versus TCR1 mice was also seen in IL-10 deficient mice (RT2/TCR1/IL-10^{KO}: 1.3±0.5% (n=18); TCR1/IL-10^{KO}: 4.7±1.7% (n=13) (Fig. 1). Therefore, one main feature of peripheral tolerance induction in RT2/TCR1 mice, the peripheral deletion of transgenic T cells, can also be observed in the absence of IL-10.

The second parameter tested was the proliferative capacity of transgenic T cells in RT2/TCR1/IL-10^{KO} and relevant control mice. Whole MLN cell suspensions were stimulated with titrated amounts of P2 peptide, which is recognized by the transgenic TCR of Id^+CD4^+ T cells. [^{3}H]-thymidine incorporation was measured as read out. Interestingly, RT2/TCR1/IL-10^{KO} mice displayed a reduced proliferative capacity to P2 stimulation like their wt counterparts (Fig. 2A). Since RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice show a four fold reduction in total transgenic T cells compared to single transgenic TCR1 mice, one could also argue that the reduced proliferative response seen in these animals is just due to the reduced numbers of responding cells. To address this question, a [^{3}H]-thymidine proliferation assay with adjusted cell numbers was carried out. Id^+CD4^+ T cells of the different genotypes were isolated by FACS sorting and mixed with MLN cells of C3HeB/FeJ mice to obtain a final concentration of 2% transgenic T cells. As depicted in Fig. 2B, also under these conditions, RT2/TCR1 mice proficient or deficient of IL-10 showed a strong decrease in their proliferative response compared to the respective TCR1 controls. RT2/TCR1/IL-10^{KO} mice required 100-fold higher concentrations of Ag to mount a comparable proliferative response to that of TCR1/IL-10^{KO} mice.

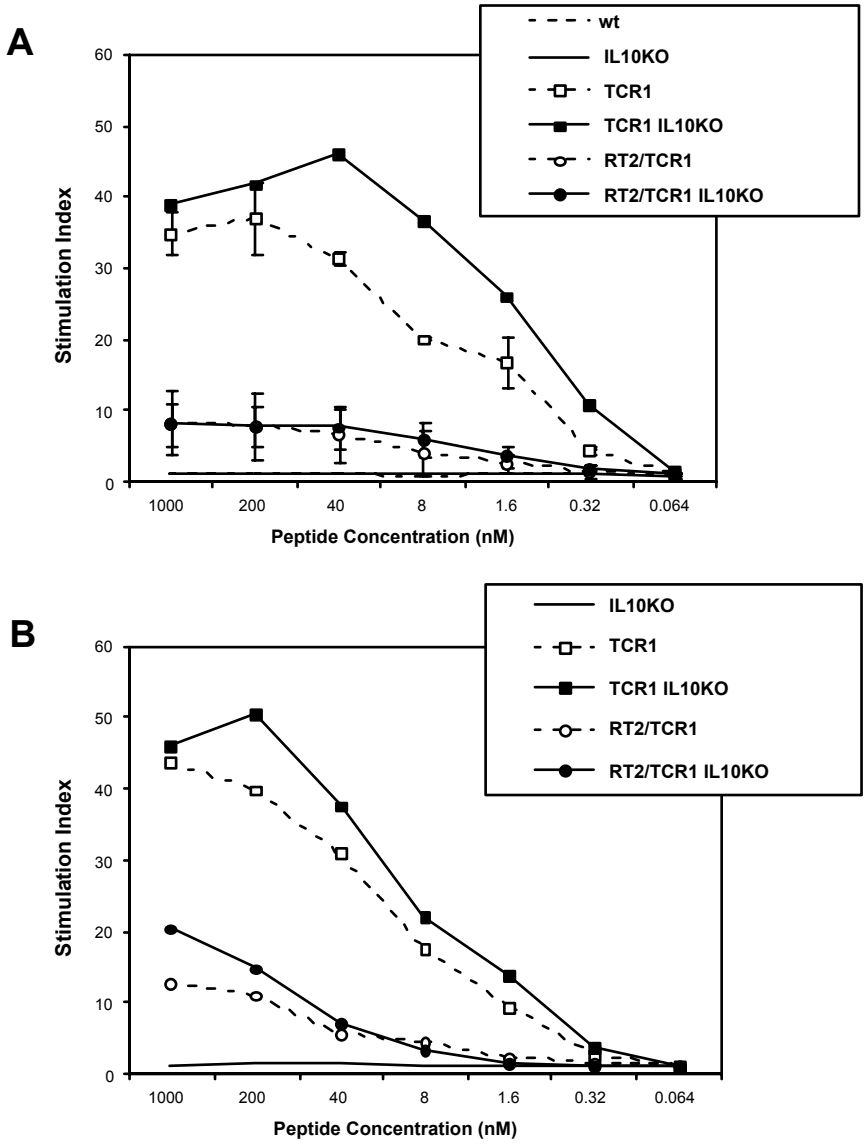

Figure 2: SV40 T Ag-specific $CD4^+$ T cell tolerance in RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice.
A) *In vitro* [^{3}H]-thymidine proliferation assay with $2x10^5$ MLN cells of the respective genotypes (wt: n=1; IL-10^{KO}: n=1; TCR1: n=2; TCR1/IL-10^{KO}: n=1; RT2/TCR1: n=2; RT2/TCR1/IL-10^{KO}: n=2). Given are mean values ± standard deviation (SD) obtained from the different genotypes. Data are representative of more than three independent experiments
B) *In vitro* [^{3}H]-thymidine proliferation assay with adjusted numbers of Id^+CD4^+ MLN cells. Pooled samples of different genotypes (IL-10^{KO}: n=2; TCR1: n=2; TCR1/IL-10^{KO}: n=1; RT2/TCR1: n=4; RT2/TCR1/IL-10^{KO}: n=2) were FACS-sorted for Id^+CD4^+ cells. $2x10^5$ MLN cells of wt mice were mixed with $4x10^3$ Id^+CD4^+ cells from each group and stimulated with titrated amounts of P2 for 64h. Cultures were performed in triplicates and mean values are shown. The results are representative of three independent experiments.

Since the proliferative response to peptide stimulation was not totally abolished in RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice, it remained to be determined whether all transgenic T cells in double transgenic mice showed an impaired response, or whether the population was split into proliferating and non-proliferating, resting transgenic T cells. To address this point, CFSE proliferation assays were carried out. CFSE is a fluorescent vital dye, used for *in vitro* labeling of cells. It covalently binds to proteins and is stably retained within the labeled cell. During cell division, CFSE is distributed equally among the daughter cells, resulting in a 50% reduction of fluorescent intensity. Using flow cytometric analysis, each division round can be detected. The advantage of CFSE versus [^{3}H]-thymidine proliferation measurement lies in the possibility to further characterize the proliferating cells by cell surface markers and additionally to determine the number of cell divisions undergone by the respective cell population. For detailed analysis of the residual proliferative *in vitro* response seen in double transgenic mice, MLN of double transgenic and control mice were labeled with CFSE, cultured for 2 days with P2 peptide and subsequently analyzed by flow cytometry. As illustrated in Fig. 3, transgenic T cells of TCR1 and TCR1/IL-10^{KO} mice underwent up to four rounds of divisions and only 10.6% ± 6.5% (n=6) of cells were resting. In contrast Id^+CD4^+ T cells of RT2/TCR1 or RT2/TCR1/IL-10^{KO} mice showed an asynchronous pattern of cell division- previously described for memory T cells (Lee et al., 1998)- and 37% ± 22% (n=7) of cells stayed out of cycle.

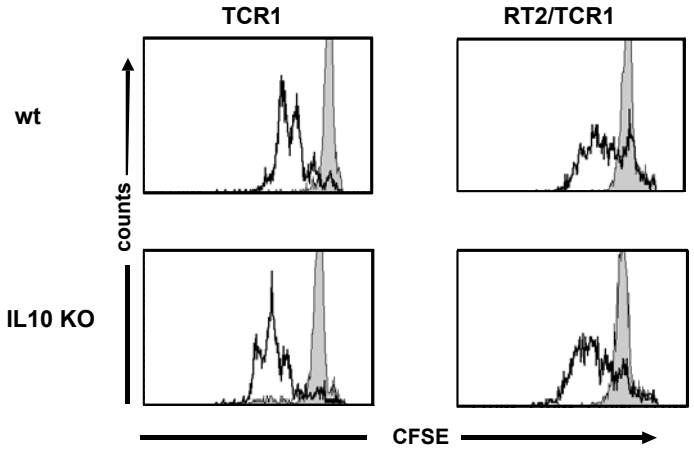

Figure 3: Cell division rate of Id^+CD4^+ T cells from wt and IL-10^{KO} mice.
Flow cytometric analysis of CFSE-labeled Id^+CD4^+ T cells. MLN cells were CFSE-labeled and cultured with P2 or medium for 2 days. Histograms are gated on Id^+CD4^+ cells showing unstimulated cells (gray filled histograms) and P2 stimulated cells (black line histograms). Representative histograms are shown. A total of 9 TCR1, 4 TCR1/IL-10^{KO}, 10 RT2/TCR1, and 8 RT2/TCR1/IL-10^{KO} mice were analyzed in 3 independent experiments.

Therefore, double transgenic mice contain two different populations of transgenic T cells. One part of the Id^+CD4^+ T cells can proceed through the cell cycle after peptide stimulation, but they are limited in their response (asynchronous pattern of cell division) compared to naïve T cells from single transgenic mice. The other part of Id^+CD4^+ T cells from double transgenic mice is totally unresponsive to peptide stimulation.

To further characterize the transgenic T cells in RT2/TCR1/IL-10^{KO} mice, different cell surface markers were analyzed. CD25 (IL-2Rα chain), CTLA-4 (cytotoxic T lymphocytes associated antigen-4, CD152), and CD69 (activation inducer molecule) are expressed on activated T cells. CD25 and CTLA-4 have also been associated with regulatory T cell function (Sakaguchi et al., 1995; Read et al., 2000; Takahashi et al., 2000). Memory T cells, like effector T cells, express high levels of integrins and CD44 (Pgp-1). This allows their migration to peripheral sites of inflammation and their retention at these sites. Naïve cells are characterized by high levels of CD62L (L-selectin) an important homing receptor mediating entry into lymph nodes, and CD45RB (isoform of the CD45 leukocyte common antigen). CD62L and CD45RB are down-regulated on antigen experienced/memory T cells. Overall there were no significant differences regarding surface marker expression on Id^+CD4^+ T cells of RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice, both showing an activated/memory T cell phenotype (Fig. 4). Interestingly, Id^+CD4^+ T cells from tolerant RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice showed high levels of CD25 and CTLA-4 expression. $CD69^+Id^+CD4^+$ T cells can only be found in LNs of the local environment of the pancreas in both types of RT2/TCR1 mice (Förster and Lieberam, 1996 and Fig. 4). It is therefore thought that Id^+CD4^+ T cells see their Ag and get activated in LNs draining the site of Ag expression. In contrast, $CD25^+Id^+CD4^+$ T cells can be detected systemically in RT2/TCR1 mice (data not shown), hence it can be assumed that CD25 in this context is not solely an activation marker but points to regulatory function of these cells.

In conclusion, developmental induction of peripheral tolerance to endogenously expressed SV40 T Ag in RT2/TCR1 mice, as manifested by deletion of autoreactive T cells and induction of unresponsiveness of the remaining transgenic T cells, can also occur in the absence of IL-10.

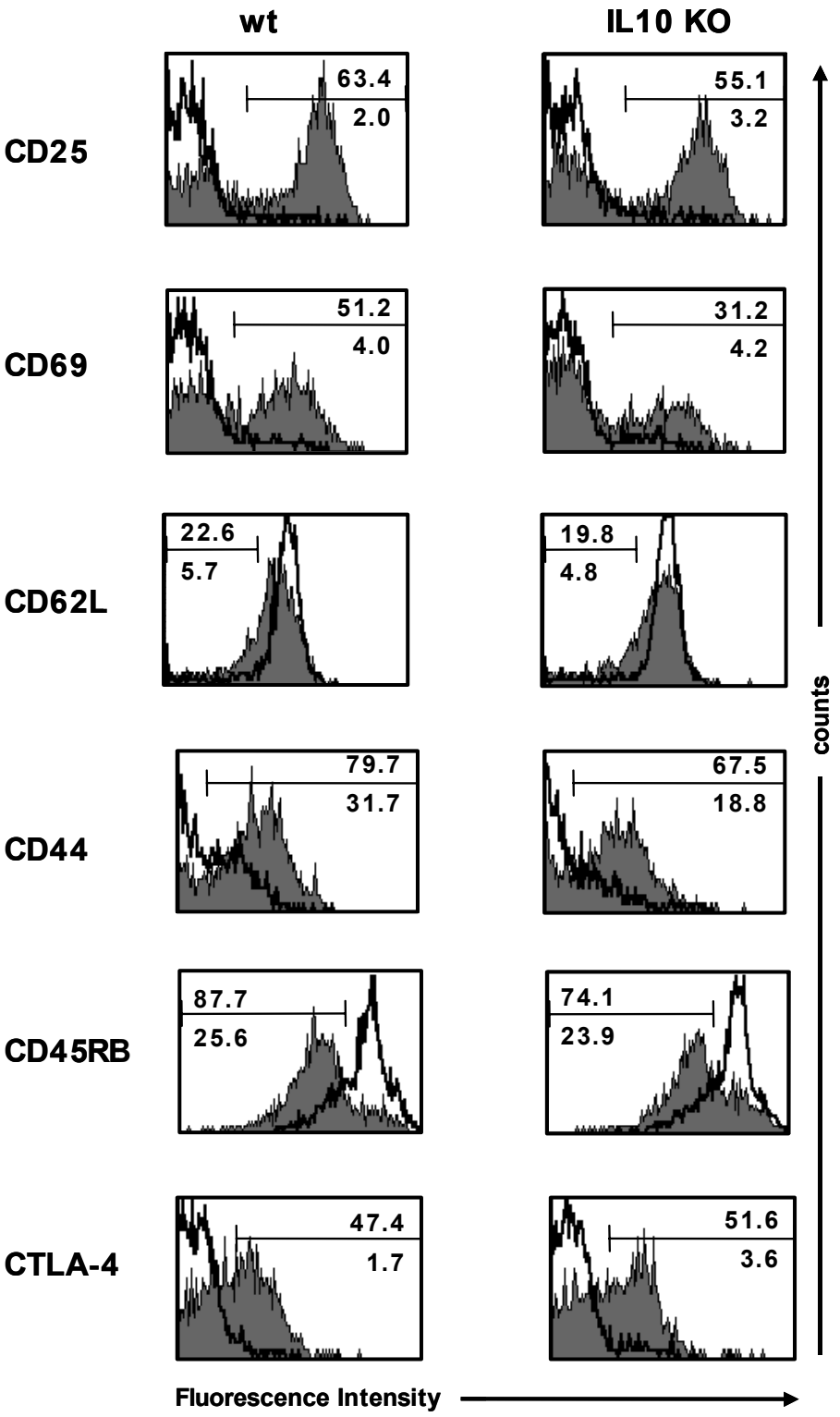

Figure 4: Expression of activation/memory markers on Id^+CD4^+ T cells in RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice.

Analysis of surface marker expression on Id^+CD4^+ MLN T cells of wt TCR1 (open histograms) and RT2/TCR1 (gray filled histograms) (left), and TCR1/IL-10^{KO} (open histograms) and RT2/TCR1/IL-10^{KO} mice (gray filled histograms) (right). Shown are histograms gated for 3000 Id^+CD4^+ T cells. Numbers indicate the percentage of cells in each fluorescence window (RT2/TCR1 upper numbers; TCR1 lower numbers). Representative histograms from one out of three independent experiments are shown.

3.1.2 Breakage of tolerance in RT2/TCR1/IL-10^{KO} mice by single antigenic challenge

Although peripheral tolerance induction in RT2/TCR1 mice was independent of IL-10, I further assessed whether the tolerance status in RT2/TCR1/IL-10^{K0} mice was as stable as that seen in wt RT2/TCR1 animals. In the literature the important regulatory function of IL-10 has been demonstrated predominantly in an *in vivo* environment (Powrie et al., 1996), whereas in numerous *in vitro* set-ups IL-10 had no influence on regulation of T cell responses by regulatory T cells (Thornton et al., 1998; Takahashi et al., 1998). Therefore, an *in vivo* test was used to analyze the role of IL-10 in maintenance of peripheral tolerance. Mice were challenged i.p. with 100 µg P2 and the percentages of Id$^+$CD4$^+$ T cells in the blood before and after peptide stimulation were compared (Fig. 5A). As expected, naïve Id$^+$CD4$^+$ T cells from TCR1 and TCR1/IL-10^{K0} mice exhibited a strong expansion following antigenic stimulation. In contrast, Id$^+$CD4$^+$ T cells from tolerant RT2/TCR1 mice were anergic to peptide stimulation *in vivo*. Surprisingly, transgenic T cells from RT2/TCR1/IL-10^{K0} mice showed a four-fold expansion similar to the Id$^+$CD4$^+$ T cells from TCR1/IL-10^{K0} mice.

To test whether this *in vivo* expansion of Id$^+$CD4$^+$ T cells from RT2/TCR1/IL-10^{K0} mice would also cause an increased responsiveness to peptide restimulation *in vitro*, [^{3}H]-thymidine proliferation assays were carried out. Remarkably, after *in vivo* peptide priming, transgenic T cells from RT2/TCR1/IL-10^{K0} mice mounted an *in vitro* response to P2, which was even higher than the response seen in wt peptide treated TCR1 mice (Fig. 5B). Therefore, in the absence of IL-10 the tolerance status of RT2/TCR1 mice cannot be maintained when strong antigenic stimulation is provided. Interestingly, this finding can only be seen in the living organism and does not occur under normal *in vitro* culture conditions.

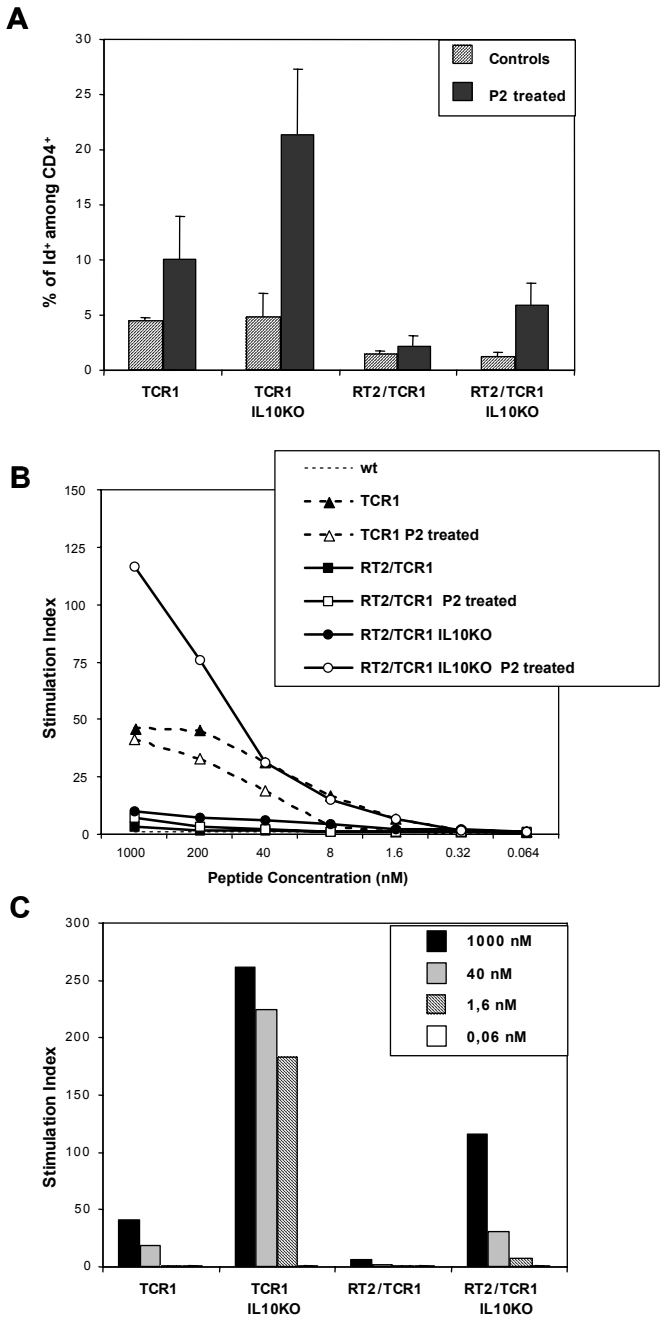

Figure 5: Loss of SV40 T Ag-specific tolerance upon antigenic challenge

A) *In vivo* peptide stimulation leads to expansion of SV40 T Ag-specific $CD4^+$ T cells in RT2/TCR1/IL-10^{KO} mice. Mice were injected i.p. with 100 µg of SV40 T Ag-peptide P2 (controls injected with PBS) and MLN cells were analyzed on d3 after peptide injection. Shown are the percentages of Id^+ cells among the $CD4^+$ T cell population for control (hatched columns) and peptide-treated animals (filled columns). Given are mean values ± SD obtained from three individual mice, except for RT2/TCR1/IL-10^{KO} P2-treated (n=4) resulting from three independent experiments.

B) *In vitro* [^{3}H]-thymidine proliferation assay with MLN cells of *in vivo* peptide-stimulated and control mice.

C) Results from animals treated with peptide *in vivo* are depicted to visualize the increased responsiveness of TCR1/IL-10^{KO} and RT2/TCR1/IL-10^{KO} T cells in direct comparison to their wt counterparts. Cultures were performed in triplicates and mean values are shown for individual mice. One representative experiment out of three independent experiments is shown.

Furthermore, TCR1/IL-10^{KO} animals showed an increased response to *in vivo* peptide stimulation compared to their wt controls (Fig. 5C), which was not detected under *in vitro* P2 stimulation without prior *in vivo* peptide priming (Fig. 2). IL-10 has been implicated in modification of APC function by downregulation of MHC and costimulatory molecules (reviewed in Moore et al., 2001). It was possible that in the absence of IL-10, APCs express generally increased levels of MHC or costimulatory molecules, thereby leading to more efficient Ag presentation and T cell stimulation. To address this point, different subsets of APCs from wt and IL-10^{KO} mice were analyzed for their expression levels of MHC II, CD80 and CD86 (Fig. 6). In general – with the exception of peritoneal macrophages- there were no significant differences in expression of the analyzed cell surface proteins between wt and IL-10^{KO} mice. Regarding MHC II expression on MLN B cells even lower levels were detected on B cells from IL-10^{KO} mice compared to wt mice. The only APC subset showing increased MHC II expression were peritoneal macrophages. Considering that the peptide is administered i.p., it cannot be excluded that peritoneal macrophages influence the *in vivo* response in the experimental set-up used.

