Aus dem Institut für Immunologie
der Ludwig-Maximilians-Universität München
Direktor: Prof. Dr. Thomas Brocker

Mechanisms of central and peripheral T cell tolerance to an antigen of the central nervous system

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Lei Wang
aus
Henan, China

München, 2016

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Betreuer: Prof. Dr. Ludger Klein

Zweitgutachter: Priv. Doz. Dr. Klaus Dornmair

Dekan: Prof. Dr. med. dent. Reinhard Hickel

Tag der mündlichen Prüfung: 22.02.2017

Eidesstattliche Versicherung

Wang, Lei

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Mechanisms of central and peripheral T cell tolerance to an antigen of the central nervous system

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 22.02.2017

Ort, Datum

Wang, Lei

Unterschrift Doktorandin/Doktorand

I dedicate this work to my family.

Table of Contents

Summary

Myelin reactive T cells are central in the development of the autoimmune response leading to central nervous system (CNS) destruction in Multiple Sclerosis (MS) and its animal model, Experimental Autoimmune Encephalomyelitis (EAE). The underlying cellular and molecular mechanisms, however, are not fully understood. In previous mouse studies, we showed that tolerance to the major component of the myelin sheath, myelin proteolipid protein (PLP), is crucially dependent on its expression in the thymus where central tolerance induction takes place. To analyze the phenotypic and functional changes taking place during the induction of tolerance in the thymus, we investigated the fate of PLP autoreactive $CD4^+$ T cells in TCR-PLP11 transgenic mice, which express a transgenic TCR specific for the dominant $PLP_{174-181}$ epitope in B6 mice, a EAE-resistant mouse strain of the $H\text{-}2^b$ haplotype. In previous work we found that a fraction of $CD4^+$ T cells specific for this region appear to escape from tolerance induction. Our data showed that in TCR-PLP11 PLP^{WT} mice, where PLP is transcribed in the thymus similar numbers of $CD4^+$ thymocytes developed, compared to TCR-PLP11 PLP^{KO} mice where PLP expression in the thymus is absent. This indicated that $PLP_{174-181}$-specific thymocytes were not negatively selected. In the periphery, the $PLP_{174-181}$-specific T cells displayed a naïve phenotype and therefore were not tolerized by clonal deletion or anergy induction. Potentially autoreactive $CD4^+$ T cells were found in the spleen and lymph nodes of TCR-PLP11 mice but only became activated when stimulated *in vitro.* These cells were not spontaneously activated *in vivo*, indicating that PLP is not expressed/presented in the periphery. TCR-PLP11 mice do not develop any clinical or histological signs of EAE. Therefore, ignorance but not deletional tolerance is considered as main tolerance mechanism to avoid $CD4^+$ T cell-mediated autoimmunity in our system. That means that naïve autoreactive $CD4^+$ T cells ignore PLP antigens and recirculate in the periphery without causing damage. In contrast, immunization of TCR-PLP11 PLP^{WT} mice with the $PLP_{174-181}$ peptide in Complete Freund´s Adjuvant (CFA) reversed this state of immune ignorance as judged by the clinical manifestations of EAE in these mice. Furthermore, TCR-PLP11 PLP^{WT} mice develop spontaneous EAE after being bred onto a RAG^{KO} background, leading us to the speculation that, besides immunological ignorance, dominant mechanism of PLP tolerance are crucial for the prevention of CNS autoimmunity. Taken together, this

study establish a novel model of immunological tolerance towards a self-antigen expressed in the central nervous system involving antigen ignorance of $CD4^+$ T cells.

Somatic recombination of TCR genes in thymocytes not only results in the production of useful TCR specificities, but also produces potentially autoreactive specificities. Autoreactive $CD4^+$ T cells are censored by two mechanisms, the so-called clonal deletion and the deviation into regulatory T cells in the thymus both requiring the same stimulus, namely the interaction with self-peptide. In order to study the mechanisms of central and peripheral tolerance that operate to shape the CD4 T-cell repertoire, we have generated a TCR transgenic mouse that expresses the α- and β-chains of a PLP_{11-18}-reactive TCR (TCR-PLP1) in the context of $H\text{-}2^b$. Using this novel TCR-PLP1 transgenic mouse model, we investigated the modalities of central tolerance induction to a self-antigen expressed in the thymus at physiological levels. We found that Plp1-specific T cells undergo clonal deletion and Treg differentiation concomitantly upon encounter of the cognate self-antigen PLP. Medullary thymic epithelial cells (mTECs) express and present the endogenous antigen PLP and mediate tolerance in an autonomous manner, whereas thymic dendritic cells are dispensable for central tolerance induction to PLP. Although central tolerance induction to PLP is very potent, it is not complete as a proportion of autoreactive T cells also escape to the periphery. However despite the presence of potentially dangerous cells in the periphery, the mice do not develop autoimmunity indicating that additional tolerogenic mechanisms promote tolerance to PLP in the periphery. We could further show that autoreactive Plp1-specific T cells are deleted or become functionally inactivated (anergy) by recognition of self peptide on dendritic cells in the periphery. In a set of bone marrow transplantation experiments we found that PLP was expressed by radioresistant stromal cells and subsequently cross-presented by dendritic cells. Taken together our results indicate a complementing role of the thymus and the periphery for tolerance induction to PLP.

Zusammenfassung

Myelin reaktive T Zellen sind von zentraler Bedeutung in der Entwicklung von Autoimmunantworten welche zu der Destruktion des Zentralen Nerven Systems (ZNS) führen und in Multiple Sklerose (MS) oder dem korrespondierenden Tiermodell, Experimentelle Autoimmune Enzephalomyelitis (EAE), resultiertn. Jedoch ist noch nicht völlig klar verstanden welche grundlegenden zellulären und molekularen Mechanismen diese Krankheiten auslösen. In früheren Mausstudien konnten wir zeigen, dass die Toleranz gegen die Hauptkomponente der Myelinscheide, dem Myelin Proteolipid Protein (PLP) entscheidend davon abhängt, ob PLP im Thymus, wo die Induktion der Zentrale Toleranz statt findet, exprimiert wird. Um die phänotypischen und funktionellen Änderungen welche während der Toleranzinduktion im Thymus statt finden zu erforschen, untersuchten wir PLP autoreaktive $CD4^+$ T Zellen von T Zell Rezeptor (TZR)-PLP11 transgenen B6 Mäusen. Diese Mäuse sind EAE resistent und exprimieren einen transgenen TZR spezifisch für das dominante $PLP_{174-181}$ Epitop im Kontext vom $H\text{-}2^b$ Haployp. In vorhergehenden Studien, fanden wir heraus, dass eine Fraktion von $CD4^+$ T Zellen spezifisch für diese Region die Zentrale Toleranz umgingen. Unsere Daten zeigten, dass in TZR-PLP11 PLP^{WT} Mäuse, welche PLP im Thymus exprimieren, im vergleich zu TZR-PLP PLP^{KO} Mäusen welche PLP im Thymus nicht exprimieren, eine ähnliche Anzahl von $CD4^+$ Thymozyten entstehen. Dies deutet darauf hin, dass $PLP_{174-181}$-spezifisch T Zellen nicht negative selektioniert werden. In der Peripherie haben $PLP_{174-181}$-spezifisch T Zellen einen naiven Phänotyp was darauf hin deutet, dass die Toleranz nicht durch Klonale Eliminierung oder durch die Induktion von Anergie etabliert wird. Potentiell autoimmune $CD4^+$ T Zellen konnten in der Milz und in den Lymphknoten von TZR-PLP11 Mäusen gefunden werden und konnten nur durch *in vitro* Stimulation aktiviert werden. *In vivo* jedoch wurden diese Zellen nicht spontan aktiviert, was darauf hin deutet, dass in der Peripherie, PLP nicht exprimiert oder präsentiert wird. TZR-PLP11 Mäuse entwickeln keine klinischen oder histologische Zeichen von EAE. Demzufolge nehmen wir an, dass in unserem System Ignoranz und nicht deletionale Toleranz dafür verantwortlich ist, dass $CD4^+$ T Zell-vermittelte Auto-Immunität verhindert wird. Dies bedeutet, dass naive autoreaktive $CD4^+$ T Zellen das PLP-Antigen ignorieren und in der Peripherie rezirkulieren ohne Schaden zu verursachen. Im Gegensatz dazu, die Immunisierung von TCR-PLP11 PLP^{WT}

Mäuse mit dem $PLP_{174-181}$-Peptid in Komplettem Freund's Adjuvans kehrte diesen Status der immunen Ignoranz um, beurteilt durch die klinische Manifestation von EAE in diesen Mäusen. Außerdem, TCR-PLP11 PLP^{WT} Mäuse entwickelten spontane EAE nach dem diese auf einen RAG^{KO} Hintergrund gezüchtet wurden. Dies brachte uns zu der Spekulation dass neben der Immunologischen Ignoranz, dominante Mechanismen der PLP Toleranz wichtig für die Verhinderung von ZNS-spezifischer Autoimmunität sind. Zusammengefasst, in dieser Studie wurde ein neues Model von Immunologischer Toleranz gegen ein Eigen-Antigen welches im ZNS exprimiert wird etabliert und die Antigen-Ignoranz von $CD4^+$ T Zellen beinhaltet.

Die somatische Rekombination von T Zell Rezeptor (TZR) Genen in Thymozyten resultiert nicht nur in der Produktion von brauchbaren TZRs sondern auch in TZRs mit potentiell autorreaktiven Spezifitäten. Generell werden autoreaktive $CD4^+$ T Zellen im Thymus durch zwei Mechanismen zensiert. Zum einen durch die sogenannte Klonale Eliminierung und zum anderen durch die Differenzierung in regulatorische T Zellen (Treg), wobei beide Mechanismen den gleichen Stimulus, die Interaktion mit Eigen-Peptiden, benötigen. Um die Mechanismen der zentralen und peripheren Toleranz, welche das CD4 T Zell Repertoire formt zu untersuchen, haben wir eine TZR transgene Mause generiert welche die alpha und beta Kette von einem TZR spezifisch für das PLP_{11-18} im Kontext von $H\text{-}2^b$ exprimiert (TZR-PLP1). Durch die Verwendung von diesem neuen TZR-PLP1 transgenen Mausmodell, konnten wir die Modalitäten der Zentralen Toleranz Induktion anhand eines Eigen-Antigens untersuchten, welches im Thymus physiologisch exprimiert wird. Wir fanden heraus, dass Plp1-Spezifische T Zellen welche durch ihr spezifisches Eigen-Antigen PLP stimuliert wurden gleichermaßen in Treg Zellen differenzierten als auch der klonale Eliminierung unterlagen. Unsere Studie zeigte, dass dendritische Zellen im Thymus unwesentlich zur Induktion der zentralen Toleranz für PLP bei tragen. Im Gegensatz dazu; Epithelzellen in der Medulla des Thymus (mTECs) exprimieren und präsentieren das endogene Antigen PLP und vermitteln Toleranz in einer autonomen Art und Weise. Obwohl die Zentrale Toleranz für PLP sehr potent ist, ist sie nicht komplett und eine kleine Fraktion von autoreaktiven T Zellen kann in die Peripherie entweichen. Obwohl in der Peripherie potentiell gefährliche Zellen vorhanden sind, entwickeln die Mäuse keine Zeichen von Autoimmunität. Dies weist darauf hin, dass in der Peripherie, zusätzliche Mechanismen die Zentrale Toleranz gegen PLP

unterstützen. Wir konnten zeige, dass autoreaktive Plp1-spezifische T Zellen eliminiert oder funktionell inaktiviert werden, sobald diese Eigen-Peptid, präsentiert auf peripheren dendritischen Zellen, erkennen. In Experimenten von Knochenmarktransplationen fanden wir heraus, dass PLP auf radioresistenten Stroma Zellen exprimiert und anschließend von dendritischen Zellen kreuzpräsentiert wird. Zusammengefasst, unsere Resultate deuten darauf hin, dass die zentrale Toleranz für PLP durch ein Zusammenspiel zwischen Thymus und Peripherie etabliert wird.

1. Introduction

1.1 T-cell development

T cell development from hematopoietic cells takes place in the thymus, which is an evolutionarily conserved primary lymphoid organ that provides a highly specialized microenvironment[1-3]. The thymus consists two main cellular zones: the major outer zone, the cortex, and the smaller central zone, the medulla. Each of which are responsible for the keys stages in the thymocytes development. The cortical region is populated by pre-T lymphocytes and medullary region contains mature T lymphocytes[4-7]. The thymic microenvironment forms a complex network of interaction that comprises non lymphoid cells (e.g., thymic epithelial cells, TEC) that are capable of attracting lymphoid progenitor cell, specifying these cells to the T cell lineage, and orchestrating positive and negative selection events to complete thymocytes development and ensuring central tolerance and in turn, developing thymocytes critically regulate the development of TECs[8-10]. Therefore, the lympho-stromal communication is a crosstalk between architectural stromal cells and traveling thymocytes[11, 12]. Two chemokine, CC-chemokine ligand 21 (CCL21) and CCL25, and adhesive interaction between platelet (P)-selectin glycoprotein ligand 1 (PSG1) and P-selectin are involved in thymus colonization[13-16].

1.1.1 Early T-cell development

Homing of bone marrow (BM)-derived lymphoid progenitors to the thymus is essential for T cell development. Early intrathymic progenitor cells are found within the most immature subset of thymocyte precursors, which lack CD4 and CD8 expression and are referred to as double negative (DN) cells[17, 18]. During intrathymic differentiation, the immature DN subset is subdivided into four defined developmental stages (DN1-4) on the basis of the expression profiles of adhesion molecular CD44 and CD25 (Interleukin-2 (IL-2) receptor alpha subunit)[19]. Differential expression of these markers reflects developmental changes in the thymocytes when they enter the thymus at the cortico-medullary junction (CMJ) and subsequently migrate to the subcapsularzone of the thymic cortex[20, 21] (Figure 1).

Differentiation to the DN1 stage, the earliest chronological subset is recognized as a $CD44^{high}C\text{-}Kit^{+}CD25^{-}$ population[22], proceeds in proximity to the site of thymic entry[23]. The DN1 cell population is a heterogeneous mixture while c-Kit^{high} DN1 cells have been shown to possess most T progenitor potential[24]. In addition to T cell precursor activity, early thymic progenitors (ETPs) have the potential to give rise to αβ T cells, γδ T cells, dendritic cells, natural killer (NK) cells, macrophages, and B cells when transferred intravenously into irradiated hosts[25, 26].

DN1 cells begin to proliferate with concomitant expression of CD25 and mark the progression to the T lineage–specified DN2 stage showing the $CD44^{high}C\text{-}Kit^{+}CD25^{+}$ phenotype[27]. DN2 cells migrate toward the outer thymic cortex under the influence of CXCL12, CCL19 and CCL12 produced by cortical thymic epithelial cells (cTECs). These DN2 cells still express considerable numbers of ‘legacy’ stem cell genes, cTEC continue to deliver strong Notch signals favoring T-lineage commitment and differentiation[28, 29]. IL-7 is essential for the survival and maturation of the IL-7Rα-expressing DN2 and DN3 cells that follow them. IL-7-deficiet mice exhibit an abrupt block at DN2 stage of thymocytes[30-32]. A transcription factor, Sox13, has been associated with DN2 cell commitment[33].

The DN2 thymocytes then start to rearrange their T cell receptor (TCR) genes and downregulate the expression of CD117 and CD44 to become $CD44^{low}C\text{-}kit^{low}CD25^{+}$ DN3 subset[34]. Thymocytes undergo recombination-activating gene (RAG)-mediated somatic rearrangements of the TCRβ, TCRγ, and TCRδ loci, which are required for the assembly of the TCR[35-37]. At the DN3 stage, the final commitment to the αβ and γδ T lineages is made[38]. While only rearrangement of the TCRβ locus is completed to initiate αβ T-cell maturation, both TCRγ and TCRδ must be productively rearranged to generate functional γδ T lineages[39]. However, how the commitment decision is made is still little understood due to the difficulty in distinguishing between these cells prior to TCR expression[40]. Sox13 is the only specific γδ-T cell lineage transcription factor identified so far. In mice, deficiency for Sox13 has impaired γδ-T cell development. For cells that proceed along the αβ TCR pathway, the newly formed functional TCR-β chains together with the invariant pre-TCR α chain[41-44] and CD3 to form the pre-TCR complex[43, 45]. This checkpoint is known as β-selection, which is the result from the formation and expression of the pre-TCR complex on

DN3 thymocytes: proliferation, rescue from apoptosis, allelic exclusion at the TCR-β gene locus, initiation of TCR-α gene expression, upregulation of CD4 and CD8 expressions, and downregulation of CD25 expression[46-47] ensures that only those thymocytes that have a successful TCRβ gene-segment rearrangement, initiation of TCR-α gene expression, upregulation of TCR on their surface are permitted to survive and to undergo further differentiation[48]. At least two additional signals, Notch1 and CXCR4 via cTEC ligands contribute to the differentiation and expansion at the β-selection checkpoint[49, 50].

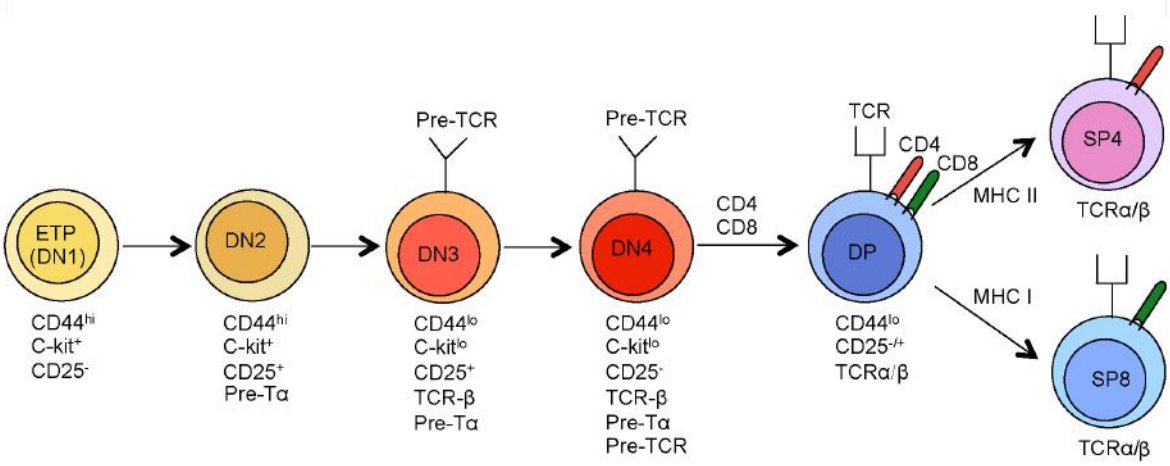

Figure 1. Schematic view of early T-cell development. T-cell development from less mature to more mature cells with distinct phenotype proceeds from left to right. DN, double negative; RAG, recombination-activating gene; DP, double positive; SP, single positive; TCR, T-cell receptor; MHC I, major histocompatibility complex class I; MHC II, major histocompatibility complex class II.

Thymocytes that emerge from β-selection undergo TCRα-VJ rearrangement, recombine and express a functional TCRα chain what leads to the second component chain of the mature αβ antigen receptor. They stop to express receptors characteristic of hematopoietic cells, and become unresponsive to cytokine signals, e.g., interleukin-7 (IL-7)[51, 52]. They also initiate CD4 and CD8 expression what further drives DN4 cells to become double-positive (DP) immature T cells is referred to the DN4 or pre-DP stage[53-55]. If a rearranged β-chain does not lead to any signaling, the cell may die by neglect[56]. In this stage, the cells become phenotypically $CD44^{-}CD25^{-}$ and migrate to the outermost cortex, the subcapsular zone. As soon as DPs express a functional αβ-TCR on their surface, cells undergo two major rounds of selection: positive and negative selection[57]. Along with positive

selection, DP cells become committed either to CD4 single positive (SP) or to CD8 SP thymocytes[22, 38, 56, 59] depending on the ability of their TCR to bind to peptide-MHC class II or peptide-MHC class I complexes, respectively[60, 61].

During the specification and commitment processes, numerous signaling molecules and transcription factors must be completed before cells reach DN4 stage. To date, a number of molecules have been identified involved in T-cell development by "loss-of-function" and "gain-of-function" approaches. Notch is a signaling receptor molecule to Delta or Jagged ligands, and the Notch signaling is involved in many aspects of development[28, 29, 62-64]. Notch1-delta-like 4 (DL4) signaling has been shown to be required for DN1-DN2 transition. The absence of Notch-1 led to arrest T cell development at early stage and to ectopic differentiation of intrathymic B cells [65, 66]. An interesting observation in mice in which Notch1 is deleted by CD4-Cre was the lack of perturbed T-cell development from late DN3 stage, suggesting that Notch1 is involved in maintaining lineage integrity in early, but not late and mature, thymocyte development[67]. Other transcription factors, including Runx1, GATA-3 and two E-proteins (E2A and HEB), cooperate with Notch1, play multiple roles during T cell development[68-71]. We still do not understand how these transcription factors function within the same transcriptional network and whether there is a single factor that acts as the master regulator in T lineage, similar to Pax5 in B cells[72]. To investigate this, more efforts will be required.

1.1.2 T cell selection

The population of DP-thymocytes contains the unselected T-cell repertoire. The recognition of αβ TCR with peptide: MHC (pMHC) complexes presented in the cortical microenvironment is regarded as the central event in positive and negative selection, leading to the fate decision of DP thymocytes. The selection procedure is known as positive and negative selection[56, 73, 74]. Only those thymocytes that receive low avidity TCR interactions with self-pMHC will receive a signal for survival and differentiate into single-positive (SP) thymocytes. In contrast, high-avidity interactions elicit signals that lead to the deletion of tissue-specific-antigen-reactive T cells by negative selection or induced the differention into $Foxp3^+$ regulatory T cells and thereby avoiding autoimmunity[75-77] (Figure 2).

1.1.2.1 Positive selection

To generate peptides for MHC class I presnetation, cTECs uniquely express the proteasome subunit β5t[56]. Proteasomes are multicatalytic protease complexes responsible for producing antigenic peptides that can bind efficiently to MHC class I molecules as well as degradating cytoplasmic proteins[78, 79]. β5t-containing proteasomes, termed thymoproteasomes, favoring the production of peptide that are less stably bound to MHC class I molecules compared with the other types of identified proteasomes, one is β5i-containing immuno-proteasomes, the other is standard proteasomes containing β5 subunits[80]. The reason is because β5t have the different catalytic properties from those of β5i/β5. The importance of the unique catalytic activity of β5t was showed by the analysis of $\beta5t^{-/-}$ mice. These mice exhibited a substantially reduced positive selection of MHC class I-restricted $CD8^+$ T cells and had an altered CD8 T-cell repertoire[79]. These data indicates that β5t-dependent peptides are essential for positive selection of $CD8^+$ T cells and also critical to generate of an immunocompetent repertoire of $CD8^+$ T cells. Furthermore, the study showed by Katsuhiro et al., demonstrated that unique cleavage motifs in β5t-dependent MHC class I-associating peptides are enriched with low-affinity TCR ligands that efficiently induce positive selection. Taken together, these aspects indicate that cTECs regulate positive selection of CD8 T cells by producing a unique set of MHC class I-associating peptides that exhibit low affinity for TCR[80-83].

With respect to the positive selection of MHC II-restricted $CD4^+$ T cells, many lysosomal proteases produce peptide antigens. cTEC highly but not exclusively express lysosomal proteases Prss16 (also known as thymus-specific serine protease (Tssp))[84] and cathepsin L[85], which are necessary for optimal positive selection of CD4 T cells[86, 87]. Analyses of mice deficient in Prss16 have indicated a defective positive selection of $CD4^+$ T cells with certain TCR specificities, including diabetogenic self-reactive $CD4^+$ T cells[88]. Cathepsin L-deficient mice also show a greatly reduced repertoire, which is manifested by a reduced number and diversity of MHC-II restricted $CD4^+$ T cells[89]. It is also shown that macroautophagy is required for the generation of pMHC complexes for positive selection[90-92], which is a protein degradation process that facilitates loading of intracellular antigens onto MHC II

molecules. Mice lacking the essential autophagy gene Atg5 showed altered repertoire selection of the $CD4^+$ T cell compartment[93, 94]. These data strongly supports the idea that cTECs display a specific set of unique self-peptides to induce positive selection of a functionally competent repertoire of $CD4^+$ T cells.

1.1.2.2 Negative selection

Negative selection is based on the interaction of self-peptides presented by MHC molecules, i.e. high affinity and/or avidity interaction between the TCR and self-peptie-MHC complexes will undergo apoptosis[57]. The process of negative selection enriches 'useful' T cells that are potentially reactive to foreign antigens, but not to self-antigens, presented by self-MHC molecules and thereby avoiding autoimmunity[95].

To achieve successful negative selection, thymocytes interact with stromal cells presenting self-antigens that are expressed ubiquitously or are tissue-restricted in the thymic microenvironment. Medullary thymic epithelial cells (mTECs) and thymic dendritic cells (tDCs) are the key players for negative selection (also see section 1.1.3). mTECs as the main stromal cell subset in the medulla are capable of expressing a large number of tissue restricted self-antigens (TRAs)[96]. This phenomenon has been termed promiscuous gene expression and is mediated at least partially by the autoimmune regulator (AIRE)[97, 98]. While mTEC express and present TRAs both on MHC class I molecular and MHC class II molecular, tDC are important for cross-presentation of mTEC-derived TRAs. Both populations require B7:CD28 interactions to promote clonal deletion of T cells reactive to TSAs[99-101]. However, evidence for an autonomous role of mTEC as negatively selecting APC has obtained in several mouse models. It has been shown that siRNA-mediated reduction of MHC class II expression in mice rescues CD4SP compartment from clonal deletion[102]. Furthermore, genetic ablation of DCs in mice also showed that tDCs are capable to delete autoreactive $CD4^+$ T cells without the contribution of mTECs[103, 104]. It has been shown that after transferring TCR transgenic $CD8^+$ H-Y T cells into recipients, T cells recognizing the male antigen were detected in the periphery of female mice, but are deleted in males, and highlights the efficiency of negative selection in preventing the release of autoreactive T cells into the

periphery[105]. In contrast to mTECs, there is little evidence to support an autonomous role of cTECs in the promotion of clonal deletion *in vivo*[106].

1.1.3 Antigen presenting cells in the thymus

Within the discrete thymic microenvironments, developing T cells interact with individual stromal cells which display self-antigen-derived epitopes on their surface and are involved in T cell development and seletion, particularly shape the repertoire of pMHC complexes on their surface and therefore in the development and generation of T cells. Thus, it is important to understand the contribution of the various thymic APC subsets and their distinct properties regarding antigen presentation. APCs in the thymus consists of cTEC, mTEC, DCs and also B cells. However, compared with other APC lineages, B cells only present as a tiny population in the thymus[84, 107] and their role in negative selection remains elusive[73, 108].

Cortical thymic epithelial cells (cTEC)

cTECs are the essential component that forms the architecture of the thymic cortex and supports early T-cell development and positive selection of immature thymocytes. Besides that, some studies indicated that cTECs also contribute to negative selection[106] as well as to induction of regulatory T cells[109, 110] (Figure 2). However, the mechanism how cTECs induce TCR-mediated positive selection is unknown. One of the concepts addressed this selection paradox using an ‘altered peptide’ model. It suggested that cTECs present positively selecting “specially tailored” peptides and might be different from those tolerance-inducing APCs in the medulla[111]. Two other hypotheses claimed an affinity/avidity model, which predicted the quality/quantity of TCR-peptide-MHC interaction and therefore shaping lymphocyte repertoires somatically[112, 113]. The first evidence of the peptide machinery in cTECs was addressed to cathepsins in $CD4^+$ T cells. Interestingly, cTECs preferentially express cathepsin L but not cathepsin S, which is expressed by other haematopoietic APCs and mTECs. The phenotype of $Ctsl^{-/-}$ mice indicate that lysosomal proteases are necessary for positive selection of CD4SP cells [88, 114, 115]. Although cTECs express high levels of MHC class II molecules[116-118], they are

inefficient in presenting exogenous proteins using the classical endocytic pathway. Instead, cTECs use macroautophagy to deliver the intracellular antigens to the MHC II pathway to generate a functionally competent repertoire of $CD4^+$ T cell compartment[119].

Medullary thymic epithelial cells (mTEC)

mTECs are the unique cell type capable of expressing a broad range of tissue-restricted antigens in a promiscuous fashion[120, 121]. Beside that, mTECs constitutively express MHCII and CD80 on their surface. Thus, one can distinguish two subsets of mTEC with respect to these markers: $mTEC^{lo}$ and $mTEC^{hi}$ expressing low to intermediate and high levels of MHC class II and CD80, respectively[122, 123]. It has been proposed that $mTEC^{hi}$ are the most mature, terminally differentiated subset of mTEC with antigen presentation characteristics of professional APC[124, 125]. The transcription factor Aire is primarily found in lymphoid organs, particularly in the nuclei of mature, highly MHC II–expressing mTECs in the thymus and is the only known regulator that induces the expression of some but not all TRAs. The initial report by Anderson et al. suggested that Aire promotes the promiscuous expression of TRAs in mTECs[101]. The importance of Aire controlling the transcription of TRAs in mTEC and thus in T-cell tolerance is highlighted by the fact that mutations in Aire gene lead to the human autoimmune syndrome known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)[126, 127]. Similarly, mice with mutations in the Aire gene suffer from spontaneous multi-organ autoimmune disease characterized by multiorgan lymphocytic infiltration and autoantibody production[128]. In addition, Aire has been described to enhance the antigen-presentation capability of mTEC[101].

Despite the low frequency (1–3%) of mTECs expressing a particular TRA[98, 129, 130], it would still be feasible that antigen expression and direct presentation by mTECs is sufficient for the induction of both dominant and recessive modes of central tolerance. However, such a mandatory division in mTECs is still a matter of intensive research. TCR transgenic mice specific for human C-reactive protein (hCRP) rises evidence that promiscuous expression of hCRP in mTECs acts autonomously to tolerize $CD4^+$ T cells speicifc for an endogenous antigen[99]. In another system where the function of

mTEC for the induction of dominant tolerance was shown is the AIRE-HA model. In this model, mTEC-specific expression of hemagglutinin (HA) led to the deviation of antigen specific T cells into the regulatory T cells (Treg) lineage in a cell autonomous way, independent of antigen transfer and presentation by haematopoietic APCs[102, 131].

Since very few mTECs express a given TRA in the medulla, there is another scenario that explains how TRAs are presented to developing thymocytes. This thought argues for the cross-presentation of mTEC expressed self-antigens presented by DCs. Although mTECs synthesize the TRAs, they do not directly present antigen to delete TRA-reactive T cells. Instead, mTECs serve as TRAs suppliers, eventually spread these antigens in the thymic medulla where neighbouring DCs would present these antigens and increase the probability of TRAs being encountered by SP T cells. This concept received experimental support, utilizing the RIP-mOVA system, the authors showed that intercellular antigen transfer from mTECs to BM-derived APCs is necessary for the deletion of autoreactive CD4SP and CD8SP T cells[132]. It seems highly plausible that both antigen presentation by mTEC and DCs can mediate negative selection and Treg cell differentiation to establish central tolerance (Figure 2).

Dendritic cells (DC)

The dendritic cells (DCs) are highly specialized APCs in the medullary region of the thymus. Thymic DCs are subdivided into two major subsets based on their cell surface markers expression and functional properties: conventional $CD11c^{high}$ DC (cDC) and $CD11c^{mid}CD45RA^{+}$ plasmacytoid-derived DC (pDC). cDCs can be further divided into lymphoid resident and migratory DCs according to Sirpα expression[133]. $Sirp\alpha^{-}$ cDC develop from intrathymic precursor cells, while $Sirp\alpha^{+}$ DCs and pDCs immigrate from the periphery, home to the thymus at steady state and therefore are called migratory DCs[134-136].

DCs present a broad range of self-antigens, including TRAs expressed and transferred from mTEC, circulating antigens captured by thymic DCs from the blood and antigens acquired from peripheral tissues by $Sirp\alpha^{+}$ DCs and pDCs homing to

the thymus[132, 137-139]. DCs play an important and sufficient role in negative selection of $CD4^+$ thymocytes. Accordingly, in mice that lack antigen presentation specifically in DCs diminished negative selection of bulk polyclonal CD4SP T cells was found[103, 140]. Using transgenic mouse models also confirmed an indispensible role of DCs in negative selection[104, 141]. DCs interact with antigen-specific thymocytes with high affinity, could also drive the interacting thymocytes to become Treg cells[142]. However, the underlying mechanisms by which thymic DCs mediate negative selection and Tregs induction remain to be better established.

1.2 Central tolerance

A major challenge for the immune system is to preclude the release of self-reactive thymocytes. If they proceed through the terminal maturation stage and migrate to the periphery, they could recognize the body's own components and attack host tissues leading to autoimmunity. In order to prevent autoimmunity, T cell development needs to be controlled by the mechanisms of central tolerance, which occurs in the thymus and peripheral tolerance, which occurs in the secondary lymphoid tissue.

The central tolerance mechanisms are distinguished into recessive (negative selection/clonal deletion) and dominant (Treg generation). Central tolerance can be regarded as a consequence of minimizing the release of functionally competent autoreactive T cells from the thymus. In contrast, dominant tolerance involves generation of a subset of $CD4^+$ T cells with immunosuppressive function (regulatory T-cell; Treg), which can dampen the activation and expansion of potentially hazardous cells that have avoided negative selection and enter the peripheral tissue[144-146].