Taken together, it can be stated that in IL-10^{KO} mice no general dysregulation of MHC II and costimulatory levels on APC can be detected compared to wt APC. To further underline this finding, the stimulatory capacity of MLN feeder cells from either wt or IL-10^{KO} mice were tested. FACS sorted Id^{+}CD4^{+} T cells from TCR1 mice were cultured on different feeder layers from wt or IL-10^{KO} mice. After peptide stimulation the proliferative response of Id^{+}CD4^{+} T cells was similar irrespective of the origin of feeder cells (data not shown), ruling out differences in the stimulatory capacity between MLN cells of wt or IL-10^{KO} mice. One plausible explanation for the discrepancy between *in vivo* and *in vitro* peptide stimulation seen in TCR1/IL-10^{KO} and RT2/TCR1/IL-10^{KO} mice could be that different subsets of APCs are involved during the response. Presumably, DCs are the main stimulators during *in vivo* peptide stimulation, whereas *in vitro*, B cells will serve as APC (reviewed in Jenkins et al., 2001).

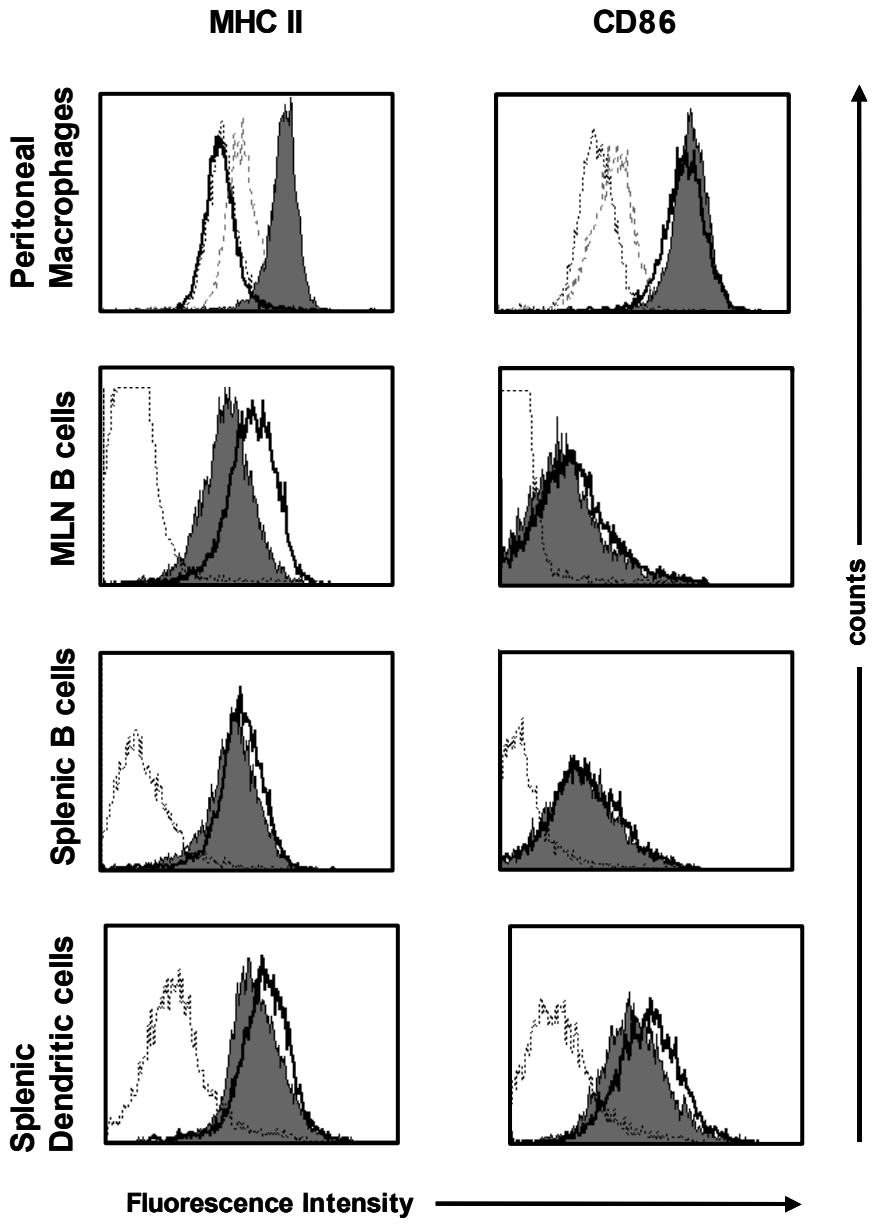

Figure 6: Expression of MHC class II and costimulatory molecules on APC of wt versus IL-10^{KO} mice

Flow cytometric analysis of APC from IL-10^{KO} (gray filled histograms) and wt mice (open solid line histograms). Splenic DCs were stained for CD11c and purified via MACS. Depicted is MHC class II and CD86 expression on gated F4/80^{+} cells (peritoneal macrophages), B220^{+} cells (MLN B cells, splenic B cells) and CD11c^{+} cells (DCs). Isotype matched antibodies were used as negative controls (dotted lines) and were identical for IL-10^{KO} and wt mice, with the exception of peritoneal macrophages (wt isotype control: dotted lines; IL-10^{KO} isotype control: gray lines).

3.1.3 Lack of peptide-induced T cell tolerance in TCR1/IL-10^{KO} mice

In RT2/TCR1 mice peripheral tolerance is established towards an endogenously expressed neo-antigen, the SV40 T Ag. Another approach to study peripheral tolerance is to treat naïve mice systemically with peptide in the absence of adjuvant (Dresser, 1976). This can be also done with TCR transgenic mice, provided that the transgenic T cells are present at low frequency (Kearney et al., 1994), which is the case in TCR1 mice. Since developmental induction of peripheral tolerance in RT2/TCR1 mice towards endogenously expressed SV40 T Ag was established in the absence of IL-10, it was additionally tested whether peptide-induced tolerance could also be induced in TCR1 mice deficient for IL-10.

TCR1 and TCR1/IL-10^{KO} mice were subjected to a tolerizing peptide injection protocol. The animals were treated twice (d0 and d15) with 100 µg P2 peptide in the absence of adjuvant and the percentages of Id$^+$CD4$^+$ T cells in the blood were determined during the course of the experiment. As illustrated in Fig. 7A, both TCR1 and TCR1/IL-10^{KO} mice showed an expansion of transgenic T cells after the first peptide injection (see also Fig. 5), which was more pronounced in TCR1/IL-10^{KO} mice. At d11 after the first injection, the transgenic T cell pool had contracted in both types of mice. Surprisingly, after the second peptide injection, Id$^+$CD4$^+$ T cells from IL-10 deficient mice expanded as vigorously as after the first peptide injection. At d22, the animals were analyzed and the percentages of Id$^+$CD4$^+$ T cells in the MLN were determined. TCR1 mice showed a reduction of transgenic T cells by 68% (1.7±1.1% (n=14)) compared to untreated TCR1 mice (5.3±1.3% (n=39)), whereas the proportion of Id$^+$CD4$^+$ cells in peptide-treated TCR1/IL-10^{KO} mice (5.8±3.9% (n=11)) was on average slightly higher than that of the untreated controls (4.7±1.7% (n= 13)) (Fig. 7A and data not depicted). Thus, no deletion of transgenic T cells took place in TCR1/IL-10^{KO} mice following injection of soluble peptide.

Next, the *in vitro* responsiveness to P2 stimulation of T cells derived from peptide-treated or untreated TCR1 and TCR1/IL-10^{KO} mice was tested (Fig. 7B). A minimal proliferative response was detected from MLN cells of peptide-treated TCR1 animals. Thus, peripheral tolerance could be induced by two sequential injection of peptide in TCR1 mice. In contrast, T cells from peptide-treated TCR1/IL-10^{KO} mice were as responsive to peptide stimulation as their untreated controls. Consequently, peptide-induced tolerance cannot be achieved in TCR1 mice in the absence of IL-10.

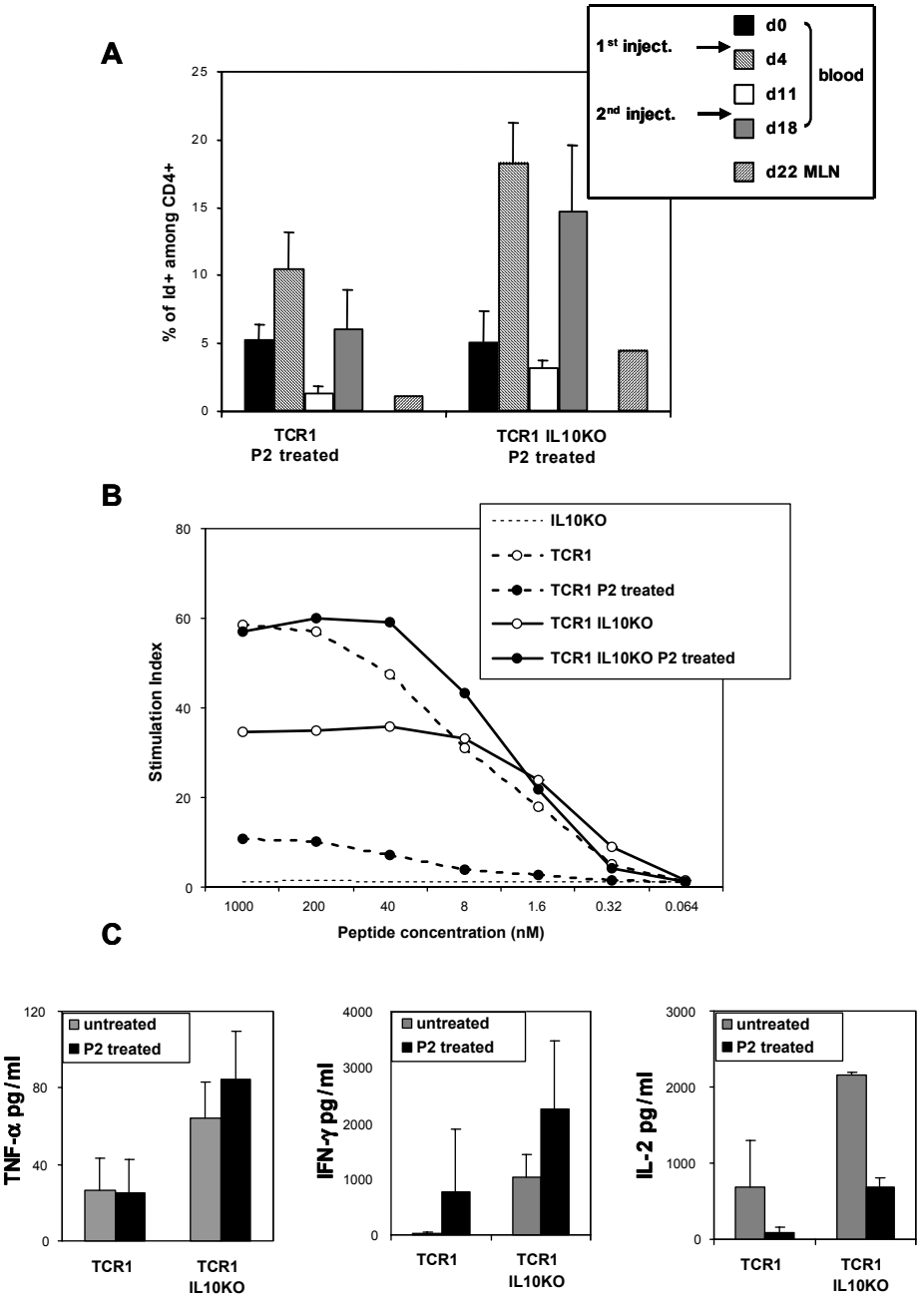

Figure 7: Lack of peptide induced T cell tolerance in IL-10 deficient mice.

A) Frequency of Id^+CD4^+ T cells in the blood (d0, d4, d11, d18) and MLN (d22) of peptide-treated animals. Mice were injected twice (d0, d15) i.p. with 100 µg of SV40 T Ag-peptide P2 and MLN cells were analyzed on d22. Mean values ±SD are given for blood samples (TCR1: n=9; TCR1/IL-10^{KO}: n=2). Values for MLN were obtained from pooled MLN cells of all mice in each group so that SD cannot be calculated in this case. Frequencies of Id^+CD4^+ cells in MLN of untreated and P2-treated mice given in the text are mean data obtained from all experiments performed.

B) *In vitro* [^{3}H]-thymidine proliferation of MLN cells at d22. Samples were pooled according to treatment and genotype (IL-10^{KO}: n=2; TCR1: n=5; TCR1 P2-treated: n=9; TCR1/IL-10^{KO}: n=2; TCR1/IL-10^{KO} P2-treated: n=2). Cultures were performed in triplicates and mean values are shown.

C) Cytokine production of MLN cells after *in vitro* peptide stimulation for 2 days. Mean values ±SD are given (TCR1: n=4; TCR1 P2-treated: n=5; TCR1/IL-10^{KO}: n=2; TCR1/IL-10^{KO} P2-treated: n=3).

Additionally, the concentrations of the cytokines TNF-α, IFN-γ and IL-2 were determined in the supernatants after peptide restimulation *in vitro* (Fig. 7C). Generally, TCR1/IL-10^{KO} mice showed elevated levels of TNF-α and IFN-γ. A reduction of cytokine production after *in vivo* peptide treatment was seen only for IL-2, but even in this case MLN cells from peptide-treated TCR1/IL-10^{KO} mice produced the same amount of IL-2 as those from untreated TCR1 animals.

As mentioned earlier, the [^{3}H]-thymidine incorporation assay provides no information whether the responding cells are anergic to peptide stimulation, or whether there are just lower numbers of responding cells. To address this question, CFSE proliferation assays were carried out in addition. MLN cells were labeled with CFSE and after 2 days of culture analyzed by flow cytometry. First, the percentages of Id^+CD4^+ T cells at the end of the culture were determined (Fig. 8A). Given the lower percentages of transgenic T cells found in MLN of peptide-treated TCR1 mice, even after 2 days of *in vitro* restimulation the proportion of Id^+CD4^+ T cells was reduced compared to untreated mice, although they had expanded to the same extent. *In vivo* peptide-treated TCR1/IL-10^{KO} mice showed identical responses compared to untreated TCR1/IL-10^{KO} mice. In general, there were no differences in peptide-treated versus untreated animals regarding the number of cell divisions undergone by Id^+CD4^+ T cells (Fig. 8B). However, IL-10 deficient mice showed a tendency towards a higher rate of cell division. After the 2 day culture period more than 25.7±6.4 (n= 4) of transgenic T cells from TCR1/IL-10^{KO} mice had divided three times, whereas less than 6.9±4.7 % (n= 9) of Id^+CD4^+ T cells from TCR1 mice had undergone three divisions (Fig. 8C).

In conclusion, peptide-induced tolerance in TCR1 mice is mainly achieved by deletion of transgenic T cells and the reduced percentage of Id^+CD4^+ T cells accounts for the impaired response to peptide restimulation. The tolerizing protocol applied does not induce anergy of the remaining cells in TCR1 mice. Interestingly, sequential peptide injections do not lead to deletional tolerance in TCR1 mice in the absence of IL-10, once again pointing towards an important regulatory role of IL-10 in the context of the living organism.

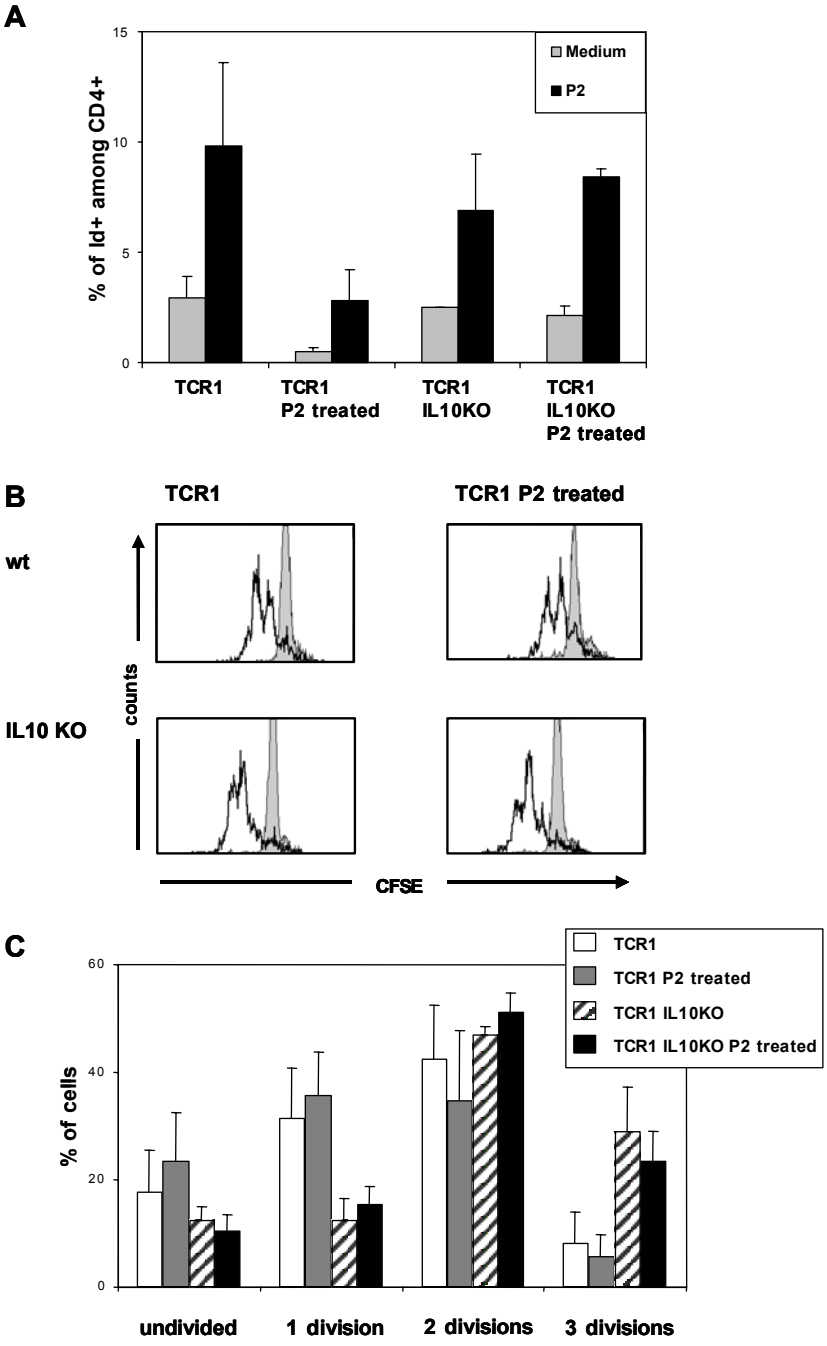

Figure 8: Quantification of cell division rates of Id^+CD4^+ T cells from TCR1 and TCR1/IL-10^{KO} mice following *in vivo* peptide treatment.

Mice were treated as described in Fig. 7. MLN were isolated on d22, CFSE-labeled and cultured with 1µM P2 or medium for 2 days. **A)** Frequency of Id^+CD4^+ after 2 days of *in vitro* culture. Mean values ±SD are given for each *in vivo* treatment group.

B) Flow cytometric analysis of CFSE-labeled Id^+CD4^+ cells. Histograms are gated on Id^+CD4^+ cells showing unstimulated cells (gray filled histograms) and P2-stimulated cells (black line histograms). Representative histograms are shown.

C) Detailed analysis of division numbers of CFSE-labeled, P2-stimulated Id^+CD4^+ cells. The percentages of cells, which had undergone zero to three divisions, were calculated by gating on the respective CFSE division peaks. Mean values ±SD are given for each *in vivo* treatment group (TCR1: n=4; TCR1 P2-treated: n=5; TCR1/IL-10^{KO}: n=2; TCR1/IL-10^{KO} P2-treated: n=3).

3.1.4 Increased sensitivity to bacterial superantigens in IL-10^{KO} mice

Since peptide-induced tolerance could not be established in TCR1 mice deficient for IL-10, it was important to examine whether this phenomenon was a particularity seen in TCR transgenic mice, or whether it was a general characteristic of IL-10^{KO} mice. Therefore a tolerance induction model applicable in normal, non-TCR transgenic mice was chosen, which is the Staphylococcal Enterotoxin B (SEB) model of tolerance induction. *Staphylococcus aureus* is a gram-positive bacterium, which produces different enterotoxins also commonly known as superantigens. These superantigens are potent T cell mitogens, which induce T cell proliferation at concentrations of 10^{-9} M. Irrespective of the TCR specificity, superantigens bind to a particular V_β region of the TCR and to non-polymorphic regions of MHC II molecules on APCs. Thereby, a large population of T cells is activated, constituting up to 20% of the total T cell population. This broad activation leads to increased cytokine production *in vivo* and clinical symptoms similar to septic shock. SEB can be used in high doses to study acute shock situations whereas SEB mediated tolerance induction can be studied by applying low doses. SEB-induced tolerance is characterized by deletion of T cells bearing the specific V_β TCR (V_β7, 8.1-8.3, 17 in the case of SEB) and unresponsiveness of the remaining cells to SEB restimulation *in vitro*.

First, different doses of SEB were tested in wt and IL-10^{KO} mice to determine optimal experimental conditions. Unexpectedly, IL-10^{KO} mice showed a strongly increased susceptibility to SEB induced shock, since already a single injection of 10 µg of SEB led to 100% lethality (4 out of 4 animals died within one day after SEB injection). A study on IL-10^{KO} mice of the C57BL/6 background had previously demonstrated that IL-10 deficiency resulted in higher sensitivity to SEB (Hasko et al., 1998). Nevertheless, IL-10^{KO} mice on the C57BL/6 background could be treated with a dose up to 300 µg causing no lethality, which is 30 fold higher than the dose tolerated by IL-10^{KO} mice on the C3HeB/FeJ background. Therefore, the following experiments were set up with low doses and repeated injections of SEB. Tolerance in wt animals could be established by two consecutive injections of 2 µg SEB within 2 weeks. As shown in Fig. 9A mice treated with SEB *in vivo* showed a strongly reduced proliferative response when restimulated with SEB *in vitro* compared to untreated controls. However, this procedure also led to lethality in IL-10^{KO} mice after the second SEB injection.

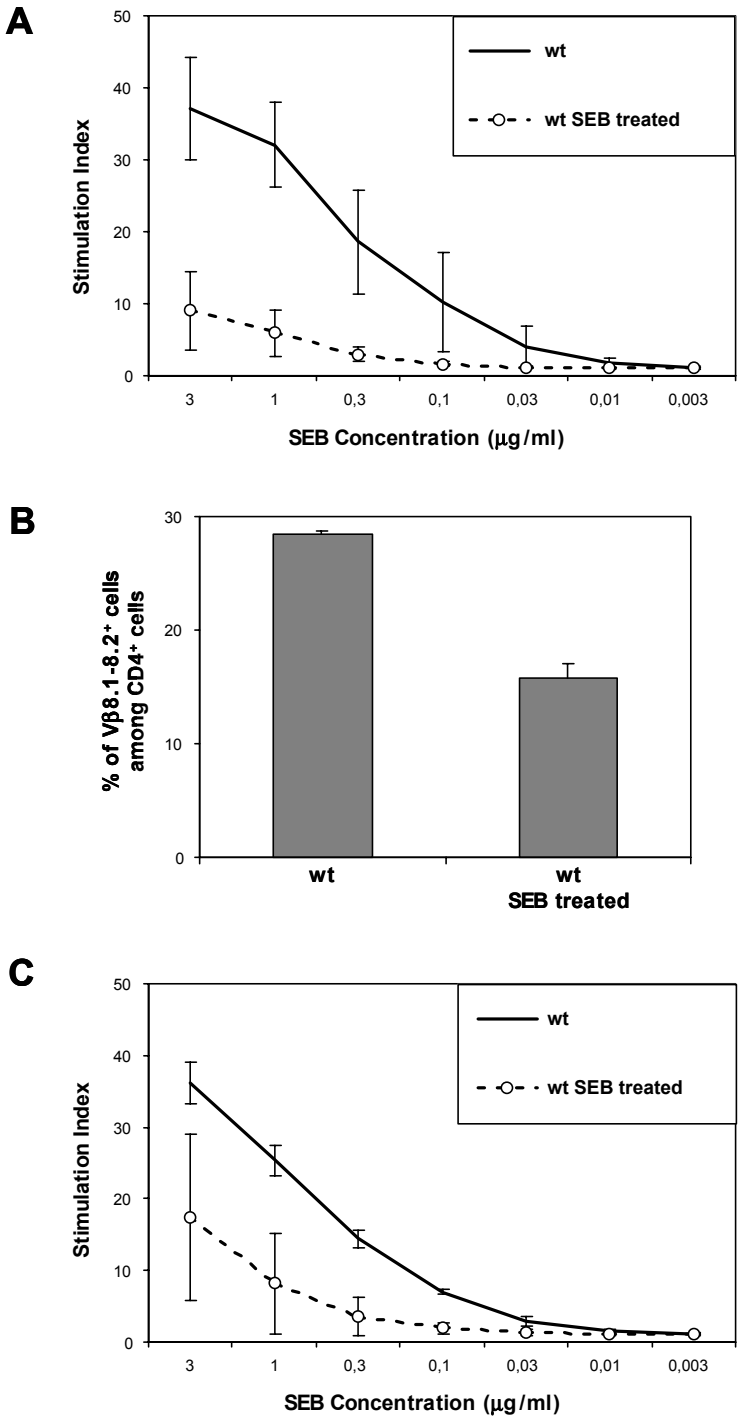

Figure 9: SEB induced tolerance in wt animals.

A) *In vitro* [^{3}H]-thymidine proliferation assay with SEB treated or untreated wt animals. 2 µg SEB was injected i.p. (d0 and d7) and the animals were analyzed on d14. Mean values ± SD are given (wt: n=3; wt SEB treated: n=3).

B) Frequency of V_β8.1-8.3 positive cells among CD4$^+$ T cells. 1 µg SEB was injected i.p. (d0 and d6) and the animals were analyzed on d13. Mean values ± SD are given (wt: n=3; wt SEB treated: n=3).

C) *In vitro* [^{3}H]-thymidine proliferation assay with SEB treated or untreated wt animals. 1 µg SEB was injected i.p. (d0 and d6) and the animals were analyzed on d13. Mean values ± SD are given (wt: n=3; wt SEB treated: n=3).

The dose of SEB was consequently lowered to 1 µg SEB given twice and tested first in wt animals. Unfortunately, this regimen was not sufficient to induce profound tolerance in wt animals. A significant reduction in V_β8.1-8.3 positive cells could be induced, but the proliferative response to SEB restimulation *in vitro* showed high fluctuations (Fig. 9B and C). Furthermore, also with a low dose of 1 µg SEB given twice, one of three IL-10^{KO} mice died after the second SEB injection. Due to highly differential sensitivity of wt and IL-10^{KO} mice to SEB treatment *in vivo*, the experiments were stopped at that point. As conclusion it can be stated that IL-10 deficient mice show a much higher responsiveness to super-antigen stimulation resulting in strongly increased susceptibility to SEB induced shock. This increased lethality is much more apparent in IL-10^{KO} mice on the C3HeB/FeJ compared to the C57BL/6 background. Although no direct conclusion regarding the induction of SEB-induced tolerance can be derived from these experiments, they support the notion that IL-10^{KO} mice exhibit a much higher susceptibility to TCR-mediated T cell activation than wt mice.

3.1.5 Combined treatment of RT2/TCR1/IL-10^{KO} mice with peptide and CpG-ODN to induce tumor immunity

As described above, the tolerance status of tumor specific T cells in RT2/TCR1/IL-10^{KO} mice could be broken by a single intervention with P2 peptide. To test whether peptide treatment could lead to a long-term effect on tumor immunity in RT2/TCR1/IL-10^{KO} mice, animals were treated with two sequential peptide injections two weeks apart. Even after this treatment, RT2/TCR1/IL-10^{KO} mice had elevated levels of transgenic T cells compared to RT2/TCR1 controls and these cells retained their increased proliferative capacity (data not shown). Nevertheless, no significant differences were observed regarding the tumor progression of peptide- versus untreated RT2/TCR1/IL-10^{KO} mice. To induce effective tumor immunity it is thus not sufficient to expand the respective population, but one also has to boost the effector function of the T cells to get a potent tumor response. This could presumably be achieved by additional activation of APCs during peptide treatment through immunostimulatory agents. One such agent is CpG-ODN containing un-methylated cytidine-guanidine sequences, frequently found in bacterial DNA. CpG-ODN belongs to a class of molecular bacterial patterns recognized by the innate immune system leading to its activation in order to form a first line of defense against bacterial infection. Stimulation of DCs with CpG-ODN leads to

upregulation of MHC II and costimulatory molecules as well as increased IL-12 production, thereby boosting Ag-specific responses.

A combined treatment with CpG-ODN and P2 peptide was set up for RT2/TCR1 and RT2/TCR1/IL-10^{KO} mice where the animals should be injected twice within two weeks and analyzed later on. During the course of the experiments, blood samples were taken and analyzed for the percentages of transgenic T cells to follow the induced T cell response. As already seen in SEB treated IL-10^{KO} mice, also RT2/TCR1/IL-10^{KO} mice showed a detrimental responsiveness to CpG-ODN stimulation, resulting in lethality after a single injection of 5 nmol CpG-ODN together with 100 µg P2 peptide. This overshooting immune response was dependent on T cells expressing the transgenic TCR, since IL-10^{KO} mice treated with 5 nmol CpG-ODN and P2 peptide showed no lethal response. The CpG-ODN concentration was consequently reduced to 1 nmol in the following experiments. Strikingly, also a repeated injection of 1 nmol CpG-ODN and P2 peptide led to a lethal shock in the TCR1/IL-10^{KO} mouse, whereas the RT2/TCR1/IL-10^{KO} animal survived. Therefore, in the case of naïve TCR1/IL-10^{KO} mice, peptide stimulation together with CpG-ODN as potent adjuvant induces a fulminant T cell response, which cannot be controlled in the absence of IL-10, leading to a lethal shock. Tolerant RT2/TCR1/IL-10^{KO} mice however possess reduced total numbers of transgenic T cells, which are impaired in their *in vitro* peptide response compared to TCR1/IL-10^{KO} mice and thus are able to resist the combined CpG-ODN/P2 treatment. Interestingly, after repeated stimulation with peptide and CpG-ODN *in vivo* transgenic T cells from RT2/TCR1/IL-10^{KO} were fully responsive to peptide restimulation *in vitro*. In contrast, CpG-ODN/P2 *in vivo* treatment of RT2/TCR1 mice could not break the tolerance status. These mice were still unresponsive to peptide stimulation *in vitro* (Fig. 10).

A further conclusion to be drawn from these data is that tolerance induction does not take place in TCR1 mice after repeated stimulation with CpG-ODN/P2 *in vivo*, whereas repeated *in vivo* peptide stimulation induces tolerance (see Fig. 7B). This is in agreement with the common view that tolerance is induced by T cell stimulation in the absence of costimulation, which is the case for P2 treatment *in vivo*. In contrast, an effective T cell response is induced in animals treated simultaneously with CpG-ODN and P2 *in vivo*.

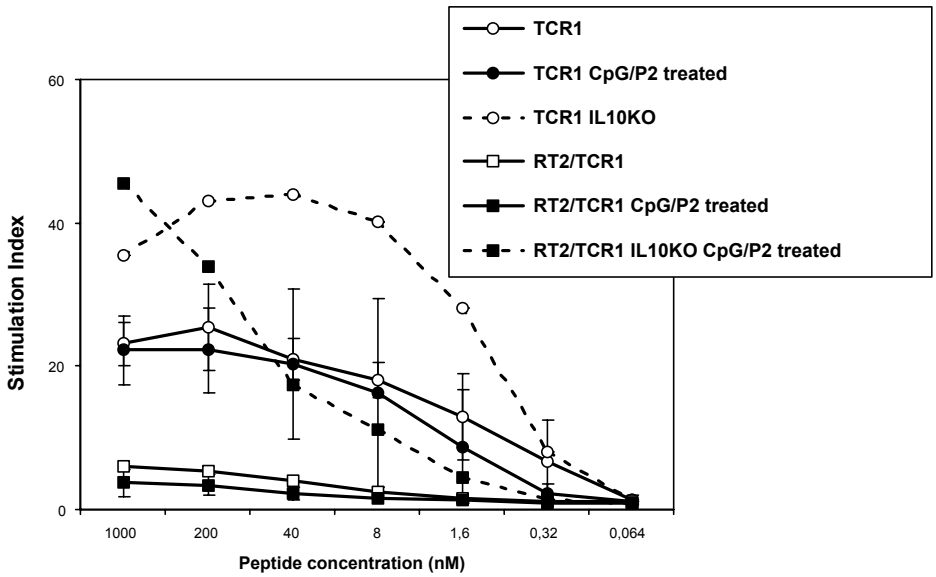

Figure 10: Sustained responsiveness of Id^+CD4^+ T cells from RT2/TCR1/IL-10^{KO} mice following longterm *in vivo* CpG-ODN/peptide treatment.
In vitro [^{3}H]-thymidine proliferation assay with CpG-ODN/P2 treated or untreated mice. Animals were injected twice (d0, d12) i.p. with 1nmol CpG-ODN and 100 µg SV40 T Ag-peptide P2 and analyzed on d19. Cultures were performed in triplicates and mean values are shown.

As described above, the treatment of RT2/TCR1/IL-10^{KO} mice with CpG-ODN/P2 *in vivo* led to a strong and sustained T cell response. The remaining question was whether this response had any effect on tumor development in these mice. Developing tumors in RT2 and RT2/TCR1 mice produce increasing amounts of insulin, thereby leading to reduced systemic glucose levels. By measuring the blood glucose levels, one can get further information on tumor progression in the respective animals. Therefore, glucose levels in the blood were analyzed during the course of the experiment. A reduction in blood glucose could be determined in treated und untreated RT2/TCR1 mice indicating that CpG-ODN/P2 treatment had no significant influence on tumor progression. During the course of the experiment it became evident unfortunately that it was not possible to correlate the blood glucose levels in RT2/TCR1/IL-10^{KO} mice with tumor progression since also untreated TCR1/IL-10^{KO} mice showed a reduction in blood glucose levels. The general reduction of blood glucose seen in IL-10^{KO} mice is probably due to problems in nutritient resorption induced by the general intestinal pathology seen in IL-10 deficient animals.

Taken together, a combination of peptide treatment together with CpG-ODN leads to a strong T cell response in RT2/TCR1 mice in the absence of IL-10. This is probably an efficient way to induce tumor immunity in the RT2/TCR1 model, however, conclusive experiments in RT2/TCR1/IL-10^{KO} are difficult to carry out, since these animals show a general increased sensitivity to CpG-ODN as adjuvant. As mentioned above, an additional limitation to long-term studies carried out in IL-10^{KO} mice is the general intestinal pathology seen in these animals. IL-10 deficient animals develop an intestinal inflammation similar to IBD (Kuhn et al., 1993). The underlying mechanism is the unregulated activity of macrophages towards enteric bacteria (Berg et al., 1996). Due to alterations in the intestine IL-10^{KO} mice have a disturbed nutrient resorption leading to growth retardation and anemia. Depending on the housing conditions of the animals, the disease severity varies. In our breeding colony, first signs of IBD (diarrhea) were noticed at an age of 5-7 weeks. With increasing age, the IL-10^{KO} mice showed profound weight loss indicating progressive intestinal inflammation. Therefore, long-term studies were limited in RT2/TCR1/IL-10^{KO} by the general intestinal pathology induced in the absence of IL-10.

The next task was to transfer the findings from RT2/TCR1/IL-10^{KO} mice into a therapeutical set-up. Would it be possible by transiently blocking IL-10 in RT2/TCR1 mice to break tolerance with combined CpG-ODN/P2 treatment?

The blocking of IL-10 *in vivo* can be achieved by administration of an Ab directed against the IL-10 receptor (IL-10R). After purification of the anti-IL-10R from hybridoma supernatants, its biological function was tested *in vitro*. As test system TNF-production of raw macrophages after cultivation with different stimuli was chosen. Stimulation of raw macrophages with LPS induces strong TNF-α production. Addition of IL-10 during LPS stimulation downregulates the LPS induced TNF-α production. This downregulatory effect of IL-10 should be blocked in the presence of the anti-IL-10R. As illustrated in Fig. 11A, the anti-IL-10R efficiently blocked the function of IL-10. But also addition of anti-IL-10R alone led to TNF-α production. This presumably is due to blocking of autocrine production of IL-10 by raw macrophages.

The next step was the treatment of RT2/TCR1 mice with anti-IL-10R in combination with CpG-ODN/P2. Animals were injected with 500 µg anti-IL-10R one day prior to injection of CpG-ODN/P2 to ensure efficient systemic blockade of IL-10 during peptide priming. To analyze the immune response induced by this treatment, MLN cells were

isolated 4 days after *in vivo* CpG-ODN/P2 stimulation and restimulated *in vitro* in the presence of P2 or medium. CpG-ODN/P2 treatment alone induced a minimal proliferation of $CD4^+$ T cells of RT2/TCR1 mice after peptide restimulation *in vitro* (Fig. 11B). In contrast, in combination with blockade of IL-10 a strong $CD4^+$ T cell response was induced. However, also *in vivo* anti-IL-10R treatment alone induced over 10% of the total lymphocyte population to proliferate even without restimulation *in vitro*. This experiment indicates that blockade of IL-10 during *in vivo* peptide priming can break the tolerance status of RT2/TCR1 mice. However, besides the peptide-specific population expanded by this treatment, also proliferation of lymphocytes with an unknown specificity is induced. Thus, IL-10 is continuously needed in the living organisms to maintain a certain level of unresponsiveness of the immune system. Since we did not see systemic autoimmune pathology in IL-10 deficient mice, this may reflect an adaptation of the immune system in $IL\text{-}10^{KO}$ mice.

Taken together, peptide priming in the absence of IL-10 is an efficient approach to break tolerance in RT2/TCR1 mice. Longterm studies will be carried out in future to determine whether the combination of peptide stimulation and blockade of IL-10 not only leads to expansion of transgenic T cells but also induces effective tumor responses in RT2/TCR1 mice.

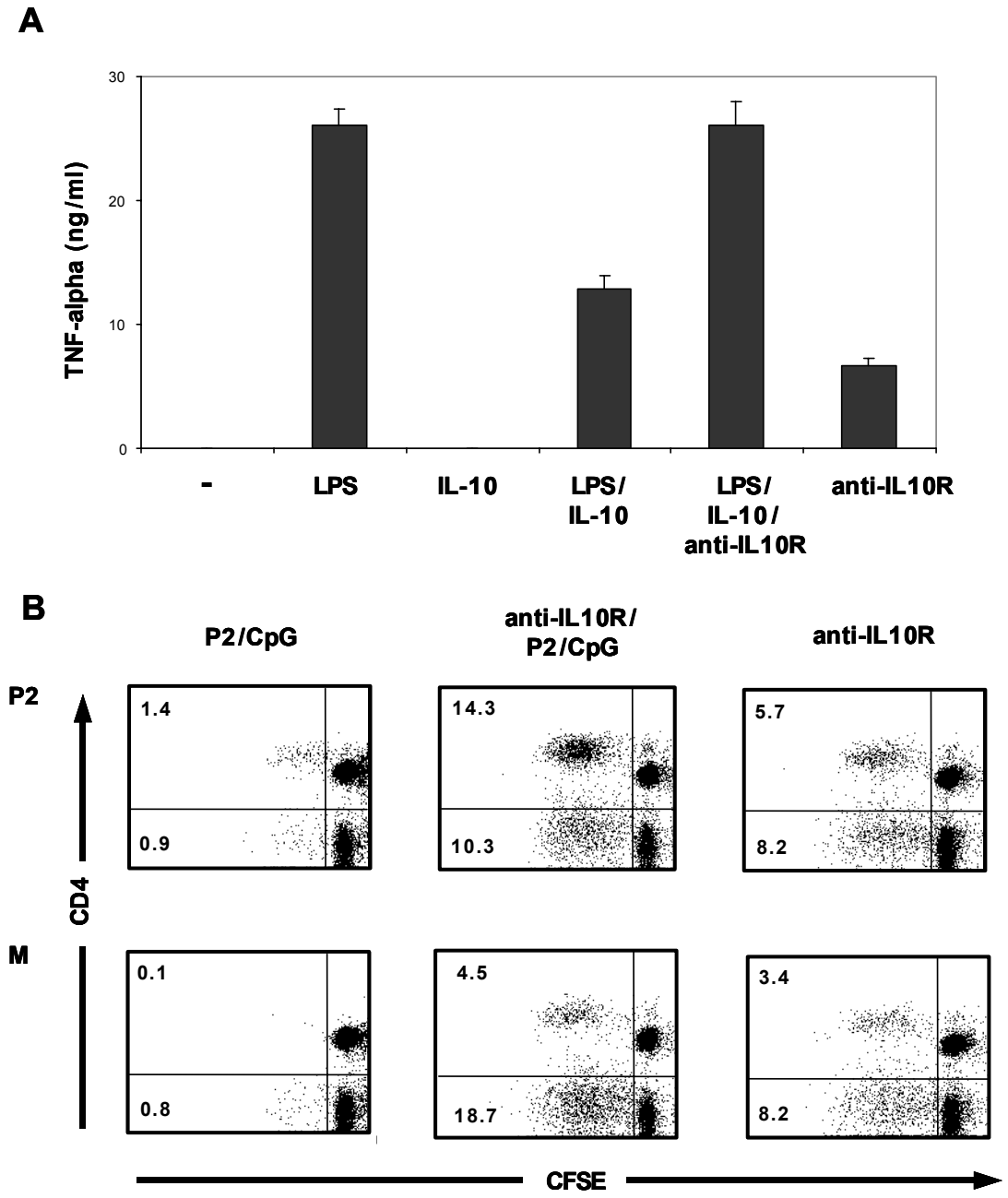

Figure 11: Blocking of IL-10 during *in vivo* peptide stimulation.

A) *In vitro* assay to test the biological activity of the anti-IL10R. TNF-alpha production of $1.5x10^6$ raw macrophages was measured in culture supernatants after 4 h of stimulation with 100 ng/ml LPS, 10 ng/ml IL-10, or 10 ng/ml anti-IL-10R Ab.

B) Flow cytometric analysis of CFSE-labeled MLN cells from *in vivo* treated RT2/TCR1 mice. Animals were systemically treated with 500 µg anti-IL-10R Ab one day prior to i.p. injection of 1 nmol CpG-ODN and 100 µg SV40 T Ag-peptide P2. Control animals received either the anti-IL-10R Ab alone or the combined CpG/P2 treatment. MLN cells were taken on d5 after the first injection, CFSE-labeled and cultured with P2 or medium for 3 days. Representative dot plots are shown.

3.2 $CD25^+Id^+CD4^+$ T cells of RT2/TCR1 mice have regulatory function *in vitro*

Developmental induction of peripheral tolerance in RT2/TCR1 mice correlates with the appearance of $CD25^+Id^+CD4^+$ T cells. The cell surface marker CD25 has been associated in different experimental models with regulatory T cell function (Takahashi et al., 1998; Asseman et al., 1999). In unmanipulated mice about 5-9 % of peripheral $CD4^+$ T cells are CD25 positive and these cells can suppress T cell proliferation of naïve $CD4^+$ T cells *in vitro*. Remarkably, in tolerant RT2/TCR1 mice more than 50% of the remaining Id^+CD4^+ T cells are $CD25^+$ (Fig. 4). It could be assumed that the unresponsiveness of Id^+CD4^+ T cells to peptide stimulation *in vitro* is due to active suppression of the T cell response by these $CD25^+Id^+CD4^+$ T cells. To test this hypothesis, Id^+CD4^+ T cells of tolerant RT2/TCR1 mice were FACS sorted into $CD25^+$ and $CD25^-$ cells. The two populations were stimulated *in vitro* with peptide in the presence of irradiated splenocytes as APCs. Additionally, the respective populations were mixed with naïve Id^+CD4^+ T cells from TCR1 mice to test whether they could inhibit proliferation of naïve T cells. As depicted in Fig. 12A and B $CD25^+$ as well as $CD25^-Id^+CD4^+$ T cells from RT2/TCR1 mice showed minimal responsiveness to peptide stimulation compared to naïve Id^+CD4^+ T cells from TCR1 mice. Interestingly, in coculture experiments, only $CD25^+Id^+CD4^+$ T cells from RT2/TCR1 mice could suppress proliferation of naïve Id^+CD4^+ T cells from TCR1 mice. Therefore, the regulatory function within the Id^+CD4^+ T cell population from RT2/TCR1 mice segregates with CD25, whereas both $CD25^+$ as well as $CD25^-Id^+CD4^+$ T cells are impaired in their responsiveness to peptide stimulation. The regulatory function and anergic state of $CD25^+Id^+CD4^+$ T cells from RT2/TCR1 mice could be broken by IL-2 added during peptide stimulation (data not shown), which has been described in the literature as one characteristic of regulatory T cells (Thornton et al., 1998; Takahashi et al., 1998).

One essential remark has to be made at this point: The suppressive activity of $CD25^+Id^+CD4^+$ T cells could only be observed under very specific *in vitro* conditions. During the assays described above, identical numbers of feeders as well as effector T cells were used. Initial assays were carried out with 20 fold more irradiated splenocytes than effector T cells.