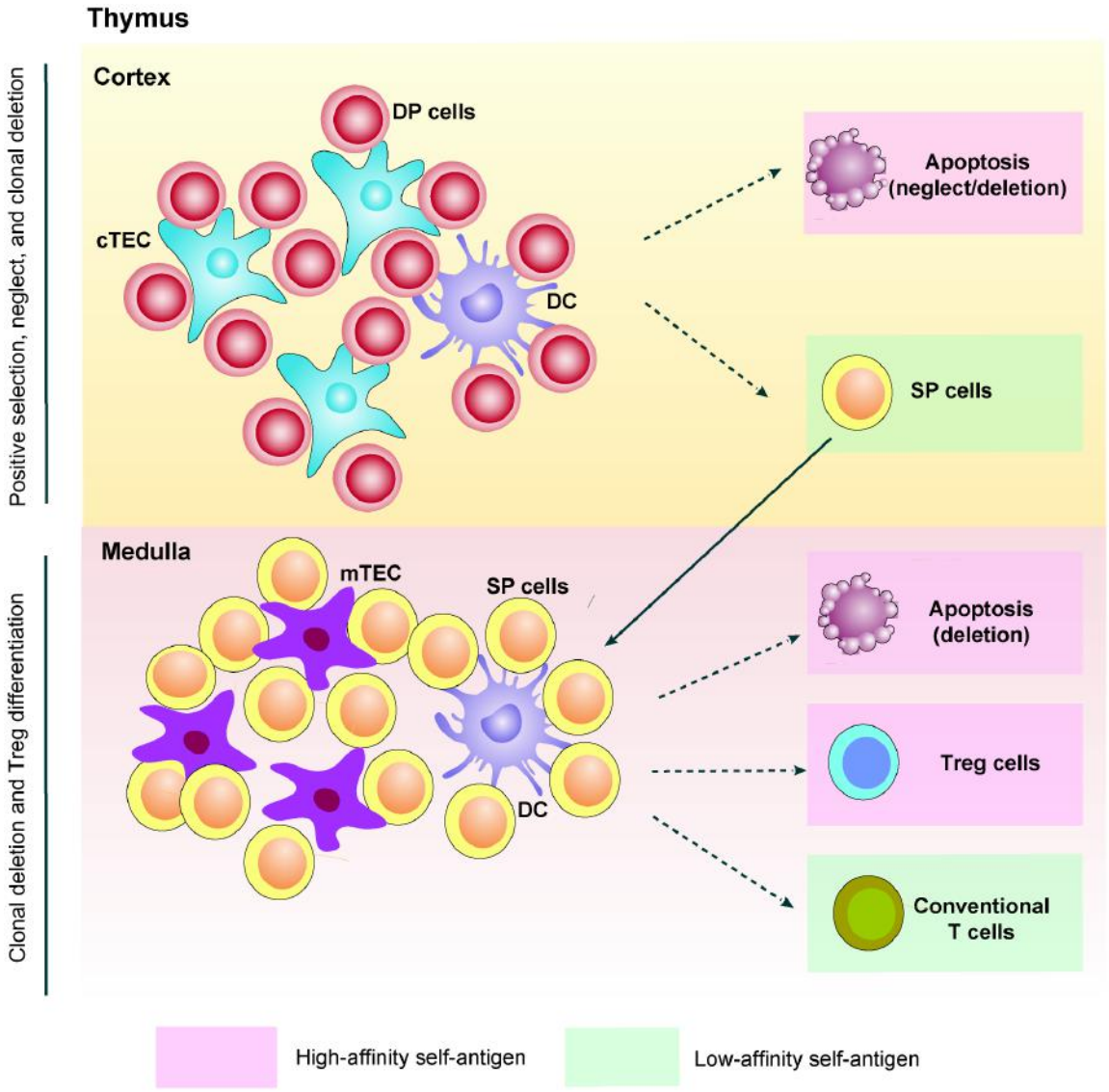

Figure 2. T cells undergo selection and maturation processes on the basis of their TCR reactivity. cTECs are uniquely responsible for inducing positive selection of functionally distinct T cells. T cells recognize low-affinity self peptide-MHC complexes inducing survival signals and further differentiation into CD4SP or CD8SP thymocytes as they migrate from the cortex to the mudulla. This process is referred to as positive selection. The remaining T cells, which TCR bind too strongly or do not even recognize pMHC complexes are destined to die through apoptosis. mTECs and DCs are the key players for negative selection. Self-reactive T cells bearing TCRs with high affinity for self-peptide: MHC complexes are deleted. Alternatively, strong TCR signals can induce CD4SP cells to differentiate into regulatory (Treg) T cells. Figure adapted from Li et al.[143].

1.2.1 Clonal deletion

The so-called ‘clonal selection theory’, a seminal landmark of modern immunology, was published in 1959 by Frank Macfarlane Burnett. It proposed that lymphocytes that are potentially dangerous self-reactive will be eliminated from the T-cell repertoire to prevent autoimmunity[147]. The elimination of autoreactive lymphocytes is implicated in the process of clonal deletion or negative selection is T-cell progenitors expressing TCRs with high affinity (that is, above a certain quantifiable threshold) for self-antigens will die by induced apoptosis[148-150]. There was outstanding experimental support for the ‘clonal deletion model’ by Marrack et al. They demonstrated that superantigen (SAg)-specific T cell expressing Vβ17a TCR were efficiently eliminated in mice expressing SAg derived from the mouse mammary tumor virus, when SAg expression was lacking, the same T cells escaped clonal deletion and migrated to the periphery[144]. Furthermore, many TCR transgenic mouse models expressing T cell receptor specific for a self-antigen was generated to validate the clonal deletion model. In these model systems, mice were designed to express antigens from transgenes, for example, Hemagglutinin (HA); in some others TCR transgenic mice recognized a naturally expressed antigen, e.g. H-Y[57].

The fundamental questions of clonal deletion are where does self-reactive T cells undergo deletion and at which stages of thymocytes are removed? The questions which relevant APCs and what the molecular signals are involved have been studied broadly. The medulla is generally thought to be the place for negative selection, which provides the most complex ligandome as well as a wide range of restricted tissue-specific antigens. However, whether clonal deletion also occurs in the cortex is controversial. It is clear that the nature of thymocyte TCR and self-antigen expression have an effect on the timing of clonal deletion. For example, Hogquist et.al recapitulated the H-Y TCRa expression at the physiological DP stage (H-Y^{cd4} mice)[151]. It was shown that thymocytes deletion occurs at the transition from DP to SP. Thus, in general, polyclonal thymocytes are specific for ubiquitous self-antigens seem to be deleted in the cortex. In contrast, deletion occurs in the medulla when those cells are restricted to tissue-specific antigens, superantigens and circulating antigens. The process of negative selection was mediated by mTECs and tDCs via presentation of TRAs. mTECs express and present TRAs on MHC class I and MHC

class II, while tDCs are essential for cross-presentation of mTEC-derived TRAs. It has been described that several TCR co-stimulatory molecules are contribute to apoptosis, among these are CD5, CD28, CD43 and Fas[152, 153]. More work is needed to understand how the different affinity ligands can be discriminated by a TCR to induce the distinct outcomes of positive and negative selection. With respect to the proximal TCR signaling events, several molecules have been identified to regulate negative selection, including the mitogen activated protein kinase (MAPK) family members Jun N-terminal kinase (JNK) and p38, which is initiated in part by pro-apoptotic BCL2 family members BIM[154, 155]. Another important activator of the JNK/p38 with a fundamental role in negative selection is Misshapen/Nck interacting kinase (NIK)-related kinase (MINK) and Grb-2[156]. Furthermore, Nur77, an orphan nuclear receptor, has been found to interact with the anti-apoptotic molecule Bcl-2 in the mitochondria, thereby leading to cell death[157]. More recently it was found that CTLA-4 signaling diminish the efficacy of clonal deletion of thymocytes[158, 159].

1.2.2 Clonal diversion

Self-reactive T cells bearing TCRs with high affinity for self-peptide: MHC complexes are deleted. However, negative deletion is an inevitably incomplete process and raises the question whether tolerance is maintained by additional tolerance mechanisms. In the late eighties, the so-called dominant tolerance had been discovered, which represented a yet unknown mode of tolerance at that time. The elegant work from Le Dourain has implicated that the existence of dominant tolerance operating in the thymus[160, 161]. They found that transplantation of embryonic tissues from quail into age-matched chicken embryos induced the rejection of graft soon after birth. Importantly, this graft rejection would be prevented by simultaneous transplantation of limb buds with embryonic thymi. In such chimeras, embryonic thymi were grafted before when they had been colonized by hematopoietic precursors, which indicated that tissue-specific tolerance induction was established by thymic epithelium (TE). Since a recessive tolerance induction mechanism could not explain why the transplanted limb was accepted, this unexpected finding revealed that a special type of T cells could be generated in the thymus and have the capacity to inhibit graft-reactive T cells[162]. Sakaguchi and colleagues identified a subset of T cells with a regulatory function (hereafter referred

to as Treg) and mediate dominant tolerance that is essential to prevent autoimmunity. Those $CD4^+$ T cells constitutively express the CD25 with the capacity to suppress potentially harmful cells that are activated upon encounter their cognate antigen[149, 163]. In 2003, the transcription factor forkhead box protein P3 (Foxp3) has been demonstrated to be the key regulator and is required for Treg development and function in thymus as well as in periphery[77, 164, 165]. This comes from the finding that in the 'scurfy' mouse and in humans carrying a mutation in the gene encoding Foxp3 suffers from severe autoimmune manifestations due to impaired Treg induction[166, 167].

TCR interaction with self-peptide-MHC complexes in the thymus is regarded as the essential driving force for thymocytes development, this raises the interesting question how these autoreactive thymocytes avoid clonal deletion and deviate into the Treg lineage? Except the affinity model, several studies indicate that avidity might play a role in thymic selection. Direct evidence for autonomous, DC-independent contribution of mTECs in both negative selection and deviation of Tregs was observed using mTEC-specific Ciita silencing in the TCR-HA x Aire-HA model[131, 168]. In C2TAkd mice, the presentation of mTEC is diminished to about 10% of the wide type amounts. For mTECs mediated negative selection, C2TAkd mice have enlarged polyclonal CD4SP population (increase of -20%) and enhanced selection of Tregs (increase of -46%)[102]. Thus, how Tregs are rescued from clonal deletion and what particular features of APC are needed for Treg differentiation remains to be determined. In addition to TCR signals, CD28-B7 signaling has a cell-intrinsic role in Treg differentiation. With genetic ablation of either CD28 or its ligands, thymic Tregs were strongly reduced in these mice[169, 170]. Cytokine signaling co-operating with a TCR stimulus was found to crucially contribute to the maintenance and survival of Tregs. IL-2//IL-15- or STAT5-deficiency in mice will inhibit effector T cell proliferation, and therefore, will hinder an immune response[171-174]. Furthermore, it has been proposed that TGFβ signaling is required for Treg generation, a requirement that is later compensated for by IL-2. However, the combined deficiencies in both TGF-β and IL-2 signaling led to the complete absence of thymic Tregs[175].

1.3 Peripheral tolerance

Although central tolerance very efficiently deletes T cell precursors whose TCRs

have high avidity for self-pMHC complexes expressed on DCs and mTECs, it is an imperfect process. In part because not all self-antigens are expressed in the thymus, and by the existence of self-reactive, functional T cells in the periphery could induce autoimmune disease both in human and mice. Therefore, the T-cell selection process does not end with emigrating in the thymus, rather T cells undergo further selection process after entering the periphery to maintain unresponsiveness to self-antigens that are expressed outside of the thymus. The escaping autoreactive T cells are controlled by peripheral tolerance mechanisms that mainly include the functional unresponsiveness of anergic T cell, deletion of peripheral T cells, ignorance and regulatory T cells conversion[176] (Figure 3). Thus, multiple mechanisms will help to control T-cell responses and maintain tolerance in the periphery.

1.3.1 Anergy

T cells encounter with self-antigen might lead to intrinsic functional inactivation, but these cells remain alive in a long-term hyporesponsive state, termed as anergy[177]. Anergic T cells are characterized by a variety of functional limitations, including cell differentiation, cell division and cytokine production. The anergic state of $CD4^+$ T cells can be induced through TCR ligation in the absence of co-stimulation or high in co-inhibition signals such as cytotoxic T lymphocyte antigen-4 (CTLA-4)[178]. Co-stimulation provides a second signal to T cells in conjunction with signaling via their TCR upon recognition of antigen presented by MHC. However, costimulatory signals can also function as a negative regulator that inhibit T cell responses and mediate tolerance[179]. The CD28/B7 pathway of co-stimulation is critical in preventing anergy induction[180]. The development of anergic T cells is antagonized by CD28 signaling, which induces copious amounts of IL-2 and facilitates subsequent PI3K/AKT-mTOR dependent anergy reversal. Nonetheless, the role of CD28 ligands co-stimulatory ligands B7-1 (CD80) and B7-2 (CD86) in the induction of anergy is still unclear. McConnell et al. demonstrated that blocking the CD80 and CD86 inhibited tolerance instead of promoting it, was resolved by the observation that CTLA-4 engagement was required to induce anergy *in vivo*[181, 182] (Figure 3).

CTLA-4, express at a late stage in T cell activation, binds to CD80 and CD86 with higher affinity than CD28, which plays an essential role in maintaining

unresponsiveness. Genetic CTLA-4 deficiency shows autoimmunity and lethal lymphoproliferative disorders[183-185]. Notably, Ctla4$^{-/-}$ CD4^{+} T cells and wild-type T cells resist anergy induction when these cells are treated with CTLA-4-specific mAb following soluble antigen administration in the absence of adjuvant or infection[186]. Although results from Wing et al showed that CTLA-4 is required for natural Tregs to suppress immune responses, adoptive transfer of OVA-specific CD4^{+} T cells from CTLA-4$^{-/-}$ DO11.10 Tg mice into RIP-mOVA Rag^{ko} recipients induce acute insulitis and diabetes, whereas CTLA-4$^{+/+}$ DO11.10 T cells are unable to break tolerance[187].

Programmed death 1 (PD-1) molecular is an immunoinhibitory receptor, as another candidate for regulating anergy induction. Animals deficient for PD-1 or its ligand PD-L1 and PD-L2 exhibit a breakdown of peripheral tolerance and demonstrate autoimmune disorders[188-190]. PD-1 signaling can inhibit cytokine secretion as well as block tissue migration 'stop signals' that are necessary for productive TCR engagements. As more members of the CD80/CD86 family and their rececptors emerge[191], we might have a better chance to discover how T cell anergy is controlled through TCR and other cell-surface receptors.

Tolerogenic DCs sample self-antigens and present it to antigen-specific T cells but cannot deliver adequate costimulatory signals inducing anergic and IL-10-producing T cells with regulatory properties. In an immunosuppressive environment, immunosuppressive cytokines IL-10 and TGF-β support the generation of tolerogenic DCs[192]. Mature DCs efficiently initiate effector T cell response, while immature DCs are involved in silencing T cell-mediated immune responses[193]. In steady state, it is believed that tolerogenic DCs are generated by incomplete maturation. On CD4^{+} T cells, the expression of ICOS, an activation-induced member of the CD28 family, which can also contribute to induction of anergy. The mechanism by which DCs promote tolelogenic responses involves the express of the enzyme indoleamine 2,3-dioxygenase (IDO), which is induced through ligation of CTLA-4 by CD80/CD86. On one hand, IDO catalyzes the degradation of the essential amino acid tryptophan, which leads to the inhibition of T cell proliferation. On the other hand, IDO^{+} regulatory DCs and Tregs might interact and suppress local T-cell responses and promoting systemic tolerance[194].

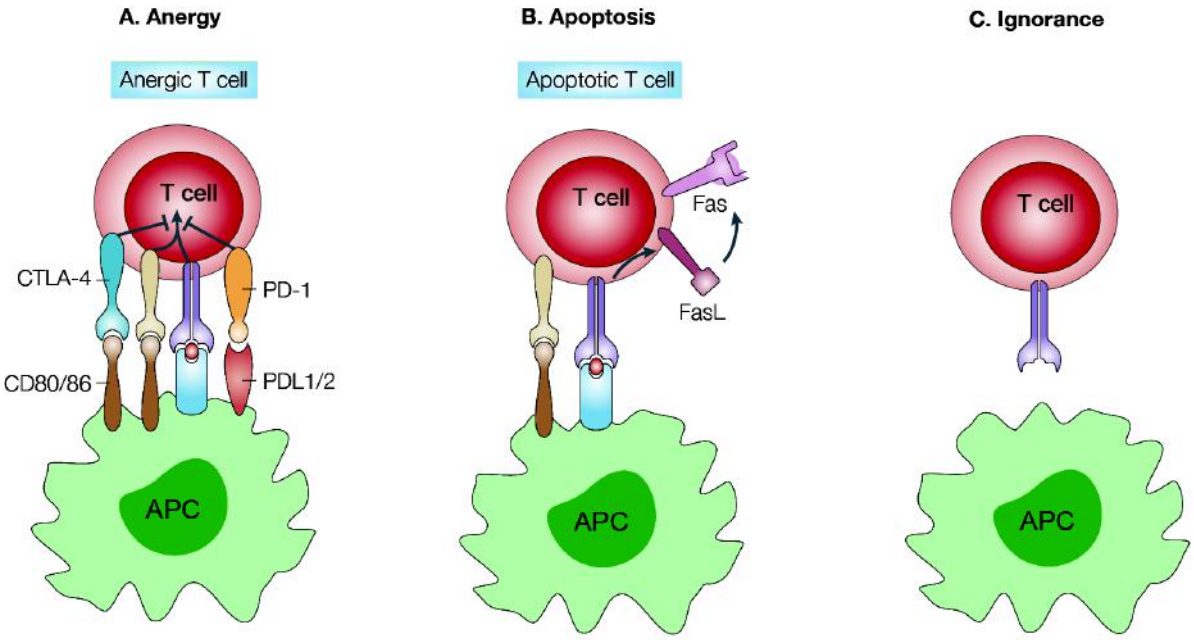

Figure 3. Mechanisms to maintain peripheral tolerance. A. T cells encounter with self-antigen might lead to intrinsic functional inactivation, termed as anergy, possibly involving interaction of the T-cell molecules such as CTLA-4 or PD-1 with their ligands (CD80/86, PDL1/2). B. Self-reactive lymphocytes engaged by self-pMHC complexes die by apoptosis, which occurs through a combination of the death receptor Fas and its ligand, FasL. C. Naïve self-reactive autoaggressive T cells might never encounter the self-protein they recognize, termed as immunologic ignorance. CTLA-4; cytotoxic T-lymphocyte-associated antigen 4; PD-1; programmed cell death 1; PDL, PD-1 ligand; FasL, Fas ligand (Figure modified from Walker et al.[176]).

1.3.2 Peripheral deletion

Another important mechanism to maintain peripheral tolerance is peripheral deletion. Self-reactive lymphocytes engaged by self-pMHC complexes die by apoptosis, a process called 'Activation induced cell death' (AICD) which occurs through a combination of the death receptor Fas (CD95) and its ligand, FasL (CD178)[195, 196] and Bim-dependent triggering of the Bcl-2 and Bcl-xL-mediated mitochondrial pathway of apoptosis (Figure 3). Although Fas- and Bim-mediated AICD are mechanistically different, these pathways are coordinated and cooperate in killing mature T cells that are stimulated by self-antigens.

Surprisingly, AICD of peripheral T cells is regulated by the Fas signaling pathway which is enhanced by IL-2. IL- 2 is traditionally thought to be a survival and growth-promoting cytokine[197]. Interest in this pathway came from the observation that T cells

from two strains of mice with defects in Fas (Fas^{lpr}MRL mice) and FasL (gld mice) fail to undergo peripheral deletion and develop spontaneously lymphoproliferative disease[198]. Interestingly, some studies indicated that the death of activated T cells during the shutdown of an acute immune response is mediated Bim, but not Fas[199, 200]. Bim is a natural antagonist of the survival protein Bcl2. Bim binds and activates Bax and Bak, lead to the permerbilization in the mitochondrial outer membrane and subsequent caspase activation and what eventually leads to cell death. Experiments with Bim-deficient mice have shown that antigen-specific T cells accumulation in the spleen and lymph node as well as development of autoimmunity[201]. Thus, for at least some self-pMHC complexes, the induction of peripheral deletion is an important contributor to peripheral tolerance.

1.3.3 Ignorance

Naïve self-reactive autoaggressive T cells are readily found in disease-free individuals, termed as immunologic ignorance, and establishes a barrier to self-pMHC complex recognition. This situation is thought to result mainly from the physical segregation of autoreactive T cells from most non-lymphoid tissues. The low expression level of the target autoantigen does not reach the threshold and/or the avidity of the T cells that are specific for a given autoantigen is too low, which is required to trigger a T-cell response (Figure 3). In a pioneering study of the development of murine diabetes, where they showed antigen-presenting cells are absent or deficient in the connective tissue between the blood vessles and the islets, T cells do not encounter pancreatic antigen in processed and recognized form[202]. Nevertheless, by peripheral immunization of rodents with organ-specific self-peptides has shown that the reversal of ignorant reigns could lead, in susceptible strains, to the development of organ-specific autoimmune disease[203].

1.4 Experimental Autoimmune Encephalomyelitis

Animal models have been used extensively in investigating molecular mechanisms of neuroinflammation and development of new therapeutic options. Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system[204]. The classical animal model to mimic MS-like symptoms in the CNS is experimental

autoimmune encephalomyelitis (EAE), which can be induced either by injecting myelin sheath protein with Complete Freund's Adjuvant (CFA) or by passive transferring Th1 cell lines specific for the myelin proteins into susceptible animals. In addition, spontaneous models that make use of T cell receptor (TCR) transgenic T cells exist[205-207]. The genetic background of mouse strains and the nature of proteins of the CNS used for immunization determine the pathology and the disease course. In EAE, the three main myelin proteins, including proteolipid protein (PLP), myelin basic protein (MBP), and myelin oligoglycoprotein (MOG) have been shown to induce autoaggressive T cells.

1.4.1 Target autoantigen-Proteolipid protein (PLP) in EAE

The most abundant CNS myelin protein proteins are PLP constituting 50% of whole myelin proteins composition. PLP is a highly hydrophobic and integral transmembrane protein of the myelin membrane and encoded on the X chromosome[208]. In mice, two main transcripts of PLP has been described: one encodes for the full-length 276 amino acid isoform, the other DM20 isoform lacks the residues 116-150 in the cytosolic loop of PLP. In different species, PLP/DM20 is highly conserved in its amino acid sequence, which suggests that the protein plays an important role in forming myelin sheaths[209] (Figure 4). The differential peripheral expression of one major encephalitogenic and immunodominant $PLP_{139–151}$ peptide that is present in full-length PLP, but is absent in the splice variant of PLP-DM20, results in the escape of $PLP_{139-151}$ reactive cells from central tolerance[210]. Furthermore, SJL/J TCR transgenic mice specific for PLP do develop spontaneous disease[206].

Naturally occurring mouse mutants such as Jimpy and the $Jimpy^{msd}$ mouse, or the myelin-deficient rat, exhibited myelin defects such as dysmyelination and hypomyelination[211]. In other species, some of these myelin defects resulted in Pelizaeus-Merzbacher (PMD) and the X-linked spastic paraplegia (SPG-2) disease[212, 213]. Due to point mutations, the primary structure of PLP is altered. This leads to misfolded polypeptides are incapable of exiting form intracellular compartment, interfering with oligodendrocyte differentiation and its survival. Surprisingly, mutant mice that lack expression of a targeted PLP gene do not

develop the known dysmyelinated phenotype[214].

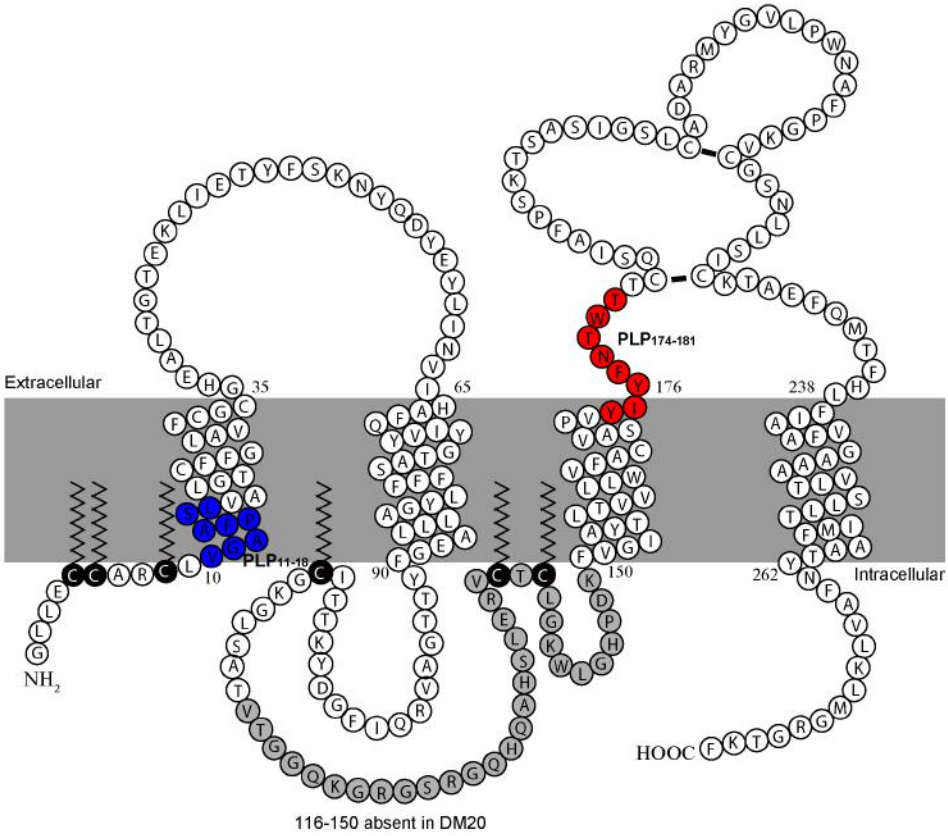

Figure 4. Topoloy representation of PLP and its isoform DM20. PLP is a highly hydrophobic and integral transmembrane protein of the myelin membrane[204]. In different species, PLP/DM20 is phylogenetically highly conserved in its amino acid sequence[205]. The amino acids of the molecule that are absent in the splice variant of DM20 (residues 116–150) are shaded dark grey. The core position of PLP_{11-18} (Plp1) and $PLP_{174-181}$ (Plp11) is highlighted in blue and red, respectively. Figure modified from Greer et al.[215].

1.4.2 Central tolerance to PLP

The expression of PLP might play an important role in the induction of central tolerance to PLP. A number of studies identified that the expression of full-length PLP is mainly located to the brain and spinal cord, whereas the DM20 isoform is predominantly expressed in peripheral lymphoid organs[216]. It has been identified that immunization with synthetic PLP epitopes can induce EAE in several strains of mice[217]. In the SJL/J (H-2^s) strain, there are two major encephalitogenic epitopes of PLP, $PLP_{139–151}$ and $PLP_{178-191}$. Both of these epitopes bind highly with I-A^s molecule, but the immune response to $PLP_{139–151}$ is dominant. Immunization of SJL/J mice with

either of these two epitopes can induce EAE, indicating that low-affinity binding of these autoantigenic peptides to I-A^s molecule or formation of unstable pMHC complexes could form the autoreactive repertoire in the periphery[218]. Actually, different mouse strains show differences in their susceptibility to EAE. Whereas SJL/J mice are highly susceptible, C57BL/6 strain (H-2^b) are relatively resistant to the development of EAE when immunizing them with the same protein/peptide[217].

Using thymus transplantation experiments in B6 PLP^{KO} animals, Klein et al. showed that the intrathymic expression of PLP in radioresistant thymic stromal cells is sufficient for tolerance induction[219]. In the C57BL/6 strain, the full spectrum of the immunogenic regions of PLP in the context of H-2^b was identified. Previous work was done by Klein et al. where they immunized B6 PLP^{WT} and B6 PLP^{KO} mice with purified PLP protein and subsequently re-stimulated the primed lymph node T cells with a set of overlapping peptides (24 amino-acids in length) which span the whole PLP protein with a shift of 16 amino-acid residues, revealed four immunogenic regions of PLP react against $CD4^+$ T cells. When the authors used 8-12 amino acids to re-stimulate the draining lymphocytes further indicated four core-epitopes in the context of H-2^b, termed PLP_{11-18} (Plp1), $PLP_{174-181}$ (Plp11), $PLP_{205-213}$ (Plp13), and $PLP_{240-247}$ (Plp15). Importantly, subsequent to immunization of B6 PLP^{WT} animals with whole protein, restimulation with the individual core regions demonstrated a residual response to the epitope Plp11, indicating incomplete tolerance towards this region. By contrast, no detectable recall response could be elicited against other three identified epitopes (Figure 5). Indeed, immunization of C57BL/6 mice with epitope Plp11 can induce EAE, consistent with this other three epitopes fail to induce EAE (unpublished data).

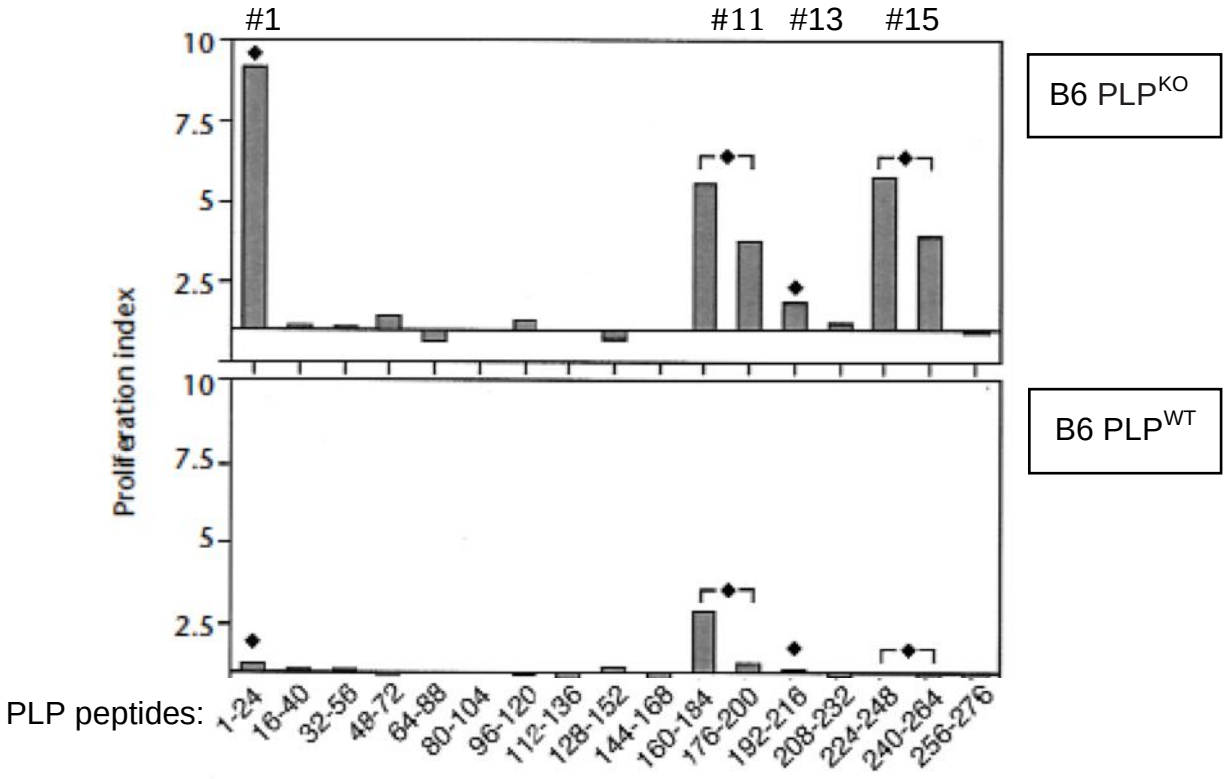

Figure 5. The four immunogenic regions of PLP react against CD4$^+$ T cells in the context of H-2^b. B6 PLP^{WT} and B6 PLP^{KO} mice were immunized with purified PLP protein and subsequently re-stimulated the primed lymph node T cells with a set of overlapping peptides (24 amino-acids in length) which span the whole PLP protein with a shift of 16 amino-acid residues, revealed these four immunodominant regions. Tolerance induction to PLP epitope1 was very efficient, while tolerance induction to PLP epitope 11 is leaky in B6 PLP^{WT} mice. Figure adapted from Klein et al. [219].

1.5 Aim of the thesis

This study was undertaken with the following objectives:

1) Why tolerance induction to $PLP_{174-181}$ is leaky in B6 WT mice?
2) How do $PLP_{174-181}$-reactive T cells escape thymic deletion?
3) How is tolerance to PLP maintained in the presence of $PLP_{174-181}$ specific T cells?
4) Whether $PLP_{174-181}$-specific T cells have different cell fates compared with PLP_{11-18}-specific T cells?
5) What are the contributions of the individual thymic antigen presenting cell types to central and periphery tolerance to PLP?

2. Results

2.1 Lack of tolerance induction to a self-antigen in the central nervous system

2.1.1 Generation of a $PLP_{174-181}$-specific TCR-transgenic mouse

One of the dominant PLP epitopes in H-2^b mice is contained within the amino acid sequence 174-181 of PLP. T cells recognizing this epitope are restricted to antigen recognition in the context of MHC class II I-A^b molecules. Previous research has shown that a fraction of CD4 T cells specific for this region was not tolerized against $PLP_{174-181}$ and induced autoimmunity upon EAE induction[219]. We sought to determine the mechanisms of tolerance induction towards $PLP_{174-181}$, therefore, we have constructed a transgenic mouse expressing genes encoding a rearranged T cell receptor specific for $PLP_{174-181}$.

2.1.1.1 Production of T Cell hybridomas specific for $PLP_{174-181}$ peptide

In order to obtain a TCR specific for $PLP_{174-181}$, we produced $PLP_{174-181}$-specific T cell hybridomas. To do so, lymph node cells of PLP^{KO} mice 9 days after immunization with the 24-mer peptide $PLP_{160-184}$ in CFA were stimulated *in vitro* with $PLP_{174-181}$ peptide. Subsequently, antigen-specific T cells were restimulated with irradiated spleen cells and antigen. After one round restimulation *in vitro*, T cell blasts were fused with BW5147 cells in order to generate T cell hybridoma. BW5147 cells lack functional TCR-α and -β genes, and have been used to analyze the specificity of TCRs expressed by heterogeneous populations of T cells[220]. Once produced, the T cell hybridomas were cloned and screened for specificity and expression of antibody stainable TCR variable Vα and Vβ regions. The clone A43-11-5 was chosen for the generation of the TCR transgenic mouse after being tested for specificity and TCR-α and -β expression.

2.1.1.1.1 A43-11-5 hybridoma is specific for PLP protein and $PLP_{174-181}$ peptide

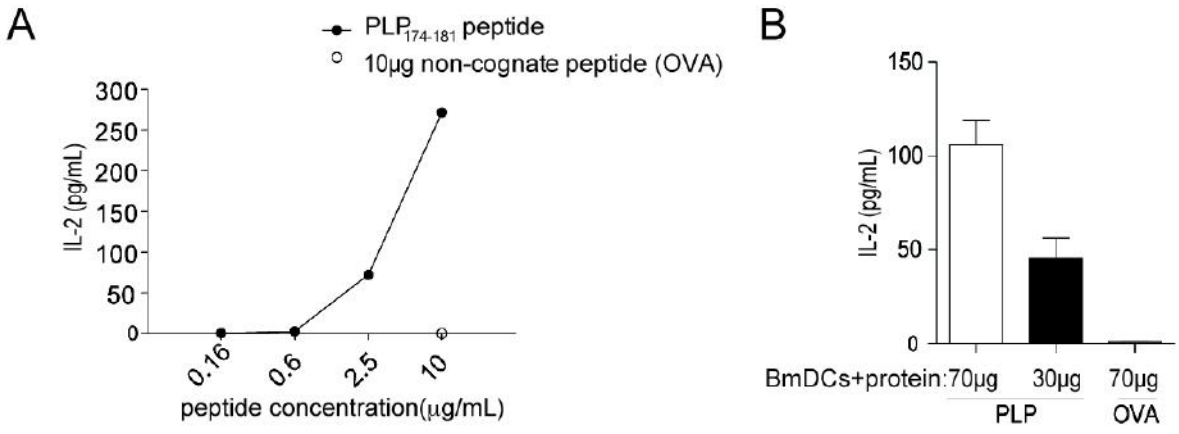

Figure 6. Reactivity of A43-11-5 hybridomas against self-MHC-peptide ligands. A) IL-2 production of A43-11-5 hybridomas (10^5 cells/well) was measured after 48 hours incubation in wells coated with $PLP_{174-181}$ peptide together with splenocytes (10^6 cells/well), as described in Materials and Methods. A43-11-5 hybridoma responded highly to stimulation with its cognate antigen in a dose-dependent manner. The hybridoma cells were stimulated with OVA as negative control. B) IL-2 secretion of A43-11-5 hybrid T cells were stimulated with PLP protein and OVA protein, respectively. The T cell hybrid responded to stimulation with PLP protein in correlation with the amount of protein that was given, but not to stimulation with OVA protein. The results are representative of at least three independent experiments.

One crucial criteria for the selected T cell hybridoma clone A43-11-5 was its specificity for $PLP_{174-181}$. To test this, we stimulated the hybridoma clone with titrated concentrations of $PLP_{174-181}$ peptide and measured IL-2 secretion. The result in Figure 6A demonstrates a dose-dependent reactivity and specificity for the desired peptide. As expected, cells did not produce significant levels of IL-2 in response to stimulation with non-cognate peptide (OVA), showing its specificity to $PLP_{174-181}$. Subsequently the A43-11-5 clone was also tested for the capacity to specificaly recognice PLP protein. For that bone-marrow derived dendritic cells (BmDCs) were pulsed with PLP protein and control OVA protein respectively and co-cultured with the A43-11-5 clone *in vitro*. Supernatants were collected 48 hours later and IL-2 cytokine levels were measured. As shown in Figure 6B, PLP protein specifically induced the production of IL-2. The strength of the stimulation response correlated to the amount of PLP protein given to the BmDCs culture. In contrast, antigens that did not specifically interact with the hybridoma, such as OVA, did not induce production

of the IL-2. Taken together, the A43-11-5 hybridoma clone was specific to PLP protein as well as to the relevant $PLP_{174-181}$ peptide.

2.1.1.1.2 A43-11-5 hybridoma expresses TCRAV2 and TCRBV14

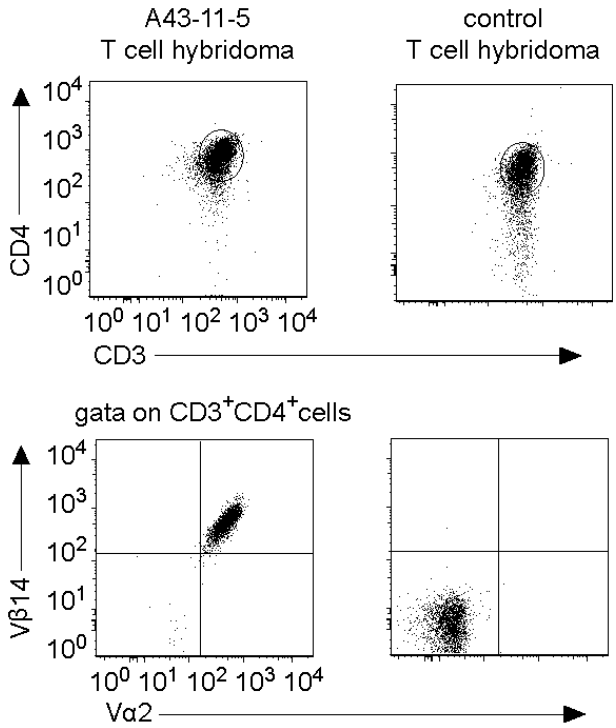

Figure 7. Flow cytometry staining of the TCR of T cell hybridoma clone A43-11-5. A43-11-5 clone express TCRAV2 and TCRBV14 at their surface.

To be able to visualize the transgenic T cells in $PLP_{174-181}$ transgenic mice, the transgenic TCR must be stainable by available TCR antibodies. To that end hybridomas were analyzed for the expression of CD4 and TCR using flow cytometry showing that the $PLP_{174-181}$-specific A43-11-5 clone expressed both the TCRAV2- and TCRBV14-TCR gene segments to which specific antibodies were commercially available (Figure 7). A hybridoma, which did not express a known TCR at its surface, was used as negative control. The ability for staining both TCR variable chains via antibodies, facilitated to trace the fate of transgenic T cells in the TCR-PLP11 mouse by flow cytometry.

Taken together, the T cell hybridoma clone A43-11-5 was an optimal candidate for the generation of the $PLP_{174-181}$-specific TCR-transgenic mouse since it was highly

responsive and specific to the relevant $PLP_{174-181}$ peptide (Figure 6), and stainable by available of TCR-α and-β antibodies (Figure 7).

2.1.1.2 Cloning of full-length TCR pairs used by the $Vα2^+Vβ14^+$ $PLP_{174-181}$-specific T cell hybridoma

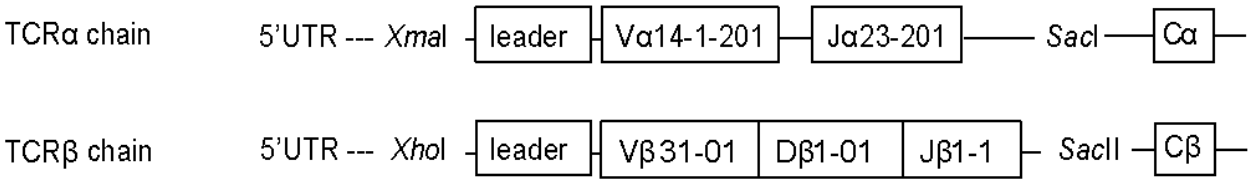

Figure 8. Diagram depicting cassette vectors pTα and pTβ. pTα/pTβ cassette vectors contain V-region (Vα and Vβ, respectively) promoter and the complete constant-region (Cα and Cβ, respectively) gene sequences[221]. Vector sequences were removed by a combined *Xma*I and *Sac*I restriction enzyme digest for TCRα chain and by a combined *Xho*I and *Sac*II restriction enzyme digest for TCRβ chain.

To generate a transgenic mouse line that expresses a TCR recognizing $PLP_{174-181}$ peptide in association with $H\text{-}2^b$, genomic DNA fragments, including rearranged $TCRV_α\text{-}J_α$ and $TCRV_β\text{-}D_β\text{-}J_β$ sequences, were obtained from A43-11-5 hybridoma DNA, which has TCR complexes composed of Vα14-1-201 and Jα23-201 for the α chain, and Vβ31-01, Dβ1-01, and Jβ1-1 for the β chain, which were annotated in *Ensembl* [www.ensembl.org]. Using primers complementary to the upstream region of the TCRVα14-1-201 gene segment and to the downstream UTR of the TCRJα23-201 gene segment, the rearranged TCRVα-Jα gene segments were cloned and *Xma*I and *Sac*I restriction sites were induced, respectively. Similarly, the TCRβ Chain was cloned using *Xho*I and *Sac*II restriction sites, respectively. These Vα14-1-201 and Jα23-201 and Vβ31-01, Dβ1-01, and Jβ1-1 PCR products were then subcloned into unique pTα and pTβ cassette expression vectors, containing V-region (Vα and Vβ, respectively) promoter and the complete constant-region (Cα and Cβ, respectively) gene sequences[221], generating a pTα and pTβ DNA transgene expression constructs, respectively (Figure 8). The designation Vα2 and Vβ14, which were mentioned below, refer to the antibodies that specifically stained the rearranged Vα- and Vβ-regions, respectively.

2.1.1.3 *In vitro* verification of the expression and functionality of the TCR-PLP11 cassette vectors

In order to test expression and functionality of the cloned Vα2 and Vβ14 cassette vector respectively, the recombinant pTα-PLP11 and pTβ-PLP11 vectors were digested with *Sac*I and *Kpn*I, respectively and linearized prior to microinjection of TCR α and β gene constructs into fertilized eggs of C57BL/6 mice.

Surface expression on 293 T cells

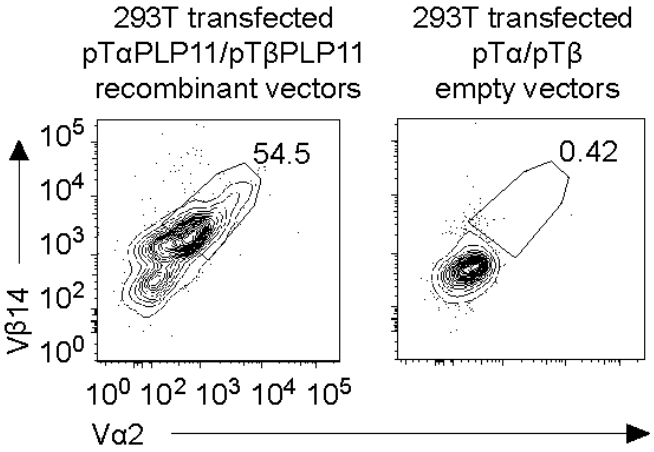

Figure 9. 293T cells transfected with linearized pTα-PLP11 and pTβ-PLP11 vectors or the empty pTα/pTβ cassette vectors were stained with mAbs against TCR Vα2 and Vβ14. Flow cytometry showing the expression of cloned TCR on the surface of transfected 293T cells.

The linearized recombinant pTα-PLP11 and pTβ-PLP11 vectors were transiently co-transfected at a ratio of 1:1 into 293T cells with the calcium phosphate method. Vα2 and Vβ14 expression (54.5%) were detectable on the surface of HEK293T cells after transfection (Figure 9). Signals of the TCR expression was not detected on the cell surface of HEK293T cells transfected with empty pTα/pTβ cassette vectors.

Specificity

To further test the functionality and specificity of the recombinant vector, A5 T-cell hybridomas were transduced with linearized pTα-PLP11 and pTβ-PLP11 vectors encoding Vα2 and Vβ14, respectively. A5 is a derivative of the T helper line 16.2, which is specific for a hemagglutin in peptide of influenza virus presented by class II I-E^d MHC molecules[222]. These $CD4^+$ T cells also contained an NFAT (Nuclear Factor of Activated T cells) linked to green fluorescent protein (GFP) and therefore, NFAT-activation could be determined by analyzing induction of GFP expression[223]. Stable transfectants were selected in medium containing puromycin and were subsequently screened for the expression of the introduced TCR on the surface. In TCR-PLP11 transfected A5 T cell hybridoma cells, more than 80% of cells expressed $PLP_{174-181}$-specific TCR was detected by staining with antibodies for TCR Vα2 and TCR Vβ14 (Figure 10A). Among the $Vα2^+Vβ14^+$ cells, transfected hybridomas showed antigen-induced NFAT activity in the presence of $PLP_{174-181}$ peptide as detected by GFP expression (Figure 10B). Taken together, recombinant pTα-PLP11 vector and pTβ-PLP11 vector have been tested successfully for functionality of the TCR and reactivity to $PLP_{174-181}$.

These constructs were co-microinjected into fertilized C57BL/6 eggs to generate TCR-PLP11 transgenic mice. Offspring were screened by PCR and transgenic offspring was crossed to PLP^{KO} mice to generate TCR-PLP11 PLP^{KO} mice.

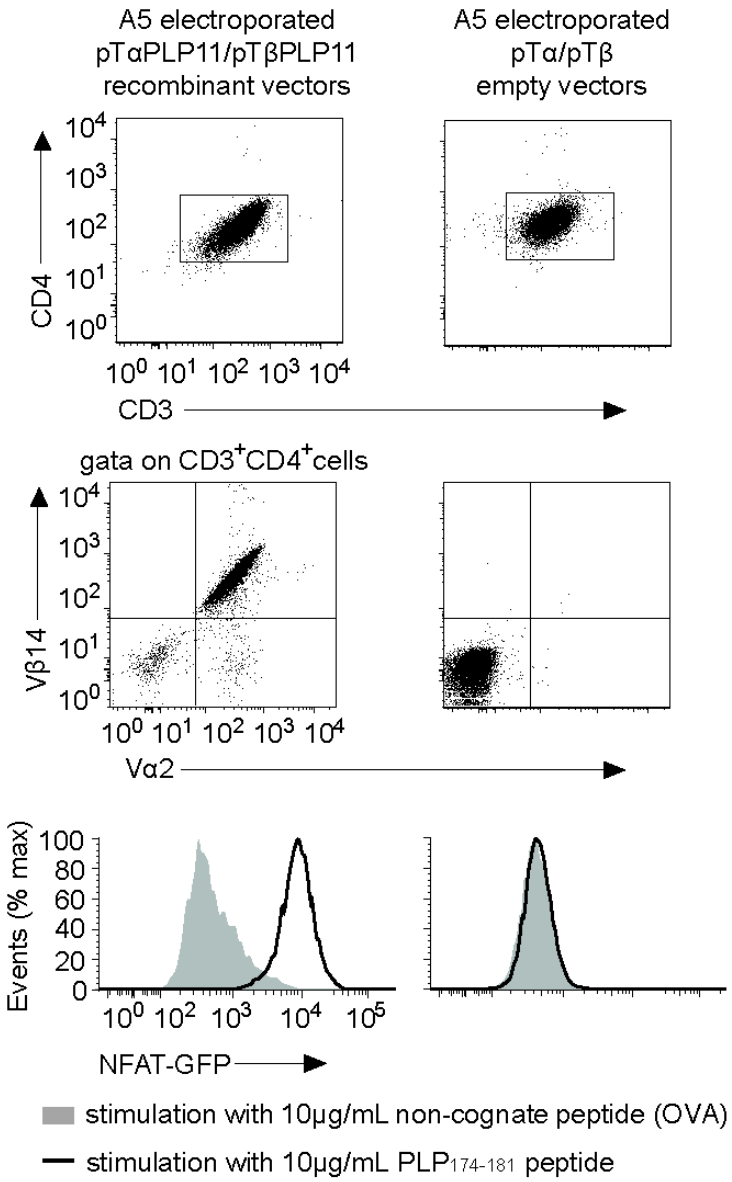

Figure 10. Flow-cytometric analysis of $PLP_{174-181}$-specific TCR expression and GFP expression in A5 cells by electroporation with linearized pTα-PLP11 and pTβ-PLP11 vectors. The transfected A5 cells were stimulated with non-cognate peptide used as negative control. A) TCR-PLP11 transfected A5 cells showed expression of the $PLP_{174-181}$-specific TCR ($Vα2^+$ and $Vβ14^+$) on the cell surface. B) When TCR-PLP11 transfected A5 cells were stimulated with $PLP_{174-181}$, leading to activation of the hybridoma cells, this is translated into an NAFT driven GFP expression. The transfected A5 cells did not respond to non-cognate peptide (OVA), confirming the functionality and specificity of the pTα-PLP11 and pTβ-PLP11 cassette vectors.

2.1.2 Analysis of T cell subsets in TCR-PLP11 mice

We studied the thymocyte cellularity and the developmental cell subsets from TCR-PLP11 Tg mice to determine whether thymic clonal deletion participated to PLP tolerance. Central tolerance eliminates thymocytes that recognize self-peptide: MHC avidly. Thymic cellularity of TCR-PLP11 PLP^{WT} thymus was 161 × 10^6 cells, on average, which was comparable to that of TCR-PLP11 PLP^{KO} thymus (126 × 10^6) (Figure 11A). Morover, the percentage of single positive $CD4^+$ thymocytes (30%, on average) was similar in the presence and absence of cognate antigen. The frequency and number of cells in each thymic developmental subset (DN, DP, CD8SP, CD4SP) were undistinguishable in TCR-PLP11 Tg mice (Figure 11B).

Furthermore, CD4SP thymocytes in both groups equally expressed the transgenic TCR (Figure 11C). Within the transgenic CD4SP population no difference was observed with respect to the maturation stage of those cells in PLP^{WT} and PLP^{KO}. (Figure 11D). Thus, despite the expression of the PLP self-antigen in the thymus, there was no evidence for intrathymic deletion or modulation of TCR expression of PLP-specific $CD4^+$ T cells in TCR-PLP11 PLP^{WT} mice. We also examined the development of TCR-PLP11 Tg $Foxp3^+$ regulatory T cells, however also this subset was not affected by the presence of PLP in the thymus. Thus no Plp11-specific Treg cells were induced in PLP^{WT} mice.

Taken together, by comparing T cell development of TCR-PLP11 Tg mice in the presence and absence of the cognate self-antigen PLP, we can conclude that there is no central tolerance induction to PLP in TCR-PLP11 mice.

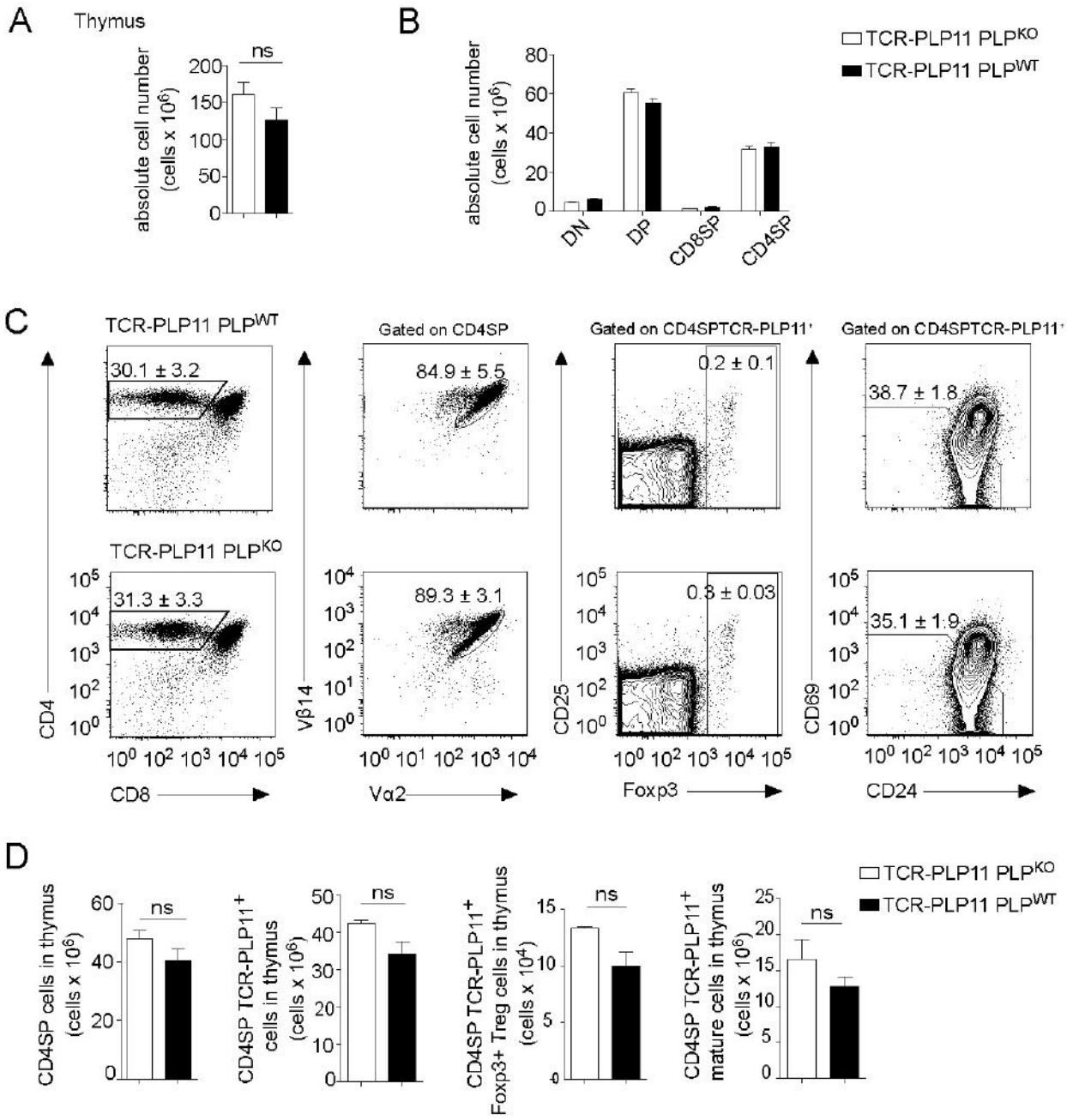

Figure 11. Flow cytometry analysis of the thymocytes of TCR-PLP11 PLP^{WT} and TCRPLP11 PLP^{KO} mice. A and B) The absolute numbers of the total thymus (A) and the absolute number of double negative (DN), double positive (DP), CD4 single positive (SP), CD8 single positive (SP) in TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} mice at 3 weeks with the standard error of the mean (SEM). A) No difference was observed in total thymic cellularity in TCR-PLP11 PLP^{KO} and TCR-PLP11 PLP^{WT} mice. (B) Absolute cell number of thymocytes in the respective compartment of T cell development: DN, DP, CD8SP and CD4SP cells were also demonstrated no difference. C and D) Thymocytes were enumerated and analyzed by flow cytometry. The percentage of thymocytes in each subset is indicated for each quadrant (C) and in absolute cell numbers (D). Anti-CD4 and CD8 staining of thymocytes (first column), level of expression of the TCR Vα2Vβ14 on CD4SP cells (second column), Foxp3^{+} Tregs in the TCR-PLP11^{+} cell population (third column) and mature cells within the TCR-PLP1^{+} cell population in the thymus (fourth column). It indicated that absence of thymic deletion in TCR-PLP11 PLP^{WT} mice.

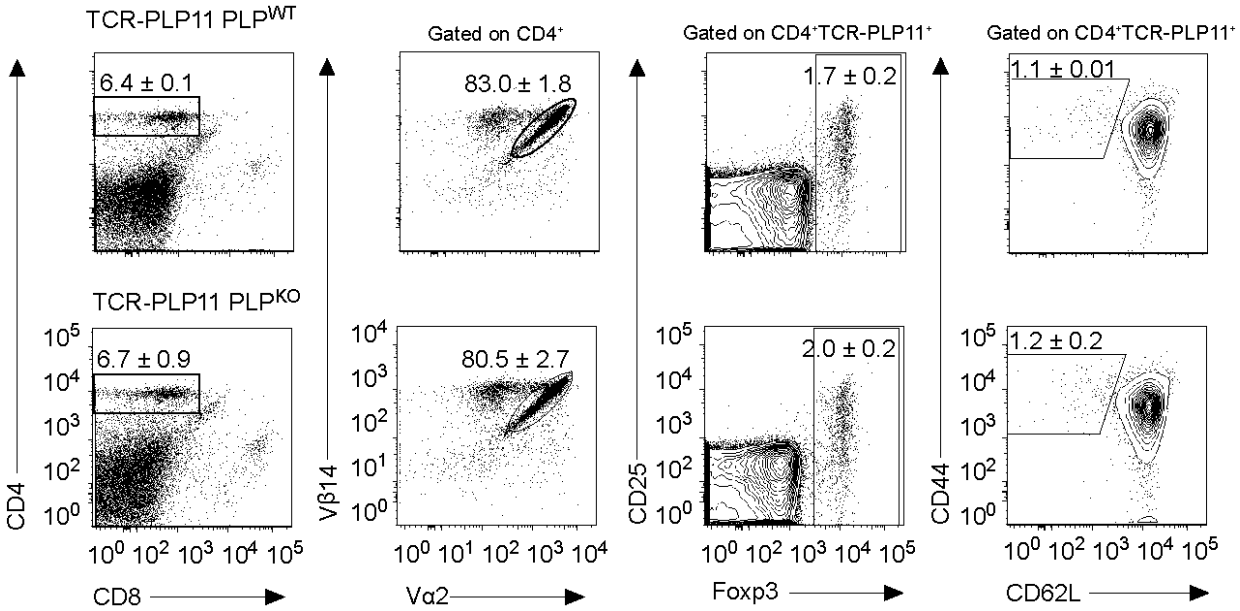

Figure 12. Flow cytometry analysis of peripheral phenotype in PLP-TCR11 transgenic mice. Anti-CD4 and CD8 staining of splenocytes (first column) from TCR-PLP11 PLP^{KO} (n=8) and TCR-PLP11 PLP^{WT} mice (n=8), level of expression of TCR Vα2Vβ14 on $CD4^+$ T cells (second column), $Foxp3^+$ Tregs in the TCR-$PLP11^+$ cell population (third column), and $CD62L^-CD44^{hi}$ antigen experienced T cells in the TCR-$PLP11^+$ cell population (fourth column). It indicated that no deletion of PLP-specific T cells in TCR-PLP11 PLP^{WT} mice. The numbers above the gates represent the mean average ± the standard error of the mean (SEM).

In the periphery, presentation of autoantigen can lead to the deletion of autoreactive T cells[224, 225]. We thus analyzed the frequency and number of $CD4^+$ subset in spleen of TCR-PLP11 Tg mice. Our results showed that the proportion, as well as the absolute number of $CD4^+$ T lymphocytes present in peripheral lymphoid organs is not different between TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} mice, and that these cells express similar levels (83.0 ± 1.8% vs 80.5 ± 2.7%) of the transgenic TCR (Figure 12). Again, a very small population of Plp11-specific Tregs were detectable in the periphery of TCR-PLP11 Tg PLP^{WT} and PLP^{KO} mice. Moreover, $CD4^+$ TCR-$PLP11^+$ T cells appeared naïve, as they displayed mainly a $CD62L^{high}CD44^{low}$ phenotype (Figure 12). Therefore, there is no deletion of PLP-specific T cells in TCR-PLP11 mice, and central or peripheral deletion is not the main tolerance mechanism operating in TCR-PLP11 Tg mice.

2.1.3 Transgenic T Cells from TCR-PLP11 Tg mice proliferate in response to $PLP_{174-181}$

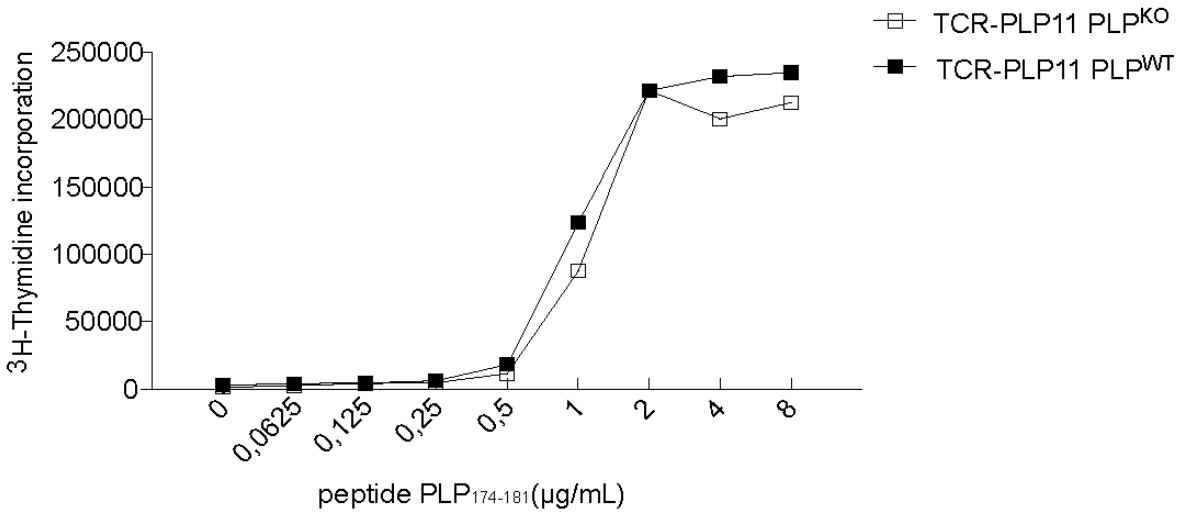

Figure 13. Proliferation of Plp11-specific T cells in response to $PLP_{174-181}$. Plp11-specific T cells were established from TCR-PLP11 Tg mice and cultured with spleen cells with $PLP_{174-181}$ with different concentration for 48 hours, and ^3H thymidine uptake was measured over an additional 16 hours.

To determine whether Plp11-specific T cells can recognize naturally processed PLP peptides, as well as exogenously added $PLP_{174-181}$ in the context of $I\text{-}A^b$, splenocytes from TCR-PLP11 PLP^{WT} mice and TCR-PLP11 PLP^{KO} mice were cultured with $PLP_{174-181}$ peptide. Splenocytes isolated from TCR-PLP11 mice proliferated in response $PLP_{174-181}$. Dose response proliferation assays revealed no difference in the dose-response curve of TCR-PLP11 PLP^{WT} mice and TCR-PLP11 PLP^{KO} mice resepectively (Figure 13). Our results indicated that Plp11-specific $CD4^+$ T cells were functional in response to the cognate antigen *in vitro*.

2.1.4 Plp11-specific T cells can proliferate specifically *in vivo*

Because Plp11-specific T cells are not deleted, we speculated that one possible mechanism of tolerance in our mouse model was ignorance, defined by the non-detection of Ag by the immune system. We thus investigated whether the specific $PLP_{174-181}$ peptide was readily available for TCR recognition in the periphery. To this end, we performed adoptive transfer of purified CFSE-labeled $CD4^+$ T cells from TCR-PLP11 PLP^{WT} mice or TCR-PLP11 PLP^{KO} mice into congenic B6 PLP^{WT} mice. $CD4^+$TCR-$PLP11^+$ cells were analyzed four days later for proliferation by flow cytometry. We did not observe any T cell proliferation after transfer into B6 PLP^{WT} recipients (Figure 14A), no matter whether the T cells came from TCR-PLP11 PLP^{WT} or TCR-PLP11 PLP^{KO} background.

Next, we want to know whether the cells are at all capable of proliferation and not affected by the CFSE labeling procedure. So we immunize the recipient mice with $PLP_{174-181}$ peptide in CFA. The lymphocytes from the immunized recipient mice proliferate specifically as early as two days after immunization (Figure 14B and 14C). At day 1, Plp11-specific T cells remained undivided; the first cell divisions occurred at day 2, at day 3, a marked T cells division was observed and continued to day 4. This indicated that the T cells from TCR-PLP11 mice proliferate specifically *in vivo*. These results suggest that the PLP-specific T cells in TCR-PLP11 Tg mice remained ignorant either as a consequence of low TCR affinity and/or low levels of $I\text{-}A^b$: $PLP_{174-181}$ complex expression on APCs failed to reach the threshold of T-cell stimulation.