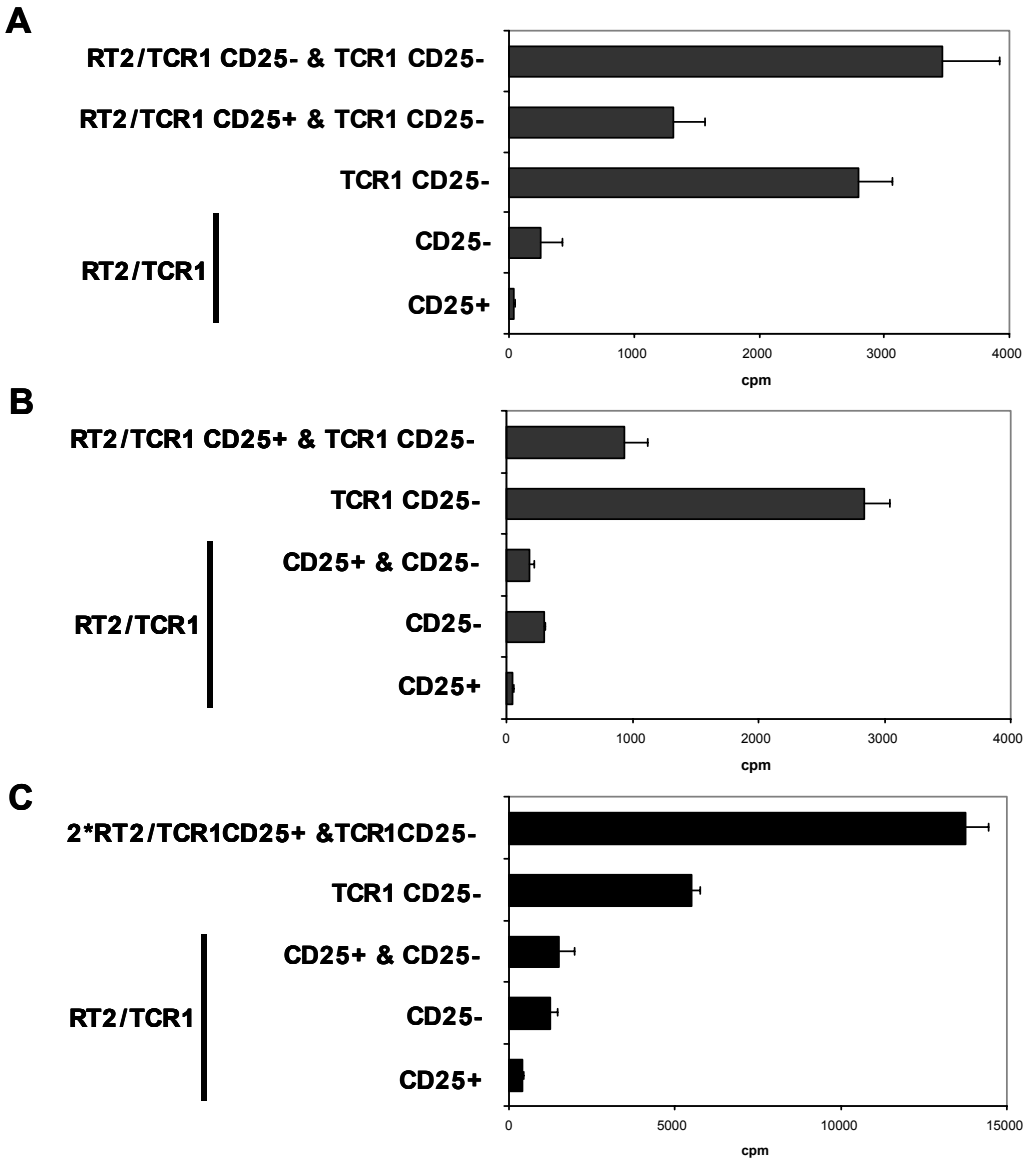

Figure 12: CD25$^+$Id$^+$CD4$^+$ T cells of tolerant RT2/TCR1 mice show regulatory function *in vitro*.
Id$^+$CD4$^+$ splenocytes of tolerant RT2/TCR1 mice were FACS sorted into CD25$^+$ and CD25$^-$ fractions. As naïve control cells FACS purified CD25$^-$Id$^+$CD4$^+$ T cells from TCR1 mice were taken.
A) and **B)** 10 000 sorted cells were cultured alone or cocultured at a ratio of 1:1 in the presence of P2 peptide along with 10 000 irradiated spleen cells as APCs. Cultures were performed in triplicates and mean values ± SD are shown. The results are representative of three independent experiments.
C) Cultures were performed as in A and B with the exception of higher numbers of irradiated splenocytes (200 000).

Under these conditions, Id^+CD4^+ T cells from RT2/TCR1 mice were still impaired in their response to peptide stimulation, but the $CD25^+Id^+CD4^+$ T cells no longer showed any suppressive activity, even when mixed at a ratio of 2:1 with Id^+CD4^+ T cells from TCR1 mice (Fig. 12C). Since the regulatory function of $CD25^+CD4^+$ T cells has been shown to be cell-cell contact dependent (Thornton et al., 1998; Takahashi et al., 1998), one simple explanation for the observed finding would be that by addition of high numbers of APCs, $CD25^+$ Id^+CD4^+ T cells are sterically hindered to exert their immunosuppressive effect on naïve T cells.

It would have been interesting to also analyze $CD25^+Id^+CD4^+$ T cells from RT2/TCR1/IL-10^{KO} mice for their suppressive activity *in vitro*. To carry out these assays, several mice (at least 6) of the same age were needed to obtain sufficient numbers of sorted $CD25^+Id^+CD4^+$ T cells. Unfortunately, this experiment was not possible to perform, since RT2/TCR1/IL-10^{KO} mice were not available in sufficient numbers. Due to the general pathology seen in IL-10^{KO} mice, the breeding had to be carried out with heterozygous breeding pairs. Since also the RT2 and TCR1 transgenes had to be present, the statistical probability to obtain RT2/TCR1/IL-10^{KO} mice was only 1 in 16.

3.3 Role of peripheral lymph nodes and LTβR in peripheral tolerance induction

As illustrated above tolerance in RT2/TCR1 mice is achieved by three principal mechanisms: deletion of autoreactive T cells, induction of anergy, as well as active suppression by regulatory $CD25^+Id^+CD4^+$ T cells. So far, an essential role of IL-10 could be determined for maintenance of tolerance in RT2/TCR1 mice, but many aspects on how tolerance is established in this model are still unknown. In RT2/TCR1 mice local activation of transgenic T cells can be seen in the pancreatic draining LNs and MLNs already at 1 week after birth and the percentage of activated Id^+CD4^+ T cells increases up to the age of 3 weeks (Förster and Lieberam, 1996). Several studies on autoimmunity have stressed the importance of the local lymphoid environment for induction of effector as well as regulatory cell function (Gagnerault et al., 2002; Kurts et al., 1997). To investigate whether peripheral tolerance can also be established in the absence of lymphoid structures, RT2/TCR1 mice were crossed into a lymphotoxin β receptor (LTβR) deficient background. LTβR is a member of the TNF receptor superfamily and is expressed by follicular dendritic cells, macrophages and stroma cells (Browning et al., 1997; Fütterer et al., 1998). Its ligands ($LT\alpha_1\beta_2$ and LIGHT) are expressed by peripheral lymphocytes and LTα, LTβ as well as TNF deficient mice show abnormalities regarding the development of secondary lymphoid structures (reviewed in von Boehmer, 1997). $LT\beta R^{KO}$ mice have been shown to lack all peripheral LNs (Fütterer et al., 1998) thereby representing an ideal tool to analyze the influence of lymphoid structures in the RT2/TCR1 model.

3.3.1 LTβR deficient mice on the C3HeB/FeJ background contain rudimentary MLN structures

LTβR deficient animals have been initially described on the C57BL/6 background (Fütterer et al., 1998). Since our TCR1 model is restricted to $I\text{-}A^k$, the $LT\beta R^{KO}$ mice had to be backcrossed to the C3HeB/FeJ background and were then intercrossed with RT2/TCR1 mice. While analyzing RT2/TCR1/$LT\beta R^{KO}$ animals, it was found that in contrast to LTβR deficient animals on the C57BL/6 background, which lack all peripheral and mucosal LN, $LT\beta R^{KO}$ mice on the C3HeB/FeJ background possessed mesenteric lymph node-like structures (Fig. 13A).

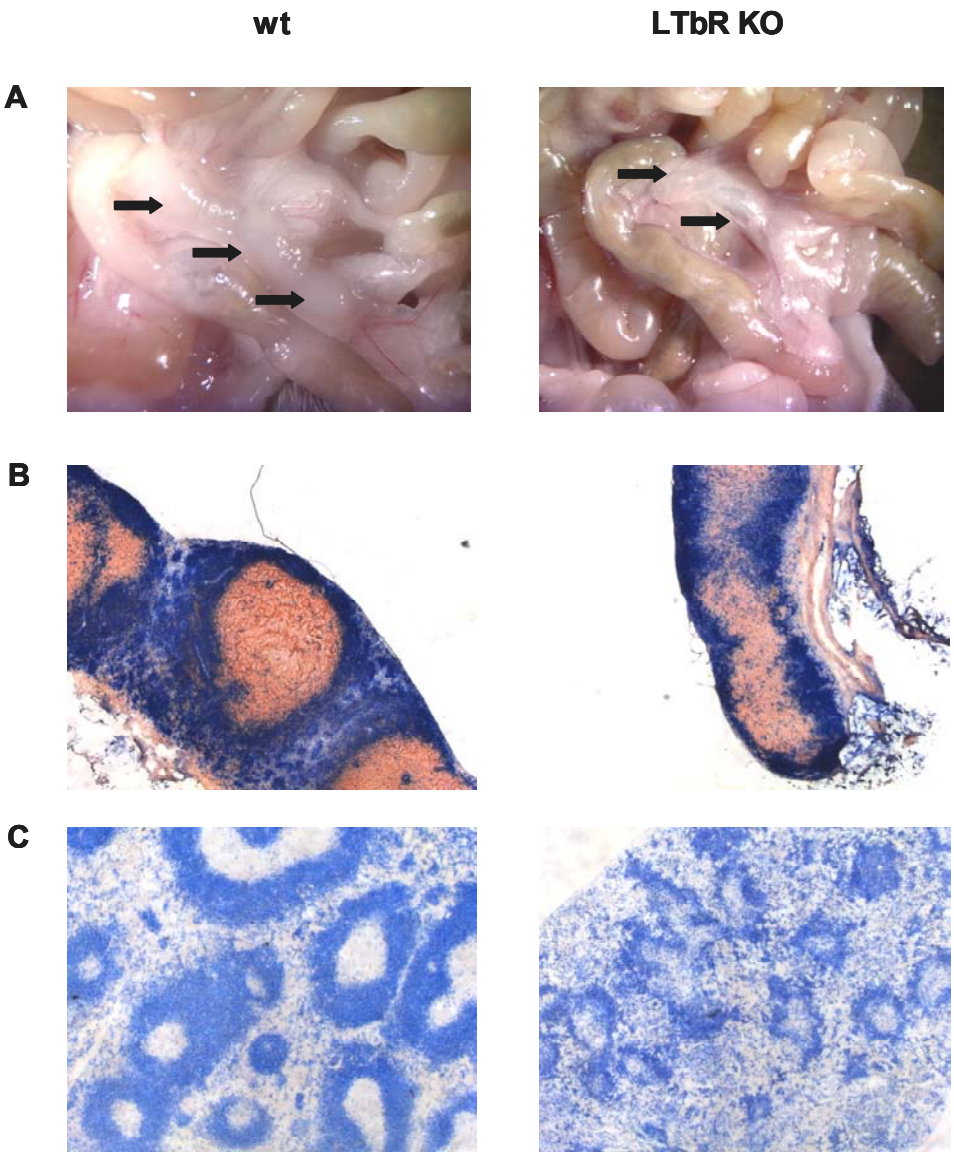

Figure 13: LTβR^{KO} mice possess lymph node-like mesenteric structures.

A) Mesenterial regions of wt C3HeB/FeJ and LTβR^{KO} C3HeB/FeJ mice. The arrows indicate the MLNs and LN like structures

B) Immunohistological analysis of MLNs of wt C3HeB/FeJ mice and rudimentary MLN of LTβR^{KO} C3HeB/FeJ mice. Staining was carried out for CD3 (red staining) and B220 (blue staining).

C) Immunohistological analysis of the spleen of wt C3HeB/FeJ and LTβR^{KO} C3HeB/FeJ mice. Staining was carried out for B220 (blue staining).

These structures contained strongly reduced cell numbers compared to MLN of wt animals ($LT\beta R^{KO}$: $5.0x10^5 \pm 7.0x10^5$ (n=33); wt: $2.3x10^7 \pm 0.7x10^7$ (n=27)). Additionally, the typical anatomical segregation of B and T cells in distinct areas of the LN is disturbed in rudimentary MLN of $LT\beta R^{KO}$ C3HeB/FeJ mice (Fig. 13B) like it is also observed in the spleen (Fig. 13C).

FACS analysis revealed that LN like structures of $LT\beta R^{KO}$ mice showed a bias towards increased B cell numbers, as it has also been seen in the blood of $LT\beta R^{KO}$ animals compared to wt controls (Fig. 14A, and Fütterer et al., 1998).

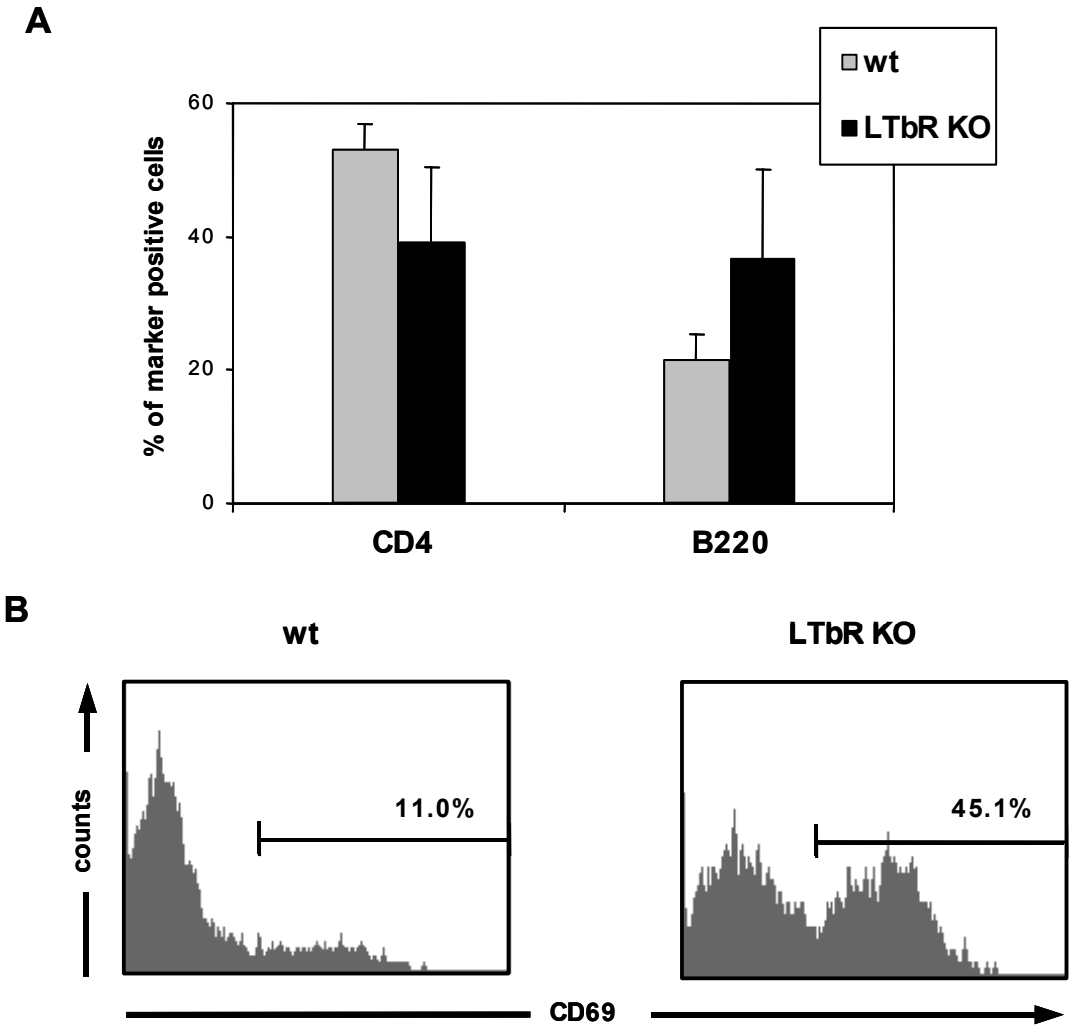

Figure 14: Cellular composition of MLN and rudimentary MLN of wt versus $LT\beta R^{KO}$ mice
A) Cellular distribution of $CD4^+$ and $B220^+$ cells in wt MLN and $LT\beta R^{KO}$ rudimentary MLN. Mean values ±SD are shown for pooled data of three independent experiments (wt n=12; $LT\beta R^{KO}$ n=19).
B) Increased numbers of $CD69^+CD4^+$ T cells in rudimentary MLN of $LT\beta R^{KO}$ mice. Flow cytometric analysis of CD69 expression on $CD4^+$ T cells. Histograms are gated for $CD4^+$ T cells and the numbers indicate the percentage of cells in each fluorescence window. Representative histograms are shown.

Furthermore, 46.6%±11.8% (n=16) of the $CD4^+$ T cells in rudimentary MLN of $LT\beta R^{KO}$ mice showed an activated phenotype as determined by CD69 expression in contrast to 11.2%±1.9% (n=10) of $CD4^+$ T cells in wt MLN (Fig. 14B and data not depicted). Thus, the different genetic background enabled the presence of rudimentary MLN-like structures, but apart from this difference, the phenotype of $LT\beta R^{KO}$ mice in the C3HeB/FeJ background was similar to that of C57BL/6 $LT\beta R^{KO}$ mice.

The presence of mesenteric lymph node-like structures has been reported for $LT\alpha^{KO}$ mice, but only in 30% of the animals (Banks et al., 1995) and it is not clear, which factors control the different outcome. Interestingly, animals deficient in LTβ possess MLN (Alimzhanov et al., 1997).

3.3.2 Lack of SV40 T Ag-specific T cell tolerance in RT2/TCR1/$LT\beta R^{KO}$ mice

The three principal mechanisms of tolerance in RT2/TCR1 mice –deletion, induction of anergy and presence of regulatory T cells- were tested in RT2/TCR1/$LT\beta R^{KO}$ mice.

First the percentages of transgenic T cells in the thymus versus periphery were compared. Similar to wt TCR1 and RT2/TCR1 animals about 4% of single positive $CD4^+CD8^-$ thymocytes were Id^+ on the $LT\beta R^{KO}$ background (TCR1 4.1%±0.8% (n=8); TCR1/$LT\beta R^{KO}$ 3.6%±1.1% (n=8); RT2/TCR1 3.7%±0.5% (n=12); RT2/TCR1/$LT\beta R^{KO}$ 4.4%±1.0% (n=13)) (Fig. 15 and data not depicted). Because of the deletional peripheral tolerance in RT2/TCR1 mice, these animals possessed reduced percentages of Id^+CD4^+ T cells in the spleen compared to single transgenic TCR1 wt mice. In RT2/TCR1/$LT\beta R^{KO}$ mice lower frequencies of Id^+CD4^+ splenocytes can also be found compared to TCR1/$LT\beta R^{KO}$ mice but this accounts only for a 2 fold reduction compared to a 4 fold reduction in RT2/TCR1 versus TCR1 wt animals (TCR1 3.7%±1.2% (n=12); TCR1/$LT\beta R^{KO}$ 3.9%±0.8% (n=11); RT2/TCR1 1.0%±0.3% (n=16); RT2/TCR1/$LT\beta R^{KO}$ 2.3%±0.7% (n=25)) (Fig. 15 and data not depicted).

In addition, RT2/TCR1/$LT\beta R^{KO}$ mice had comparable levels of transgenic T cells in rudimentary MLN compared to TCR1 and TCR1/$LT\beta R^{KO}$ controls (TCR1 5.9%±2.3% (n=8); TCR1/$LT\beta R^{KO}$ 4.0%±0.9% (n=8); RT2/TCR1 1.7%±0.4% (n=10); RT2/TCR1/$LT\beta R^{KO}$ 5.8%±1.7% (n=14)). In view of these data, it cannot clearly be

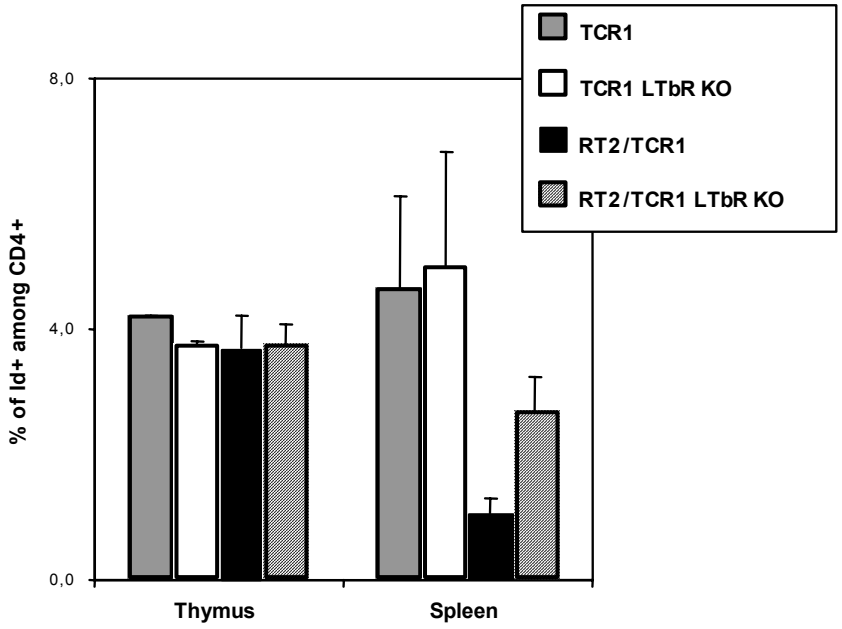

Figure 15: Frequency of Id^+CD4^+ T cells in thymus and spleen of wt and $LT\beta R^{KO}$ mice.
Percentages of Id^+ among $CD4^+CD8^-$ thymocytes and $CD4^+$ splenocytes were determined via flow cytometric analysis. Given are mean values ±SD obtained from two (TCR1; TCR1 $LT\beta R^{KO}$) or three individual mice (RT2/TCR1; RT2/TCR1/$LT\beta R^{KO}$). Data are representative of three independent experiments.

stated whether peripheral Id^+CD4^+ T cells are deleted in RT2/TCR1/$LT\beta R^{KO}$ mice or not. In the spleen, a partial deletion of Id^+CD4^+ T cells in RT2/TCR1/$LT\beta R^{KO}$ mice can be found. However, regarding the percentages of Id^+CD4^+ T cells in rudimentary LNs of RT2/TCR1/$LT\beta R^{KO}$ mice, no differences can be seen compared to TCR1/$LT\beta R^{KO}$ controls, but this could reflect an increased recruitment of Id^+CD4^+ T cells to this structure, since also a general increase in activated T cells can be found.

To determine whether T cell anergy was established in RT2/TCR1/$LT\beta R^{KO}$ mice, the responsiveness of Id^+CD4^+ T cells to peptide stimulation was assessed by CFSE proliferation assays. As depicted in Fig. 16A, splenic T cells from RT2/TCR1/$LT\beta R^{KO}$ mice possessed full responsiveness to peptide stimulation after 3 days of *in vitro* culture. As in TCR1 and TCR1/$LT\beta R^{KO}$ controls, distinct peaks of cell divisions by Id^+CD4^+ T cells from RT2/TCR1/$LT\beta R^{KO}$ mice can be detected, whereas RT2/TCR1 mice showed an asynchronous pattern of cell division typical for memory or anergic T cells (Lee et al., 1998) (Fig. 16B, and Fig. 3).

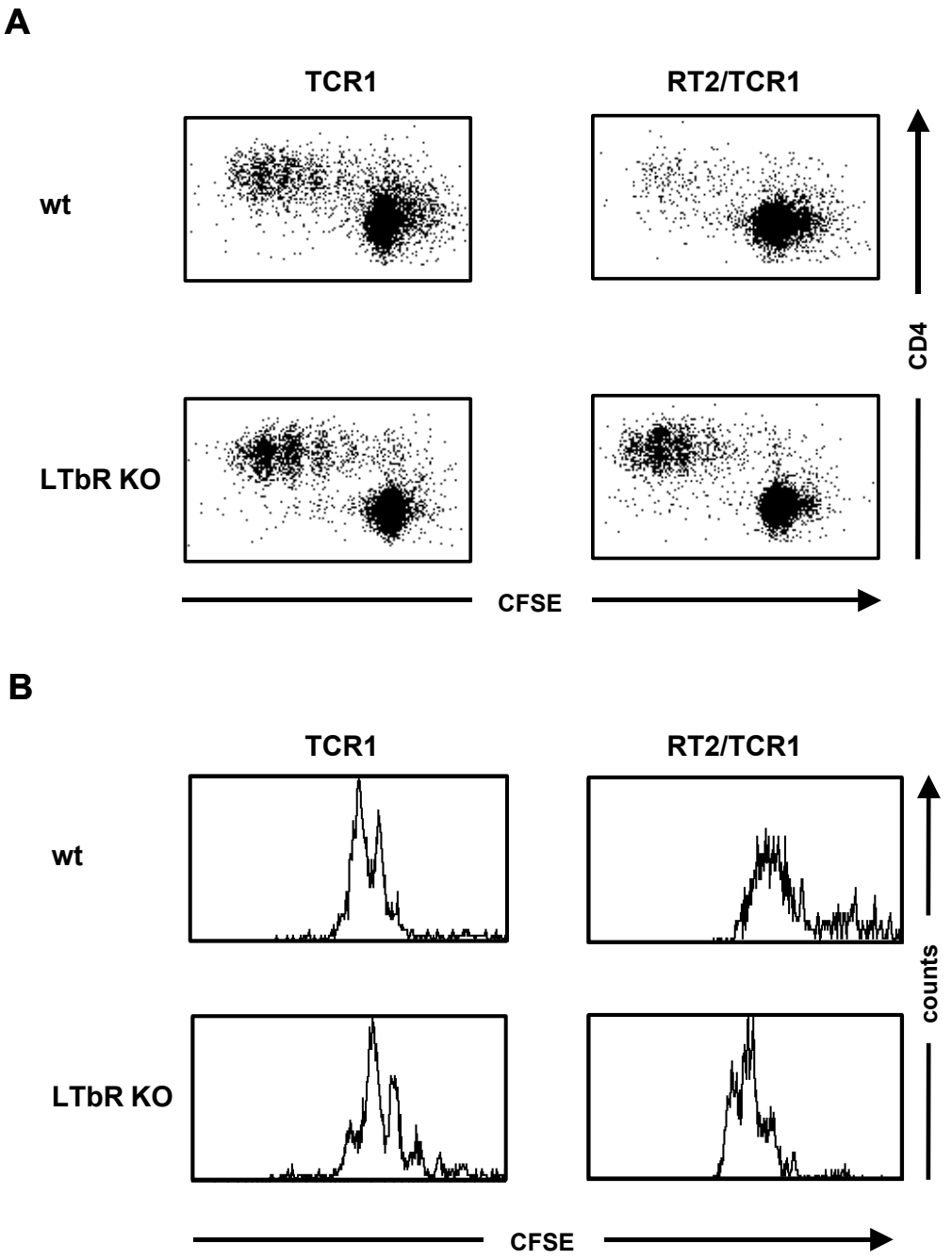

Figure 16: Id$^+$CD4$^+$ T cells of RT2/TCR1/LTβR^{KO} mice are full responsive to peptide stimulation *in vitro*.

Splenocytes were CFSE-labeled, cultured with P2 or medium for 3 days and subsequently analyzed by flow cytometry.

A) Representative dot plots gated on CD4$^+$ T cells stimulated with P2 are shown.

B) Representative histograms showing cell division patterns of Id$^+$CD4$^+$ T cells. Data are representative for three independent experiments.

Furthermore, in RT2/TCR1 mice up to 50% of the Id^+CD4^+ T cells stayed out of cycle after 2 days of stimulation (see Fig. 3), whereas in RT2/TCR1/LTβR^{KO} mice the majority of Id^+CD4^+ T cells were cycling (data not shown). Thus it can be concluded that anergy induction in RT2/TCR1 mice is dependent on the presence of the LTβR, presumably because of the lack of normal peripheral LN.

The third characteristic of tolerance induction in the RT2/TCR1 model is the appearance of regulatory $CD25^+Id^+CD4^+$ T cells. To test this aspect, the expression levels of CD25 on Id^+CD4^+ T cells of RT2/TCR1/LTβR^{KO} mice were determined. Transgenic T cells from RT2/TCR1/LTβR^{KO} mice possessed only 4.0%±1.6% (n=10) $CD25^+Id^+CD4^+$ T cells compared to 36.2%±7.0% (n=8) $CD25^+Id^+CD4^+$ T cells in RT2/TCR1 wt animals (TCR1: 1.2%±0.8% (n=5); TCR1/LTβR^{KO}: 0.9%±0.5% (n=5)) (Fig. 17A). Therefore, the strong response of Id^+CD4^+ T cells from RT2/TCR1/LTβR^{KO} mice to peptide stimulation seen *in vitro* could be due to the absence of regulatory $CD25^+Id^+CD4^+$ T cells.

The next question addressed was whether the lack of induction of $CD25^+Id^+CD4^+$ T cells in RT2/TCR1/LTβR^{KO} mice was specific for the transgenic TCR, or whether LTβR^{KO} mice had a general defect in generating regulatory T cells. For this purpose, the percentages of $CD25^+CD4^+$ T cells were determined in the spleen of wt and LTβR^{KO} mice. Strikingly, LTβR^{KO} mice had overall reduced frequencies of $CD25^+CD4^+$ T cells (5.2%±1.8%; n=26) compared to wt controls (13.6%±1.6%; n=26) (Fig. 17B). Nevertheless, the existing $CD25^+CD4^+$ T cells of LTβR^{KO} had normal regulatory function as determined by *in vitro* suppression assays (Fig. 17C). Like their wt counterparts, $CD25^+CD4^+$ T cells of LTβR^{KO} inhibited proliferation of $CD25^-CD4^+$ T cells after CD3 stimulation. The results demonstrate that in the absence of LTβR, peripheral tolerance cannot be established in the RT2/TCR1 model. Furthermore, the entire population of regulatory $CD25^+CD4^+$ T cells in LTβR^{KO} mice appears to be strongly reduced compared to wt animals.

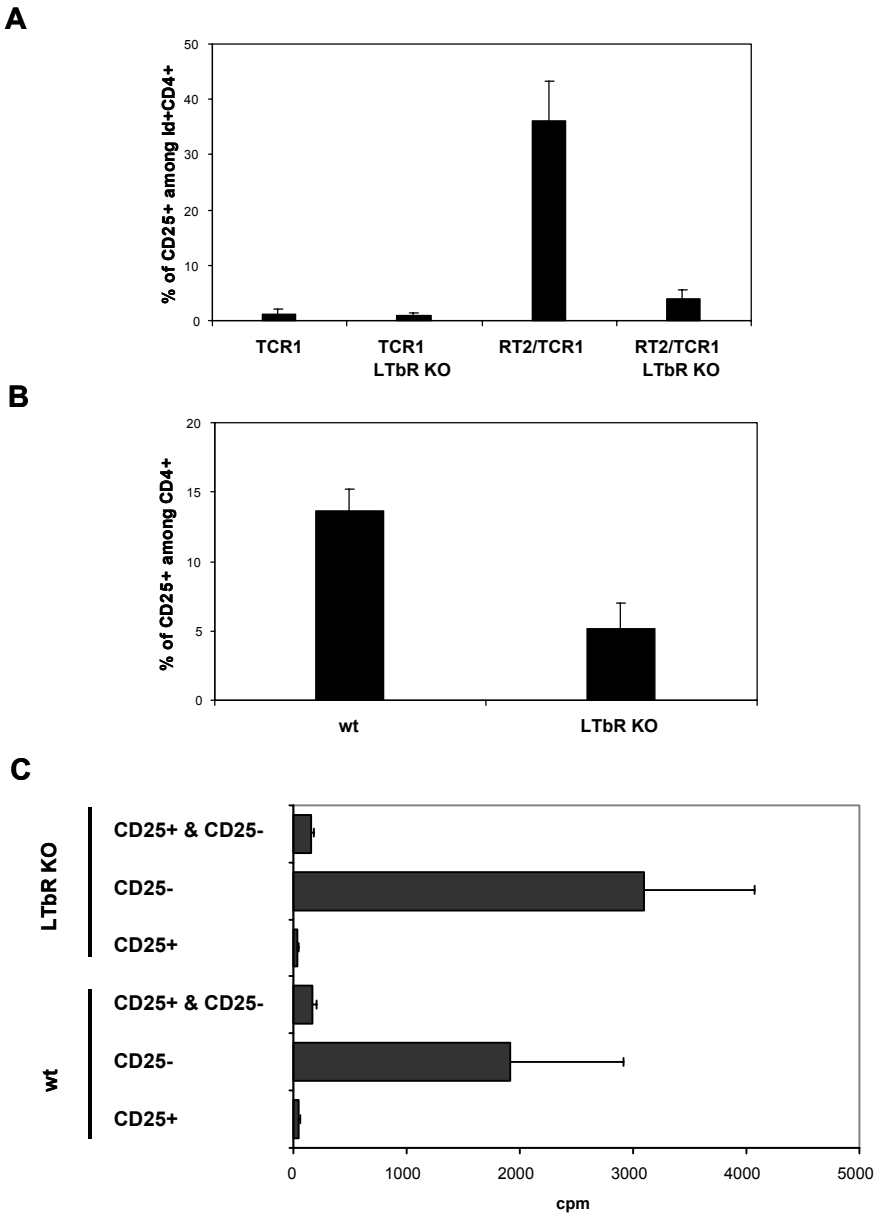

Figure 17: Reduced frequency of $CD25^+CD4^+$ T cells in $LT\beta R^{KO}$ mice.

A) and **B)** Flow cytometric analysis of CD25 expression of splenocytes.

A) Gates were set for Id^+CD4^+ T cells and the frequency of $CD25^+$ cells was determined. Mean values ±SD are given (TCR1 n=5; TCR1/$LT\beta R^{KO}$ n=5; RT2/TCR1 n=8; RT2/TCR1/$LT\beta R^{KO}$ n=10). Data are pooled from several independent experiments.

B) Percentage of $CD25^+$ cells among $CD4^+$ T cells. Mean values ±SD are given (wt n=26; $LT\beta R^{KO}$ n=26). Data are pooled from several independent experiments.

C) $CD25^+CD4^+$ T cells of $LT\beta R^{KO}$ mice possess regulatory function *in vitro.* Splenocytes were enriched for CD4 by MACS and cell sorted into $CD25^+$ and $CD25^-$ cells pools. $2.5x10^4$ $CD25^+$ or $CD25^-CD4^+$ cells were cultured alone or cocultured at a ratio of 1:1. $2.5x10^4$ irradiated splenocytes were added as APC. Cultures were stimulated with α-CD3 for 3 days, performed in triplicates and mean values ± SD are shown. The results are representative of three independent experiments.

3.3.3 Role of the thymus in the generation of regulatory $CD25^+Id^+CD4^+$ T cells in RT2/TCR1 and RT2/TCR1/LTβR^{KO} mice

The thymus is the organ of central tolerance induction where the majority of autoreactive T cells are eliminated by negative selection. Recently, there has been accumulating evidence that the thymus also plays an important role in the generation of regulatory $CD25^+CD4^+$ T cells. It could be shown that $CD25^+CD4^+$ T cells are continuously produced by the thymus and these thymocytes have similar properties to peripheral $CD25^+CD4^+$ T cells as they are anergic to TCR stimulation and suppress the proliferation of naïve T cells (Itoh et al., 1999; Jordan et al., 2001).

Given the reduced percentages of peripheral $CD25^+CD4^+$ T cells in LTβR^{KO} mice, it cannot be excluded that these animals already have a defect in the generation of $CD25^+CD4^+$ T cells in the thymus. Therefore, the percentages of $CD25^+CD4^+CD8^-$ thymocytes within the single positive $CD4^+CD8^-$ thymocyte population of LTβR^{KO} mice were compared to those of wt mice. In both groups the percentages of $CD25^+CD4^+CD8^-$ thymocytes were nearly identical (wt: 6.9%±1.4% (n=18); LTβR^{KO}: 6.6%±1.1% (n=20)). However, thymi of LTβR^{KO} mice differed in their cellular composition from wt controls since they had increased numbers of single positive (SP) thymocytes, indicating that thymic selection processes are dysregulated (Fig. 18A). The total thymocyte number did not differ significantly in wt versus LTβR^{KO} mice (wt $0.9x10^8$±$0.3x10^8$ (n=26); LTβR^{KO} $1.3x10^8$±$0.5x10^8$ (n=26)). Therefore, in total numbers, more $CD25^+CD4^+CD8^-$ thymocytes are present in LTβR^{KO} in comparison to wt mice.

To check whether tolerant RT2/TCR1 animals also possess $CD25^+$ transgenic T cells in the thymus, and whether there were differences in the absence of LTβR, the expression levels of CD25 by $Id^+CD4^+CD8^-$ thymocytes were assessed (Fig. 18B). Remarkably, 13.8%±4.0% (n=12) of the $Id^+CD4^+CD8^-$ thymocytes expressed CD25 in RT2/TCR1 mice. Also in RT2/TCR1/LTβR^{KO} mice 8.7%±4.2% (n=13) of the transgenic thymocytes were CD25 positive. In contrast, only 4.6%±2.3% (n=8) and 3.3%±2.0% (n=8) of $Id^+CD4^+CD8^-$ thymocytes from TCR1 and TCR1/LTβR^{KO} animals expressed CD25. Since the standard errors in these experiments were quite high, a cell sort was carried out to selectively enrich for $CD4^+$ single positive thymocytes and reduce background fluorescence. $CD4^+CD8^-$ thymocytes were sorted and subsequently analyzed for CD25 and Id expression on an analytic flow cytometer.

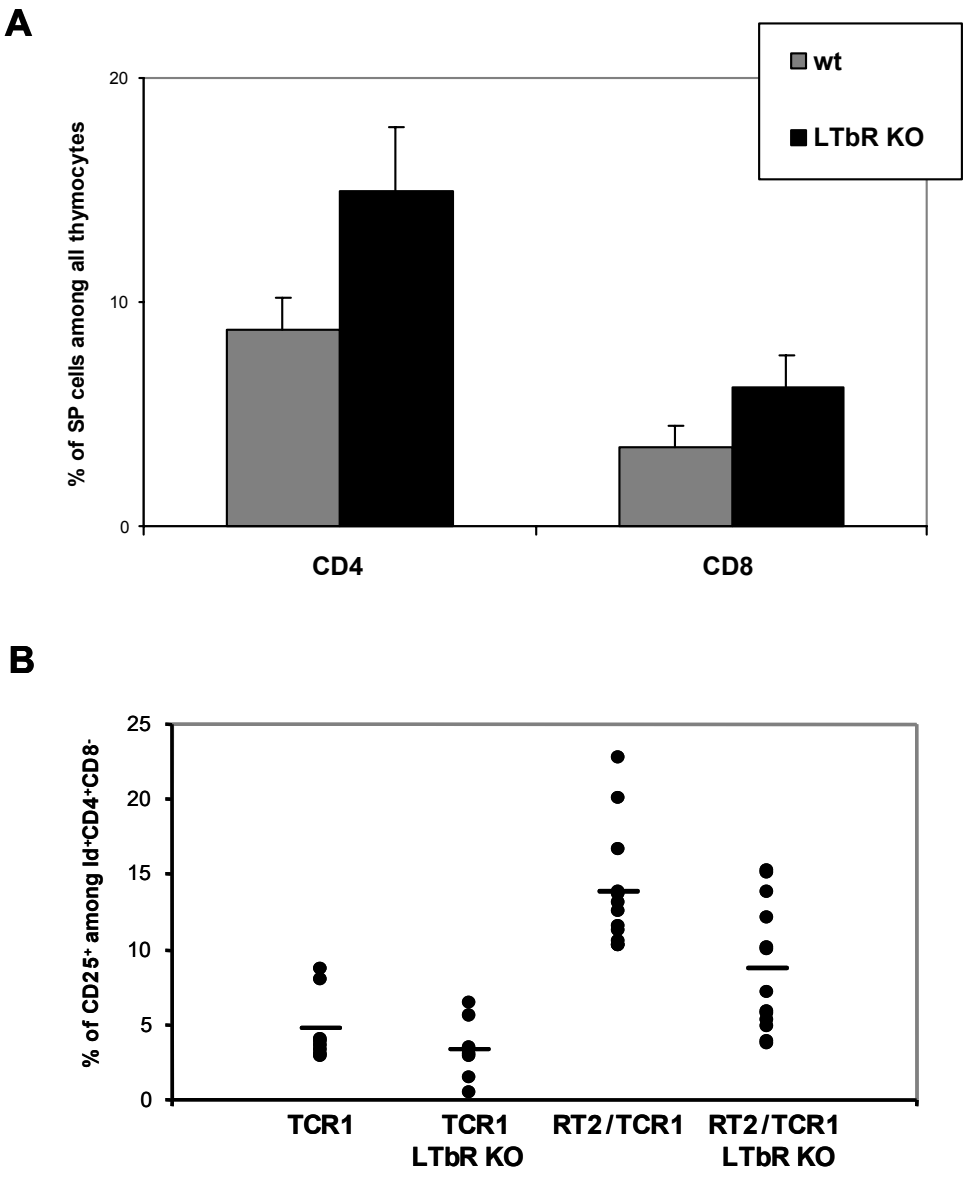

Figure 18: Thymic cell distribution in wt and LTβR^{KO} mice.
A) Percentage of single positive CD4^{+}CD8^{-} or CD4^{-}CD8^{+} thymocytes from wt and LTβR^{KO} mice. Data are pooled from three independent experiments (wt n=19; LTβR^{KO} n=20).
B) Frequency of CD25^{+} cells among Id^{+}CD4^{+}CD8^{-} thymocytes. Data are pooled from three independent experiments (TCR1 n=6; TCR1/LTβR^{KO} n=6; RT2/TCR1 n=9; RT2/TCR1/ LTβR^{KO} n=9).

In general, the cell sort experiment confirmed the data obtained previously (TCR1 3.9%±0.02% (n=2); TCR1/LTβR^{KO} 1.7%±0.07% (n=2); RT2/TCR1 13.7%±1.1% (n=3); RT2/TCR1/LTβR^{KO} 8.2%±1.6% (n=3)). Thus, RT2/TCR1 mice showed an increase of CD25 expressing Id^{+}CD4^{+}CD8^{-} thymocytes compared to TCR1 mice, irrespective of LTβR genotype. Nevertheless, RT2/TCR1/LTβR^{KO} mice had slightly lower levels compared to RT2/TCR1 mice. Since it has been described that transgenes

expressed under the rat insulin promoter are also expressed in the thymus (von Herrath et al., 1994; Jolicoeur et al., 1994), it can be assumed that transgenic T cells in RT2/TCR1 mice see their Ag already during thymic development. A certain percentage of transgenic T cells may therefore be induced to become regulatory thymocytes. How this process may be affected by the LTβR remains to be elucidated.

3.3.4 Lack of tolerance in RT2/TCR1/LTβR^{KO} mice does not lead to tumor immunity

Since RT2/TCR1/LTβR^{KO} mice were not tolerant to the SV40 T Ag, the next question addressed was whether these animals would develop autoimmunity and mount an effective anti-tumor response. To investigate this aspect, the pancreata of RT2/TCR1/LTβR^{KO} and RT2/TCR1 mice were examined for autoimmune infiltration. The different stages of pancreatic inflammation in RT2/TCR1 mice are well defined (Förster and Lieberam, 1996). These animals undergo an initial infiltration of the pancreas composed mainly of CD4^{+} and B220^{+} cells, which peaks around 3 weeks of age. At this stage the animals develop destructive insulitis, which is overcome by outgrowth of SV40 T Ag transformed β-cells. At end stages of tumorigenesis de novo formed lymphoid structures can be found in the pancreas of RT2/TCR1 mice. These structures have also been described in different models of diabetes (Ludewig et al., 1998; Wu et al., 2001) and they are a main characteristic of an ongoing immune response in the pancreas. Strikingly, lymphoid structures cannot be found in RT2/TCR1 mice deficient for LTβR (Fig. 19). However, the pancreata of RT2/LTβR^{KO} as well as RT2/TCR1/LTβR^{KO} mice were not devoid of lymphocytes since a general infiltration of CD4^{+} as well as B220^{+} cells could be found. Nevertheless, these infiltrating lymphocytes cannot form organized follicular structures in the absence of the LTβR. This phenomenon has also been described in a diabetes model, where NOD mice were treated with LTβ receptor-immunoglobulin fusion protein (LTβR-Ig) (Wu et al., 2001). Disruption of LTβR signaling by this treatment led to abrogation of autoimmune destruction of the pancreas.