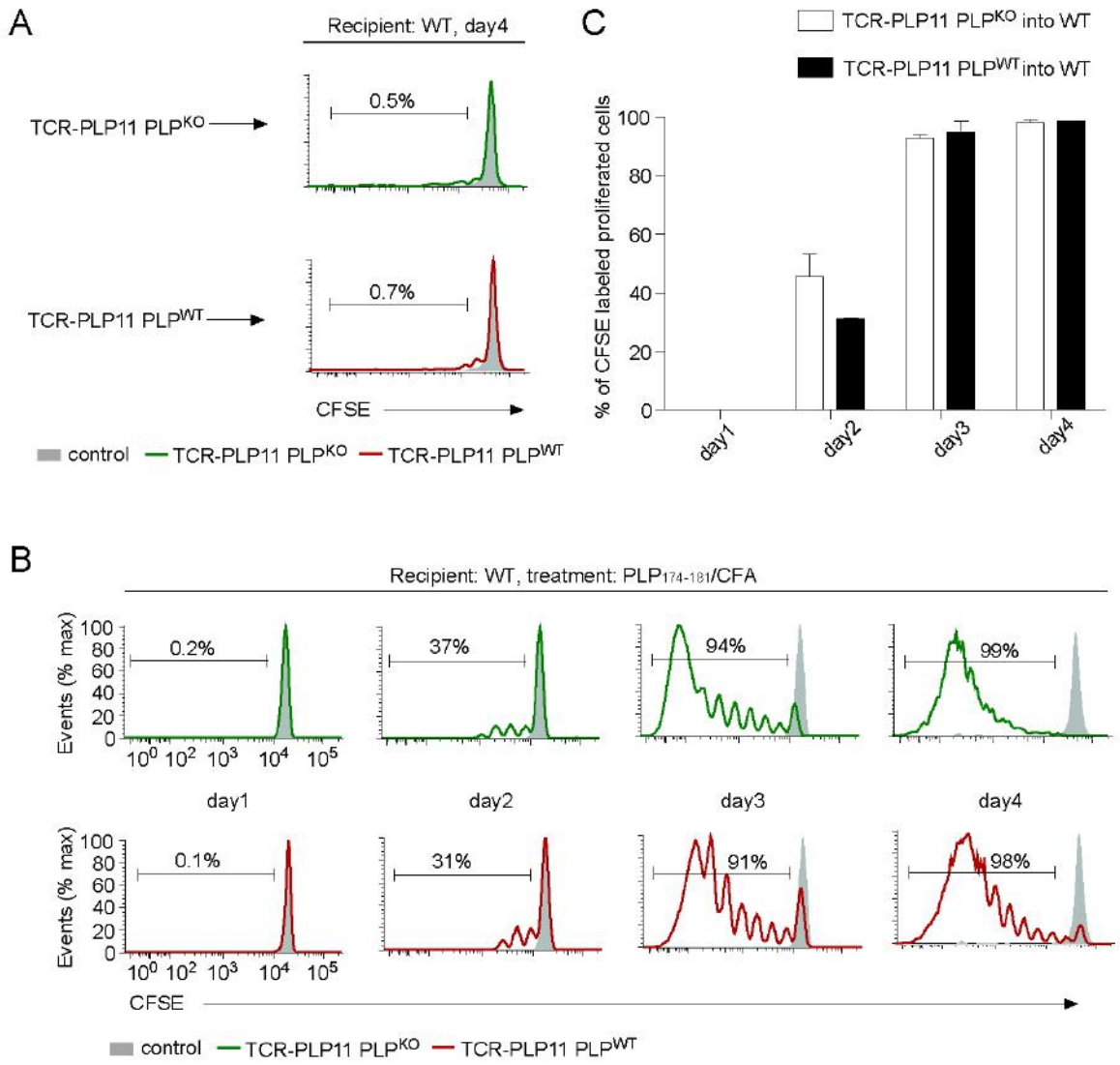

Figure 14. Tracking Plp11-specific T cell division using CFSE. (A) $CD4^+$ Plp11-specific cells were labelled with CFSE and then 5 × 10^6 cells were adoptively transferred i.v. into congenic B6 mouse. Three days later, various lymph nodes were harvested and analyzed by flow cytometry. Histograms are gated on $CD4^+$TCR-PLP11$^+$$CFSE^+$ cells. (B) PLP11-specific T cells proliferate in the draining lymph nodes of B6 mice immunized with $PLP_{174-181}$. $CD4^+$ Plp11-specific T cells were labeled with CFSE and then 5 × 10^6 cells were adoptively transferred i.v.into congenic B6 mice. Before transferred the cells, mice were immunized with 50ug $PLP_{174-181}$ via the footpad for 1, 2, 3, 4 d before being harvested inguinal lymph nodes and analyzed by flow cytometry. Histograms are gated on $CD4^+$TCR-PLP11$^+$$CFSE^+$ cells. Results are representative of three experiments with five mice per groups. (C) The proportion of cells in each division cycle that were $CD4^+$TCR-PLP11$^+$ $CFSE^+$ cells at each time point.

2.1.5 TCR-PLP11 mice are susceptible to EAE

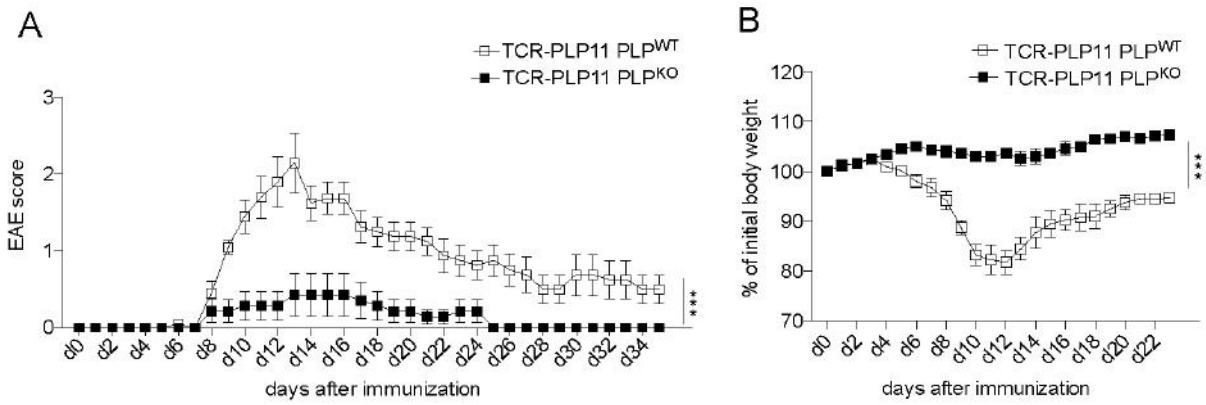

Figure 15. EAE induction in TCR-PLP11 transgenic mice. (A) TCR-PLP11 PLP^{WT} mice and TCR-PLP11 PLP^{KO} mice littermates were immunized with $PLP_{174-181}$ in CFA plus pertussis toxin and observed for the development of EAE over time. The data are shown as the Mean ± SEM of five independent experiments. (B) TCR-PLP11 mice are susceptible to EAE and also accompanied by an impressive loss in body weight.

Experimental autoimmune encephalomyelitis (EAE) is a well-characterized murine model of multiple sclerosis (MS) that is extensively used to understand the role of specific molecules and cell subsets the disease pathology of MS. We determined whether EAE could be induced in the TCR-PLP11 Tg mice by the standard immunization protocol that comprises the immunization with $PLP_{174-181}$ peptide in CFA and additionally injection of two doses of pertussis toxin at days 0 and 2. The severity and incidence of disease were monitored. TCR-PLP11 PLP^{WT} mice and TCR-PLP11 PLP^{KO} mice were mixed littermates that were clinically scored without prior knowledge of their genotype. PLP-immunized TCR-PLP11 PLP^{WT} mice developed EAE in which first symptoms of disease were observed around day 8 post-immunization. At the peak of the disease, at day 13, the TCR-PLP11 PLP^{WT} mice displayed most severe symptoms. The clinical data from more than 5 weeks of observation of five independent experiments (Figure 15) indicated that immunization of TCR-PLP11 mice with PLP11 can induces EAE and also accompanied by an impressive loss in body weight only in TCR-PLP11 PLP^{WT} mice. A total of five independent experiments confirmed comparable days of onset, maximal clinical scores and 100 % incidence in TCR-PLP11 PLP^{WT} after immunization with specific peptide. Of note, TCR-PLP11 PLP^{KO} mice also showed light symptoms of EAE,

which might be a result of the immunization procedure is the background of immunization. These results indicate that TCR-PLP11 mice are susceptible to EAE.

2.1.6 TCR-PLP11 mice lacking endogenous TCR α and β chains develop EAE spontaneously

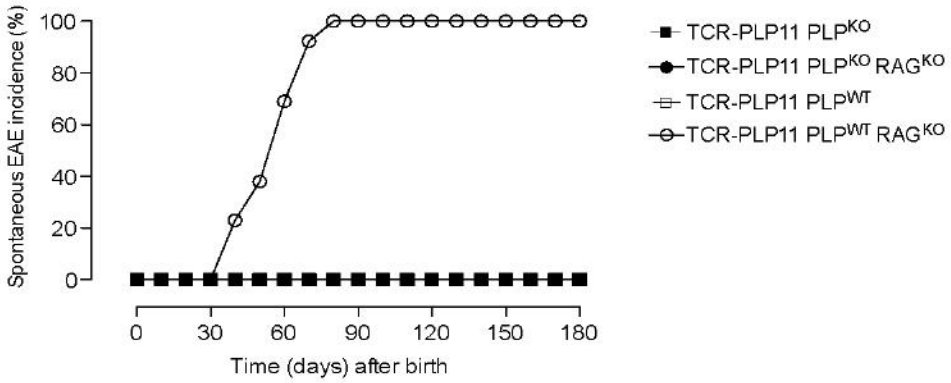

Figure 16. Spontaneous EAE was observed in TCR-PLP11 PLP^{WT}RAG^{KO} mice with 100% incidence (n=18). Age-matched TCR-PLP11 PLP^{KO}RAG^{KO} (n=20), TCR-PLP11 PLP^{WT} (n=50) and TCR-PLP11 PLP^{KO} (n=50) were free of clinical disease during the same observation period. Data indicate the percentage of mice that developed EAE within each group.

Despite the lack of tolerance towards PLP, the vast majority of TCR-PLP11 mice never developed EAE spontaneously. To eliminate the effect of endogenous TCR rearrangements on thymic and peripheral development of Plp11-specific CD4$^+$ T cells, the TCR-PLP11 mice were crossed with RAG^{KO} mice. Surprisingly, 100% of TCR-PLP11 PLP^{WT}RAG^{KO} mice developed EAE spontaneously. The disease onset was accelerated, with approximately 23% of the TCR-PLP11 PLP^{WT}RAG^{KO} mice first exhibiting symptoms of EAE by the age of 40 days, and the disease often remained stable. All mice developed EAE by 80 days of age (Figure 16). In contrast, TCR-PLP11 PLP^{KO}RAG^{KO}, TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} remained disease-free during 6-month observation period under the same conditions. These data suggest that TCR-PLP11 PLP^{WT} mice lacking endogenous TCR α and β chains develop EAE spontaneously.

2.1.7 Rag1-deficient TCR-PLP11 mice do not express Foxp3

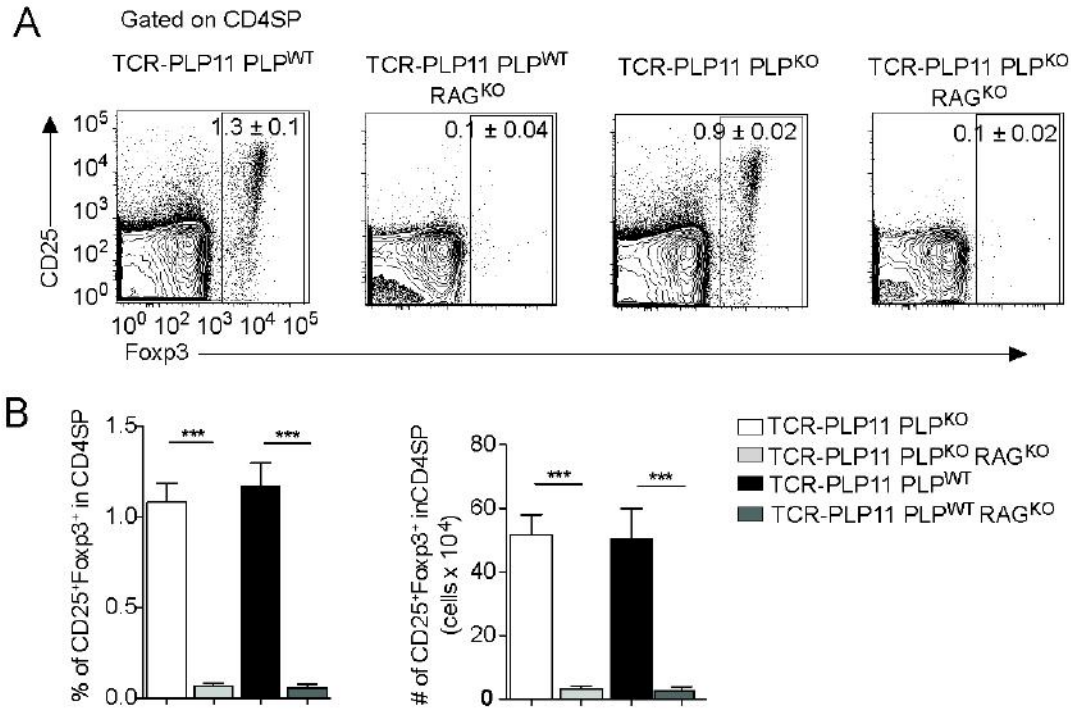

Figure 17. Analysis of thymic Foxp3$^+$ regulatory T cells in TCR-PLP11 and Rag1-deficient TCR-PLP11 mice. (A) Foxp3 analysis was gated on CD4$^+$CD8$^-$ cells. The numbers above the gates represent the mean average ± the standard error of the mean (SEM). (B) The percentage of Foxp3$^+$ Tregs (first column) and absolute cell numbers (second column) in CD4SP cells in the thymus of TCR-PLP11 and Rag1-deficient TCR-PLP11 mice. Results are representative of three independent experiments with five mice per groups.

Rag1 deficient TCR-PLP11 mice display a monoclonal Plp11-specific CD4$^+$ T cell repertoire in both TCR-PLP11 PLP^{WT}RAG^{KO} and TCR-PLP11 PLP^{KO}RAG^{KO} mice (data not shown). It has been known that TCR-PLP11 healthy mice harbor non-tolerant autoreactive CD4$^+$ T cells, these cells might be kept under control by one, or a combination of tolerance mechanisms. Therefore, we measured the presence of Foxp3$^+$ regulatory T cells in the thymus, which might be more effective at controlling self-reactive cells and hence prevent autoimmunity. When we analyzed the frequency as well as the absolute number of CD25$^+$Foxp3$^+$ thymocytes within CD4$^+$ T cells, thymocytes from Rag1-deficient TCR-PLP11 mice produced virtually no Foxp3$^+$ T cells. In contrast, a clearly expression of Foxp3 was detectable in 3-week-old TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} mice (1.3 ± 0.1% vs 0.9 ± 0.02%) within CD4$^+$ compartment, this population was comparable and independent on the

expression of PLP in the thymus (Figure 17A and 17B). Taken together, no Foxp3$^+$ T cells are generated in Rag1-deficient TCR-PLP11 mice.

2.1.8 Foxp3$^+$ T cells presence in CD4$^+$ T cells expressing TCR encoded by the endogenous TCR loci

Since TCR-PLP11 mice and TCR-PLP11 mice on Rag1-deficient mice differ only in their capacity to express endogenous TCR chains, which lead to the differences in EAE susceptibility. In Rag1-sufficient TCR-PLP11 mice, approximately 90% of CD4$^+$ T cells express the Plp11-specific TCR, the remaining 10% of CD4$^+$ T cells express endogenous TCR-α or –β genes (Figure 11 and Figure 18). When we analyzed TCR expression within CD4$^+$ compartment in TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} mice, we observed the dominant Vβ14 together with three populations of Vα2: Vα2^{high} cells that express exclusively the transgene-encoded Plp11-specific TCR; Vα2^{intermediate} cells that express two α chains, one is transgene-encoded α chain and the other is encoded by the endogenous TCR loci; Vα2^{low} cells that express TCR-α chain encoded by endogenous loci. To correlate the EAE susceptibility with the T cell repertoire, we stained Foxp3 of the three Vα2 populations. We observed a high frequency of Foxp3$^+$ T cells among Vα2^{intermediate} and Vα2^{low} populations both in TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} mice. However, in Vα2^{high} cells, the frequency of Foxp3$^+$ T cells is remarkably low compared with WT mice (Figure 18). These data further suggest that CD4$^+$ T cells expressing TCR encoded by the endogenous TCR loci have a protect TCR-PLP11 mice from spontaneous EAE.

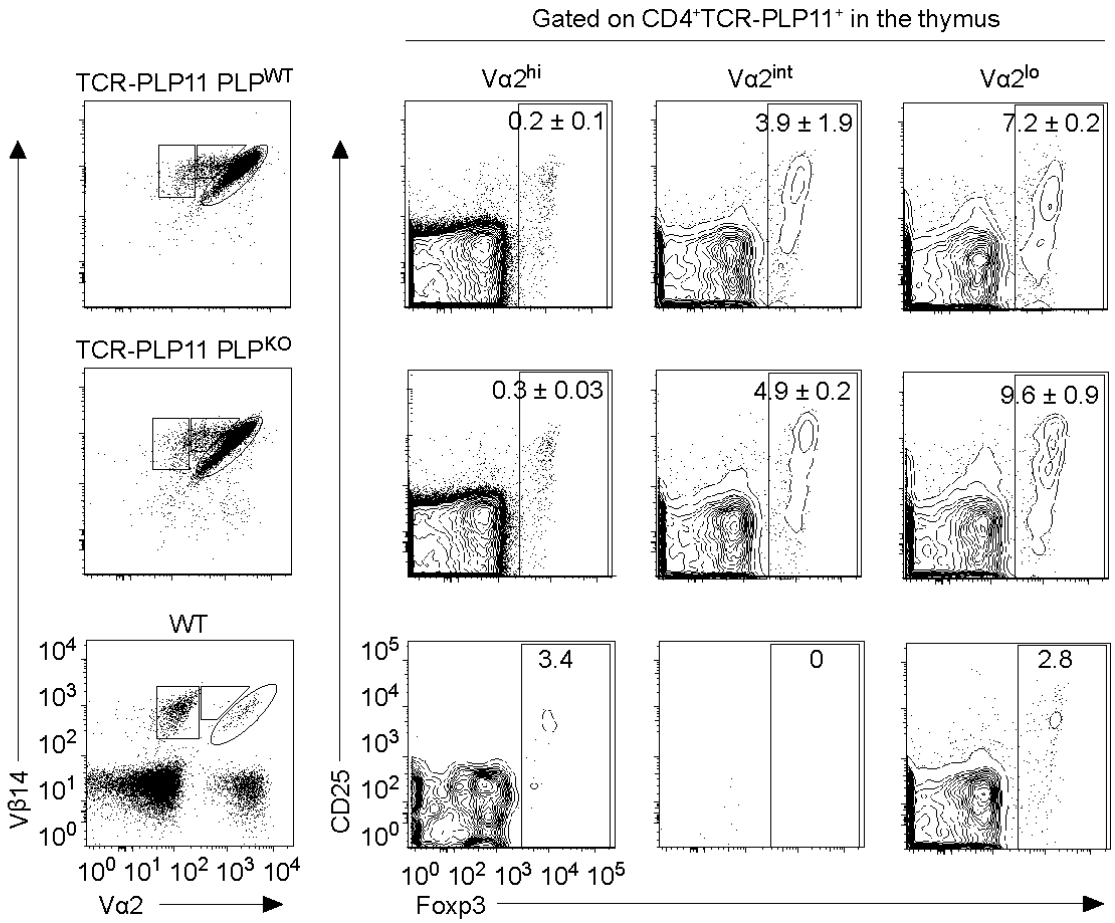

Figure 18. Foxp3 analysis of thymocytes of TCR-PLP11 Tg mice expressing different levels of Vα2. The numbers above the gates represent the mean average ± the standard error of the mean (SEM).

2.2 Mechanisms of central and peripheral T cell tolerance to an antigen of the central nervous system

2.2.1 Thymic development of PLP_{11-18}-specific T cells in TCR-PLP1 mice

Previous work performed by Klein et al. using a set of overlapping 24-mer peptides subsequent to immunization of PLP^{KO} mice with purified PLP protein, revealed four immunogenic MHCII-PLP epitopes[219]. Among these four regions, PLP_{11-18} yielded a strong recall response in PLP^{KO} mice, whereas no response in PLP^{WT}, indicating a tightly controlled tolerance towards this region. We want to understand the tolerance mechanism to PLP_{11-18}. For this reason, a TCR Tg mouse model specific for PLP1 was generated in our lab (Winnewisser J., PhD thesis). Briefly, a CD4 PLP_{11-18}-specific T cell clone (D9-11-9) was derived from B6 PLP^{KO} mice upon immunization with PLP. The D9-11-9 clone expressed a TCR composed of Vα3.2 and Vβ6. The rearranged TCRα and TCRβ chain DNA segments were subcloned into the cassette vectors pTα and pTβ[221], respectively and injected into B6 oocytes ($H\text{-}2^b$) to generate TCR-PLP1 TCR transgenic mice.

Age-matched TCR-PLP1 PLP^{KO} thymocytes were analyzed in parallel. The thymic expression of PLP in TCR-PLP1 PLP^{WT} had little effect on the percentage of DN and DP thymocytes, but the proportions of CD4SP cells (4.0 ± 0.4%) were significantly reduced in TCR-PLP1 PLP^{WT} mice compared with TCR-PLP1 PLP^{KO} mice (16.9 ± 1.9%) in Figure 19. This profile indicated the presence of negative selection of thymocytes expressing TCR-PLP1 within the thymus in PLP^{WT} mice. We then compared the abundance of transgenic TCRα and TCRβ chain expressing CD4 T cells between TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} mice. Staining with anti-Vα3.2 and anti-Vβ6 demonstrated that 85.5 ± 3.2% of the CD4SP cells of TCR-PLP1 PLP^{KO} mice were found to express PLP1-speicifc T cell receptor, compared with a reduction of Plp1-specific $CD4^+$ T cells in TCR-PLP1 PLP^{WT} mice (51.1 ± 4.1%) (Winnewisser J., PhD thesis).

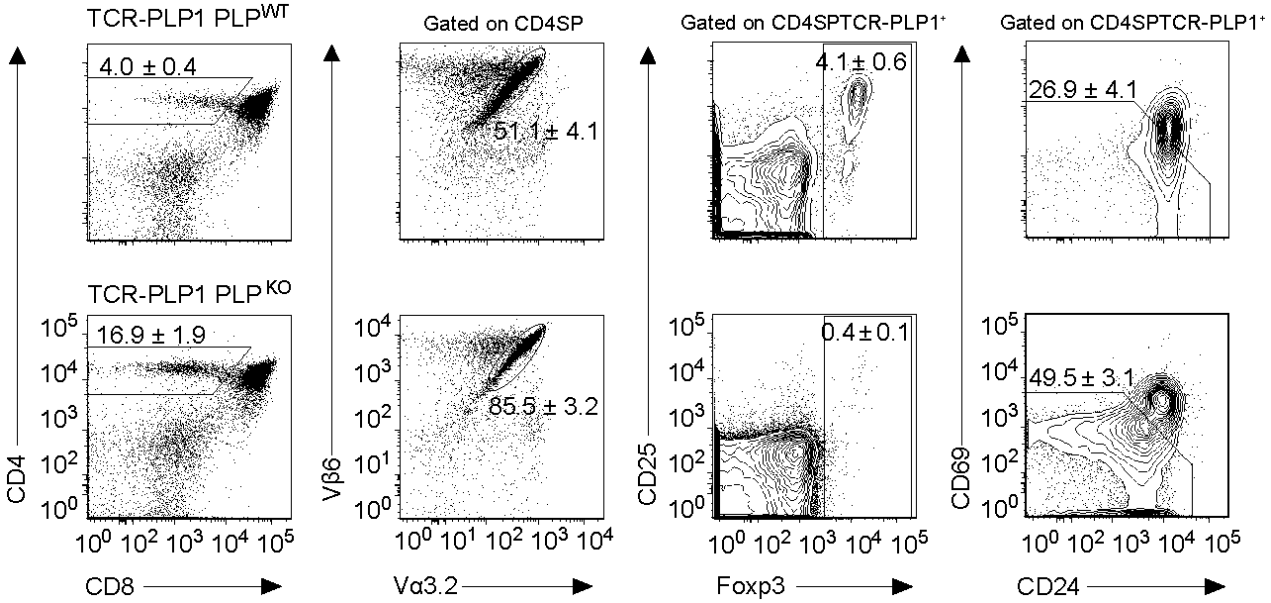

Figure 19. Flow cytometry analysis of the thymocytes of TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} mice. Thymocytes were enumerated and analyzed by flow cytometry. Anti-CD4 and CD8 staining of thymocytes (first column), level of expression of the TCR Vα3.2Vβ6 on CD4SP cells (second column), Foxp3$^+$ Tregs in the TCR-PLP1$^+$ cell population (third column) and mature cells within the TCR-PLP1$^+$ cell population in the thymus (fourth column). The numbers above the gates represent the mean average ± the standard error of the mean (SEM). TCR-PLP1 PLP^{WT}: n=10; TCR-PLP1 PLP^{KO}: n=8.

Previous work showed that negative selection is linked clonal deletion of autoreactive thymocytes with thymus-derived regulatory T cells (Tregs)[231]. Tregs expressing Foxp3 and high levels of CD25 are required for controlling immune responses by inhibiting the activation of effector T cells. Furthermore, Tregs development in the thymus seem to depend on the presence of self-antigen recognition, When we analyzed the induction of regulatory T cells in thymi, while in TCR-PLP1 PLP^{KO} mice only a small fraction of TCR-PLP1$^+$ T cells was deviated into Foxp3$^+$ Treg cells (0.4 ± 0.1%), in TCR-PLP1 PLP^{WT} mice was 10-times higher (4.1 ± 0.6%) (Figure 19) (Winnewisser J., PhD thesis). Taken together, deletion of TCR-PLP1$^+$ CD4$^+$ T cells and selection of T regulatory cells both operate for this important self-antigen PLP in central tolerance.

2.2.2 Contribution of thymic antigen presenting cells to tolerance induction to PLP

Presentation of self-antigens by thymic APCs result in different cell fates of the autoreactive T cells through positive selection[74], negative selection[141] and induction of Tregs[149]. The role of thymic APCs in mediating tolerance has been studied showing both specialized and overlapping functions among them[117, 232, 233]. Central tolerance to PLP is operated by two mouse models (TCR-PLP1 x Foxn1-Cre x $PLP^{fl/fl}$ mice and TCR-PLP1 PLP^{WT} ΔDC mice), which have been recently shown by us (Winnewisser J., PhD thesis).

2.2.2.1 Expression and presentation of PLP by mTEC is sufficient to mediate negative selection and concomitant Treg induction

Firstly, we investigated that whether PLP expression and presentation in radioresistant cells are sufficient and necessary to induce tolerance. We crossed TCR-PLP1 Tg mice to Foxn1-Cre x $PLP^{fl/fl}$ mice (hereafter called TCR-PLP1 PLPΔTEC mice). The resulting Foxn1-Cre x $PLP^{fl/fl}$ mice ablated PLP expression only in TEC. Our study demonstrated that PLP expression by TECs is essential and sufficient for negative selection and concomitant Treg induction in the thymus (Figure 20).

2.2.2.2 Thymic Dendritic cells do not present PLP for tolerance induction

It has been demonstrated that thymic DCs are very efficient in mediating negative selection of developing thymocytes[106, 234-236]. To study DCs contribution to T cell homeostasis and maintenance of tolerance, we crossed TCR-PLP1 Tg mice to ΔDC mice. The loss of DCs in TCR-PLP1 Tg mice does not exhibit defective negative selection (Figure 21). It indicated that hematopoietic antigen presenting cells such as medullary DCs which cross-present mTEC-derived antigens, do not contribute negative selection in our experiment system.

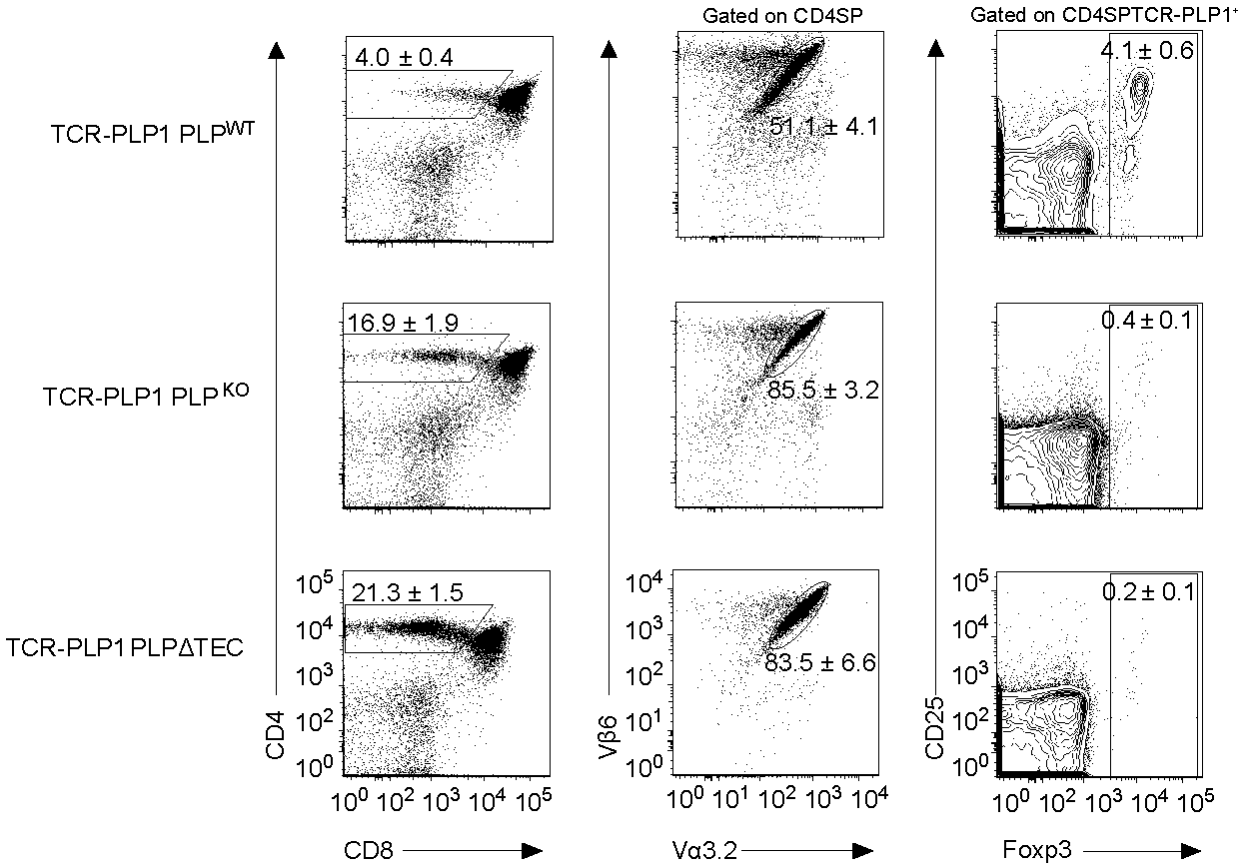

Figure 20. Deletion of PLP expression exclusively in TECs abrogated central tolerance to PLP. CD4 T-cell development in 3-week-old TCR-PLP1 PLP^{WT} mice, TCRPLP1 PLP^{KO} mice and TCR-PLP1 PLPΔTEC mice. The plots depict that the average percentage ± the standard error of the mean (SEM) of CD4SP profiles (first column), level of expression of the TCR Vα3.2Vβ6 among CD4SP cells (second column) and the percentage of $Foxp3^+$ Tregs in the TCR-$PLP1^+$ cell population (third column). TCR-PLP1 PLP^{WT}: n=10; TCR-PLP1 PLP^{KO}: n=8; TCR-PLP1 PLPΔTEC: n=2.