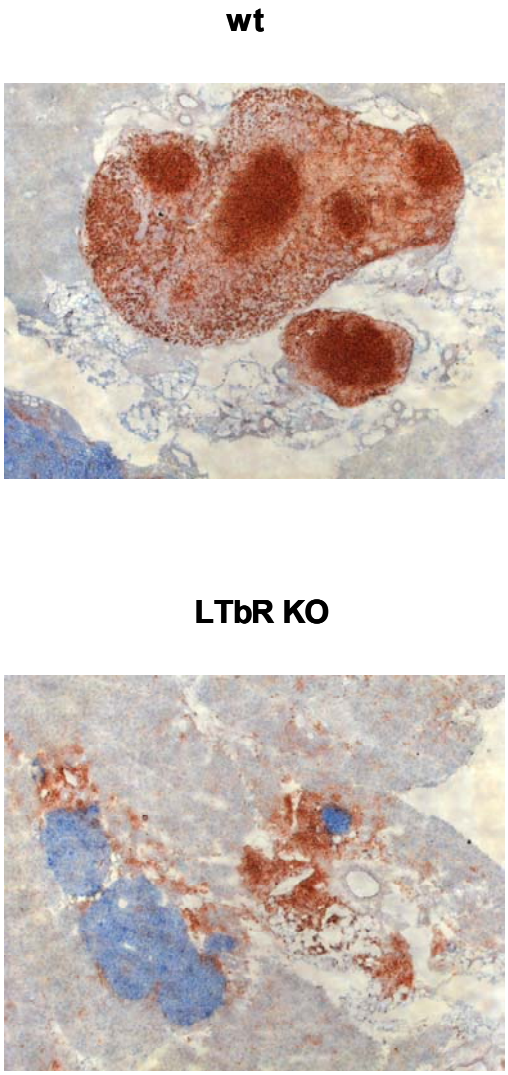

Figure 19: Lack of de novo formed lymphoid structures in pancreata of RT2/TCR1/LTβR^{KO} mice.

Immunohistological analysis was carried out with cryosections of pancreata from the respective genotypes. Staining was carried out for CD4 (red staining) and SV40 T Ag (blue staining).

Taken together, even though RT2/TCR1/LTβR^{KO} mice have reactive transgenic T cells able to mount a tumor response, this does not result in increased infiltration of the pancreas at end stages of tumorigenesis nor to enhanced tumor destruction. One could imagine different limitations associated with the LTβR^{KO} phenotype, involving Ag presentation or interaction of different lymphocyte subsets, which could account for this finding. Also abnormalities in chemokine patterns found in the absence of LTβR signaling (Ngo et al., 1999) may lead to impaired recruitment of lymphocytes as well as disturbed organization of infiltrating lymphocytes in the target organ. Therefore, the RT2/TCR1/LTβR^{KO} model led to further knowledge on the induction of regulatory T cells, but could give only limited information on mechanisms to induce an anti-tumor response.

3.3.5 Additional findings: LN metastasis found in animals bearing the RT2 transgene

Several different lines of transgenic mice expressing the SV40 T Ag have been described (mostly generated in the lab of D. Hanahan). The line used in this work was the RIP1-Tag2 (RT2) line, where the transgene is expressed already early during embryonic development (starting at embryonic d10). RT2 animals establish profound tolerance against SV40 T Ag and develop tumors proceeding through defined stages. Metastases are usually not found in RT2 mice of the C57BL/6 background (Perl et al., 1998). However, during analysis of RT2 mice in the C3HeB/FeJ background crossed to different strains analyzed during this work, it became evident that already at an age of 12 weeks 35% of RT2 animals (n=20) had metastasis in MLNs. The percentages of metastasis bearing RT2 mice increased dramatically at an age of 13-15 weeks, such that more than 60% of 14 weeks old RT2 animals (n=26) showed metastasis. Since these animals develop hypoglycemia with increasing tumor mass, RT2 animals were sacrificed around the age of 14 weeks. Intriguingly, the presence of transgenic T cells in RT2/TCR1 mice strongly reduced the incidence of metastasis (14 week old animals 3.7% (n=27)), although these animals were impaired in their responsiveness to peptide derived from the SV40 T Ag. At this point it is not clear whether the reduced metastasis rate found in RT2/TCR1 animals compared to RT2 mice is just due to a delay in tumor growth, since RT2/TCR1 mice show destructive insulitis at an age of 3 weeks. However, even 15-16 weeks old RT2/TCR1 animals still have reduced numbers of

metastasis bearing mice (1 of 13 animals) compared to RT2 animals at an age of 13 weeks (9 of 26 animals). Since RT2/TCR1 mice also develop hypoglycemia, older animals were not analyzed for ethical reasons.

Intriguingly, RT2/LTβR^{KO} mice showed a higher incidence of MLN metastasis compared to RT2 animals already at an age of 13 weeks (RT2/LTβR^{KO} 12 of 15 mice; RT2 9 of 26 mice). Also in the LTβR deficient background, the presence of the transgenic TCR inhibited MLN metastasis development.

Fig. 20 summarizes the data mentioned above. The percentages of MLN tumor bearing mice at an age of 12-14 weeks are depicted. This figure does not reflect the differences regarding the appearance of tumors in RT2 versus RT2/LTβR^{KO} mice, but it nicely shows the influence of the transgenic TCR on tumor metastasis.

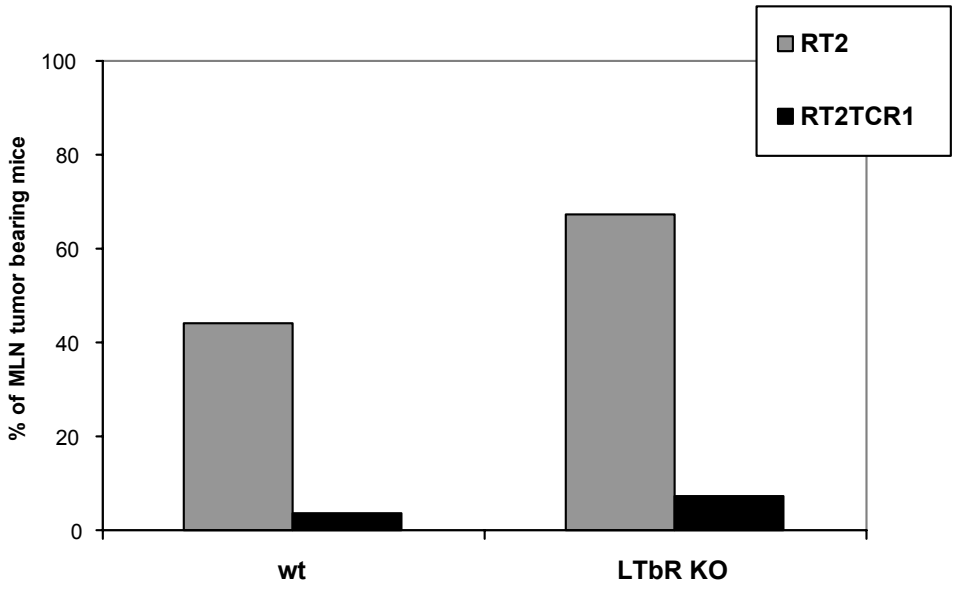

Figure 20: Incidence of MLN metastasis in RT2 transgenic mice.
Mice at an age of 12-14 weeks were analyzed for the presence of MLN tumors (RT2: n=72; RT2/TCR1: n= 54; RT2/LTβR^{KO}: n= 46; RT2/TCR1/LTβR^{KO}: n=27).

4 DISCUSSION

RT2/TCR1 mice represent an animal model to study peripheral tolerance induction during ontogeny in a naturally developing immune system. In these mice peripheral tolerance is established towards an endogenously expressed neo-oncogene (Förster et al., 1994). Three different factors were analyzed during this PhD work for their influence on tolerance induction in the RT2/TCR1 model. In the first part, the role of the immunosuppressive cytokine IL-10 was investigated. The second part was addressing the question whether regulatory T cells were involved in the establishment of SV40 T Ag specific tolerance. Finally, the third part focused on the anatomical environment needed for peripheral tolerance induction.

4.1 Role of IL-10 in peripheral tolerance induction

IL-10 is an anti-inflammatory cytokine involved in down-modulation of immune responses (Moore et al., 2001). The immuno-suppressive effect of IL-10 is probably due to interference with APC activation, since IL-10 directly inhibits APC function by down-regulation of costimulatory molecules and MHC II (de Waal Malefyt et al., 1991). Furthermore, IL-10 mediates direct anti-proliferative effects on T cells (Groux et al., 1997). However, since IL-10 is a pleiotropic cytokine it also has immuno-stimulatory properties and can increase recruitment and cytotoxicity of $CD8^+$ T cells. The latter effect may explain that transgenic expression of IL-10 in the pancreas of NOD mice led to acceleration of disease (Balasa et al., 1996). In contrast, transgenic expression of a viral analogue of IL-10 normally encoded by the Epstein-Barr virus, protected NOD mice from diabetes (Kawamoto et al., 2001). Viral IL-10 has extensive sequence and structural homology to cellular IL-10 (84% identity on the amino acid level) (Liu et al., 1997), but it lacks several of the immuno-stimulatory activities of cellular IL-10. Strikingly, it could be demonstrated recently that a single amino acid determines the immuno-stimulatory activity of IL-10 (Ding et al., 2000).

Despite several reports on the important role of IL-10 in the limitation of inflammatory responses to pathogens as well as during autoimmune responses (Standiford et al., 1995; Katsikis et al., 1994; Mignon-Godfrey et al., 1995) it was not known whether IL-10 had any impact on the development or maintenance of peripheral tolerance towards self-Ag. To determine the role of IL-10 in peripheral tolerance induction, RT2/TCR1 animals deficient for IL-10 were analyzed in comparison to wt RT2/TCR1 mice.

Peripheral tolerance in wt RT2/TCR1 mice is established within the first 6 weeks of life. Like wt RT2/TCR1 mice, RT2/TCR1/IL-10^{KO} mice had reduced percentages of transgenic T cells in the periphery compared to TCR1/IL-10^{KO} mice at an age of 6-8 weeks (Fig. 1). Furthermore, these cells were anergic to TCR stimulation *in vitro* (Fig. 2). Therefore, peripheral tolerance in RT2/TCR1 mice as characterized by deletion and induction of anergy can also be established in the absence of IL-10. However, a single intra-peritoneal injection of P2-peptide without adjuvant was able to break tolerance in RT2/TCR1/IL-10^{KO} mice. *In vivo* peptide treatment induced vigorous expansion of transgenic T cells in RT2/TCR1/IL-10^{KO} mice and these cells were fully responsive to peptide restimulation *in vitro* (Fig. 5). In contrast, *in vivo* peptide treatment of wt RT2/TCR1 mice had no effect on the tolerance status.

The increased responsiveness to *in vivo* peptide stimulation seen in RT2/TCR1/IL-10^{KO} mice was also found in single transgenic TCR1/IL-10^{KO} mice. MLN cells from naïve TCR1/IL-10^{KO} mice exhibited similar responses to peptide stimulation *in vitro* compared to wt TCR1 mice, however, *in vivo* peptide treatment led to a stronger expansion of transgenic T cells in the absence of IL-10 (Fig. 5). How can this discrepancy between *in vitro* and *in vivo* peptide stimulation found in IL-10 deficient animals be explained? Differences in the presentation of the peptide *in vitro* versus *in vivo* could account for this finding. Thus, different types of APCs could be involved and/ or the activation status of APCs could be altered.

Regarding the type of APCs, *in vitro* systems indeed differ from the *in vivo* situation (reviewed in Jenkins et al., 2001). The initial antigen presentation to naïve $CD4^+$ T cells *in vivo* is carried out by DCs residing in the T cell areas of secondary lymphoid organs. In contrast, during *in vitro* cultures the most abundant APCs are B cells, which in lymphoid organs are anatomically separated from naïve T cells. Thus, *in vitro* cultures differ from the *in vivo* environment since the distinct spatial relationship between T cells and APCs found in lymphoid organs is destroyed. Furthermore, also chemokine gradients maintained in lymphoid structures or other factors secreted by lymphoid stroma would be missing during *in vitro* cultures. For example, IL-10^{KO} mice have chronically increased systemic levels of the proinflammatory cytokine TNF-α (Rennick et al., 1997), which could affect the initial antigen presentation *in vivo*. Increased TNF-α would not be present during the initiation of the *in vitro* response since soluble factors are washed away during preparation of single cell suspensions needed for *in vitro* cultures.

The second aspect is the activation status of APCs. Several *in vitro* studies have illustrated the inhibitory function of IL-10 on the expression of MHC II and costimulatory molecules of APCs (de Waal Malefyt et al., 1991; Ding et al., 1993). In contrast, *ex vivo* analysis of the expression levels of CD80, CD86 and MHC II from different subsets of APCs of wt and IL-10^{KO} mice indicated no general differences, with the exception of peritoneal macrophages (Fig. 6). Hence, APCs of IL-10^{KO} mice have a normal activation status *in vivo*, regarding the costimulatory molecules analyzed, although it cannot be excluded that other molecules are affected. The contradictory *in vivo* versus *in vitro* findings may reflect an adaptation of the homeostatic regulation of APC function *in vivo* in the absence of IL-10. Alternatively, IL-10 may not be required for the steady state regulation of APCs, but it may be essential to control the magnitude of APC activation during an ongoing immune response.

In line of the latter hypothesis, peritoneal macrophages of IL-10^{KO} mice had higher levels of MHC II expression compared to wt mice. Given that IL-10^{KO} mice develop chronic enterocolitis, the increased levels of MHC II on peritoneal macrophages presumably reflect an ongoing immune response in the intestine. The route of peptide delivery for *in vivo* experiments was peritoneal injection of the P2-peptide. Therefore, it is possible that peritoneal macrophages influence the *in vivo* response. Taken together, different subsets of APCs are involved during peptide stimulation *in vivo* versus *in vitro*. B cells serve as APCs during *in vitro* cultures, whereas DCs prime *in vivo*. The different outcome of *in vivo* versus *in vitro* priming found in IL-10 deficient mice could consequently be due to different regulation of T cell responses by B cells versus DCs in the absence of IL-10. Interestingly, the discrepancy between the regulatory role of IL-10 *in vivo* compared to *in vitro* situations was also found in studies on regulatory $CD4^+$ T cells. During *in vitro* cultures, IL-10 had no impact on the suppressive function of regulatory $CD4^+$ T cells (Thornton et al., 1998; Takahashi et al., 1998), whereas it was necessary to promote suppression *in vivo* (Asseman et al., 1999).

The crucial regulatory role of IL-10 during *in vivo* Ag encounter was further underlined by the finding that peptide-induced peripheral tolerance failed to be established in TCR1/IL-10^{KO} mice. Sequential peptide injections did not induce deletion of transgenic T cells of TCR1/IL-10^{KO} mice in contrast to wt TCR1 animals (Fig. 7A). TCR1/IL-10^{KO} mice -whether or not treated with peptide *in vivo*- showed a tendency towards higher proliferation rates (Fig. 8C). Furthermore, increased levels of pro-inflammatory cytokines like TNF-α and IFN-γ could be detected in cultures of MLN cells from TCR1

mice deficient for IL-10 after restimulation with peptide *in vitro* (Fig. 7C). Similar to the absence of peptide-induced T cell tolerance found in TCR1/IL-10^{KO} mice, Greenwald et al. demonstrated that transgenic T cells deficient for CTLA-4 are also resistant to peptide-induced peripheral tolerance (Greenwald et al., 2001). As seen with TCR1/IL-10^{KO} mice also transgenic CTLA-4^{KO} T cells showed increased expansion after peptide stimulation *in vivo* compared to wt transgenic T cells. Additionally, transgenic CTLA-4^{KO} T cells proceeded faster through the cell cycle, which I could also observe for T cells from TCR1/IL-10^{KO} mice (Fig. 8C). One of the mechanisms proposed for the resistance of CTLA-4^{KO} T cells to tolerance induction was the preferential engagement of CD28, thereby inducing sustained activation instead of anergy. Indeed, the similarities between the experiments described with transgenic CTLA-4^{KO} T cells and the TCR1/IL-10^{KO} mice are striking. It is difficult however to postulate that similar mechanisms of T cell activation are acting in these two systems. In the case of CTLA-4 deficient T cells the defect is cell autonomous, whereas this is unlikely for IL-10 deficient T cells. Increased levels of pro-inflammatory cytokines are produced in cultures from TCR1/IL-10^{KO} after peptide stimulation, and this could lead to sustained stimulation of T cells by APCs. Furthermore, IL-10^{KO} mice have chronically increased systemic levels of TNF-α (Rennick et al., 1997). Therefore, it could be suggested that the resistance to tolerance induction seen in TCR1/IL-10^{KO} mice is due to dysregulated pro-inflammatory cytokine production, which leads to sustained T cell activation and thereby prevents tolerance induction.

Aberrant cytokine production of IL-10^{KO} T cells after strong TCR triggering can also explain the increased sensitivity of IL-10^{KO} mice to bacterial superantigens. SEB is a potent mitogen for T cells and can be used to study T cell tolerance as well as acute shock syndromes depending on the dose applied. SEB-induced shock depends on IL-2 and IFN-γ production by T cells (Miethke et al., 1992). A publication by Hasko et al. describes in great detail the changes in the plasma cytokine profile induced by SEB injection in C57BL/6 mice (Hasko et al., 1998). According to these authors, the early consequences of SEB treatment are the release of pro-inflammatory cytokines like TNF-α, IL-2 and IFN-γ. These cytokines can be detected systemically already 2 hours after SEB injection. IL-10 can be detected in the blood with a delayed kinetic compared to the pro-inflammatory cytokines (peak around 4 hours) (Hasko et al., 1998). $CD4^+$ T cells are the major source of IL-10 as well as TNF-α (Florquin et al., 1994). Studies on

IL-10^{KO} mice have demonstrated the essential role of IL-10 in limiting the shock-inducing inflammatory response elicited by SEB. IL-10^{KO} mice have increased levels of TNF-α, IL-2 and IFN-γ after SEB treatment compared to wt mice (Hasko et al., 1998). Thus, lack of IL-10 leads to aberrant cytokine production in this system and in consequence IL-10^{KO} mice are more susceptible to lethal shock induced by SEB.

Strikingly, the genetic background influences the severity of SEB induced shock in IL-10^{KO} mice. Although IL-10^{KO} mice on the C57BL/6 background are more susceptible to SEB induced shock then wt C57BL/6 mice (Hasko et al., 1998), they tolerate a 30 fold higher concentration of SEB compared to IL-10^{KO} mice on the C3HeB/FeJ background. Furthermore, the genetic background has a strong influence on the severity of intestinal pathology found in IL-10^{KO} mice. For example, BALB/c and C3H have been shown to be permissive backgrounds for the development of enterocolitis, whereas C57BL/6 is non-permissive (Bristol et al., 2000; Berg et al., 1996). Since the intestinal inflammation found in IL-10^{KO} mice is generated against commensal bacteria, the different susceptibility to the development of enterocolitis seen in various backgrounds could be due to varying reactivity against bacterial components through the innate immune system.

The important regulatory role of IL-10 in the feedback inhibition of immune responses was also demonstrated by treatment of RT2/TCR1/IL-10^{KO} and TCR1/IL-10^{KO} mice with peptide and CpG-ODN. Un-methylated CpG-ODN is recognized as a danger signal by the immune system through binding to Toll-like receptor 9 (Hemmi et al., 2000), leading to maturation and activation of DCs as well as other cells of the innate immune system. The combination of peptide and CpG-ODN thus facilitates antigenic stimulation of T cells by fully activated DCs. Hence, as in the SEB system, a strong signal through the TCR is given. In the absence of IL-10, RT2/TCR1 as well as TCR1 animals were highly sensitive and frequently developed a lethal shock syndrome to the combined peptide/CpG-ODN treatment. The susceptibility to the development of a shock reaction correlated with the responsiveness and the percentages of the transgenic T cells. TCR1/IL-10^{KO} mice were highly susceptible to this treatment, whereas RT2/TCR1/IL-10^{KO} tolerated higher concentrations of CpG-ODN. These experiments demonstrate that the transgenic T cells are the effector T cells mediating the detrimental response to peptide/CpG-ODN treatment in IL-10^{KO} mice. However, the amplitude of the initiated response is regulated by the activation status of the APC.

Taken together, it could be demonstrated that IL-10 is not required for tolerance induction towards self-Ag during ontogeny. Since many cytokine pathways are redundant, IL-10 may be replaced by another regulatory cytokine under normal homeostatic conditions. Alternatively, IL-10 may simply not be involved in peripheral tolerance induction. In contrast, IL-10 is necessary to maintain immunological tolerance in situations where strong antigenic stimulation is provided. These findings correlate with the immunopathology found in IL-10^{KO} mice. IL-10 deficient mice develop intestinal inflammation associated with uncontrolled cytokine production of macrophages and $CD4^+$ T cells (Berg et al., 1996). The intestinal immune system is continuously confronted with large amounts of environmental Ag including bacterial components. IL-10 is thus needed to dampen unwanted reactivity of the immune system to these Ags.

As outlined above, IL-10 is crucial for controlling immune responses induced by strong antigenic stimulation. Regarding the induction of effective tumor immunity this finding may be translated into a therapeutic application. Thus, unresponsiveness of the immune system to tumor Ags may be overcome by transient blockade of IL-10 during stimulation with tumor Ags.

In RT2/TCR1/IL-10^{KO} mice *in vivo* peptide stimulation efficiently expanded tumor specific transgenic T cells (Fig. 5A), however these cells were not sufficient to control the tumor growth. Thus, to induce tumor immunity, the tumor specific T cells not only have to be expanded, but they also have to acquire effector function. In addition to triggering a $CD4^+$ T cell response, activation of $CD8^+$ T cells may also be necessary to induce tumor immunity in most cases. Several studies have shown that eradication of tumors can be achieved through unspecific stimulation of the immune system. For example, peritumoral injections of CpG-ODN led to eradication of transplanted tumors by NK- and $CD8^+$ T cells (Kawarada et al., 2001). Also combined treatment of CpG-ODN with anti-IL10R led to rejection of transplanted tumors (Vicari et al., 2002). In both studies, the underlying mechanism is the activation of DCs, which then can efficiently present tumor Ag. However, the immunostimulatory CpG-ODN had to be injected directly into the tumor or into the vicinity of the tumor. Systemic application of CpG-ODN had only partial or no effect on the tumor growth. This approach is thus limited since the tumor site is in many situations not directly accessible. Administration of tumor specific Ag combined with immunostimulatory CpG-ODN and blockade of

IL-10 may provide a means to induce systemic tumor immunity even towards endogenously growing tumors. This treatment broke tumor specific tolerance in RT2/TCR1 mice, but also induced expansion of lymphocytes with an unknown specificity (Fig. 11B). Thus, by increasing the activation status and Ag presentation capacity of APCs also unwanted activity is generated. How harmful the generation of nonspecific effector cells is in this context remains to be determined, but clearly demonstrates the small grade between induction of tumor-immunity and auto-immunity.

4.2 Regulatory T cells in the RT2/TCR1 model

The essential role of regulatory T cells for maintenance of peripheral tolerance has been demonstrated in several animal models (reviewed in Lafaille et al., 2002). The concept of regulatory T cells originated from studies carried out in the 1970s. In the early experiments tolerance was induced by treatment of mice with foreign Ag, and T cells from treated animals could transfer Ag-specific unresponsiveness to naïve mice. Thus, it was demonstrated that T cells were able to transfer tolerance and this phenomenon was termed infectious tolerance (reviewed in Cobbold and Waldmann, 1998). During the last decade, a specific T cell population able to confer infectious tolerance was defined as a subpopulation of $CD4^+$ cells constitutively expressing CD25 (Sakaguchi et al., 1995; Asano et al., 1996).

Intriguingly, tolerance induction in the RT2/TCR1 model correlates with the systemic appearance of $CD25^+$ transgenic T cells. More then 50% of the transgenic T cells found in MLN of tolerant RT2/TCR1 mice constitutively express CD25, whereas CD25 is not expressed on transgenic T cells from non-tolerant TCR1 mice (Fig 4). Furthermore, half of the population of transgenic T cells from RT2/TCR1 mice also express CTLA-4 (Fig 4), an additional marker associated with regulatory T cell function. *In vitro* suppression assays revealed that $CD25^+$ transgenic T cells of tolerant RT2/TCR1 mice indeed had regulatory function (Fig 12). In these assays, transgenic T cells of tolerant RT2/TCR1 mice were sorted into $CD25^+$ and $CD25^-$ cell populations and tested whether they could suppress the proliferation of naïve transgenic T cells from TCR1 mice. Only the $CD25^+$ transgenic T cells could suppress proliferation of naïve T cells in cocultures. Interestingly, also purified $CD25^-$ T cells were impaired in their responsiveness to peptide stimulation when cultured alone compared to naïve TCR1 mice. Consequently, elimination of regulatory T cells per se does not lead to restored responsiveness of

$CD25^-$ transgenic T cells from RT2/TCR1 mice to antigenic stimulation in this experimental set-up. This may indicate that suppression mediated by $CD25^+$ transgenic T cells *in vivo* induces a profound state of unresponsiveness in $CD25^-$ transgenic T cells, which is sustained during peptide restimulation *in vitro*. Taken together, during peripheral tolerance induction a population of $CD25^+$ transgenic T cells is generated in RT2/TCR1 mice and these cells can actively maintain tolerance via inhibition of naïve T cells.

The mechanism how regulatory T cells suppress naïve T cell responses still remains to be elucidated, but several lines of evidence stress the important role of IL-2 during this process. In accordance to published data (Thornton et al., 1998; Takahashi et al., 1998), addition of exogenous IL-2 during peptide stimulation could break the anergic state as well as the suppressive function of regulatory $CD25^+$ transgenic T cells from tolerant RT2/TCR1 mice (own observation). Thus, limiting amounts of IL-2 during Ag responses may be one of the essential prerequisites for regulatory T cells to accomplish their function. Indeed, the suppression exerted by regulatory T cells results in inhibition of IL-2 production by effector T cells (Thornton et al., 1998; Takahashi et al., 1998).
The second requirement necessary for suppression is direct cell contact between the regulatory and the naïve T cell (Thornton et al., 1998; Takahashi et al., 1998). However, the test systems used to address this point exclude the role of APCs during this interaction. To test for cell contact dependency, the different T cell populations are separated by means of a membrane (transwell system) during stimulation. This also implicates that suppressor and effector T cells are stimulated by different APCs. It is therefore not possible to determine whether a direct cell contact between regulatory and naïve T cell is needed, or if the $CD25^+CD4^+$ T cell acts through the APC as an intermediate. Regulatory T cells may induce downregulation of costimulatory molecules and MHC II on APCs, thereby limiting the priming capacity of the APCs. Nevertheless, Thornton et al. could demonstrate that despite the presence of regulatory T cells upregulation of co-stimulatory molecules on APCs occurred normally (Thornton et al., 2000). In the same study, the suppression mediated by regulatory T cells could not be overcome by addition of an excess of activated APCs. This finding stands in contrast to observations made in our system. Regulation by transgenic $CD25^+$ T cells from RT2/TCR1 mice could not be maintained in the presence of an excess of APCs. The differences in the two models may be due to different APC cell numbers used.

Thornton et al. cocultured just 2 fold more APCs then effector T cells in the presence of regulatory T cells, and under these conditions, the suppressive effect is maintained. However, by culturing naïve T cells and regulatory T cells in the presence of 20 fold more APCs as done in this PhD work the suppressive activity of regulatory T cells was abrogated. Two different factors could account for this finding. Firstly, if direct cell contact between the regulatory and naïve T cell is needed increasing the numbers of APCs would hinder the interaction between the two types of T cells. Assuming that the mobility of the T cells in this assay is limited, the addition of an excess of APCs would thus abrogate the suppressive effect. Secondly, increasing the numbers of APCs may also lead to an increased cytokine production by APCs after interaction with effector T cells. Since limited amounts of IL-2 are essential to maintain the suppressive function of regulatory T cells (Thornton et al., 1998; Takahashi et al., 1998), increased cytokine levels produced by APCs could abrogate suppression. This may also include IL-6 production by activated APCs, which was recently shown to block regulatory T cell function (Pasare and Medzhitov, 2003).
In conclusion, the regulatory function of $CD25^+$ transgenic T cells from RT2/TCR1 mice can be demonstrated under specific conditions *in vitro*, further strengthening the notion that regulation needs direct interaction between regulatory and naïve T cells.

Having demonstrated that the $CD25^+$ transgenic T cells found in tolerant RT2/TCR1 mice have regulatory function it remained to be addressed how these cells are generated. Since the percentage of $CD25^+$ transgenic T cells increases in the periphery during tolerance induction, interactions in peripheral lymphoid organs presumably promote this effect. On the other hand, several reports documented an important role of the thymus in the generation of regulatory T cells (Papiernik et al., 1998; Itho et al., 1999). Tolerance induction in RT2/TCR1 mice does not imply negative selection as mechanisms of central tolerance, since TCR1 as well as RT2/TCR1 mice have the same percentages of transgenic T cells among single positive $CD4^+CD8^-$ thymocytes (Fig. 1 and Fig. 14). However, a significant increase of $CD25^+$ transgenic $CD4^+CD8^-$ thymocytes could be found in RT2/TCR1 mice compared to TCR1 mice (Fig. 18 B). Several organ-specific Ags have recently been shown to be expressed by thymic medullary epithelial cells (Derbinski et al., 2001). Also the expression of transgenes under the control of the rat insulin promoter does lead to a weak ectopic expression in the thymus (Jolicoeur et al., 1994). In a different model carrying transgenic T cells with

a high affinity for hen egg lysozyme (HEL) expressed under control of the rat insulin promoter this ectopic expression was sufficient to induce deletion of the majority of transgenic T cells through negative selection (Liston et al., 2003). Additionally, 25% of the remaining transgenic $CD4^+CD8^-$ thymocytes expressed CD25. In this model it was concluded that the increase in the percentage of $CD25^+$ transgenic thymocytes is secondary to negative selection in accordance to the assumption that $CD25^+$ T cells are more resistant to clonal deletion (Papiernik et al., 1998). In contrast, in the RT2/TCR1 system, clonal deletion is not achieved in the thymus, but CD25 expression is induced on a small percentage of transgenic $CD4^+CD8^-$ thymocytes, implicating an altered selection of these cells. Jordan and coworkers demonstrated that a TCR with high affinity for self-Ags is required for selection of $CD25^+CD4^+$ thymocytes. In contrast to the findings by Liston (Liston et al., 2003), the selection of $CD25^+CD4^+$ thymocytes in the latter model also did not imply deletional mechanisms (Jordan et al., 2001). In addition, not only the affinity of the TCR is a critical factor but also the expression level of the self-Ag. Thus, a certain threshold of avidity (TCR affinity and TCR numbers per cell together with the Ag density) may be necessary to induce CD25 expression, but this level is not sufficient to induce clonal deletion (reviewed in Coutinho et al., 2001). The avidity model may also explain the different outcomes of thymic selection in various animal models of organ-specific TCR transgenic mice (Lafaille et al., 1994; Akkaraju et al., 1997; Jordan et al., 2001). The impact of thymic $CD25^+$ transgenic T cells during peripheral tolerance induction in RT2/TCR1 mice has not been defined yet. However, thymic generation of $CD25^+$ transgenic T cells may not be sufficient for tolerance induction since RT2/TCR1 mice are not per se tolerant to the SV40 T Ag but peripheral tolerance is induced during ontogeny (Förster and Lieberam, 1996). During this process the percentage of peripheral $CD25^+$ transgenic T cells increases, implicating the requirement of additional interactions in the periphery.

4.3 Dependency of peripheral tolerance induction on LTβR signaling

Peripheral tolerance induction in the RT2/TCR1 model is achieved by three main mechanisms since clonal deletion, anergy induction and active suppression by regulatory T cells can be observed. Tolerance induction via clonal deletion and anergy requires Ag recognition by the autoreactive T cells (Schwartz, 2003). Generally it is assumed that self-Ags are constitutively presented in the lymph nodes draining the side of Ag expression as it could be demonstrated for various pancreatic beta-cell antigens (Kurts et al., 1996; Hugues et al., 2002). This constitutive expression of peripheral self-Ags has been proposed to be required for induction and maintenance of peripheral tolerance (Seddon et al., 1999; Garza et al., 2000; Scheinecker et al., 2002). These findings suggest the following sequence of events: self-reactive T cells see their Ag and get activated in LNs draining the site of Ag expression, but under nonpathological conditions, this Ag encounter leads to deletion and/or anergy induction of the autoreactive T cells.

Tolerance induction in RT2/TCR1 mice is indeed preceded by local activation of transgenic T cells in the LNs draining the pancreas (Förster and Lieberam, 1996). This local activation, indicated by expression of CD69, is sustained only in peritoneal LNs of tolerant RT2/TCR1 mice, whereas $CD25^+$ transgenic T cells can be found systemically in tolerant animals. Furthermore, peripheral transgenic T cells of tolerant RT2/TCR1 mice express cell surface markers typical for an antigen experienced/memory phenotype (Fig. 4) implicating that they have been or are continuously stimulated by antigen. It is therefore suggested that the pancreatic draining LNs are required for systemic tolerance induction in the RT2/TCR1 model (Förster and Lieberam, 1996).

To test this hypothesis, RT2/TCR1 mice were crossed with mice lacking all peripheral LNs due to deficiency of the lymphotoxin beta receptor (LTβR) (Fütterer et al., 1998). Thus, the role of draining LNs during tolerance development could be investigated in the RT2/TCR1 model.

$LT\beta R^{KO}$ mice lacking all peripheral LNs were initially described on the C57BL/6 background. Since the transgenic TCR of TCR1 mice is restricted to $I\text{-}A^k$, the $LT\beta R^{KO}$ mice were backcrossed to the C3HeB/FeJ background and then intercrossed with RT2/TCR1 mice. Intriguingly, $LT\beta R^{KO}$ mice on the C3HeB/FeJ background possessed mesenteric lymph node-like structures (Fig. 13). These structures presumably cannot fulfill the same function as normal LNs, since they have a different morphology and

also differ in their cellular composition. T and B cell rich areas can be defined but the typical segregation of lymphoid follicles found in wt animals is missing. In addition, increased B cell percentages can be detected and nearly 50% of the $CD4^+$ T cells are activated (Fig. 14). Animals deficient for the monomers (LTα and LTβ) of one of the ligands of LTβR ($LT\alpha_1\beta_2$) generally lack peripheral LNs but show divers patterns of mesenteric lymphoid development. 30% of $LT\alpha^{KO}$ mice possess mesenteric LN-like structures (Banks et al., 1995), whereas MLNs can be found in 100% of $LT\beta^{KO}$ mice (Alimzhanov et al., 1997; Koni et al., 1997). The presence of MLNs in $LT\beta^{KO}$ mice compared to the total lack of peripheral LNs in $LT\beta R^{KO}$ mice was attributed to a role of LIGHT -the second ligand for LTβR- during MLN genesis. It could recently be demonstrated that LIGHT deficient animals have normal peripheral LNs (Scheu et al., 2002). However, LIGHT was shown to cooperate with LTβ in the organogenesis of MLNs, since only 25% of the double KO mice had MLNs. In addition to the divers findings regarding MLN organogenesis in LTα, LTβ, LIGHT and $LT\beta R^{KO}$ deficient mice it could now be demonstrated that also the genetic background has an influence in the generation of rudimentary MLN like structures.

RT2/TCR1 mice deficient for LTβR were subjected to comparative analysis with wt RT2/TCR1 mice. Strikingly, RT2/TCR1/$LT\beta R^{KO}$ mice did not develop SV40 T Ag-specific peripheral tolerance. RT2/TCR1/$LT\beta R^{KO}$ mice showed only partial deletion of transgenic T cells in the spleen compared to wt RT2/TCR1 mice (Fig. 15) and splenic T cells of RT2/TCR1/$LT\beta R^{KO}$ mice were fully responsive to peptide stimulation *in vitro* (Fig. 16). Furthermore, only 4 % of transgenic T cells expressed CD25 in RT2/TCR1/$LT\beta R^{KO}$ mice compared to over 30 % in RT2/TCR1 mice (Fig. 17). Taken together, all three mechanisms of peripheral tolerance induction- namely clonal deletion, clonal anergy and induction of regulatory T cells – established in RT2/TCR1 mice were impaired in RT2/TCR1/$LT\beta R^{KO}$ mice.

From a reductionist view it can be concluded from these findings that peripheral LNs are essential for peripheral tolerance induction. Tolerance induction in the RT2/TCR1 model is preceded by a phase of local activation of transgenic T cells in the draining LNs. This initial activation of antigen-specific T cells is commonly observed in various tumor models and is driven mainly via cross-presentation of the Ag in draining LNs (Nguyen et al., 2002). Thus, in the absence of peripheral LNs the initial encounter of naïve autoreactive T cells with peripheral Ag does not take place. Therefore, peripheral

tolerance mechanisms like clonal deletion and clonal anergy, which depend on Ag recognition by T cells, are not induced. However, LTβR^{KO} mice possess rudimentary MLN like structures and it cannot be excluded that the partial deletion of transgenic splenic T cells in RT2/TCR1/LTβR^{KO} mice may be initiated in these structures. Nevertheless, the presence of MLN-like structures in RT2/TCR1/LTβR^{KO} mice is not sufficient to induce peripheral tolerance to the SV40 T Ag.

Additionally, also the generation of regulatory T cells appears to depend on the presence of peripheral LNs since only a small percentage of transgenic T cells in RT2/TCR1/LTβR^{KO} mice express CD25 (Fig. 17). Studies carried out by Papiernik et al. suggest that during clonal deletion preferentially CD25^{-} T cells are eliminated. Consequently, CD25^{+} T cells are enriched in the remaining population (Papiernik et al., 1998). According to this theory, the reduced frequency of CD25^{+} transgenic T cells found in RT2/TCR1/LTβR^{KO} mice may be explained by impaired deletion of transgenic CD25^{-} T cells. But despite reduced frequencies of CD25^{+} transgenic T cells found in RT2/TCR1/LTβR^{KO} mice also a general reduction in the regulatory CD25^{+}CD4^{+} T cell pool could be detected in LTβR deficient animals (Fig. 17B). Seddon and Mason demonstrated that the generation of regulatory T cells depends on peripheral recognition of self-Ag (Seddon and Mason, 1999). Self-Ags are constitutively presented in the lymph nodes draining the side of Ag expression (Scheinecker et al., 2002). Thus, in the absence of peripheral LNs, the normal regulatory T cell pool cannot be maintained since the mandatory recognition of self-Ag is missing. Therefore, interactions in the peripheral LN are essential for the maintenance of a normal peripheral regulatory T cell pool. I also determined whether the small population of CD25^{+}CD4^{+} T cells found in LTβR^{KO} mice were indeed regulatory T cells or represented a population of recently activated T cells, since CD25 is also transiently induced shortly after T cell activation. To address this question, T cell suppression assays were carried out. Splenocytes of LTβR^{KO} and wt mice were sorted into CD25^{+} and CD25^{-} T cell pools and CD25^{+}, CD25^{-} or CD25^{+} and CD25^{-} T cells were stimulated in the presence of anti-CD3. CD25^{+}CD4^{+} T cells of LTβR^{KO} mice were not impaired in their regulatory function since they could suppress the proliferation of naïve CD25^{-} T cells (Fig. 17C). Thus, a general reduction of CD25^{+}CD4^{+} T cells can be found in LTβR^{KO} mice, but the remaining CD25^{+}CD4^{+} T cells have full regulatory function.

Since tolerance is not established against the SV40 T Ag in RT2/TCR1/LTβR^{KO} mice an interesting question was whether these animals were able to mount a tumor response. The formation of lymphoid follicular structures is a characteristic feature of an ongoing inflammation in the pancreas. These structures can be found in different models of diabetes (Ludewig et al., 1998; Wu et al., 2001) and are also seen in RT2/TCR1 mice. In RT2/TCR1/LTβR^{KO} mice lymphoid follicular structures could not be detected in the pancreas. However, a general infiltration of the pancreas containing CD4^{+} T cells and B cells could be found as it has been described for various organs in LTβR^{KO} mice (Fütterer et al., 1998). Thus, although RT2/TCR1/LTβR^{KO} mice possess transgenic T cells fully reactive to the tumor Ag, this does not lead to significant tumor immunity. Naïve T cells are restricted to secondary lymphoid organs and can only be activated in these sites (Jenkins et al., 2001). The lack of peripheral LNs could consequently induce a state of T cell ignorance towards antigens normally presented in peripheral LN. The essential role of draining LNs in the generation of effector T cells could be demonstrated in the NOD model (Gagnerault et al., 2002) as well as in a model of T cell-dependent contact hypersensitivity (Rennert et al., 2001). Therefore the absence of LNs in RT2/TCR1/LTβR^{KO} mice not only inhibits the development of peripheral tolerance towards the SV40 T Ag, but it also prevents the activation of tumor specific T cells in these organs.

This hypothesis can also be applied for the maintenance of peripheral tolerance towards self-Ags in LTβR^{KO} mice. Since reduced numbers of regulatory T cells are found in the absence of LTβR one could speculate that this may lead to increased susceptibility to develop autoimmune diseases. However, this is not the case. Infiltrations of lymphocytes around perivascular regions can be found in various organs of LTβR^{KO} mice (Fütterer et al., 1998), but this does not lead to autoimmunity. Thus, despite reduced levels of regulatory T cells, LTβR^{KO} mice do not develop autoimmunity, since presumably peripheral priming of effector T cells is also impaired.

LTβR^{KO} mice provide an attractive model to investigate the role of peripheral LNs during immune responses. However, LTβR deficiency also leads to additional alterations of the immune system. Therefore, the data obtained with LTβR^{KO} mice can be explained by the lack of peripheral LN, but it could also be due to the lack of direct signals provided by the LTβR.

Despite the lack of peripheral LN, LTβR^{KO} mice have a disturbed splenic microarchitecture and aberrant formation of splenic germinal centers (Fütterer et al., 1998). Blocking of LTβR signaling in wt mice by an LTβR-Fc fusion protein leads to the same phenotypic splenic alterations as found in LTβR^{KO} mice (Mackay et al., 1997). Continuous signaling through the LTβR is thus essential for the maintenance of splenic organization. Stromal cells in the spleen express LTβR and have been shown to secrete distinct types of chemokines (Cyster et al., 1999), which are essential for recruitment and retention of lymphocytes. Stromal cells found in T cell areas mainly produce secondary lymphoid tissue chemokine (SLC, CCL21) and EBV-induced molecule 1 ligand chemokine (ELC, CCL19). Naïve T cells express the specific receptor for SLC and ELC, which is CCR7. Thus, ELC and SLC expression guides naïve T cells to the T cell zones. Follicular stromal cells produce B lymphocytes chemoattractant (BLC) and the receptor for BLC, CXCR5 is expressed by recirculating B cells and induced on T cells after activation. Taken together, chemokine gradients are essential to maintain the distinct splenic architecture. It could be demonstrated that LT$\alpha_1\beta_2$ signaling is required for stromal cell expression of homing chemokines in B and T cell areas of the spleen (Ngo et al., 1999).

Thus, the finding that RT2/TCR1/LTβR^{KO} mice did not develop de novo formed lymphoid structures, may not only be due to absence of activation of tumor-specific T cells, but also due to inefficient recruitment and retention of these cells in the pancreas. De novo formed lymphoid structures can also be induced in the pancreas by transgenic expression of ELC in pancreatic beta cells (Luther et al., 2000). The development of these structures was dependent on B cells and lymphotoxin $\alpha_1\beta_2$. The essential role of LTβR signaling in lymphoid neogenesis was also demonstrated in a model of diabetes (Wu et al., 2001). In this study, the formation of lymphoid follicular structures in the pancreas normally associated with diabetes progression could be completely blocked by soluble LTβR. Thus, by affecting chemokine expression as well as expression of adhesion molecules (Mackay et al., 1997; Mackay et al., 1998) lymphotoxin mediated signals can inhibit the induction of ectopic lymphoid tissues.

Additional to the essential role of chemokines released by stromal cells in the organized interaction of peripheral T and B cells, also regulation of thymic selection processes occurs through interaction of thymocytes with thymic stromal cells. Generally, LTβR^{KO} mice had increased percentages of single positive cells in the thymus (Fig. 18A),

pointing towards altered selection processes. Regarding the transgenic T cells in the thymus of RT2/TCR1/LTβR^{KO} mice a slight reduction of CD25 expression in single positive transgenic thymocytes could be detected compared to wt RT2/TCR1 mice. At this point it is unclear whether this difference has any impact on the peripheral transgenic T cell pool. The percentages of CD25^{+}CD4^{+}CD8^{-} thymocytes of LTβR^{KO} mice were similar to wt controls. Taken together, LTβR^{KO} mice not only lack peripheral LNs but also display several defects in thymic as well as splenic organization. To which extend these alterations are important in regulating T cell responses or affecting tolerance mechanisms remains to be determined.

4.4 Outlook / Future perspective

In this PhD work I could demonstrate that IL-10 is essential to maintain tolerance in situations of strong antigenic stimulation. Future work will focus on the translation of this finding into a therapeutical set-up for the induction of specific tumor immunity. Thus, it will be analyzed if effective tumor immunity can be achieved by transient blockade of IL-10 during stimulation with tumor Ag. To achieve a strong antigenic stimulation, two different approaches will be used. In a first approach RT2/TCR1 mice will be treated with anti-IL10R, tumor peptide and CpG-ODN in combined therapy. I already could demonstrate that this treatment was effective in breaking tolerance towards the SV40 T Ag and it will be determined if tumor immunity can be induced. In a different approach, strong antigenic stimulation will be achieved by treatment of RT2/TCR1 mice with a recombinant modified vaccinia virus Ankara (MVA) expressing transformation deficient SV40 T Ag. Recombinant MVA induces potent cell-mediated immunity and MVA-based constructs are developed as vaccines for a variety of diseases (Schneider et al., 1998; Drexler et al., 1999; Allen et al., 2000). By boosting the immune system with recombinant SV40 T Ag expressing MVA epitopes recognized by CD8 cells will be presented, so that direct cytotoxicity by CTLs may be induced. Also in this experimental set-up the effect of transient blockade of IL-10 will be analyzed.