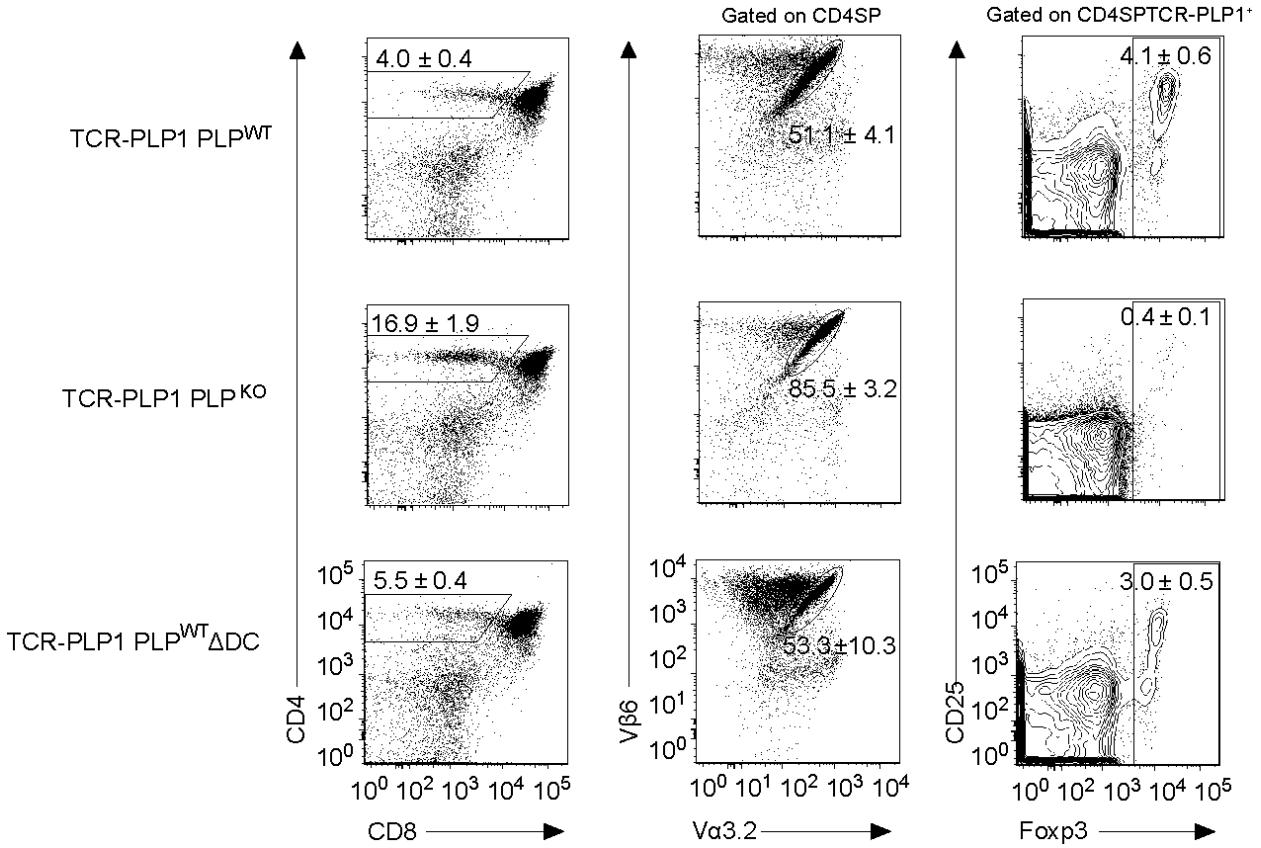

Figure 21. Central tolerance to PLP is not dependent on DCs. CD4 T cell development in 3-week-old TCR-PLP1 PLP^{WT} mice, TCR-PLP1 PLP^{KO} mice and TCR-PLP1 PLP^{WT}ΔDC mice. The plots depict that the average percentage ± the standard error of the mean (SEM) of CD4SP profiles (first column), level of expression of the TCR Vα3.2Vβ6 among CD4SP cells (second column) and the percentage of $Foxp3^+$ Tregs in the TCR-$PLP1^+$ cell population (third column). TCR-PLP1 PLP^{WT}: n=10; TCR-PLP1 PLP^{KO}: n=8; TCR-PLP1 PLP^{WT}ΔDC: n=6.

2.2.3 Peripheral tolerance to PLP carried out by deletion

In accordance with thymic characteristics, splenic profiles demonstrated that proportions of $CD4^+$ T cells were significantly lower in TCR-PLP1 PLP^{WT} mice (1.2 ± 0.2%) than those of TCR-PLP1 PLP^{KO} mice (5.8 ± 1.3%). Regulatory T cells are required for maintaining peripheral tolerance to self-antigen by inhibiting the activation of effector T cells. Therefore, we also examined the frequency of $Foxp3^+$ Treg cells in the TCR-PLP1 Tg mice. Notably, TCR-$PLP1^+$ $Foxp3^+$ T cells were significantly enhanced in the spleen of TCR-PLP1 PLP^{WT} mice (38.4 ± 1.7%) when compared to the low percentage of TCR-$PLP1^+$ $Foxp3^+$ Treg cells in TCR-PLP1 PLP^{KO} mice (1.2 ± 0.3%) (Figure 22). Taken together, in the periphery we observed pronounced reduction of TCR-$PLP1^+$ T cells and increased frequencies of Tregs in the presence of the cognate self-antigen PLP.

In order to assess contribution of the periphery to tolerance to PLP we abrogated central tolerance by using TCR-PLP1 PLPΔTEC. In this mouse model we previousely showed that in the absence of PLP in thymic epithelium central tolerance was eliminated. When the splenic profiles of the TCR-PLP1 PLPΔTEC mice were compared with TCR-PLP1 PLP^{KO} mice, the proportions of $CD4^+$ cells (1.9 ± 0.3%) were obviously reduced in the spleen of TCR-PLP1 PLPΔTEC mice (Figure 22), indicating the deletion of autoreactive T cells of the spleen when PLP expression was ablated in TEC. In addition, while in TCR-PLP1 PLP^{WT} mice a large proportion of Tregs was present (38.4 ± 1.7%), in TCR-PLP1 PLPΔTEC mice, this population was not observed (2.5 ± 1.1%), and had a similar size as in the complete absence of PLP in PLP^{KO} mice.

To study DCs contribution to peripheral tolerance, we analyzed the splenocytes in TCR-PLP1 PLP^{WT}ΔDC. The numbers of splenocytes as well as the percentage of $CD4^+$ T cells in TCR-PLP1 PLP^{WT}ΔDC mice were reduced (0.8 ± 0.1%), which is similar compared with DC-sufficient TCR-PLP1 PLP^{WT} littermates (1.2 ± 0.2%). Furthermore, the induction of TCR-$PLP1^+$ $Foxp3^+$ regulatory T cells were comparable between TCR-PLP1 PLP^{WT}ΔDC (43.3 ± 2.1%) and TCR-PLP1 PLP^{WT} mice (38.4 ± 1.7%) (Figure 22).

Altogether, peripheral deletion in response to PLP is one mechanism by which the immune system could eliminate PLP-specific T cells that escape thymic deletion.

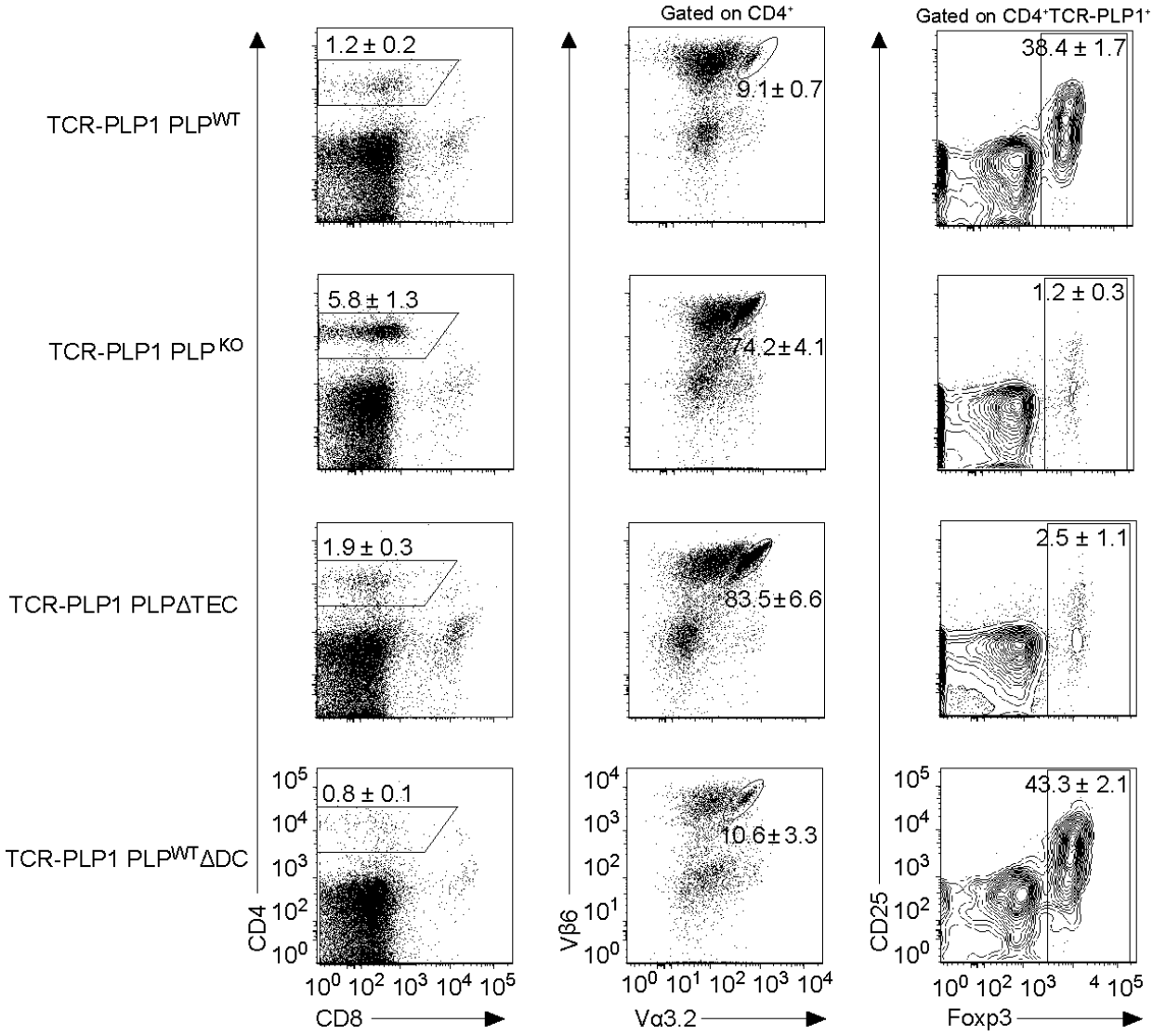

Figure 22. Flow cytometry analysis of peripheral phenotype in TCR-PLP1 Tg mice. Anti-CD4 and CD8 staining of splenocytes (first column) from TCR-PLP1 PLP^{KO} (n=8), TCR-PLP1 PLP^{WT} mice (n=10), TCR-PLP1 PLPΔTEC mice (n=2) and TCR-PLP1 PLP^{WT}ΔDC mice (n=6) level of expression of the TCR Vα3.2Vβ6 on $CD4^+$ cells (middle column), $Foxp3^+$ Tregs in the TCR-$PLP1^+$ cell population (third column). The numbers above the gates represent the mean average ± the standard error of the mean (SEM).

2.2.4 ICOS and FR4 are highly expressed on TCR-PLP1 anergic CD4$^+$ T cells

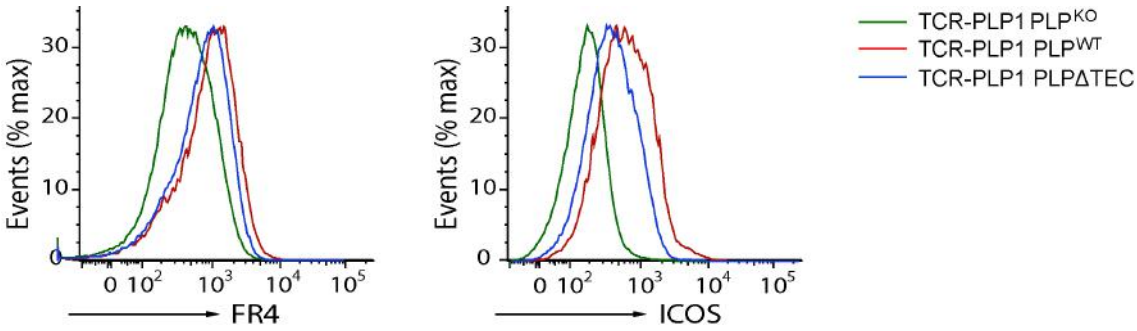

Figure 23. Flow cytometric analysis of ICOS and FR4 expression among CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells in TCR-PLP1 PLP^{WT} (red line histogram), TCR-PLP1 PLP^{KO} mice (green line histogram) and TCR-PLP1 PLPΔTEC mice (blue line histogram). Data is representative of results obtained from six indicated mice analyzed in two independent experiments.

Albeit central and peripheral deletion of PLP reactive CD4$^+$ T cells takes place, there is still a large proportion of autoreactive T cells that seem to escape tolerance induction. Despite this fact, surprisingly, the mice do not develop CNS autoimmune disease. We wondered whether additional mechanisms are required to silence self-reactive periphery T cells, such as anergy induction. T cell anergy is defined as a defect in TCR-dependent proliferation in response to challenge with antigen[237]. First of all, we examined phenotypic characteristics of this escapee population. FACS cytometric analysis of surface inducible costimulator (ICOS) and Folate receptor 4 (FR4), two novel anergy marker[238, 239], expression among CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells was performed. As you seen in Figure 23, while low levels of ICOS and FR4 showed low expression in TCR-PLP1 PLP^{KO} mice (green line histogram) compared with high expression on CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells both in TCR-PLP1 PLP^{WT} (red line histogram) and TCR-PLP1 PLPΔTEC mice (blue line histogram).

2.2.5 Anergy is another mechanism of periphery tolerance to PLP

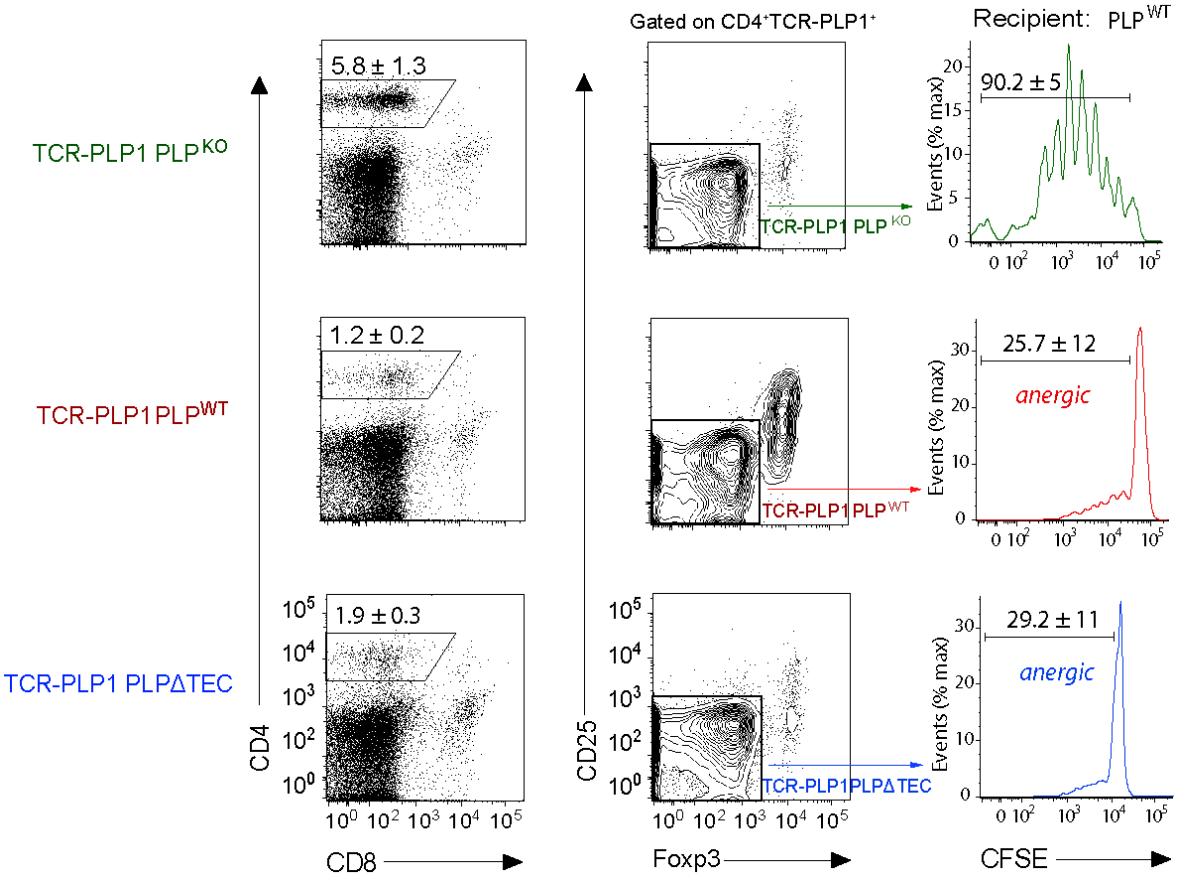

Figure 24. CFSE-labeling CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells from different mouse strains for *in vivo* monitoring of adoptively transferred cells. 5 x 10^6 of CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells were sorted and labeled with 0.5 µM CFSE and adoptively transferred into C57BL/6 recipient. Three days later, splenocytes and lymph node cells were analyzed for cell division. Significant proliferation was observed when the cells come from TCR-PLP1 PLP^{KO} mice measured as decreasing flurosence of CFSE (green histogram), or undivided cells with a single, bright CFSE peak were seen both in TCR-PLP1 PLP^{WT} mice (red histogram) and TCR-PLP1 PLPΔTEC mice (blue histogram).

Next, we performed functional analysis to directly compare CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells among different mouse strain. To do so, congenically marked CD4$^+$ TCR-PLP1$^+$ T cells were depleted from Foxp3$^+$ Treg cells, labeled with CFSE and transferred into PLP^{WT} mice (5 x 10^6 cells/recipient) that express PLP in the periphery. Proliferation of CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells was examined by CFSE dilution 3 days later. In case of TCR-PLP1 PLP^{KO} mice, when naïve CD4$^+$ T cells were transferred into PLP^{WT} recipient, CD4$^+$TCR-PLP1$^+$ T cells readily recognized PLP-MHC complexes and proliferated, so the first conclusion would be the PLP epitope was expressed and presented in the periphery. The second conclusion

would be the Plp1-specific T cells proliferate specifically and heavily (90.2 ± 5%). In marked contrast, if we took the cells from TCR-PLP1 PLP^{WT} mice, TCR-PLP1 PLP^{WT} T cells had a strongly impaired proliferative capacity (25.7 ± 12%) even when antigen was obviously presented in the periphery of the host (Figure 24). We concluded that clonal anergy was induced in the periphery of our model system. Similarly, when the $CD4^+$TCR-$PLP1^+$ T cells were taken from TCR-PLP1 PLPΔTEC mice, where PLP was presented in the periphery, but self-antigen PLP was ablated in the thymus, the proliferation response of CFSE-labeled $CD4^+$TCR-$PLP1^+Foxp3^-$ T cells to PLP was significantly reduced (29.2 ± 11%), meaning the $CD4^+$ T cells are anergic from TCR-PLP1 PLPΔTEC mice (Figure 24).

These results indicated that escaping $CD4^+$TCR-$PLP1^+$ T cells from both TCR-PLP1 PLP^{WT} and TCR-PLP1 PLPΔTEC mice are anergic, demonstrated that anergy is yet another mechanism of periphery tolerance to PLP in our model system.

2.2.6 The presence of TCR-PLP1 PLP^{WT+} T cells did not hinder TCR-PLP1 PLP^{KO+} T cells to proliferate

In addition to thymus-derived regulatory T cells (nTreg), induced regulatory T cells (iTreg) are generated from conventional $CD4^+$ T cells in the periphery. Both of them have the potential to suppress a variety of immune response in the periphery *in vitro* and *in vivo*[240]. To exclude the possibility that the impaired proliferative response of TCR-PLP1 PLP^{WT} T cells was dampened by residual PLP-specific Treg cells, $CD4^+Foxp3^-$ T cells from $CD45.1^+/CD45.2^+$ TCR-PLP1 PLP^{KO} was mixed with $CD4^+Foxp3^-$ T cells from $CD45.2^+$ homozygote TCR-PLP1 PLP^{WT} at a 1:1 ratio, using CFSE-labeled before transfer into $CD45.1^+$ homozygote WT mice. Three days after transfer, CFSE profiles and percentages of donor-derived $CD4^+$TCR-$PLP1^+$ cells were determined in recipient mice. As shown in Figure 25, TCR-PLP1 PLP^{WT+} T cells still did not proliferate in WT host. In contrast, TCR-PLP1 PLP^{KO+} T cells proliferated vigorously in the presence of PLP in the periphery (Figure 25). Therefore, the presence of TCR-PLP1 PLP^{WT+} T cells did not hinder TCR-PLP1 PLP^{KO+} T cells to proliferate. Thus, these results exclude the possible inhibition of residual PLP-specific Treg cells, which might be lead to unresponsive of peripheral TCR-PLP1 PLP^{WT+} T cells.

Taking these observations together, T cells from TCR-PLP1 PLP^{WT} mice displayed an anergic phenotype. Moreover, TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} PLP-specific T cells were functional different with respect to their proliferative capacities.

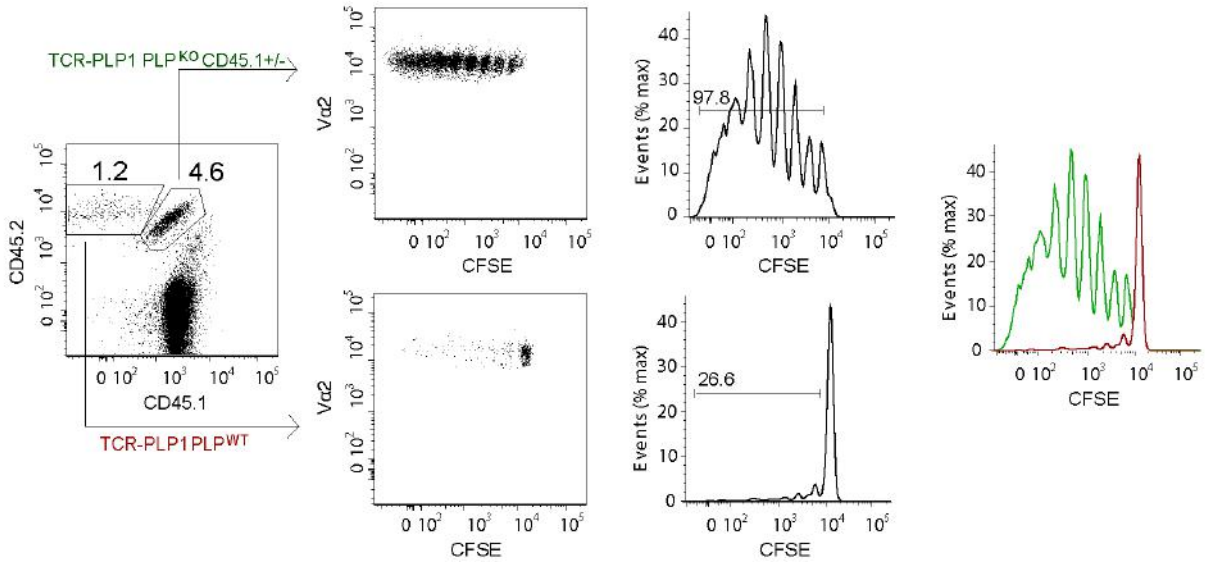

Figure 25. Co-transfer of CFSE-labeling $CD4^{+}$TCR-PLP1^{+}Foxp3^{-} T cells from TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} into WT recipient for monitoring of adoptively transferred cells. Three days later after co-transfer, splenocytes and lymph node cells were analyzed for cell division. Significant proliferation was observed when the cells come from TCR-PLP1 PLP^{KO} mice measured as decreasing flurosence of CFSE (green histogram) and undivided cells with a single, bright CFSE peak were seen in TCR-PLP1 PLP^{WT} mice (red histogram).

2.2.7 PLP expression by radioresistant cells in the periphery, but to be presented by hematopietic cells

In order to determine the source of PLP as well as the type of APC that presents PLP in the periphery, we compared PLP stimulated proliferation in various bm restonstitution experiments. We reconstituted WT or PLP^{KO} mice with $MHCII^{WT}PLP^{WT}$ or $MHCII^{KO}PLP^{WT}$ BM and afterwards adoptively transfered congenically marked $CD4^{+}$ TCR-PLP1^{+} Foxp3^{-} T cells population from TCR-PLP1 PLP^{KO} mice. In our bone marrow chimera setting, TCR-PLP1 PLP^{KO+} T cells proliferated when transferred into WT mice with MHCII expression ($MHCII^{WT}PLP^{WT}$ → PLP^{WT}, 70.9 ± 4.2%, Figure 26, first column). In the situation where hematopoietic cells were PLP^{WT} and MHCII-sufficient but PLP-deficient in radioresistant cells, those

TCR-PLP1 PLP^{KO+} T cells did not proliferate ($MHCII^{WT}PLP^{WT} \rightarrow PLP^{KO}$, 4.8 ± 1.1%, Figure 26, second column). This indicated that in the periphery, PLP was not expressed by BM-derived APCs but instead only by radioresistant cells. This is in agreement with our observation that in the thymus PLP expression by mTECs alone mattered. Importantly, TCR-PLP1 PLP^{KO+} T cells exposed to PLP environment after reconstitution of the WT mice with MHC-deficient BM showed poorly proliferation ($MHCII^{KO}PLP^{WT} \rightarrow$ WT, 4 ± 1.7%, Figure 26, third column). As negative control setting, TCR-PLP1 PLP^{KO+} T cells did not proliferate in PLP^{KO} mice reconstituted with $MHCII^{KO}PLP^{KO}$ BM ($MHCII^{KO}PLP^{KO} \rightarrow PLP^{KO}$, 4.3 ± 1.2%, Figure 26, fourth column). Thus, this result confirmed that PLP was presented by hematopoietic cells to autoreactive TCR-$PLP1^+$ T cells in the periphery. In summary, although these APCs of hematopoietic origin were not responsible for expressing PLP, they are crucial for mediating efficient MHC II-restricted PLP presentation to TCR-$PLP1^+$ T cells. Once the antigen-presentation capacity of haematopoietic APCs was impaired due to MHC deficiency, TCR-PLP^+ T cells did not proliferate.

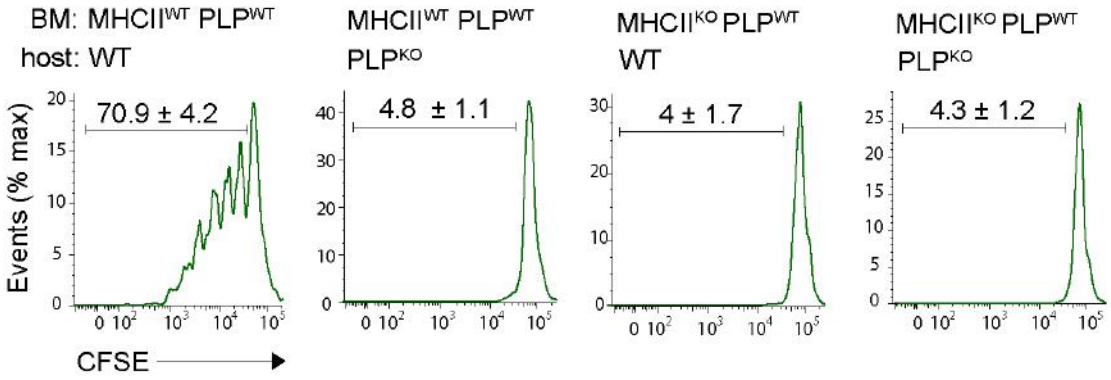

Figure 26. PLP expression by radioresistant cells in the periphery, but to be presented by hematopietic cells. Chimeric mice were generated by reconstituting WT or PLP^{KO} host mice with $MHCII^{WT}PLP^{WT}$ or $MHCII^{KO}PLP^{WT}$ bone marrow. FACS analysis of TCR-$PLP1^+$ T cells labeled with CFSE which were transferred to the indicated chimeric mice. TCR-$PLP1^+$ T cells were stained for the expression of CD4, CD8, Vα3.2 and Vβ6. CFSE dilution was assessed within $CD4^+$TCR-$PLP1^+$ population three days later in the lymph node of chimeric mice. Histograms show the mean ± SEM for each group.

2.2.8 DCs are necessary for PLP presentation and anergy induction

To identify the haematopoiectic APC that is necessary for PLP presentation we first elucidated the role of DCs. To that end, we transferred CFSE-labeled CD45.1$^+$ PLP_{11-18}-specific naïve CD4$^+$ T cells from TCR-PLP1 PLP^{KO} mice into CD45.2$^+$ WT or ΔDC mice and analyzed their proliferation. ΔDC mice lacking DC would be a useful tool to determine the role of DC in T cell priming *in vivo* and tolerance establishment. We demonstrated that Plp1-specific CD4$^+$ T cells proliferated when injected into WT recipient (Figure 27A), however, when the CD4$^+$ T cells were injected into a DC free environment, little proliferation was observed in the CD4$^+$TCR-PLP1$^+$ T cells (27.4 ± 2.7%), which confirmed that PLP is really presented by DCs (Figure 27B). The conclusion is peptide presentation by DCs is necessary for CD4$^+$ T cells proliferation in TCR-PLP1 Tg mice.

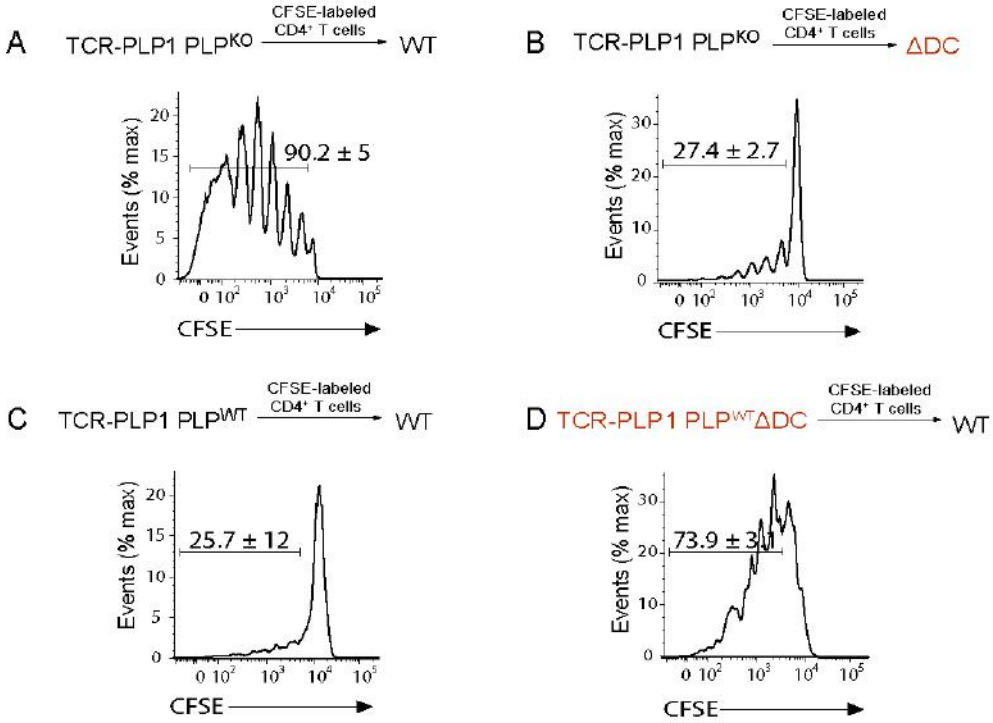

Figure 27. CFSE-labeling CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells from different mouse strains for *in vivo* monitoring of adoptively transferred cells. 5 x 10^6 of CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells were sorted from TCR-PLP1 PLP^{KO} mice and labeled with CFSE and adoptively transferred into either WT recipient (A) or ΔDC recipient (B). CD4$^+$TCR-PLP1$^+$Foxp3$^-$ T cells were sorted from either TCR-PLP1 PLP^{WT} mice (C) or TCR-PLP1 PLP^{WT}ΔDC mice (D), labeled with CFSE and adoptively transferred into WT recipient. Three days later, splenocytes and lymph node cells were analyzed for cell division.

DCs were shown that promote immune homeostasis by inducing and maintaining peripheral T cell tolerance[12]. Are DCs necessary for anergy induction? To this end, we transferred CFSE-labeled CD45.2$^+$ CD4$^+$ T cells either from TCR-PLP1 PLP^{WT} or TCR-PLP1 PLP^{WT}ΔDC mice into CD45.1$^+$ WT recipient where PLP is expressed in their periphery. We previously analyzed surface anergy marker expression (ICOS and FR4, Figure 23) and functionality among CD4$^+$TCR-PLP1$^+$ T cells in TCR-PLP1 PLP^{WT} mice, indicating the escaping Plp1-specific T cells from TCR-PLP1 PLP^{WT} are anergic (Figure 24 and Figure 27C). If DCs are responsible for anergy induction, these escaping CD4$^+$ T cells should maintain anergic state in the periphery. Therefore, when CD4$^+$ T cells were taken from TCR-PLP1 PLP^{WT}ΔDC mice (Figure 27D), with a greater percentage of the CD4$^+$TCR-PLP1$^+$ T cells demonstrating proliferation (73.9 ± 3.1%). In sum, we conclude that DCs are not only necessary for peptide presentation, but also necessary for anergy induction.