Furthermore, the requirements needed for generation of regulatory T cells will be addressed. In order to dissect the role of the thymus versus periphery in the generation of regulatory T cells thymus transplantations will be performed. Thereby, it will be possible to determine whether ectopic expression of the SV40 T Ag in the thymus is

required for induction of peripheral regulatory T cells. Thymus transplantations will be also performed in the LTβR^{KO} model, in order to analyze whether LTβR deficiency leads to altered selection of T cells in the thymus. In addition, the role of LTβR signaling during peripheral tolerance induction in wt RT2/TCR1 mice will be investigated by transient blockade of LTβR during ontogeny.

5 SUMMARY

During this PhD work the immunological recognition of a specific tumor Ag was analyzed in a transgenic mouse model. In RT2/TCR1 mice peripheral tolerance is established towards an endogenously growing neo-oncogene (SV40 T Ag). By tracking the fate of tumor specific $CD4^+$ transgenic T cells the induction of peripheral tolerance can be followed during ontogeny.

To investigate the role of the immunosuppressive cytokine IL-10 in peripheral tolerance induction, RT2/TCR1 mice were crossed into an IL-10 deficient background. Developmental induction of self-tolerance to the SV40 T Ag occurred independently of IL-10 and was characterized by deletion of the majority of autoreactive T cells and functional impairment of the remaining ones. Analogous to wt RT2/TCR1 animals, tolerance induction correlated with the appearance of $CD25^+$ transgenic T cells. However, in contrast to stable tolerance in wt mice, tolerance could be broken in transgenic T cells from IL-10 deficient mice by a single exogenous antigenic stimulation *in vivo*. In addition, also peptide-induced peripheral tolerance could not be established in the absence of IL-10 in single transgenic TCR1 mice. It can be concluded that IL-10 is crucial for maintenance but not for developmental induction of peripheral T cell tolerance.

Furthermore, it could be demonstrated that $CD25^+$ transgenic T cells found in tolerant RT2/TCR1 mice represent regulatory T cells able to suppress proliferation of naïve T cells. Thus, in addition to clonal deletion and anergy induction, also active suppression by regulatory T cells is induced during peripheral tolerance induction in the RT2/TCR1 model.

Since tolerance induction in the RT2/TCR1 model is preceded by local activation of transgenic T cells in LNs draining the site of SV40 T Ag expression it was investigated whether peripheral tolerance could also be induced in the absence of peripheral lymph nodes. To address this question, RT2/TCR1 mice were crossed into a LTβR deficient background, in which peripheral LN development is blocked. Strikingly, peripheral tolerance could not be established in RT2/TCR1/$LT\beta R^{KO}$ mice as only partial deletion of transgenic T cells occurred and the remaining transgenic T cells were fully responsive to Ag-specific T cell stimulation *in vitro*. In addition, in the absence of LTβR only a low percentage of transgenic T cells expressed CD25 in

RT2/TCR1/LTβR^{KO} mice and the peripheral pool of regulatory CD25^{+}CD4^{+} T cells was reduced compared to wt animals. Thus, it can be concluded that LTβR expression is required for peripheral tolerance induction in the RT2/TCR1 model. Most likely, the failure of peripheral tolerance induction in LTβR-deficient RT2/TCR1 mice is due to the absence of peripheral LN. However, it cannot be excluded at present that other signals transmitted through the LTβR during adult life also influence tolerance induction.

6 REFERENCES

Akkaraju, S., Ho, W. Y., Leong, D., Canaan, K., Davis, M. M., and Goodnow, C. C. (1997). A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. Immunity *7*, 255-271.

Alimzhanov, M. B., Kuprash, D. V., Kosco-Vilbois, M. H., Luz, A., Turetskaya, R. L., Tarakhovsky, A., Rajewsky, K., Nedospasov, S. A., and Pfeffer, K. (1997). Abnormal development of secondary lymphoid tissues in lymphotoxin beta-deficient mice. Proc Natl Acad Sci U S A *94*, 9302-9307.

Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., and Mathis, D. (2002). Projection of an immunological self shadow within the thymus by the aire protein. Science *298*, 1395-1401.

Asano, M., Toda, M., Sakaguchi, N., and Sakaguchi, S. (1996). Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. J Exp Med *184*, 387-396.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med *190*, 995-1004.

Balasa, B., and Sarvetnick, N. (1996). The paradoxical effects of interleukin 10 in the immunoregulation of autoimmune diabetes. J Autoimmun *9*, 283-286.

Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., Bouley, D. M., Thomas, J., Kanangat, S., and Mucenski, M. L. (1995). Lymphotoxin-alpha-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. J Immunol *155*, 1685-1693.

Berg, D. J., Davidson, N., Kuhn, R., Muller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M. W., and Rennick, D. (1996). Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest *98*, 1010-1020.

Bogen, B. (1996). Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4+ T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. Eur J Immunol *26*, 2671-2679.

Brabletz, T., Pfeuffer, I., Schorr, E., Siebelt, F., Wirth, T., and Serfling, E. (1993). Transforming growth factor beta and cyclosporin A inhibit the inducible activity of the interleukin-2 gene in T cells through a noncanonical octamer-binding site. Mol Cell Biol *13*, 1155-1162.

Bristol, I. J., Farmer, M. A., Cong, Y., Zheng, X. X., Strom, T. B., Elson, C. O., Sundberg, J. P., and Leiter, E. H. (2000). Heritable susceptibility for colitis in mice induced by IL-10 deficiency. Inflamm Bowel Dis *6*, 290-302.

Christ, M., McCartney-Francis, N. L., Kulkarni, A. B., Ward, J. M., Mizel, D. E., Mackall, C. L., Gress, R. E., Hines, K. L., Tian, H., Karlsson, S., and et al. (1994). Immune dysregulation in TGF-beta 1-deficient mice. J Immunol *153*, 1936-1946.

Cobbold, S., and Waldmann, H. (1998). Infectious tolerance. Curr Opin Immunol *10*, 518-524.

Coutinho, A., Hori, S., Carvalho, T., Caramalho, I., and Demengeot, J. (2001). Regulatory T cells: the physiology of autoreactivity in dominant tolerance and "quality control" of immune responses. Immunol Rev *182*, 89-98.

Curotto de Lafaille, M. A., and Lafaille, J. J. (2002). CD4(+) regulatory T cells in autoimmunity and allergy. Curr Opin Immunol *14*, 771-778.

Cyster, J. G. (1999). Chemokines and cell migration in secondary lymphoid organs. Science *286*, 2098-2102.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M. G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., and de Vries, J. E. (1991). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med *174*, 915-924.

Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. (2001). Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. Nat Immunol *2*, 1032-1039.

Diebold, R. J., Eis, M. J., Yin, M., Ormsby, I., Boivin, G. P., Darrow, B. J., Saffitz, J. E., and Doetschman, T. (1995). Early-onset multifocal inflammation in the transforming growth factor beta 1-null mouse is lymphocyte mediated. Proc Natl Acad Sci U S A *92*, 12215-12219.

Ding, Y., Qin, L., Kotenko, S. V., Pestka, S., and Bromberg, J. S. (2000). A single amino acid determines the immunostimulatory activity of interleukin 10. J Exp Med *191*, 213-224.

Dresser, D. W. (1976). Tolerance induction as a model for cell differentiation. Br Med Bull *32*, 147-151.

Florquin, S., Amraoui, Z., Abramowicz, D., and Goldman, M. (1994). Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. J Immunol *153*, 2618-2623.

Folkman, J., Watson, K., Ingber, D., and Hanahan, D. (1989). Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature *339*, 58-61.

Forster, I., Hirose, R., Arbeit, J. M., Clausen, B. E., and Hanahan, D. (1995). Limited capacity for tolerization of CD4+ T cells specific for a pancreatic beta cell neo-antigen. Immunity *2*, 573-585.

Forster, I., and Lieberam, I. (1996). Peripheral tolerance of CD4 T cells following local activation in adolescent mice. Eur J Immunol *26*, 3194-3202.

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M. H., and Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity *9*, 59-70.

Gagnerault, M. C., Luan, J. J., Lotton, C., and Lepault, F. (2002). Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. J Exp Med *196*, 369-377.

Garza, K. M., Agersborg, S. S., Baker, E., and Tung, K. S. (2000). Persistence of physiological self antigen is required for the regulation of self tolerance. J Immunol *164*, 3982-3989.

Gershon, R. K., and Kondo, K. (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology *18*, 723-737.

Gershon, R. K., and Kondo, K. (1971). Infectious immunological tolerance. Immunology *21*, 903-914.

Gorelik, L., and Flavell, R. A. (2000). Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity *12*, 171-181.

Greenwald, R. J., Boussiotis, V. A., Lorsbach, R. B., Abbas, A. K., and Sharpe, A. H. (2001). CTLA-4 regulates induction of anergy in vivo. Immunity *14*, 145-155.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E., and Roncarolo, M. G. (1997). A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature *389*, 737-742.

Hanahan, D. (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature *315*, 115-122.

Hanahan, D. (1998). Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. Curr Opin Immunol *10*, 656-662.

Hasko, G., Virag, L., Egnaczyk, G., Salzman, A. L., and Szabo, C. (1998). The crucial role of IL-10 in the suppression of the immunological response in mice exposed to staphylococcal enterotoxin B. Eur J Immunol *28*, 1417-1425.

Heath, W. R., and Carbone, F. R. (2001). Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol *19*, 47-64.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740-745.

Hoglund, P., Mintern, J., Waltzinger, C., Heath, W., Benoist, C., and Mathis, D. (1999). Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med *189*, 331-339.

Homann, D., Jahreis, A., Wolfe, T., Hughes, A., Coon, B., van Stipdonk, M. J., Prilliman, K. R., Schoenberger, S. P., and von Herrath, M. G. (2002). CD40L blockade

prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. Immunity *16*, 403-415.

Huang, A. Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., and Levitsky, H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science *264*, 961-965.

Hugues, S., Mougneau, E., Ferlin, W., Jeske, D., Hofman, P., Homann, D., Beaudoin, L., Schrike, C., Von Herrath, M., Lehuen, A., and Glaichenhaus, N. (2002). Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta cells. Immunity *16*, 169-181.

Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F., and Sakaguchi, S. (1999). Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J Immunol *162*, 5317-5326.

Janeway, C. A., Jr. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today *13*, 11-16.

Jenkins, M. K., Khoruts, A., Ingulli, E., Mueller, D. L., McSorley, S. J., Reinhardt, R. L., Itano, A., and Pape, K. A. (2001). In vivo activation of antigen-specific CD4 T cells. Annu Rev Immunol *19*, 23-45.

Jolicoeur, C., Hanahan, D., and Smith, K. M. (1994). T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. Proc Natl Acad Sci U S A *91*, 6707-6711.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., and Enk, A. H. (2000). Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. J Exp Med *192*, 1213-1222.

Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., and Enk, A. H. (2001). Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med *193*, 1285-1294.

Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A., and Caton, A. J. (2001). Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol *2*, 301-306.

Katsikis, P. D., Chu, C. Q., Brennan, F. M., Maini, R. N., and Feldmann, M. (1994). Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J Exp Med *179*, 1517-1527.

Kawamoto, S., Nitta, Y., Tashiro, F., Nakano, A., Yamato, E., Tahara, H., Tabayashi, K., and Miyazaki, J. (2001). Suppression of T(h)1 cell activation and prevention of autoimmune diabetes in NOD mice by local expression of viral IL-10. Int Immunol *13*, 685-694.

Kawarada, Y., Ganss, R., Garbi, N., Sacher, T., Arnold, B., and Hammerling, G. J. (2001). NK- and CD8(+) T cell-mediated eradication of established tumors by

peritumoral injection of CpG-containing oligodeoxynucleotides. J Immunol *167*, 5247-5253.

Kearney, E. R., Pape, K. A., Loh, D. Y., and Jenkins, M. K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity *1*, 327-339.

Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., and Flavell, R. A. (1997). Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. Immunity *6*, 491-500.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-274.

Kulkarni, A. B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M., and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A *90*, 770-774.

Kurts, C., Carbone, F. R., Barnden, M., Blanas, E., Allison, J., Heath, W. R., and Miller, J. F. (1997). CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. J Exp Med *186*, 2057-2062.

Kurts, C., Heath, W. R., Carbone, F. R., Allison, J., Miller, J. F., and Kosaka, H. (1996). Constitutive class I-restricted exogenous presentation of self antigens in vivo. J Exp Med *184*, 923-930.

Lafaille, J. J., Nagashima, K., Katsuki, M., and Tonegawa, S. (1994). High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. Cell *78*, 399-408.

Lee, W. T., and Pelletier, W. J. (1998). Visualizing memory phenotype development after in vitro stimulation of CD4(+) T cells. Cell Immunol *188*, 1-11.

Letterio, J. J., Geiser, A. G., Kulkarni, A. B., Dang, H., Kong, L., Nakabayashi, T., Mackall, C. L., Gress, R. E., and Roberts, A. B. (1996). Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. J Clin Invest *98*, 2109-2119.

Letterio, J. J., and Roberts, A. B. (1998). Regulation of immune responses by TGF-beta. Annu Rev Immunol *16*, 137-161.

Levings, M. K., Sangregorio, R., and Roncarolo, M. G. (2001). Human CD25+CD4+ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med *193*, 1295-1302.

Liblau, R. S., Singer, S. M., and McDevitt, H. O. (1995). Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today *16*, 34-38.

Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C. C. (2003). Aire regulates negative selection of organ-specific T cells. Nat Immunol *4*, 350-354.

Liu, Y., de Waal Malefyt, R., Briere, F., Parham, C., Bridon, J. M., Banchereau, J., Moore, K. W., and Xu, J. (1997). The EBV IL-10 homologue is a selective agonist with impaired binding to the IL-10 receptor. J Immunol *158*, 604-613.

Ludewig, B., Odermatt, B., Landmann, S., Hengartner, H., and Zinkernagel, R. M. (1998). Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue. J Exp Med *188*, 1493-1501.

Ludlow, J. W. (1993). Interactions between SV40 large-tumor antigen and the growth suppressor proteins pRB and p53. Faseb J *7*, 866-871.

Luther, S. A., Lopez, T., Bai, W., Hanahan, D., and Cyster, J. G. (2000). BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. Immunity *12*, 471-481.

Mackay, F., and Browning, J. L. (1998). Turning off follicular dendritic cells. Nature *395*, 26-27.

Mackay, F., Majeau, G. R., Lawton, P., Hochman, P. S., and Browning, J. L. (1997). Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. Eur J Immunol *27*, 2033-2042.

Maloy, K. J., and Powrie, F. (2001). Regulatory T cells in the control of immune pathology. Nat Immunol *2*, 816-822.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu Rev Immunol *12*, 991-1045.

Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ildstad, S. T., Kast, W. M., Deleo, A. B., and et al. (1995). Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med *1*, 1297-1302.

Miethke, T., Wahl, C., Heeg, K., Echtenacher, B., Krammer, P. H., and Wagner, H. (1992). T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J Exp Med *175*, 91-98.

Mignon-Godefroy, K., Rott, O., Brazillet, M. P., and Charreire, J. (1995). Curative and protective effects of IL-10 in experimental autoimmune thyroiditis (EAT). Evidence for IL-10-enhanced cell death in EAT. J Immunol *154*, 6634-6643.

Moore, K. W., de Waal Malefyt, R., Coffman, R. L., and O'Garra, A. (2001). Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol *19*, 683-765.

Morgan, D. J., Kurts, C., Kreuwel, H. T., Holst, K. L., Heath, W. R., and Sherman, L. A. (1999). Ontogeny of T cell tolerance to peripherally expressed antigens. Proc Natl Acad Sci U S A *96*, 3854-3858.

Ngo, V. N., Korner, H., Gunn, M. D., Schmidt, K. N., Riminton, D. S., Cooper, M. D., Browning, J. L., Sedgwick, J. D., and Cyster, J. G. (1999). Lymphotoxin alpha/beta and

tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. J Exp Med *189*, 403-412.

Nguyen, L. T., Radhakrishnan, S., Ciric, B., Tamada, K., Shin, T., Pardoll, D. M., Chen, L., Rodriguez, M., and Pease, L. R. (2002). Cross-linking the B7 family molecule B7-DC directly activates immune functions of dendritic cells. J Exp Med *196*, 1393-1398.

Nossal, G. J. (1994). Negative selection of lymphocytes. Cell *76*, 229-239.

Papiernik, M., de Moraes, M. L., Pontoux, C., Vasseur, F., and Penit, C. (1998). Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency. Int Immunol *10*, 371-378.

Pasare, C., and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science *299*, 1033-1036.

Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H., and Christofori, G. (1998). A causal role for E-cadherin in the transition from adenoma to carcinoma. Nature *392*, 190-193.

Pisa, P., Halapi, E., Pisa, E. K., Gerdin, E., Hising, C., Bucht, A., Gerdin, B., and Kiessling, R. (1992). Selective expression of interleukin 10, interferon gamma, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. Proc Natl Acad Sci U S A *89*, 7708-7712.

Powrie, F., Carlino, J., Leach, M. W., Mauze, S., and Coffman, R. L. (1996). A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med *183*, 2669-2674.

Powrie, F., Leach, M. W., Mauze, S., Caddle, L. B., and Coffman, R. L. (1993). Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol *5*, 1461-1471.

Rafii-Tabar, E., and Czitrom, A. A. (1986). Ontogeny of priming of cytotoxic T cells to minor alloantigens: the development of direct priming precedes that of cross-priming. Eur J Immunol *16*, 1025-1027.

Read, S., Malmstrom, V., and Powrie, F. (2000). Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med *192*, 295-302.

Rennert, P. D., Hochman, P. S., Flavell, R. A., Chaplin, D. D., Jayaraman, S., Browning, J. L., and Fu, Y. X. (2001). Essential role of lymph nodes in contact hypersensitivity revealed in lymphotoxin-alpha-deficient mice. J Exp Med *193*, 1227-1238.

Rennick, D. M., Fort, M. M., and Davidson, N. J. (1997). Studies with IL-10-/- mice: an overview. J Leukoc Biol *61*, 389-396.

Sakaguchi, S. (2000). Regulatory T cells: key controllers of immunologic self-tolerance. Cell *101*, 455-458.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

Sakaguchi, S., Takahashi, T., Yamazaki, S., Kuniyasu, Y., Itoh, M., Sakaguchi, N., and Shimizu, J. (2001). Immunologic self tolerance maintained by T-cell-mediated control of self-reactive T cells: implications for autoimmunity and tumor immunity. Microbes Infect *3*, 911-918.

Santos, L. M., al-Sabbagh, A., Londono, A., and Weiner, H. L. (1994). Oral tolerance to myelin basic protein induces regulatory TGF-beta-secreting T cells in Peyer's patches of SJL mice. Cell Immunol *157*, 439-447.

Scaglia, L., Cahill, C. J., Finegood, D. T., and Bonner-Weir, S. (1997). Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. Endocrinology *138*, 1736-1741.

Scheinecker, C., McHugh, R., Shevach, E. M., and Germain, R. N. (2002). Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. J Exp Med *196*, 1079-1090.

Scheu, S., Alferink, J., Potzel, T., Barchet, W., Kalinke, U., and Pfeffer, K. (2002). Targeted disruption of LIGHT causes defects in costimulatory T cell activation and reveals cooperation with lymphotoxin beta in mesenteric lymph node genesis. J Exp Med *195*, 1613-1624.

Schwartz, R. H. (2003). T cell anergy. Annu Rev Immunol *21*, 305-334.

Seddon, B., and Mason, D. (1999). Peripheral autoantigen induces regulatory T cells that prevent autoimmunity. J Exp Med *189*, 877-882.

Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J Immunol *163*, 5211-5218.

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., and et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature *359*, 693-699.

Smith, D. R., Kunkel, S. L., Burdick, M. D., Wilke, C. A., Orringer, M. B., Whyte, R. I., and Strieter, R. M. (1994). Production of interleukin-10 by human bronchogenic carcinoma. Am J Pathol *145*, 18-25.

Smith, K. M., Olson, D. C., Hirose, R., and Hanahan, D. (1997). Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. Int Immunol *9*, 1355-1365.

Speiser, D. E., Miranda, R., Zakarian, A., Bachmann, M. F., McKall-Faienza, K., Odermatt, B., Hanahan, D., Zinkernagel, R. M., and Ohashi, P. S. (1997). Self antigens

expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. J Exp Med *186*, 645-653.

Standiford, T. J., Strieter, R. M., Lukacs, N. W., and Kunkel, S. L. (1995). Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. J Immunol *155*, 2222-2229.

Stephens, L. A., Mottet, C., Mason, D., and Powrie, F. (2001). Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. Eur J Immunol *31*, 1247-1254.

Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. (1998). Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol *10*, 1969-1980.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W., and Sakaguchi, S. (2000). Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med *192*, 303-310.

Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Forster, I., and Akira, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity *10*, 39-49.

Teitelman, G., Alpert, S., and Hanahan, D. (1988). Proliferation, senescence, and neoplastic progression of beta cells in hyperplasic pancreatic islets. Cell *52*, 97-105.

Thornton, A. M., and Shevach, E. M. (1998). CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med *188*, 287-296.

Thornton, A. M., and Shevach, E. M. (2000). Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol *164*, 183-190.

Trudeau, J. D., Dutz, J. P., Arany, E., Hill, D. J., Fieldus, W. E., and Finegood, D. T. (2000). Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes *49*, 1-7.

Vicari, A. P., Chiodoni, C., Vaure, C., Ait-Yahia, S., Dercamp, C., Matsos, F., Reynard, O., Taverne, C., Merle, P., Colombo, M. P.*, et al.* (2002). Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody. J Exp Med *196*, 541-549.

von Boehmer, H. (1996). CD4/CD8 lineage commitment: back to instruction? J Exp Med *183*, 713-715.

von Boehmer, H. (1997). Lymphotoxins: from cytotoxicity to lymphoid organogenesis. Proc Natl Acad Sci U S A *94*, 8926-8927.

von Herrath, M. G., Dockter, J., and Oldstone, M. B. (1994). How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model. Immunity *1*, 231-242.

Wu, Q., Salomon, B., Chen, M., Wang, Y., Hoffman, L. M., Bluestone, J. A., and Fu, Y. X. (2001). Reversal of spontaneous autoimmune insulitis in nonobese diabetic mice by soluble lymphotoxin receptor. J Exp Med *193*, 1327-1332.

7 Acknowledgements

I am greatful to Prof J. Buchner for the official supervision of this PhD work.I would like to acknowledge all the members of the Förster laboratory at the Institute of Medical Microbiology, Technical University Munich. In particular, I would like to thank Prof. I Förster who has given me the opportunity to carry out this PhD work in her laboratory and who was always very supportive. Additionally I would like to acknowledge Dr. J. Alferink for finding time for discussions and providing new ideas. I also credit Mathias Schiemann for his efforts concerning FACS sorting experiments.

Finally, I would like to take the opportunity to thank my parents, my sister and Michael for their support.