2.2.9 Breakdown tolerance to PLP in TCR-PLP1 Tg mice lead to EAE

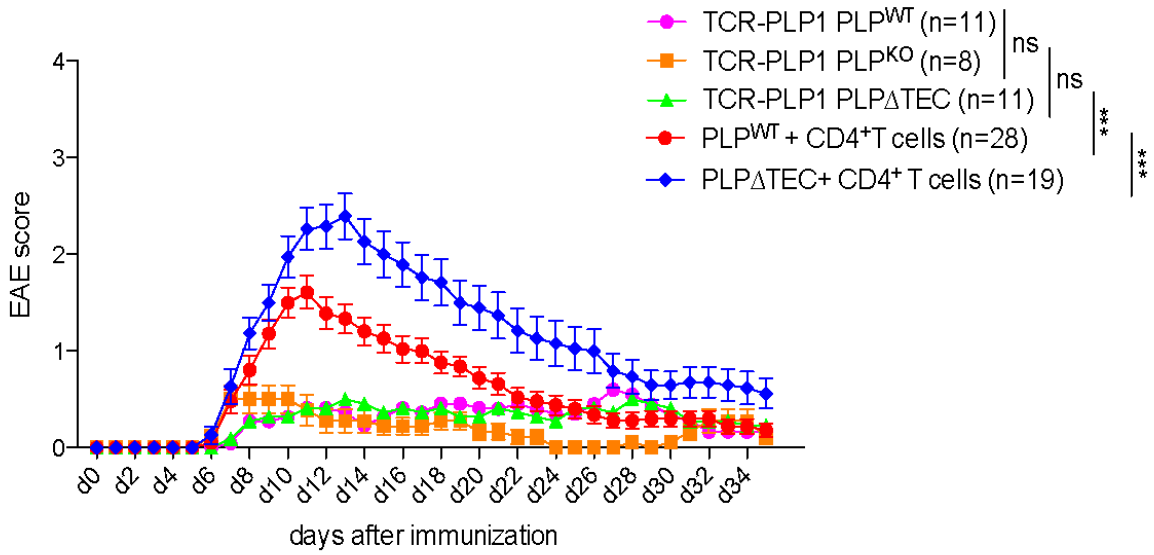

Figure 28: Differential EAE progression in TCR-PLP1 Tg mice when the tolerance induction to PLP was broken down. TCR-PLP1 PLP^{WT} Tg mice, TCR-PLP1 PLP^{KO} Tg mice and TCR-PLP1 PLPΔTEC mice were immunized with 200 µg PLP$_{11-18}$, and these mice were protected from EAE. EAE can be induced after transferring of naïve CD4$^+$ T cells derived from TCR-PLP1 PLP^{KO} mice with subsequent immunization of PLP$_{11-18}$ on WT mice and ΔTEC mice.

TCR-PLP1 Tg mice were observed for a time period longer than one year. These

mice did not develop EAE spontaneously. In order to determine the tolerance state of TCR-PLP1 Tg mice to EAE induction, we immunized TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} mice with PLP_{11-18} in CFA. CFA contains heat-inactivating Mycobacterium tuberculosis thus predominantly activating the $CD4^+$ T cells with a bias toward a Th1/Th17 response type[241]. The disease severity was monitored according to the classical EAE score. This challenge failed to induce clinical EAE development within 35 days of observation after treatment of TCR-PLP1 PLP^{WT} or TCR-PLP1 PLP^{KO} mice concluding that TCR-PLP1 mice are tolerant to EAE induction.

The interesting question is what is happening if we take away central tolerance or periphery tolerance or what if take away both central and periphery tolerance? To this end, we first immunized TCR-PLP1 PLPΔTEC mice. This mouse strain is lacking of central tolerance induction to PLP. We observed that TCR-PLP1 PLPΔTEC mice fail to evoke EAE onset after immunization with PLP_{11-18}. In order to take away periphery tolerance, $CD4^+$ splenocytes from TCR-PLP1 PLP^{KO} mice were transferred into the naïve WT recipients. 6 hours post transfer recipients were immunized with PLP_{11-18} in CFA. In this setup, WT mice receiving $CD4^+$TCR-$PLP1^+$ naïve T cells developed EAE from day 7 post immunization which was accompanied by typical weight loss. In order to take away both central tolerance and periphery tolerance, $CD4^+$TCR-$PLP1^+$ naïve T cells from TCR-PLP1 PLP^{KO} mice were transferred into ΔTEC recipients. Interestingly, all ΔTEC mice have increased severity of EAE compared to WT EAE but did not alter the disease duration (Figure 28).

These results strongly suggest that both central tolerance and periphery tolerance mechanisms, contribute to tolerance state of C57BL/6 mice to PLP.

3. Discussion

3.1 Lack of tolerance induction in Plp11-specific autoreactive T Cells

Central and peripheral tolerance prevents autoimmunity by deleting those $CD4^+$ T cells posing the greatest threat. T cells recognizing epitope $PLP_{174-181}$ are restricted to antigen recognition in the context of $I\text{-}A^b$. Previous results have shown that a fraction of $CD4^+$ T cells specific for this region appear to incomplete tolerance induction[219]. We would like to understand this phenomenon more detail, therefore, we have constructed a transgenic mouse expressing genes encoding a rearranged T cell receptor specific for $PLP_{174-181}$. Firstly, T-cell hybridomas were generated by immunization of PLP^{KO} mice with purified PLP protein. These immunizations yielded $PLP_{174-181}$-specific T-cell hybridomas from the subsequent immunized draining lymph nodes cells of the PLP^{KO} mice with $PLP_{174-181}$ peptide. Next, the TCR-α/β genes derived from selected $PLP_{174-181}$-specific T-cell hybridomas were sequenced and cloned the TCR variable regions Vα2 and Vβ14 into the pTα- and pTβ-vector for using the generation of TCR-PLP11 mice. Last, we present the characterization of a new transgenic mouse, as a novel model of immunological tolerance to PLP involving PLP ignorance for $CD4^+$ T cells, in which EAE can be induced by immunization with specific peptide $PLP_{174-181}$. Key features of this model are: 1) no deletion of the Plp11-specific T cells occurred in the thymus and periphery, 2) functional autoreactive T cells were found in the spleen and lymph nodes of TCR-PLP11 mice *in vitro* but not spontaneously activated *in vivo*, 3) development of autoimmune disease in response to PLP immunization in CFA, and 4) EAE developed spontaneously in TCR-PLP11 transgenic RAG-1-deficient mice. Taken together, these results establish a novel model of immunological tolerance towards a self-antigen expressed in the central nervous system involving antigen ignorance for $CD4^+$ T cells, which affords a unique opportunity to elucidate why and how autoreactive T cells can escape from central tolerance.

In TCR-PLP11 mice, a pronounced skewing toward the $CD4^+$ T cell population specific for epitope $PLP_{174-181}$ was expected, as the transgenic TCR genes were isolated from a MHC class II–restricted $CD4^+$ T cell clone. Despite large numbers of myelin-specific T cells, they failed to develop any clinical signs of central nervous

system autoimmunity. In order to determine the mechanisms of T cell tolerance in the thymus or periphery that could account for the missing incidence of spontaneous EAE in TCR-PLP11 mice, we monitored the fate of $CD4^+$TCR-$PLP11^+$ T cells during their development. The presence of the self-antigen PLP in the thymus of TCR-PLP11 PLP^{WT} mice did not affect the development of CD4SP T cells as wells the expression of TCR-PLP11 on the surface of those cells when compared to TCR-PLP11 PLP^{KO} mice (Figure 11). This clearly indicated that Plp11-specific thymocytes were not negatively selected in TCR-PLP11 PLP^{WT} Tg mice. This implied that periphery tolerance mechanisms might play a role in maintaining tolerance to PLP11 in TCR-PLP11 PLP^{WT} mice. Analysis of peripheral Plp11-specific $CD4^+$ T cells revealed similar numbers and frequencies of the $CD4^+$TCR-$PLP11^+$ T cells in secondary lymphoid organs of TCR-PLP11 PLP^{WT} mice as compared with TCR-PLP11 PLP^{KO} littermates. Importantly, $CD4^+$TCR-$PLP11^+$ cells from TCR-PLP11 PLP^{WT} mice displayed a naïve phenotype, and were not anergic, as they proliferated in a dose-dependent response to $PLP_{174-181}$ and comparably to Plp11-specific $CD4^+$ T cells originating from TCR-PLP11 PLP^{KO} mice when stimulated with their specific peptide *in vitro* and *in vivo* (Figure 13). Thus, on both TCR-PLP11 PLP^{WT} and the TCR-PLP11 PLP^{KO} background, the T cells were fully capable of responding to $PLP_{174-181}$ in a comparable fashion. The lack of proliferation upon adoptive transfer into PLP^{WT} mice could result from two not mutually exclusive scenarios: 1) TCR affinity of TCR-PLP11 Tg $CD4^+$ T cells is too low to reach the threshold for activation in the periphery, 2) The peptide $PLP_{174-181}$ is not efficiently presented on MHC class II molecular. We do not have proof evidence for one of these scenarios, however bioinformatic analysis of the binding of $PLP_{174-181}$ to MHC class II revealed a very poor binding capacity which might be in favour for the latter explanation. Finally, to test the tolerance state of our TCR-PLP11 Tg mice, we immunized TCR-PLP11 Tg animals with $PLP_{174-181}$ peptide in CFA. Because of the large number of Plp11-specific T cells in the periphery of TCR-PLP11 mice (Figure 12) and their activation into effector cells upon immunization the mice developed clinical signs of EAE (Figure 15).

The absence of thymic deletion in TCR-PLP11 mice could be explained either by the absence of the PLP protein in the thymus, by low avidity of the TCR-PLP-MHC interaction or by a defect in the processing/presentation of the $PLP_{174-181}$ peptide. In

the thymus, expression of PLP promotes protection through elimination of autoreactive T cells, which has been shown in TCR-PLP1 Tg mice, a mouse model that has been previously described in our lab (Winnewisser J., PhD thesis). In the presence of the self-antigen PLP in the thymus of TCR-PLP1 PLP^{WT} mice, we observed strong negative selection of TCR-$PLP1^{+}$ T cells (Figure 19). This implies that the PLP protein is indeed expressed in the thymus of TCR-PLP1 PLP^{WT} mice. Furthermore, we could verify the expression of PLP in the thymus by quantitative real time PCR, showing that PLP was expressed by mTEC as well as cTEC in the thymus (data not shown).

Next we want to determine the mechanisms of periphery tolerance preventing the development of autoimmunity in the TCR-PLP11 Tg model. In the periphery we could not find any evidence for deletion of Plp11-specific T cells nor could we detect any modification of their phenotype or alteration of their function in TCR-PLP11 PLP^{WT} mice. Our results suggest that one important mechanism for the prevention of autoimmune disease in TCR-PLP11 PLP^{WT} mice is ignorance of PLP11 self-antigen by specific T cells. Ignorance could be due to the absence of presentation or the lack of appropriate T cell activation conditions[176]. In order to determine the mechanisms of whether this state of ignorance could be due to an impaired in PLP processing and/or $PLP_{174-181}$ presentation, we adoptively transferred naïve $CD4^{+}$ T cells were labeled with CFSE from TCR-PLP11 PLP^{KO} mice into B6 mice. A high level of the CFSE fluorescence was maintained in the recipients, indicating that no cell division had occurred (Figure 14A). In control mice, the proliferation of $CD4^{+}$TCR-$PLP11^{+}$ T cells were observed for draining lymph nodes of WT mice immunized with $PLP_{174-181}$ as shown by the progressive dilution of CFSE (Figure 14B and 14C). Plp11-specific T cells rapidly underwent extensive cell division, suggesting $CD4^{+}$ T cells are activated by the antigen encounter following immunization. This observation suggested that the adoptively transferred PLP-specific cells didn't encounter PLP in the periphery of the recipients. In TCR-PLP11 mice, this situation could be related to the low level of expression of the PLP self-antigen or the low affinity of the PLP-TCR for its ligand, or to an impairment of $PLP_{174-181}$ processing/presentation.

3.2 Active EAE induction in TCR-PLP11 Transgenic Mice

EAE is a commonly used mouse model for multiple sclerosis, which can be induced either by injection of myelin antigen in CFA or by transfer of activated myelin-reactive $CD4^+$ T cells. Activating stimuli leads to an augmentation of the density of peptide-MHC complexes, which trigger the induction of a pathogenic autoimmune response. To analyze the impact of the PLP-specific T cells on EAE development, we immunized TCR-PLP11 mice with the $PLP_{174-181}$ peptide in CFA combined with injection of pertussis toxin (PT). Because TCR-PLP11 PLP^{WT} and TCR-PLP11 PLP^{KO} littermates appear physically identical, genotype-blind disease scoring and thus elimination of bias was feasible. Our results showed that the TCR-PLP11 PLP^{WT} transgenic mice were susceptible to the induction of EAE (Figure 15). As the majority of $CD4^+$ T cells expressed the transgenic T cell receptor, the accelerated disease onset could be explained by the high number of Plp11-specific $CD4^+$ T cells present in their immune repertoire. The immunized sick mice exhibited partial or complete recovery from symptoms. In contrast, due to the absence of PLP expression in TCR-PLP11 PLP^{KO} mice, the PLP-induced immune response should not induce CNS tissue damage. When TCR-PLP11 PLP^{KO} mice were immunized with $PLP_{174-181}$ peptide, all animals exhibited very low-grade EAE which likely results from immunization background (Figure 15). These data suggested that TCR-PLP11 Tg $CD4^+$ T cells were potentially pathogenic and can be activated to access and attack the CNS and induce EAE. Indeed, in a similar model[226], immunological ignorance could be overcome in mice expressing a transgenic TCR specific for a myelin-specific peptide (MBP) upon immunization with MBP accompanied by injections of pertussis toxin as well as with administration of pertussis toxin alone.

The presence of $CD4^+CD25^+$ regulatory T cells is a critical parameter for the suppression of autoimmunity[146]. To examine regulatory T cells in the TCR-PLP11 Tg mice, we determined the number of $CD4^+CD25^+$ T cells in spleens and lymph nodes by flow cytometry. Our analysis in TCR-PLP11 Tg mice revealed a generally lower number of $CD25^+Foxp3^+$ cells in the $CD4^+$TCR-$PLP11^+$ T cell population than the expected 10% in nontransgenic littermates. Moreover, there is no difference in the number of $CD4^+Foxp3^+$ T cells between TCR-PLP11 PLP^{WT} and the TCR-PLP11

PLP^{KO} mice (Figure 11). It will be of interest to investigate whether other types of regulatory T cells suppress more fulminant disease development in our system.

3.3 EAE developed spontaneously in TCR transgenic RAG-1-deficient mice

A crucial feature of the TCR-PLP11 PLP^{WT} mouse is that it spontaneously develops EAE on the Rag^{KO} background (Figure 16). The TCR-PLP11 Tg mice are particular valuable because they allow us to evaluate the pathogenic and regulatory mechanisms. By contrast, the occurrence of spontaneous EAE is not detected in our TCR-PLP11 Tg mice during routine care of the animals. The only difference between these two strains of mice is that in these immunodeficient mice, mature $CD4^+$ T cells exclusively express the transgenic TCRs specific for Plp11 and no endogenous TCRs could rearrange and consequently no Tregs were generated, which express TCRs encoded by the endogenous TCR α and TCRβ loci. In contrast, in TCR-PLP11 Tg mice include some nontransgenic lymphocytes, indicating that a protective role of a small population of non-Tg TCR specificities. Three most popular models were also reported to develop spontaneous EAE when bred onto the RAG knockout background, including TCR transgenic mice specific for myelin oligodendrocyte glycoprotein (MOG)[207] and mice specific for myelin basic protein (MBP)[205], as well as TCR transgenic mice recognizing $PLP_{139\text{-}151}$[206]. Lafaille's group demonstrated that a single administration of as few as 2×10^5 $CD4^+$ splenocytes from naïve immunocompetent mice could protect against spontaneous EAE occurring in MBP-specific TCR transgenic/$RAG1^{-/-}$ mice (referred to as T/R-)[227]. Although these studies preceded the discovery of Foxp3, which is expressed in regulatory T cells and is the specific lineage maker for their identification, the transferred $CD4^+$ splenocytes undoubtedly included Treg cells as well as other regulatory T cells. MBP-specific TCR transgenic mice (referred to as T/R+) do not develop EAE spontaneously perhaps due to the presence of small population of non-Tg TCR specificities. Actually, the presence of Tregs has been reported to impact the course of disease in different models. Hori et al., using Lafaille's model system, evaluated the ability of $CD4^+CD25^+$ Tregs to control the development of spontenous EAE. They demonstrated that MBP-specific $CD4^+CD25^+$ Tregs contributed the suppression. Meanwhile, other types of $CD4^+CD25^+$ Tregs with different specificities also indicated a protective role. The $CD4^+CD25^+$ Tregs could transfer protection of animals against

EAE significantly[228], and depletion of Treg cells with anti-CD25 antibody from EAE-susceptible SJL/J mice and EAE resistant B10.S mice exacerbated EAE[229, 230].

In TCR transgenic mice, T lymophocytes expressing the transgenic TCR-β chains results in allelic exclusion of endogenous β chains, whereas the transgenic TCR-α chains expression does not. Therefore, in TCR transgenic mice a proportion of T cells will express the transgenic β chain together with an endogenous α chain. When we analyzed the Plp11-specific TCR expression within the $CD4^+$ compartment, we observed the dominant Vβ14 together with α chains other than that the Tg Vα2, a lot of them expressing Foxp3 (Figure 18). This might partly explain why spontenous EAE is observe in TCR-PLP11 $PLP^{WT}RAG^{KO}$ mice but not in TCR-PLP11mice. A very low frequency of $CD4^+CD25^+$ T cells (<1%) in TCR-PLP11 $PLP^{WT}RAG^{KO}$ mice might be due to a restricted endogenous TCR-encoded repertoire (Figure 17). These finding suggest that regulatory TCR expressing Treg cells from TCR-PLP11 mice recongnize a not necessarily different one self-antigen, that mediate specific regulatory function. One feature of particular interest for future studies is to remove of endogenous T cells (putative regulatory), cells of PLP-specific TCR transgenic mice, and the monitor whether transfer of polyclonal T cells completely prevented EAE in TCR-PLP11 $PLP^{WT}RAG^{KO}$ mice. Moreover, It will be of very interest to address the question of whether the transferred $CD4^+CD25^+$ regulatory T cells from different antigen specificity could recruit PLP-specific T cells to regulatory function in TCR-PLP11 $PLP^{WT}RAG^{KO}$ mice. Together with our results in Rag^{KO} animals showed a potential contribution of dominant tolerance to the prevention of CNS autoimmunity in TCR-PLP11 mice.

3.4 Central tolerance to PLP is induced by clonal deletion and concomitant Treg induction of TCR-PLP1^{+} T cells

Immunological tolerance is a fundamental property of the immune system which is maintained by central and peripheral mechanisms, allowing the immune system to respond to non-self antigens and unresponsive to self-antigens. In central tolerance, the two major mechanisms include clonal deletion and clonal diversion (Treg differentiation) of thymocytes with high-affinity TCRs specific for self-peptide-MHC complexes, thereby eliminating potentially dangerous self-reactive T cells. However, self-reactive T cells can still escape central tolerance checkpoints. Therefore, peripheral tolerance exists which ensures those escaping cells remain unresponsive in peripheral organs, involving the deletion of self-reactive T cells or induction of functionally unresponsiveness (anergy) after encountering self-antigens outside of the thymus. Breakdown of either central or periphery tolerance can lead to autoimmunity.

In TCR-PLP1 Tg mice, thymocytes that bear the PLP1 specific TCR are negatively selected upon interactions with PLP-MHC class II complexes. Concomitantly, in the presence of the cognate self-antigen PLP, a large proportion of the Plp1-specific CD4^{+} T cells undergo selection to become CD4^{+}CD25^{+} Foxp3^{+} T cells. It is known that high affinity/avidity of TCRs to self-antigens is prerequisite for negative selection thymus. It is worth noting that when PLP is present in TCR-PLP11 Tg mice, the failure of thymocytes bearing the PLP11 specific TCR undergo negative selection as well as become regulatory T cells might be due to the low affinity/avidity of the TCR for PLP$_{174\text{-}181}$-MHC interaction. These data suggest that negatively selected Plp1-specific CD4^{+} T cells as well as the selection of regulartory T cells in TCR-PLP1 mice depends on the high affinity/avidity of the TCR for PLP$_{11\text{-}18}$-MHC complex.

CD4^{+} T cells bearing T cell receptors that recognize self-antigens can be eliminated by negative selection, this mode of tolerance was also observed in other TCR transgenic models. For instance, in the liver-antigen transgenic system, intrathymic hCRP expression, showing that tolerance is mediated by intrathymic deletion of immature thymocytes. Another experimental system from TCR-HA x AIRE-HA$^{131,\ 168}$, a substantial fraction of the TCR-HA^{+} thymocytes differentiates into Treg cells as well

as two-thirds of the thymocytes are subjected to negative selection. In our TCR-PLP1 mouse model, clonal deletion of the mature Plp1-specific T cells and concomitant the differentiation of antigen-specific T cells into the Treg lineage both operate for the tolerance towards self-antigen PLP.

3.5 PLP is expressed and presented by medullary thymic epithelial cells autonomously

The process of generating and presenting of self-antigens is complex. For positive selection, cortical epithelial cells are the predominant stromal cell in the cortex and are vital for that process. In contrast, multiple APCs types contribute to T cell tolerance in thymic medulla. Although previous work has suggested contributions of each APCs subset to T cell tolerance, it remained unclear if these components functioned uniquely or redundantly. We addressed the importance of thymic PLP expression by individual thymic APC subsets in mediating immune tolerance towards Plp1-specific T cells.

mTECs as the major PLP-expressing cells in the thymus

Firstly, we determined the PLP expression in specific thymic cell populations. To examine the PLP mRNA expression in hematopoietic ($CD45^+$) and stromal cell populations as well as in the thymic epithelial cell subsets ($CD45^-$) by cell separation and subsequent reverse transcriptase PCR. We found that PLP transcripts were predominately expressed in mTEC. Specifically, $mTEC^{hi}$ as the major PLP-expressing cells in the thymus in our study (Winnewisser J., PhD thesis). Using transplantation experiments, we clearly showed that expression of PLP by radioresistant stromal cells, leading to deletion and Treg cells induction of TCR-$PLP1^+$ T cells (data not shown). While TCR-$PLP1^+$ T cells were not tolerized when PLP was expressed by hematopoietic cells, indicating that expression of PLP by hematopoietic compartment was not crucial for tolerance induction to PLP.

To elucidate the essential role of this mTEC-derived PLP expression, we developed a mouse mode in which the PLP gene was specifically deleted in TECs due to the Foxn1-Cre-driven excision of the floxed PLP gene. In TCR-PLP1 PLPΔTEC mice,

we observed an impaired negative selection of TCR-PLP1^{+} T cells in the thymus and also no Plp1-specific Tregs were generated when PLP expression was abolished in TECs (Figure 20). This suggests that hematopoietic APCs (mainly thymic DCs and/or B cells) were not capable of altering the fate of PLP-specific CD4^{+} T cells. Thus, it seemed that PLP expression by TECs could orchestrate both thymocytes fates in parallel. However, we cannot rule out the possibility of tolerance induction to PLP expressed by cTECs. We found the numbers of DP thymocytes were not reduced when compared TCR-PLP1 PLP^{WT} with TCR-PLP1 PLP^{KO}, indicating that no deletion in the cortex. In contrast, Plp1-reactive T cells initiated apoptosis at the CD4SP stage in the medulla. These results showed that deletion of autoreactive PLP-specific thymocytes do require expression of PLP by mTECs to preserve tolerance.

mTEC present PLP autonomously

In theory, there are a number of ways that will allow PLP epitopes to be presented to T cells in the thymus to mediate central tolerance: bone marrow-derived APCs can take up and transport circulating PLP to the thymus; radio-resistant stromal cells, express and present PLP to the T cells directly; or PLP_{11-18} peptide derived from the PLP protein, dreived from mTECs, subsequently cross-presented by DCs to mediate the deletion of the autoreactive T cells within the thymus. We quantified the impact of BM APCs and mTECs on the processes of clonal deletion and Treg cells selection.

It has been described that mTEC-derived self-antigens spread to DCs to resolve the issue of how antigens expressed by a minor fraction of mTECs can induce T cell tolerance. Although MHC class II-bound peptides can be autologously presented on mTECs via autophagy[93, 94], DCs present peptides via classical, exogenous MHC class II loading. When the antigens are transferred from mTEC to DCs, it may blur the distinction between antigens that are displayed on DCs and APCs. This phenomenon was observed in TCR transgenic models or MHC class II tetramer at the level of individual antigens. We used a model system in which DCs could be specifically ablated, TCR-PLP1 PLP^{WT}ΔDC to rule out the possibility of mTEC-derived PLP was transferred from mTECs to DCs. We analyzed the frequency of thymocyte populations in TCR-PLP1 PLP^{WT}ΔDC and TCR-PLP1 PLP^{WT} mice (DC

deficiency versus DC sufficiency) in Figure 21. Irrespective of whether DCs were experimentally eliminated or not, this resulted in the similar frequencies of CD4SP negatively selected, indicating that medullary DCs which cross-present mTEC-derived antigens, do not contribute to negative selection in our experiment system. We wanted to directly test the PLP presentaion within different APC subsets ex vivo by using a Plp1-specific hybridoma, but PLP presenting APCs in the thymus seemed to be very rare that the sensitivity of our assay was not sufficient to measure any PLP presentation.

Previously, Hinterberger et al. demonstrated that autonomous role of mTEC in $CD4^+$ T cell tolerance by using C2TAkd mice[102]. In C2TAkd mice, MHC class II expression is diminished to approximately 10% of WT levels. We crossed C2TAkd mice with TCR-PLP1 PLP^{WT} mice, analysis of TCR-PLP1 PLP^{WT} C2TAkd mice (Winnewisser J., PhD thesis) indicated that residual MHC class II expression on mTEC, led to a diminished efficacy of negative selection, but not a complete loss of tolerance and more induction of Treg cell lineage to PLP. This observation also argued that our TCR-PLP1 model favour avidity hypothesis. *In vitro* stimulation assay revealed that $PLP_{11\text{-}18}$ peptide in the femtogram-range triggered a stimulation of $CD4^+$TCR-$PLP1^+$ peripheral T cells indicating a high affinity to PLP. In contrast, the concentration required to stimulate an equivalent response in TCR-PLP11 Tg mice was 50 times higher with the $PLP_{174\text{-}181}$, indicating $PLP_{174\text{-}181}$ peptide does not bind MHC class II efficiently. Other stromal APCs, like cortical TECs, may have the similar dual function in tolerance to PLP, which shape of the $CD4^+$ repertoire only apparent when the contribution of mTEC is diminished. Taken together, this finding is consistent with PLP expression and also shows that mTEC has impact on the antigen presentation of self-antigen PLP to auto-reactive T cells.

Taken together, these data substantiates the idea that automonous APC function of mTECs, that is expression and subsequently presentation of a self-antigen by mTEC serve an essential function to both deletion and Treg development mechanisms of tolerance induction and thymic DCs does not mediate central tolerance to PLP.

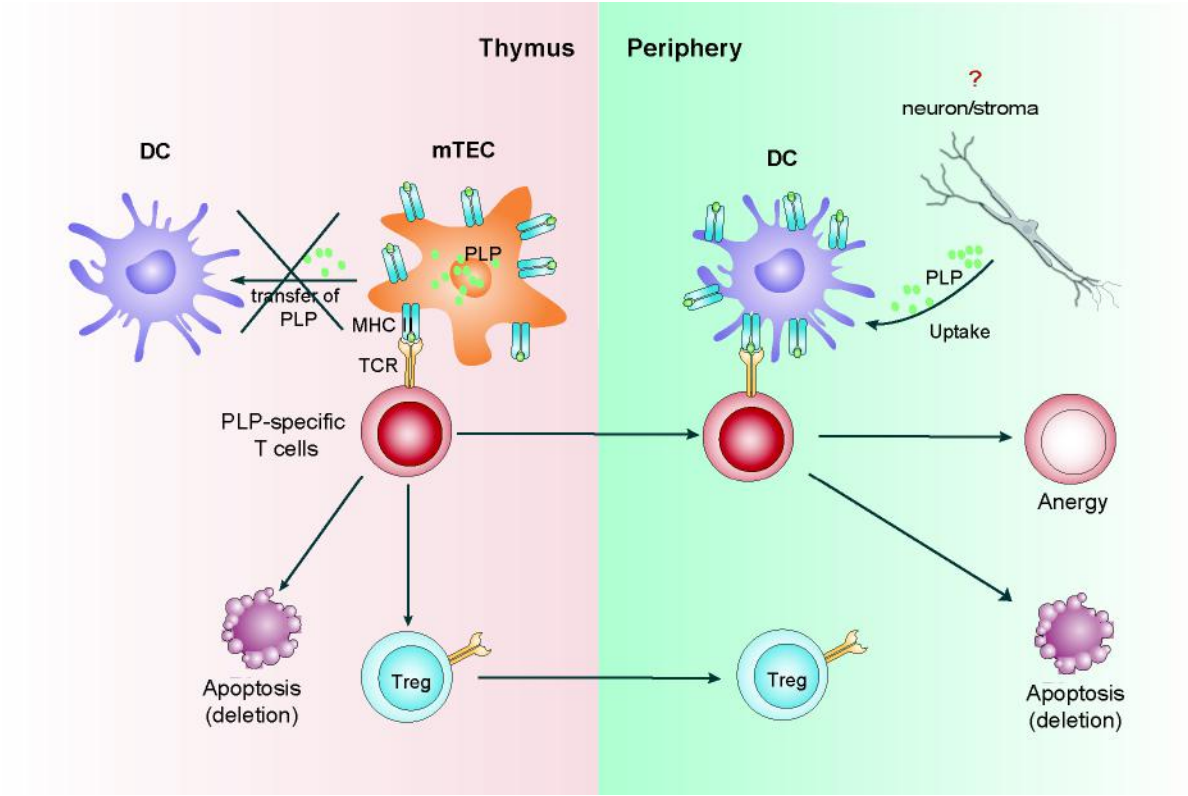

Figure 29: Graphical summary of central and peripheral tolerance mechanisms in maintaining tolerance to PLP. When TCR transgenic (TCR-PLP1) mice that bears T cells specific for the major I-Ab determinant of PLP1, we could monitor the fate of Plp1-specific $CD4^+$ T cells at physiologic levels. Moreover, we investigated the contribution of the individual thymic antigen presenting cells to central tolerance to PLP. Since autoreactive T cells can still eacape to the periphery, we could also show the mechanisms of the periphery. Furthermore, we found out the type of APC was presenting PLP to TCR-$PLP1^+$ T cells in the periphery to induce anergy. Key features of this model are: 1) Plp1-specific T cells undergo clonal deletion and Treg differentiation concomitantly upon encounter of the cognate self-antigen PLP, 2) PLP is presented by medullary thymic epithelial cells, 3) Autoreactive Plp1-specific T cells are deleted or become functionally inactivation (anergy) by recognition of PLP on dendritic cells in the periphery, 4) PLP is expressed by radioresistant stromal cells and subsequently cross-presentent by dendritic cells. Our studies demonstrate the importance of both central and peripheral mechanisms in maintaining tolerance to PLP.

3.6 Autoreactive Plp1-specific T cells are deleted or become functionally inactivation (anergy) by recognition of PLP on dendritic cells in the periphery

Central tolerance to PLP is not complete; a fraction of PLP-reactive T cells are not deleted by central tolerance and thus released into the periphery. Yet, TCR-PLP1 mice did neither develop EAE spontaneously nor was it possible to induce EAE by immunization with PLP_{11-18} (Figure 28). Therefore, many of these T cells are silenced by peripheral tolerance mechanisms, which are necessary to prevent the development of autoimmune disease. Peripheral deletion is one mechanism by which self-reactive T cells are removed that escaped thymic tolerance mechanisms. In TCR-PLP1 mice, we demonstrated that Plp1-specific $CD4^+$ T cells were deleted in the presence of the cognate self-antigen PLP as compared to the absence of PLP expression in the periphery of TCR-PLP1 PLP^{KO} mice. This view is further supported by the observation that PLP-specific $CD4^+$ lymphocytes are strongly reduced even in the absence of central tolerance in TCR-PLP1 PLPΔTEC mice. These data confirm that peripheral deletion is one mechanism by which plp specific escapees can be controlled in the periphery Noteworthy, a large fraction of the Plp1-specific $CD4^+$ T cells are deviated into regulatory T cells in TCR-PLP1 PLP^{WT} mice. However, in TCR-PLP1 PLPΔTEC mice, escaping cells are not deviated into $Foxp3^+$ Tregs cells. This suggests that peripheral Treg induction of PLP specific T cells that have escaped from negative selection in the thymus does not take place in or model system.

Another potential mechanism by which self-reactive periphery T cells are silenced is functional inactivation, referred to as anergy. Importantly, TCR-PLP1 PLP^{WT} mice as well as TCR-PLP1 PLPΔTEC mice with defective thymic clonal deletion of T cells have an increased frequency of $CD4^+$ T cells in the periphery with an anergic $ICOS^{hi}FR4^{hi}$ phenotype (Figure 23). We further characterized the functionality of $CD4^+Foxp3^-$ in TCR-PLP1 PLP^{WT} as well as TCR-PLP1 PLP^{KO} Tg mice. The adoptive transfer system studied in this investigation showed that, naive PLP-specific $CD4^+$ T cells from TCR-PLP1 PLP^{KO} mice ($CD4^+TCRPLP1^+$) expanded vigorously in recipient mice expressing physiological PLP in the periphery. In contrast, upon transfer of $CD4^+TCRPLP1^+$T cells that came from TCR-PLP1 PLP^{WT} mice, no proliferation to PLP could be detected (Figure 24). Using TCR-PLP1 PLPΔTEC T

cells confirmed that the functional inactivation of PLP specific cells was carried out in the periphery, not in the thymus. These cells represent escaping Ag-specifc T cells from the thymus and were anergized in secondary lymphoid organs. It is also possible that *in vivo* functional unresponsiveness was associated with immune deviation or suppression by residual PLP-specific Treg cells (Figure 24). We ruled out this possibility by co-transfer $CD4^{+}Foxp3^{-}$ T cells from TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} mice into PLP-expressing recipients. We demonstrated that under the co-transfer situation, which would be expected to contain putative suppressive environment, TCR-PLP1 PLP^{KO+} T cells still proliferated vigorously compared with TCR-PLP1 PLP^{WT+} T cells displayed functional defects (Figure 25). This finding highlighted the fact that TCR-PLP1 PLP^{WT} and TCR-PLP1 PLP^{KO} PLP-specific T cells were functional different with respect to their proliferative capacities. Thus, there is no evidence that suppressive mechanism contribute to the functional unresponsiveness to PLP of the tolerized TCR-$PLP1^{+}$ T cells in PLP^{WT} animals. It is worthwhile pointing out that, in KRN x NOD mice model, autoreactive T cells were likewise detected in the periphery and developed arthritis in those mice[242]. In TCR-PLP1 PLPΔTEC mice (no central tolerance), however, no signs of autoimmune disease was seen, indicating the existence of deletion and anergy as periphery tolerance mechanisms that are particularly important when central tolerance fails. It also will be important to establish the precise requirement for inducing deletion versus anergy in future experiments.

The proliferation of transferred TCR-$PLP1^{+}$ T cells was dependent on whether the host was sufficient or deficient for PLP. Bone marrow chimeras were used in which only radioresistant cells could express PLP ($MHCII^{WT}PLP^{WT}\rightarrow PLP^{WT}$, $MHCII^{WT}PLP^{WT}\rightarrow PLP^{KO}$), and only hematopoietic cells could present PLP ($MHCII^{KO}PLP^{WT}\rightarrow WT$, $MHCII^{KO}PLP^{KO}\rightarrow PLP^{KO}$). These chimeras were then transferred with naïve TCR-$PLP1^{+}$ T cells from TCR-PLP1 PLP^{KO} mouse. We observed expansion of $CD4^{+}$TCR-$PLP1^{+}$ T cells in which both hematopoietic cells and radioresistant cells can express PLP ($MHCII^{WT}$ $PLP^{WT}\rightarrow PLP^{WT}$). In contrast, in a chimera lacking PLP expression on radioresistant cells, but expressing PLP and class II on hematopoietic cells ($MHCII^{WT}PLP^{WT}\rightarrow PLP^{KO}$), TCR-PLP1 PLP^{KO+} T cells proliferation was not observed, indicating PLP was expressed only by radioresistant

cells but not expressed by hematopoietic cells (Figure 26). To determine the possible role of hematopoietic cells to present PLP to TCR-PLP1^{+} T cells in the periphery for tolerance induction, we created bone marrow chimeric mice taking advantage of MHCII^{KO} mice. Hematopoietic cells with MHCII deficiency are not able to stimulate Plp1-specific T cells in response to PLP1, allowing for the restriction of antigen presentation to hematopoietic cells but not radioresistant cells. To this end, we used class II-deficiency bone marrow to reconstituted PLP-expressing hosts (MHCII^{KO}PLP^{WT}→WT). This system did not generate a significant Plp1-specific T cell population which responded to periphery PLP of the recipients. This data indicated that PLP was present by hematopoietic cells, once its presentation capacity was impaired by the abrogation of MHCII expression, no proliferative response to PLP was observed (Figure 26). Taken together, although these APCs of hematopoietic origin were not responsible for expressing PLP autonomously, obviously they were able to pick up from non-hematopoietic cells and subsequently present and induced the robust expansion of TCR-PLP1^{+} T cells.

We speculated that peripheral DCs are playing a role in tolerance induction of PLP-specific T cells by deletion and/or anergy. To resolve such an issue, it is important to develop *in vivo* systems where DCs are not capable of presenting antigen. We performed adoptive transfers of mature naive CD4^{+} T cells from TCR-PLP1 PLP^{KO} mice into ΔDC mice. We observed the impaired capacity of TCR-PLP1^{+} T cells to proliferate to PLP in such a DC free environment. We concluded that presentation of PLP by DCs is necessary for proliferation of specific T cells. Next we asked whether DCs were also instrumental for anergy induction of autoreactive T cells. To this end, we took out the CD4^{+} T cells from either TCR-PLP1 PLP^{WT} or TCR-PLP1 PLP^{WT}ΔDC mice and transferred into PLP-expressing recipients. If DCs are responsible for anergy induction, these cells should not proliferate as the CD4^{+} T cells from TCR-PLP1 PLP^{WT} mice. As shown in Figure 27, when the cells were taken from DC free environment, CD4^{+} T cells cannot become anergic, they are capable of responding to the peptide. Therefore, the adoptive transfer experiments performed here showing in a definite and conclusive manner that peripheral DC can induce periphery tolerance of remaining TCR-PLP1^{+} T cells by anergy induction.

3.7 Both central and peripheral tolerance mechanisms in maintaining tolerance to PLP

EAE can be provoked by the immunization with self-antigen indicates that potentially autoreactive T cells escape from thymic selection and are present in the periphery T-cell repertoire of healthy individuals. To test the tolerance state of TCR-PLP1 Tg mice to EAE induction, we used a classic protocol for immunization: TCR-PLP1 Tg mice were immunized with PLP_{11-18} emulsified in CFA. Mice treated in this fashion are protected from EAE concluding that TCR-PLP1 mice are tolerant. The maintenance of tolerance to PLP_{11-18} is orchestrated by a complex sequence of tolerance mechanisms. It is important to investigate the central tolerance and periphery tolerance mechanisms mediating the tolerant state in TCR-PLP1 mice, we used three different mouse models (Figure 28). In the first scenario, we immunized TCR-PLP1 PLPΔTEC mice. This mouse strain is a lack of central tolerance to PLP. We observed that TCR-PLP1 PLPΔTEC mice do not develop EAE after immunization with PLP_{11-18}, indicating periphery tolerance mechanisms are required to prevent autoimmunity. In the second scenario, to identify the role of periphery tolerance that maintains T-cell tolerance to PLP, adoptive transfer of naïve $CD4^+$ T cells from TCR-PLP1 PLP^{KO} mice into WT recipient animals following immunization with PLP_{11-18} in adjuvant was used to break periphery tolerance. This treatment triggers mild EAE. This indicates that impair the function of periphery T cell tolerance against PLP causes PLP-specific T cells activation *in vivo* and would be poised to initiate an autoimmune attack. In an effect to break the central tolerant and periphery tolerance state of PLP1-specifc T cells, the same protocol (take away periphery tolerance as in the second scenario) was applied to ΔTEC recipients. Our results showed that all ΔTEC mice have increased severity of EAE compared to WT EAE. This interesting difference in EAE severity is a result of lacking total immune tolerance to PLP in ΔTEC mice. These observations indicate that both central tolerance and periphery tolerance mechanisms must exist to prevent autoimmunity in TCR-PLP1 Tg mice that remain healthy. The TCR-PLP1 Tg mice provided an excellent tool to investigate the extent and mechanisms underlying immune tolerance of $CD4^+$ PLP-specific T cells.

4. Materials and Methods

4.1 Materials

4.1.1 Mice

All animals used in thisTand specific pathogen-free (SPF) mouse facilities of the Institute for Immunology at the LMU Munich. PLP^{KO} mice were obtained from Klaus Nave from the Max-Planck-Institute for Experimental Medicine, Göttingen, which are described in Klugmann et al.[214]. The TCR-PLP1 transgenic mice specific for the PLP_{11-18} were generated by Hinterberger et al. (unpublished data). The TCR-PLP11 Tg mice for the $PLP_{174-181}$ were generated during this work. Pro-nuclear injections for generating the TCR-PLP Tg mouse were performed by the transgenic animal facility of the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden by Ronald Naumann. Foxn1-Cre mice were described in Gordon et al. [243]. $PLP^{fl/fl}$ mice were generously provided by Hauke Werner, Max-Planck-Institute for Experimental Medicine, Göttingen. ΔDC mice were a kind gift from David Voehringer and are described in Ohnmacht et al. [103].

Mice were analyzed at 3 weeks old and used under protocols approved by the Animals Studies Committee.

4.1.2 Antibodies

Table 1: The following antibodies were used in this study for flow cytometry. All antibodies and Second Step reagents are listed with the respective clone and conjugated fluorophore.

Specificity	Label	Clone	Isotype	Supplier
CD3	PE-Cy7	145-2C11	Armenian Hamster IgG	Biolegend
CD4	APC-Cy7	GK1.5	Rat IgG2b, κ	Biolegend
CD4	V500	RM-4-5	Rat (DA) IgG2a, κ	BD
CD8	PE-Cy5	53 - 6:7	Rat (DA) IgG2a, κ	Biolegend
CD24	Pacific Blue	M1/69	IgG2b, kappa	eBioscience
CD25	PE-Cy7	PC61	Rat IgG1, λ	Biolegend
CD44	APC-Cy7	IM7	Rat IgG2b, κ	Biolegend
CD45.1	PB	A20	Mouse(A.SW) IgG2a, κ	Biolegend
CD45.2	Alexa647	104	Mouse (SJL) IgG2a, κ	Biolegend
CD62L	APC	MEL-14	Rat IgG2a, κ	Biolegend
CD69	PE-Cy7	H1.2F3	Armenian Hamster IgG	Biolegend
Foxp3	APC	FJK-16s	IgG2a, kappa	eBioscience
TCR Vα2	PE	B20.1	Rat (LOU) IgG2a, λ	BD
TCR Vα2	Biotin	B20.1	Rat IgG2a, λ	Biolegend
TCR Vα3.2	Fitc	RR3-16	Fischer, CDF IgG2b, κ	BD
TCR Vα3.2	Biotin	RR3-16	IgG2b, κ	Biolegend
TCR Vβ6	Fitc	RR4-7	Rat IgG2b, λ	Biolegend
TCR Vβ6	PE	RR4-7	Rat IgG2b, λ	Biolegend
TCR Vβ14	Fitc	14-2	Fischer, CDF IgM, κ	BD
TCR Vβ14	Biotin	14-2	Fischer, CDF IgM, κ	Biolegend
Streptavidin	APC-Cy7			Biolegend
Streptavidin	PE-Cy7			BD
PD-1	PE-Cy7	RMP1-30	Rat IgG2b, κ	Biolegend
ICOS	FITC	C398.4A	Armenian Hamster IgG	Biolegend

FR4	PE-Cy7	12A5	Rat IgG2b, κ	Biolegend

4.1.3 Peptides

PLP peptides were synthesized by using solid-phase techniques and purified by HPLC at BioTrend in a quantity of 20 mg and a purity of >80% (HPLC). These stock solutions were stored at -20°C.

Table 2: Sequences of synthetic peptides.

Peptide	Sequence	Supplier
PLP_{1-24} peptide	GLLECCARCLVGAPFASLVATGLC	BioTrend
PLP_{9-20} peptide	CLVGAPFASLVA	BioTrend
PLP_{11-18} peptide	VGAPFASL	BioTrend
$PLP_{160-184}$ peptide	VVWLLVFACSAVPVYIYFNTWTTCQ	BioTrend
$PLP_{172-183}$ peptide	PVYIYFNTWTTC	BioTrend
$PLP_{174-181}$ peptide	YIYFNTWT	BioTrend
MOG_{35-55} peptide	MEVGWYRSPFSRVVHLYRNGK	BioTrend

4.1.4 Primers

Synthetic primers were purchased from ThermoHybaid Ulm and were delivered HPLC-purified and lyophilized. Oligonucleotides were dissolved at a concentration of 100 mmol/µl in ddH_2O. These stock solutions were stored at -20 ºC.

Table 3: List of all primer sequences used for genotyping.

Genotype	Primer name	Oligo Sequence (5'-> 3')
PLP	PLP common fwd	GAAAGGTTCCATGGTCAAGG
	PLP WT rev	CTGTTTTGCGGCTGACTTTG
	PLP KO rev	CTTGCCGAATATCATGGTGG
TCR-PLP1 Vα3.2	Vα3.2 fwd	ACAACAGAGCTGCAGCCTTC
	Vα3.2 rev	GCAGTGCTAGGAAGGGCGGC
TCR-PLP1 Vβ6	Vβ6 fwd	CCCAGAGCCAAAGAAAGTC
	Vβ6 rev	AGCCTGGTCCCTGAGCCGAA
TCR-PLP11 Vα2	Vα2 fwd	GAGTTTCCCCCAAGCTTCAGT
	Vα2 rev	GCCAGATCCTAACCAGGGAG
TCR-PLP11 Vβ14	Vβ14 fwd	AGTGCAGAGTAGACAAGCCT
	Vβ14 rev	AGACTTCTGTGTTAGCCGTCC
PLP-floxed	PLP-floxed fwd	GACATAGCCCTCAGTGTTCAGG
	PLP-floxed rev	GAATCCTGCATGGACAGACAG
Foxn1-Cre	Foxn1-Cre fwd	CTCTCCTCCGAGTATCCAATCTG
	Foxn1-Cre rev	CCCTCACATCCTCAGGTTCAG
CD11c-Cre	CD11c-Cre fwd	CGATGCAACGAGTGATGAGG
	CD11c-Cre rev	GCATTGCTGTCACTTGGTCGT
Rag1	Rag1 WT fwd	GAG GTT CCG CTA CGA CTC T
	Rag1 KO fwd	CCG GAC AAG TTT TTC ATC GT
	Rag1 Common rev	CCG GAC AAG TTT TTC ATC GT
DTA	DTA fwd	TACATCGCATCTTGGCCACG
	DTA rev	CCGACAATAAATACGACGCTG

4.1.5 Reagents and commercial kits

Table 4: List of commercial kits

Kit	Company
Fixation/Permeabilization solution	BD Bioscience
Qiaquick Gel extraction kit	Qiagen
GeneJET Plasmid Miniprep Kit	Thermo Scientific
QIAGEN Plasmid max kit	Qiagen

4.1.6 Buffers and Solutions

Table 5: Composition of buffers and solutions.

Buffer	Composition
Gitocher digestion buffer (10×)	670 mM Tris pH 8.8 166 mM ammonium sulfate 65 mM MgCl2 0.1% Gelatin
Tail digestion buffer	3 µl Proteinase K (10mg/ml stock) 2.5 µl Triton (10% stock) 5 µl Gitocher Buffer (10×) 0.5 µl β-Mercaptoethanol 39 µl H_2O
PBS (10×)	1.5 M NaCl, 30 mM KCl 80 mM Na_2HPO_4 20 mM KH_2PO_4 pH adjusted to 7.2-7.4
FACS buffer	500 ml PBS 1% bovine serum albumin (BSA) 0.1% sodium azide
PCR Red-buffer (5×)	250 mM KCl

	50 mM Tris pH8.3 43% Glycerol 7.5 mM $MgCl_2$ 2 mM Cresol Red
10x TBE	900 mM Tris 900 mM Boric acid 20 mM EDTA (pH 8)
Ack Buffer	0.15 M NH_4Cl 10 mM $KHCO_3$ 0.1 mM EDTA
LB agar	1 % tryptone 0.5 % yeast extract 10 mM NaCl 1.5 % agar

4.1.7 Cell culture media

Table 6: Cell culture media with supplements.

Medium	Supplements
cHL-1	500 ml HL-1 medium (Whittaker) 1% L-Glutamin with Penicillin /Streptomycin ((200mM, PAA) 1% MEM non-essential amino acids (100x, PAA) 1 mM Sodium Pyruvat (Gibco) 50 µM β-Mercaptoethanol (Gibco)
HAT-selection medium	500 ml cIMDM medium 12 ml Hypoxanthin/Thymidin (HT) 0.6 ml Aminopterin
cIMDM	500 ml IMDM medium containing L-Glutamin 8% Fetal calf serum (BioChrome) 1% L-Glutamin with

	Penicillin/Streptomycin (200mM, PAA) 1% MEM non-essential amino acids (100x, PAA) 1 mM Sodium Pyruvat (Gibco) 50 µM β-Mercaptoethanol (Gibco)

4.2 Methods

4.2.1 Cell Culture

4.2.1.1 Gerenal cell culture methods

General cell culture conditions were at 37°C supplemented with 5% CO_2 in complete medium consisting of IMDM supplemented with 10% FBS. Cells were passaged when 60–80% confluent at a 1:4 ratio.

4.2.1.2 Ex vivo re-stimulation of $PLP_{174-181}$-specific T cells

Nine days after immunization with $PLP_{160-184}$ peptide in CFA, mice were killed and the inguinal and poplietal lymph nodes were harvested for single cell preparations. Lymphocytes were resuspended in complete cIMDM medium (Table 6) containing 5 µg $PLP_{174-181}$ peptide and 4 x 10^6 cells/ml were seeded into 24-well plates. Cells that were cultured in medium only served as controls. At day 3 and day 7 of culture, 20 U/ml recominbant human IL-2 (hIL2) were added. On day 10, testing the specificity of the growing clones in a proliferation assay. T cells were restimulated every 10 days with 5 x 10^4 T cells with 4 x 10^5 lethally irradiated (3,000 rads) erythrocyte depleted, syngenic splenocytes or 3 x10^4 lethally irradiated bone-marrow derived dendritic cells together with 5 µg/ml cognate peptide.

4.2.1.3 Hybridoma generation

Lymph node cells from the PLP^{KO} single transgenic mice were stimulated with $PLP_{174-181}$ *in vitro*. After 3 days, cells were washed extensively to remove the serum. The hybridoma fusion partner cell line BW5147 (which is a BW-cell line that additionally carry a GFP-reporter under the NFAT-promoter, a kind gift of Dr. Dominic van Essen, Institute de Recherche sur le Cancer et le Vieillissement, Nice) was also harvested and washed. Mix the activated T cells and BW5147 cells at a ratio of 1:3 and 0.5 ml of 50% polyethylene glycol (PEG) was added slowly with gentle mixing to promote fusion. New fused hybrodoma cells are incubated in 96-well plates in the HAT (Hypoxanthine Aminopetrin Thymidine) medium. Seven days later medium was replaced with HT medium, and growing clones were expanded in normal cIMDM. After 2 weeks, only the dividing HAT-resistant T-cell hybriodoma cells are able to survive and proliferate while unfused HAT-sensitive BW tumor cells died off and unfused T cells died off as well because of the lack of exogenous IL-2.

4.2.1.4 Transfention of HEK 293T cells

Human embryonic kidney (HEK) 293T cells were transfected by the calcium phosphate method. Twenty-four hours prior to transfection, HEK 293T cells were seeded at a density of 1 x 10^6 cells in 90mm plate in 10 ml 10 mM HEPES. The following day, transfect cells. Preparing the following mix:

450 µl H_2O
12.5 to 25 µg plasmid DNA
50 µl 2.5 mM $CaCl_2$

The mix was incubated at 37°C for 5 to 7 minutes. After incubation, 37°C 500µl preheated HeBS was slowly added during vortexing. Keep 5 to 7 minutes at 37°C. Transfection mixture was carefully applied to the cells, then mix gently. Place the plate back in the incubator. 7 to 8 hours after transfection, gently changing the pre-warmed fresh medium to the plate. Do not disturb the DNA-$CaPO_4$ precipitates on the bottom of the plate. Harvest the cells 24 to 30 hours post-transfection by using ultracentrifugation.

4.2.1.5 Electroporation of cells with DNA construct

In order to express pTcassette vectors containing DNA construct in A5 cells stably, the cells were electroporated pTα cassette vector, 25 µg linearised pTβ cassette vector together with 5 µg linearised NFAT-GFP vector that contained a puromycin resistance gene with a pulse of 250 mV at RT. After 10 minutes of incubation on ice, the electroporated cells were transferred into 10 ml medium. Next day the cells were harvested and plated into 96-well on seletion medium containing 3 µg/ml puromycin. The cells that were restistant to puromycin would be selected and analyzed for TCR-expression and stimulation.

4.2.1.6 Preparation of Bone-marrow derived dendritic cells (BmDCs)

Mice were euthanized by CO_2 asphyxiation. Both hind legs were removed at just below the knee-joint through ligaments, ensuring that the epiphysis remains intact. Using lint-free tissue free paper to remove the surrounding muscles and tissue on the tibia and femur. Keeping the bones in 70% ethanol for 1 min for disinfection and then the bones were transferred to PBS for rinsing off ethanol. Both ends of the bone were trimmed and the bone marrow cells were flushed out from tibia and femur with PBS using a 25G needle. The BM cells were centrifuged and then resuspended in tris-ammonium chloride at 37°C for 5 minutes to lyse RBC. The cells were centrifuged again and then strained through a filter before being resuspended in 1 ml cIMDM medium and counted. BM Cells were adjusted at 0.2 x 10^6 cells/ml containing 10ng/ml GM-CSF. After 3 days, the cells were passaged 1/2 in fresh medium with cytokines added and replated. On day 6, loosely adherent cells were then collected on day 6 and replated in fresh medium before being harvested on day 7. Maturation of the DCs was induced by adding 300 ng/ml *E.coli*-derived LPS (Sigma) at 300 ng/ml. All cells were incubated at 37°C with 10% CO_2.

4.2.2 Molecular Biology

4.2.2.1 PCR amplication of TCR-α and TCR-β gene

Genomic DNA was prepared from 1 x 10^6 A43-11-5 T hybridoma cells by digestion with proteinase K (Sigma), followed by phenol extraction and ethanol precipitation. Briefly, equal volume of Phenol was added to DNA, thoroughly mixed and centrifuged at 10,000 rpm for 5 minutes. The aqueous layer was transferred into a clean tube. 1/10^{th} volumes of 3 M sodium acetate (Sigma) was added followed by the addition of 3 volumes of 100% ethanol (AppliChem), the reaction was incubated for 10 minutes at room temperature to precipitate the DNA. To remove ethanol solution, the DNA pellet was washed in 2 volumns of 70% ethanol. After briefly air drying the pellet, the DNA was dissolved in appropriate H_2O.

Once the genomic DNA is prepared, the PCR-based amplification process is started. Amplification is performed by adding genomic DNA 1 µl as the template, combined with primer (1.5 µl of forward and 1.5 µl of reverse primer at a concentration of 2.5 µM), 1.5 µl of dNTP, and 1 µl advantage high-fidelity DNA polymerase and incubated using the following PCR programme:

PCR programme for amplification of TCR cDNA:

2 min at 95° C

40sec at 95°C
45sec at 57°C
80sec at 72°C
} 30cycles

5 min at 72°C

PCR products were loaded to 1 % agarose gels.

4.2.2.2 Purify PCR products

For general DNA purification purposes, 5 µg DNA was digested in a 50 µl total volume including: 1-2 ml of enzyme and 1x buffer (diluted from 10 x stock) specified by the manufacture for each enzyme (The *Xma*I/*Sac*I restricted TCR-α DNA sequence and *Xho*I/*Sac*II restricted TCR-β DNA sequences), addition of acetylated BSA can sometimes improve the quality and efficiency of enzyme assay for 1 hours at 37°C followed by agarose gel electrophoresis and Qiaquick gel purification to

isolate the required DNA fragments.

4.2.2.3 Ligation

After both insert and vector were successful isolated and prepared (Figure 30), the two fragments were ligated using T4 DNA ligase using the following reaction in a microcentrifuge tube on ice:

1 µl Vector DNA (0.02pmol)
3 µl Insert DNA (0.06pmol)
1 µl T4 DNA Ligase
Up to 10 µl Nuclease-free water

Before ligation, 5'-end dephosphorylation was performed by treating alkaline phosphatase in order to prevent self ligation, according to the manufacturer's protocol.

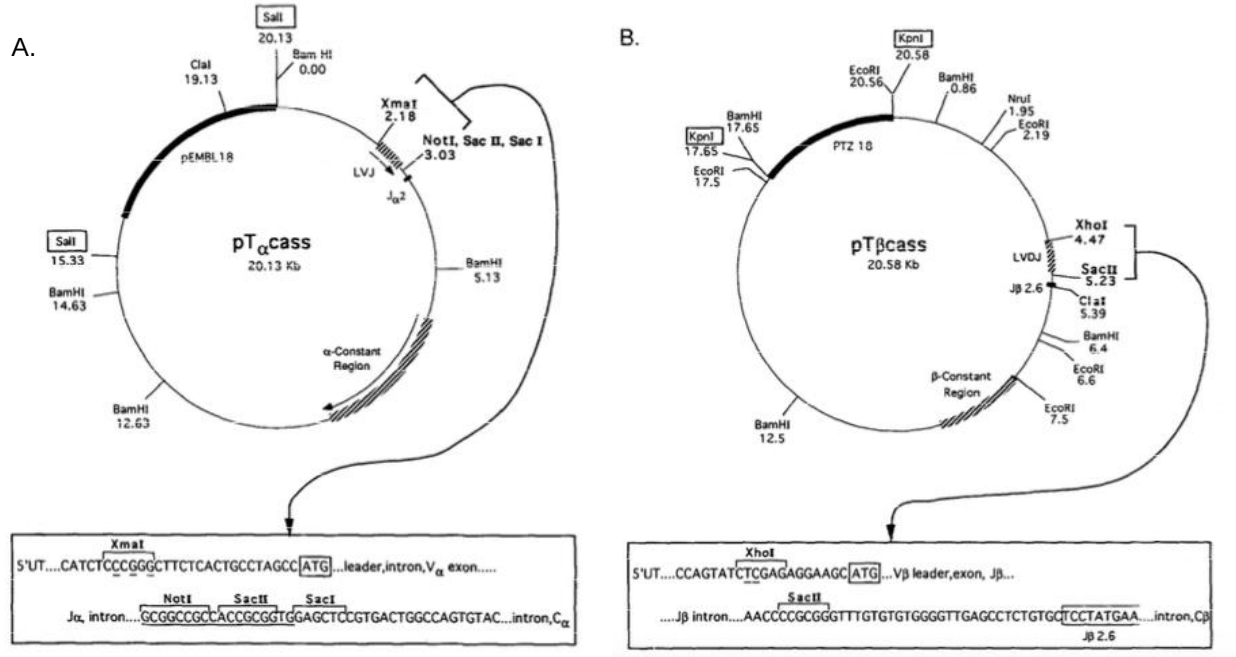

Figure 30: Map of the cassette plasmid for the expression of TCR-α (A) and TCR-β genes (B). The *Xma*I/*Sac*I restricted TCR-α DNA sequence and *Xho*I/*Sac*II restricted TCR-β DNA sequences.

4.2.2.4 Transformation

Plasmid DNA was prepared and screened for correct recombination, via transformation of the ligated DNA into competent bacterial cells. Competent *E. coli* cells were prepared as previously described in Hanahan, 1983[244]. Mix gently by pipetting up and down several times to mix the ligation mixture and the freshly thawed competent cells. The mixture placed on ice for 30 minutes followed by 42°C heat shock for 30 seconds and immediate transfer to ice to incubate for further 2 minutes. 300 µl preheated LB medium were added to the mixture. The transformed cells were incubated at 37°C for 1 hour in a shaking bath. Spread 50 µl of the cells and ligation mixture onto LB-agar plates containing 100 mg/ml Ampicillin or Kanamycin depending on the antibiotic selection gene in the plasmid construct. Immediately palce agar plates upside down at 37°C and incubate overnight for production of colonies.

4.2.2.5 Colony screening

Well isolated colonies obtained above were picked and transferred into 5 ml LB medium containing appropriate antibiotic for overnight culture for DNA preparation using a Qiaquick Miniprep kit according to the manufacture's protocol. Briefly, cells were harvested by centrifugation at 1200 rpm for 5 minutes, and resuspended the pellet bacterial cells in 250 µl buffer P1 followed by alkaline lysis in 250 µl buffer P2 and neutralized by adding 350 µl buffer P3 which precipitated protein. Precipitated protein was removed by centrifugation for 10 minutes at 13,000 rpm. Apply the supernatants containing plasmid DNA to the columns and centrifuged for 1 min to combine DNA with membrane and remaining lysate discarded. The column is washed with buffers to remove any residual impurities by centrifugation at maximum speed. Plasmid DNA was finally eluted and released from column membrane by adding 50 µl dH_2O and centrifugation at maximum speed for 1 min. Elution of DNA can be maximized by allowing the H_2O to sit in the membrane for a few minutes before centrifugation.

4.2.2.6 Detection of recombinant pTα-PLP11 vector and pTβ-PLP11 vector

To analyze the presence and orientation of the DNA insert into recombinant clones, restriction analysis was performed using appropriate restriction endonuclease enzymes. Plasmid DNA was isolated from an overnight bacterial culture and cut with different restriction endonucleases which found on the map of cloning vector. If the colony carries right orientation of the DNA insert, plasmid was sequenced with forward and reverse sequencing primers.

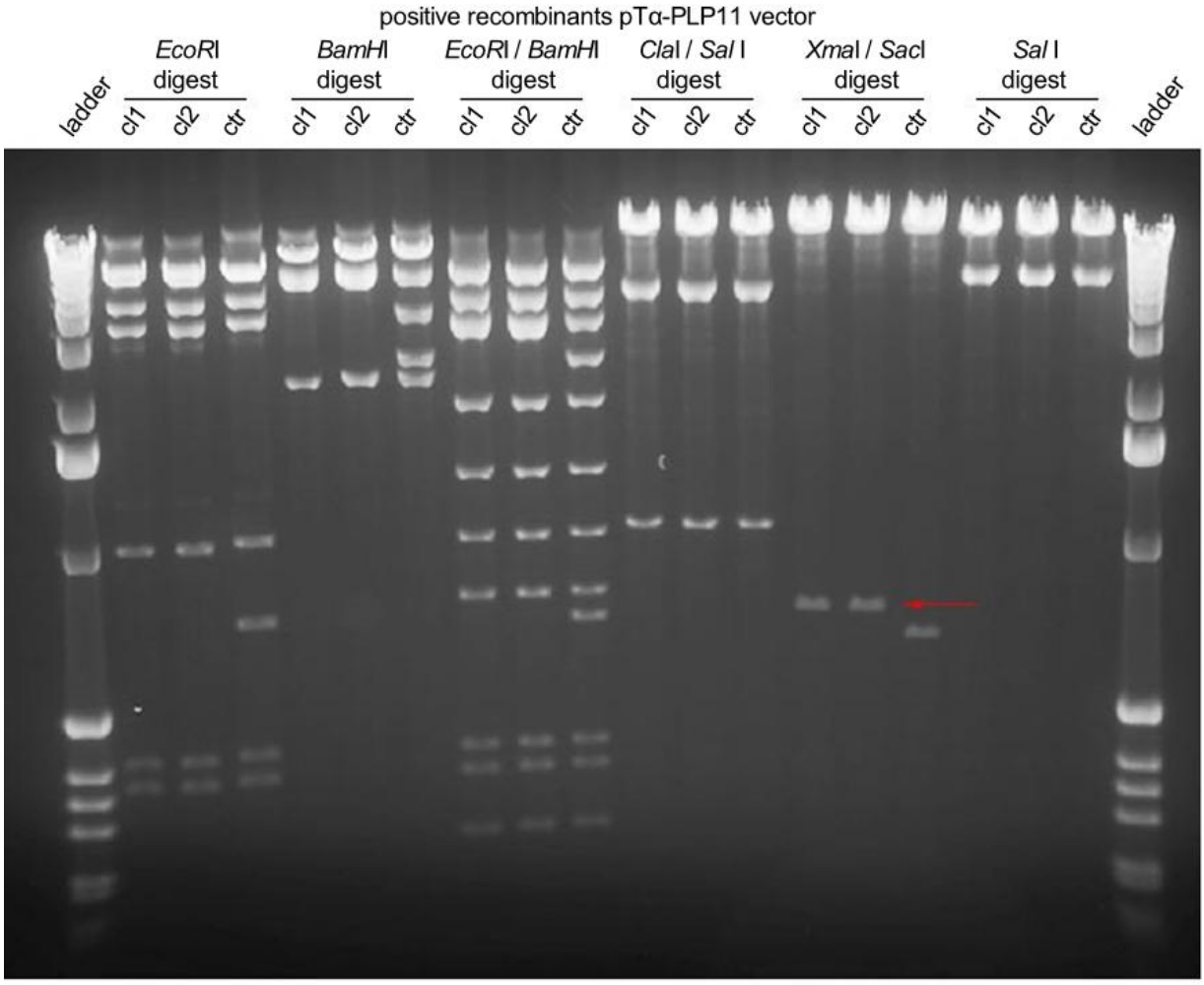

Figure 31. Restriction endonuclease analysis of the recombinant pTα-PLP11 vector. Recombinant pTα-PLP11 vector and pTα empty cassette vector as negative control was digested by *EcoR*I, *BamH*I, *EcoR*I/*BamH*I, *Cla*I/*Sal*I, *Xma*I/*Sac*I, *Sal*I, separated in a 1.5% agarose gel and stained by ethidium bromide. Line 1, 1kb DNA ladder marker; line2-3, *EcoR*I-degested DNA from two recombinant pTα-PLP11 clone and line4, pTα empty cassette vector; line5-6, *BamH*I-degested DNA from recombinant pTα-PLP11 and line7, pTa empty cassette vector; line8-9, *EcoR*I/*BamH*I-degested DNA from recombinant pTα-PLP11 and line10, pTα empty cassette vector; line11-12, *Cla*I/*Sal*I-degested DNA from recombinant pTα-PLP11 and line13, pTα empty cassette vector; line14-15, *Xma*I/*Sac*I-degested DNA

from recombinant pTα-PLP11 and line16, pTα empty cassette vector; line17-18, *Sal*I-degested DNA from recombinant pTα-PLP11 and line19, pTα empty cassette vector; line 20, 1kb DNA ladder marker. Red arrows indicate DNA fragments that belong to insertion of variable TCRVα gene at right direction.

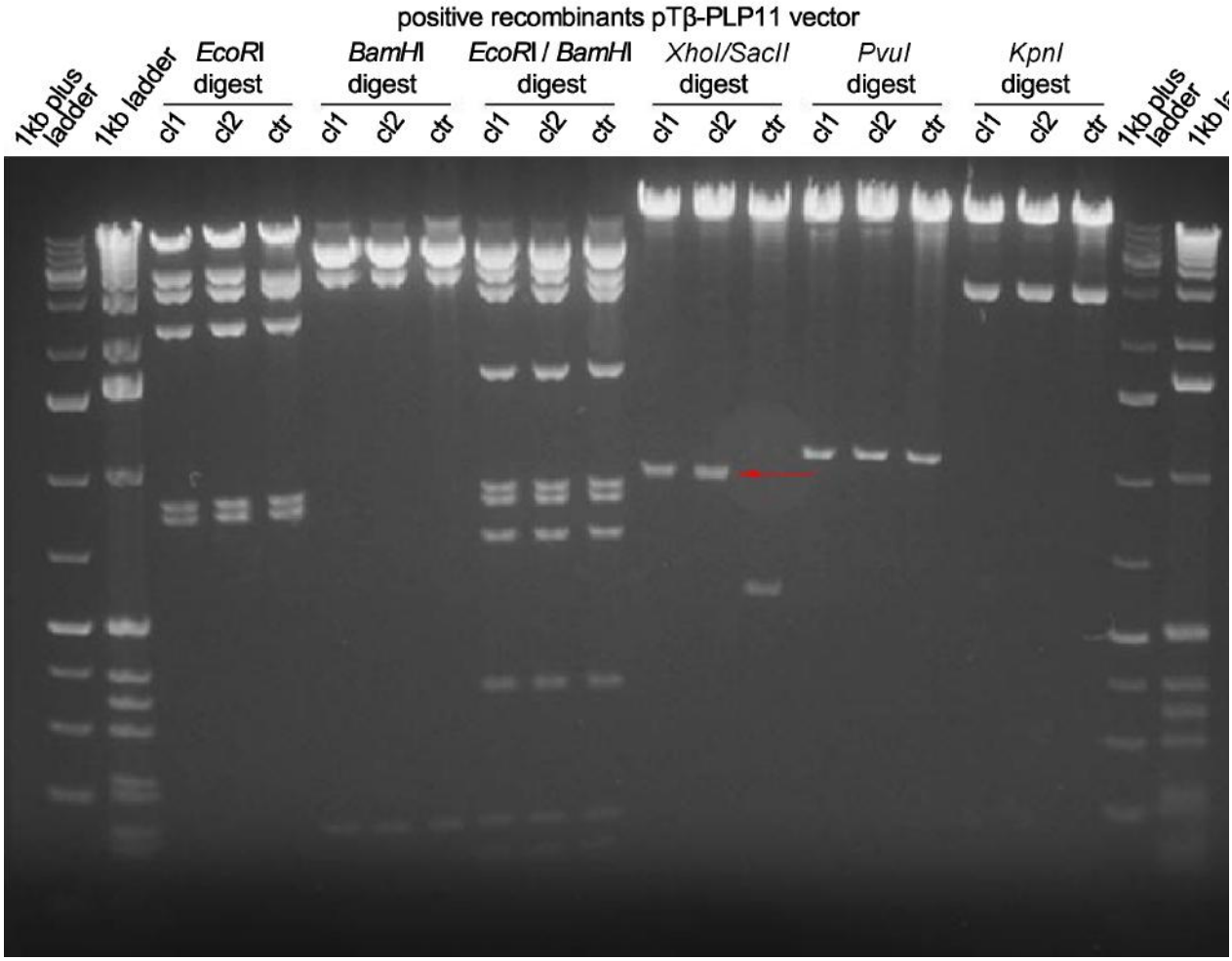

Figure 32. Restriction endonuclease analysis of the recombinant pTβ-PLP11 vector. Recombinant pTβ-PLP11 vectoe and pTβ empty cassette vector as negative control was digested by *Eco*RI, *BamH*I, *Eco*RI/*BamH*I, *Xho*I/*Sac*II, *Pvu*I, *Kpn*I, separated in a 1.5% agarose gel and stained by ethidium bromide. Line 1, 1kb plus DNA ladder marker; Line 2, 1kb DNA ladder marker; line3-4, *Eco*RI-degested DNA from recombinant pTβ-PLP11 clone (cl) and line5, pTβ empty cassette vector; line6-7, *BamH*I-degested DNA from recombinant pTβ-PLP11 and line8, pTβ empty cassette vector; line9-10, *Eco*RI/*BamH*I-degested DNA from recombinant pTβ-PLP11 and line11, pTβ empty cassette vector; line12-13, *Xho*I/*Sac*II-degested DNA from recombinant pTβ-PLP11 and line14, pTβ empty cassette vector; line15-16, *Pvu*I-degested DNA from recombinant pTβ-PLP11 and line17, pTβ empty cassette vector; line18-19, *Kpn*I-degested DNA from recombinant pTβ-PLP11 and line20, pTβ empty cassette vector; Line 21, 1kb plus DNA ladder marker; Line 22, 1kb DNA ladder marker. Red arrows indicate DNA fragments that belong to insertion of variable TCRVβ gene at right direction.

For confirmation of variable TCRVα gene position in pTα cassette vector, *Xho*I and *Sac*I restriction enzymes were selected because its restriction site is found in both variable TCRVα gene and pTa cassette vector. After digestion of pTa cassette vector containing variable TCRVα gene with *Xma*I and *Sac*I restriction enzyme, variable TCRVα DNA fragments were observed (indicated as red arrow) (Figure 31). The recombinant pTα-PLP11 vector was also cut with other restriction endonuclease enzymes which were chosen from the map of pTα cassette vectors to further confirm the recombinant pTα-PLP11 vector containing the variable TCRVα gene at right position.

Recombinant pTβ-PLP11 vector was also digested with *Xho*I and *Sac*II restriction enzyme to confirm position of variable TCRVβ gene in the vector (Figure 32). TCRVβ gene contains *Xho*I and *Sac*II restriction (indicated as red arrow), meanwhile, the backbone of the pTβ cassette vector was seen. Self ligation of pTβ-PLP11 vector was not seen the TCRVβ gene fragment after *Xho*I/*Sac*II cut. Similarly, the recombinant pTβ-PLP11 vector was also cut with other restriction endonuclease enzymesto further confirm TCRVβ gene was constructed into pTβ cassette vector at right position and direction.

As showed in the electropherogram of digestion product, which indicated recombinant pTα-PLP11 vector and recombinant pTβ-PLP11 vector had been successfully constructed.

4.2.2.7 Large scale preparation of targeted DNA and purification

Bacterial cultures for plasmid preparations should be grown from a single colony picked from a freshly streaked plate or a freshly transformed. Targeted DNA isolation was carried out according to the manufacturer recommendation of the large construct kit (Qiagen) with some modification to the supplied protocol. 500 µl of the starter culture were added into 4 flasks which contain the appropriate antibiotic in 500ml LB medium, and then incubated for 12-16 hours at 37°C 300rpm. Bacteria cells were harvested by centrifugation at 3,500g for 10 minutes at 4°C. Pellets were resuspended in 12 ml P1 buffer/RNase A. Next, 12 ml P2 buffer were added and inverted the tube gently to obtain a cleared lysate. After adding P3 buffer the solution turn white and a precipitate forms indicating butter neutralization is complete. The

mixture was centrifuged at 14,000g for 30 minutes at 4°C. Cleared supernatant was transferred to the filter colomn and allowed the column to empty by gravity flow. The column was washed twice using buffer QC to remove contaminants in the plasmid DNA. DNA was eluted with 15ml pre-warmed Buffer QF. Eluted DNA was precipitated by adding 10 ml isopropanol and then centrifuged at 3,000g for 60 minutes at 4°C. DNA pellet was washed with 5 ml 70% ethanol, and centrifuged at 15,000g for 20 minutes at 4°C. Plasmid DNA was finally eluted by adding 200 µl-500 µl dH_2O, depending on pellet size.

The purified DNA was linearized with the appropriate restriction enzyme (*Sal*I sites in pTα cass, and *Kpn*I sites in pTβ cass). Briefly, for the α chain, the targeted DNA was incubated at 37°C in the presence of 100U *SalI* in the presence of the appropriated reaction buffer in 400µl reactions. For the β chain, similarly, the targeted DNA was incubated at 37°C in the presence of 100U *Kpn*I in the presence of the appropriated reaction buffer in 400µl reactions. The reactions were verified on a 1% agarose gel. Following linearization targeted DNA was cleaned up using a standard phenol extraction.

4.2.2.8 Transgenic mouse production

Transgenic mice were generated by injection of linearized DNA into pronuclei of C57BL/6 zygotes. Microinjection was performed by Ronald Naumann at the Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany. Founders and offspring generated by backcrossing onto C57BL/6 were screened for transgene expression by PCR using genomic DNA. PCR products were visualized on 2% (w/v) agarose gels after electrophoresis. Microscopic images were acquired using Leica Application suite andfurther processed with Photoshop CS6.

4.2.3 Immunological Methods

4.2.3.1 T cell Proliferation Assay

Proliferation responses were assessed by using 4 x 10^5 T cells plus 3 x 10^4 irradiated (3,000 rads) BmDCs per well in cHL-1 medium alone as control or in the

presence of peptide at varied concentrations. Cultures were incubated for 72 hours at 37°C in 7% CO_2 in round-bottom 96-well plates. Three days later, cells were pulsed with one microCurie of thymidine ^{3}H-thyminidine for additional 20 hours and harvested onto filters according to manufacturer instructions. Measuring the amount of incorporated radioactive-labeled thymidine using a BetaPlate liquid scintillation counter (Wallac, Gaithersburg, MD).

4.2.3.2 IL-2 ELISA

Hybridoma cells (10^5/well in a 96-well plate) were incubated with 10^6 irradiated (3,000 rads) syngeneic splenocytes in medium alone or in the presence of 5 µg/ml individual peptides or 5 µg/ml ConA at 37°C, 10% CO_2. The Supernatants were collected for analysis 72 hours after stimulation from the top of the culture by quantitative capture ELISA according to the manufacturers guidelines (BD OptEIA™, BD Bioscience). Assays were performed with TMB Microwell Peroxidase Substrate and read at 450nm.

4.2.3.3 Cell surface staining

Single cell suspensions were incubated in FCS buffer with diluted fluorochrome-labeled antibodies on ice and under light protection for 30 minutes. Biotinylated antibodies were visualized by Streptavidin-PE-Cy7. After staining, cells were washed once and re-suspended in FACS buffer and analyzed on a FACSCanto (BD Bioscience). Flow cytometry data were acquired and analyzed with Flowjo software.

4.2.3.4 Intracellular Foxp3 staining

After surface staining, cells were fixed and permeabilized using the Foxp3 Staining Buffer according to manufacturer's instructions. Briefly, cells were incubated in Fixation for 45 minutes. Fixed cells were washed twice with permeabilization Buffer. The Foxp3 antibody were added and incubated for another 30 minutes at 4 °C. Washed as above twice with Permeabilization Buffer and re-suspended in FACS buffer for subsequent FACS analysis.

4.2.3.5 *In vivo* Cell Division Analysis

Total $CD4^+$ T cells were isolated from the spleens and draining lymph nodes of TCR-PLP1/TCR-PLP11 transgenic mice and stained with CFSE. A maximum concentration of 1×10^7 cells/ml were incubated in 5 µM CFSE in 0.1% BSA buffer for 10 minutes at 37°C. Cells were washed with FACS buffer and resuspended in PBS at a concentration of 2.5×10^7 splenocytes/ml. Subsequently, 5×10^6 TCR-PLP1/TCR-PLP11 $CD45.1^+$ splenocytes were injected intravenously into recipient PLP^{WT}, PLP^{KO}, and the different subsets of chimeric mice. 4 days later, pooled spleen and lymph node cells were harvested, subjected to $CD4^+$ cell enrichment, and diluted levels of CFSE fluorescence on Plp1/Plp11-specific T cells, indicative of cell division, were analyzed by flow cytometry.

4.2.4 Animal Experiments

4.2.4.1 Genotyping of mice

Transgenic animals were genotyped by PCR of genomic DNA extracted from tail biopsies. The tail biopsies were clipped after weaning and digested in 50 µl tail digestion buffer for 5 hours at 55°C, supplemented with Proteinase K (Invitrogen) at 95°C for 5 minutes. 1 µl of the lysate was directly used as template in the PCR reactions, and the total volume of the PCR was 30 µl with the following composition:

1 µl Tail genomic DNA 1 µl (10 ng-500 ng)
6 µl 10x Taq buffer with $MgCl_2$
3 µl dNTP mix (2.5 mM)
3 µl Forward Primer (10 µM stock)
3 µl Reverse Primer (10 µM stock)
1 µl Taq DNA Polymerase (5 units/µL)
13 µl Sterile dH_2O

The PCR reactions for genotyping TCR-PLP1, TCR-PLP11, CD11c-Cre, DTA, $PLP^{fl/fl}$, Foxn1-Cre, $Rag1^{KO}$ followed the TCR-program TD54x30:

3min at 94° C

45sec at 94°C
45sec at 60°C
60sec at 72°C
} 2 cycles

45sec at 94°C
45sec at 58°C
60sec at 72°C
} 2 cycles

45sec at 94°C
45sec at 56°C
60sec at 72°C
} 2 cycles

45sec at 94°C
45sec at 54°C
60sec at 72°C
} 30 cycles

10min at 72°C
forever at 15 °C

DNA products were separated by agarose (1.5 % in 1 x TBE buffer) gel electrophoresis according to their size. The gels contained 0.15 µg/ml ethidium bromide to visualize the separated DNA bands under UV light (312 nm).

4.2.4.2 Immunization of animals

Six- to eight-week-old male and female mice were immunized with 50 µg Plp1/Plp11 peptide in equal amounts of complete Freund´s adjuvant (CFA) containing Mycobacterium tuberculosis (Sigma) at a final concentration of 1 mg/ml. 50 µl of this emulsion was injected subcutaneously into the footpad of the hindleg of the mouse.

4.2.4.3 Irradiation Bone Marrow Chimeras

Lethally irradiated (950 rads) PLP^{WT} or PLP^{KO} mice were reconstituted intravenously with 10^7 bone marrow cells from the femurs of CD45.1 or CD45.2 congenic $MHC^{WT}PLP^{WT}$ or $MHC^{KO}PLP^{WT}$ mice. Lymphoid tissues from the resulting chimeric mice were harvested 8 weeks later for analysis of Plp1-specific T cells.

4.2.4.4 T Cell Transfers

Total $CD4^+$ T cells from the pooled spleen and lymph nodes of TCR-PLP1/TCR-PLP11 WT or PLP^{KO} transgenic mice were magnetically purified (Miltenyi) and 5×10^6 were transferred intravenously into CD45.1 or CD45.2 congenic WT mice. Recipient mice were immunized 6 hours ago before the cell transfer, and host TCR-PLP1/11-specific $CD4^+$ T cells were isolated and analyzed 3 days later.

4.2.4.5 Induction and evaluation of EAE

For triggering transfer EAE, $CD4^+$ T cell from TCR-PLP1 PLP^{KO} mice (3-5 x 10^6 per mouse) were injected into the tail vein of the PLP^{WT} or PLP^{KO} recipient mice. Mice were weighed and monitored daily for clinical symptoms (Table 7). The same scoring system was used for active EAE experiments.

For active EAE induction, mice were injected at base of backs with 200 µg PLP peptide emulsified in equal amounts of CFA. Pertussis toxin (400 ng) was administered intraperitoneally (i.p.) on days 0 and 2 following immunization. Animals reaching a score of 4 were eliminated.

Table 7: Classification of assessment of EAE score.

Score	Clinical symptom
0	No symptoms
0.5	Patial loss of tail tonus
1	Complete loss of tail tonus
1.5	Flaccid tail and subtle gait disturbance
2	Partial hind leg paralysis
2.5	Paralysis of a single hind limb
3	Complete hind limb paresis, mouse is able to move forward using their fore limbs
3.5	The fore limbs of mouse is partially paralysis leading to impaired forward movement
4	No moving but eating
5	No mobility/moribund.

4.2.5 Statistical analysis

Statistical analyses were performed using Prism 5.0 (Graphpad). Data is always depicted as mean ± standard error of the mean (SEM). Two-tailed Student's t test was performed to analyze the statistical significance.

5. References

1. Miller, J.F. 1961. Immunological function of the thymus. *Lancet* 2:748-749.
2. Bevan, M.J. 1977. In a radiation chimaera, host H-2 antigens determine the immune responsiveness of donor cytotoxic cells. *Nature* 269:417-418.
3. Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J Exp Med* 147:882-896.
4. Sainte-Marie, G., and C.P. Leblond. 1964. Cytologic features and cellular migration in the cortex and medulla of thymus in the young adult rat. *Blood* 23: 275-299.
5. Cantor, H., and I. Weissman. 1976. Development and function of subpopulations of thymocytes and T lymphocytes. *Prog Allergy* 20:1-64.
6. Petrie, H.T. 2003. Cell migration and the control of post-natal T-cell lymphopoiesis in the thymus. *Nat Rev Immunol* 3:859-866.
7. Gray, D.H., T. Ueno, A.P. Chidgey, M. Malin, G.L. Goldberg, Y. Takahama, and R.L. Boyd. 2005. Controlling the thymic microenvironment. *Curr Opin Immunol* 17:137-143.
8. Rossi, S.W., W.E. Jenkinson, G. Anderson, and E.J. Jenkinson. 2006. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* 441:988-991.
9. Anderson, G, E.J. Jenkinson, N.C. Moore, and J.J. Owen. 1993. MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* 362:70-73.
10. Klug D.B., C. Carter, I.B. Gimenez-Conti, and E.R. Richie. 2002. Cutting edge: thymocyte-independent and thymocyte-dependent phases of epithelial patterning in the fetal thymus. *J Immunol* 169:2842-2845.
11. Shores, E. W., W. van Ewijk, and A. Singer.1991. Disorganization and restoration of thymic medullary epithelial cells in T cell receptor-negative scid mice: evidence that receptor-bearing lymphocytes influence maturation of the thymic microenvironment. *Eur J Immunol* 21:1657-1661.
12. van Ewijk, W., E.W. Shores, and A. Singer. 1994. Crosstalk in the mouse thymus. *Immunol Today* 15:214-217.
13. Bleul, C. C. and T. Boehm. 2000 Chemokines define distinct microenvironments in the developing thymus. *Eur J Immunol* 30:3371-3379.
14. Liu, C. 2005. The role of CCL21 in recruitment of T precursor cells to fetal thymus. *Blood* 105:31-39.
15. Wurbel, M.A., M. Malissen, D. Guy-Grand, E. Meffre, M.C. Nussenzweig, M. Richelme, A. Carrier, and B. Malissen. 2001. Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell development and a reduction in T-cell receptor γδ+ gut intraepithelial lymphocytes. *Blood* 98:2626-2632.
16. Rossi, F.M. 2005. Recruitment of adult thymic progenitors is regulated by P-selectin and its ligand PSGL-1. *Nat Immunol* 6:626-634.
17. Godfrey, D.I., J. Kennedy, T. Suda, and A. Zlotnik. 1993. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3−CD4−CD8− triple negative adult mouse thymocytes defined by CD44 and CD25 expression. *J Immunol* 150:4244-4252.
18. Anderson, G., N.C. Moore, J.J. Owen, and E.J. Jenkinson. 1996. Cellular interactions in thymocyte development. *Annu Rev Immunol* 14:73-99.
19. Zúñiga-Pflücker, J.C., and M.J. Lenardo. 1996. Regulation of thymocyte development fromimmature progenitors. *Curr Opin Immunol* 8:215-224.
20. Lind E.F., S.E. Prockop, H.E. Porritt, and H.T. Petrie. 2001. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J Exp Med* 194:127-134.
21. Petrie H.T., and J.C. Zuniga-Pflücker. 2007. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu. Rev. Immunol.* 25:649-79.

22. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu Rev Immunol* 14: 29-47.
23. Dunon, D., D. Courtois, O. Vainio, A. Six, C.H. Chen, M.D. Cooper, J.P. Dangy, and B.A. Imhof. 1997. Ontogeny of the immune system: gamma/delta and alpha/beta T cells migrate from thymus to the periphery in alternating waves. *J Exp Med* 186:977-988.
24. Allman, D., A. Sambandam, S. Kim, J.P. Miller, A. Pagan, D. Well, A. Meraz, and A. Bhandoola. 2003. Thymopoiesis independent of common lymphoid progenitors. *Nat Immunol* 4:168-174.
25. Han, H., K. Tanigaki, N. Yamamoto, K. Kuroda, M. Yoshimoto, T. Nakahata, K. Ikuta, and T. Honjo. 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* 14:637-645.
26. Schmitt, T.M., and J.C. Zúñiga-Pflücker. 2002. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17:749-756.
27. Moore, T.A., and A. Zlotnik. 1995. T-cell lineage commitment and cytokine response of thymic progenitors. *Blood* 86:1850-1860.
28. Schmitt, T.M., M. Ciofani, H.T. Petrie, and J.C. Zuniga-Pflucker. 2004. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* 200:469-479.
29. Taghon, T.N., E.S. David, J.C. Zuniga-Pflucker, and E.V. Rothenberg. 2005. Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling. *Genes Dev* 19:965-978.
30. Durum, S.K., S. Candeias, H. Nakajima, W.J. Leonard, A.M. Baird, L.J. Berg, and K. Muegge. 1998. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J Exp Med* 188:2233-2241.
31. Maraskovsky, E., M. Teepe, P.J. Morrissey, S. Braddy, R.E. Miller, D.H. Lynch, and and J.J. Peschon. 1996. Impaired Survival and Proliferation in IL-7 Receptor-Deficient Peripheral T Cells receptor-deficient peripheral T cells. *J Immunol* 157:5315-5323.
32. Vissinga, C.S., D.J. Fatur-Saunders, and F. Takei. 1992. Dual role of IL-7 in the growth and differentiation of immature thymocytes. *Exp Hematol* 20:998-1003.
33. Melichar H.J., K. Narayan, S.D. Der, Y. Hiraoka, N. Gardiol, and G. Jeannet. 2007. Regulation of gammadelta versus alphabeta T lymphocyte differentiation by the transcription factor SOX13. *Science* 315:230-233.
34. Porritt, H.E., K.Gordon, and H.T. Petrie. 2003. Kinetics of Steady-state Differentiation and Mapping of Intrathymic-signaling Environments by Stem Cell Transplantation in Nonirradiated Mice. *J Exp Med* 198:957-962.
35. Godfrey, D.I., J. Kennedy, P. Mombaerts, S. Tonegawa, and A. Zlotnik. 1994. Onset of TCR-beta gene rearrangement and role of TCR-beta expression during CD3−CD4−CD8− thymocyte differentiation. *J Immunol* 152:4783-92.
36. Capone, M., R.D. Hockett, and A. Zlotnik. 1998. Kinetics of T cell receptor beta, gamma, and delta rearrangements during adult thymic development: T cell receptor rearrangements are present in CD44+CD25+ Pro-T thymocytes. *Proc Natl Acad Sci U S A* 95:12522-12527.
37. Livak, F., M. Tourigny, D.G. Schatz, and H.T. Petrie. 1999. Characterization of TCR gene rearrangements during adult murine T cell development. *J Immunol* 162:2575-2580.
38. Robey, E. and B.J. Fowlkes. 1994. Selective events in T-cell development. *Annu Rev Immunol* 12:675-705.
39. von Boehmer, H. 2005. Unique features of the pre-T-cell receptor α-chain: not just a surrogate. *Nat Rev Immunol* 5:571-577.
40. Taghon, T. and E.V. Rothenberg. 2008. Molecular mechanisms that control mouse and human TCR-αβ and TCR-γδ T cell development. *Semin Immunopathol* 30:383-398.
41. Tanaka, Y., L. Ardouin, A. Gillet, S.Y. Lin, A. Magnan, B. Malissen, and M. Malissen. 1995. Early T cell development in CD3-deficient mice. *Immunol Rev* 148:171-199.
42. Groettrup, M. 1993. A novel disulfide-linked heterodimeron pre-T cells consists of the

T-cell receptor β-chain and a 33 kD glycoprotein. *Cell* 75:283-294.
43. von Boehmer, H. and H.J. Fehling. 1997. Structure and function of the pre-T-cell receptor. *Annu Rev Immunol* 15:433-452.
44. Aifantis, I., J. Feinberg, H.J. Fehling, J.P. Di Santo, and H. von Boehmer. 1999. Early T-cell receptor-β gene expression is regulated by the pre-T-cell-receptor-CD3 complex. *J Exp Med* 190:141-144.
45. Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H.J. Fehling, and. H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266:1208-1212.
46. Levelt, C.N., and K. Eichmann. 1995. Receptors and signals in early thymic selection. *Immunity* 3:667-672
47. Wilson, A., W. Held, and H.R. MacDonald. 1994. Two waves of recombinase gene expression in developing thymocytes. *J Exp Med* 179:1355-1360
48. Vasseur, F., A. Le Campion, and C. Penit. 2001. Scheduled kinetics of cell proliferation and phenotypic changes during immature thymocyte generation. *Eur J Immunol* 31:3038-3047.
49. Trampont, P.C. 2010. CXCR4 acts as a costimulator during thymic beta-selection. *Nat Immunol* 11:162-170.
50. Maillard, I. 2006. The requirement for Notch signaling at the beta-selection checkpoint in vivo is absolute and independent of the pre-T cell receptor. *J Exp Med* 203:2239-2245.
51. Peschon, J. J. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 180:1955-1960.
52. von Freeden-Jeffry, U., P. Vieira, L.A. Lucian, T. McNeil, S.E. Burdach, and R. Murray. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* 181:1519-1526.
53. Mombaerts, P., A.R. Clarke, M.A. Rudnicki, J. Iacomini, S. Itohara, J.J. Lafaille, L.L. Wang, Y. Ichikawa, R. Jaenisch, M.L. Hooper, and S. Tonegawa. 1992. Mutations in T-cell antigen receptor genes and block thymocyte development at different stages. *Nature* 360:225-231.
54. Shinkai, Y., S. Koyasu, K. Nakayama, K.M. Murphy, D.Y. Loh, E.L. Reinherz, and F. W. Alt. 1993. Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes. *Science* 259:822-825.
55. Hoffman, E. S., L. Passoni, T. Crompton, T.M. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor -chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev* 10:948-962.
56. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1995. Positive selection of thymocytes. *Annu Rev Immunol* 13:93-126.
57. Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21:139-176.
58. Godfrey, D.I., and A. Zlotnik. 1993. Control points in early T-cell development. *Immunol Today* 14:547-553.
59. Kisielow, P., and H. von Boehmer. 1995. Development and selection of T cells: facts and puzzles. *Adv Immunol* 58:87-209.
60. Biddison, W.E., P.E., Rao, M.A Talle,., G. Goldstein, and S. Shaw. 1982. Possible involvement of the OKT4 molecule in T-cell recognition of class II HLA antigens. Evidence from studies of cytotoxic T lymphocytes specific for SB antigens. *J Exp Med* 156:1065-1076.
61. Swain, S. L. 1983. T-cell subsets and the recognition of MHC class. *Immunol Rev* 74:129-142.
62. Chi, A.W., J.J. Bell, D.A. Zlotoff, and A. Bhandoola. 2009. Untangling the T branch of the hematopoiesis tree. *Curr Opin Immunol* 21:121-126.
63. Maillard, I., T. Fang, and W.S. Pear. 2005. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu Rev Immunol* 23:945-974.
64. Radtke, F., N. Fasnacht, and H.R. Macdonald. 2010. Notch signaling in the immune

system. *Immunity* 32:14-27.
65. Radtke F., Wilson A., Stark G., Bauer M., van Meerwijk J., MacDonald H.R., Aguet M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10:547-558.
66. Pui, J.C., D. Allman, L. Xu, S. DeRocco, F.G. Karnell, S. Bakkour, J.Y. Lee, T. Kadesch, R.R. Hardy, J.C. Aster, and W.S. Pear. 1999. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* 11:299-308.
67. Wolfer, A., T. Bakker, A. Wilson, M. Nicolas, V. Ioannidis, D.R. Littman, P.P. Lee, C.B. Wilson, W. Held, H.R. MacDonald, and F. Radtke. 2001. Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development. *Nat Immunol* 2:235-241.
68. Haks, M.C. 2005. Attenuation of γδTCR signaling efficiently diverts thymocytes to the αβ lineage. *Immunity* 22:595-606.
69. Garbe, A.I., A. Krueger, F. Gounari, J.C. Zuniga-Pflucker, and H. von Boehmer. 2006. Differential synergy of Notch and T cell receptor signaling determines αβ versus γδ lineage fate. *J Exp Med* 203:1579-1590.
70. Lauritsen, J.P. 2009. Marked induction of the helix-loop-helix protein Id3 promotes the γδ T cell fate and renders their functional maturation Notch independent. *Immunity* 31:565-575.
71. Taghon, T., M.A. Yui, R. Pant, R.A. Diamond, and E.V. Rothenberg. 2006. Developmental and molecular characterization of emerging β- and γδ-selected pre-T cells in the adult mouse thymus. *Immunity* 24:53-64.
72. Cobaleda, C., A. Schebesta, A. Delogu, and M. Busslinger. 2007. Pax5: the guardian of B cell identity and function. *Nat Immunol* 8:463-470.
73. Benoist, C. and D. Mathis. 1989. Positive selection of the T cell repertoire: where and when does it occur? *Cell* 58:1027-1033.
74. Kisielow, P., H.S. Teh, H. Bluthmann, and H. von Boehmer. 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* 335:730-733.
75. Sakaguchi, S. 2005. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Ann Rev Immunol* 22:531-562.
76. Watanabe, N. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 436:1181-1185.
77. Fontenot, J.D. 2005. Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* 22:329-341.
78. Tanaka, K. and M. Kasahara. 1998. The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. *Immunol Rev* 163:161-176.
79. Murata, S. 2007. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* 316, 1349-1353.
80. Florea, B.I., M. Verdoes, N. Li, W.A. van der Linden, and P.P. Geurink. 2010. Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t. *Chem biol* 17:795-801.
81. Murata, S., Y. Takahama, and K. Tanaka. 2008. Thymoproteasome: probable role in generating positively selecting peptides. *Curr Opin Immunol* 20:192-196.
82. Nitta, T. 2010. Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells. *Immunity* 32:29-40.
83. Hsing, L.C. and A.Y. Rudensky. 2005. The lysosomal cysteine proteases in MHC class II antigen presentation. *Immunol Rev* 207:229-241.
84. Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844.
85. Nakagawa, T. 1998. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* 280:450-453.
86. Gommeaux, J. 2009. Thymus-specific serine protease regulates positive selection of

a subset of CD4+ thymocytes. *Eur J Immunol* 39:956-964.
87. Hsing, L.C. 2010. Roles for cathepsins S, L, and B in insulitis and diabetes in the NOD mouse. *J autoimmun* 34:96-104.
88. Viret, C., S. Leung-Theung-Long, L. Serre, C. Lamare, D.A. Vignali, B. Malissen, A Carrier, and S. Guerder. 2011. Thymus-specific serine protease controls autoreactive CD4 T cell development and autoimmune diabetes in mice. *J Clin Invest* 121:1810-1821.
89. Maehr, R. 2005. Cathepsin L is essential for onset of autoimmune diabetes in NOD mice. *J Clin Invest* 115:2934-2943.
90. Chicz, R.M. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* 178:27-47.
91. Brazil, M.I., S. Weiss, and B. Stockinger. 1997. Excessive degradation of intracellular protein in macrophages prevents presentation in the context of major histocompatibility complex class II molecules. *Eur J Immunol* 27:1506-1514.
92. Schmid, D., M. Pypaert, and C. Munz. 2007. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* 26:79-92.
93. Nedjic, J., M. Aichinger, J. Emmerich, N. Mizushima, and L. Klein. 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. *Nature* 455:396-400.
94. Nedjic, J., M. Aichinger, N. Mizushima, and L. Klein. 2009. Macroautophagy, endogenous MHC II loading and T cell selection: the benefits of breaking the rules. *Curr Opin Immunol* 21:92-97.
95. von Boehmer, H. and F. Melchers. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat Immunol* 11:14-20.
96. Kyewski, B. and Derbinski, J. 2004. Self-representation in the thymus: an extended view. *Nat Rev Immunol* 4:688-698.
97. Zuklys, S. 2000. Normal thymic architecture and negative selection are associated with Aire expression, the gene defective in the autoimmunepolyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). *J Immunol* 165:1976-1983.
98. Derbinski, J., J. Gäbler, B. Brors, S. Tierling, S. Jonnakuty, M. Hergenhahn, L. Peltonen, J. Walter, and B. Kyewski. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med* 202:33-45.
99. Klein, L., T. Klein, U. Ruther, and B. Kyewski. 1998. CD4 T cell tolerance to human C- reactive protein, an inducible serum protein, is mediated by medullary thymic epithelium. *J Exp Med* 188:5-16.
100. Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C.C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat Immunol* 4:350-354.100. *Rev Immunol* 4:688-698.
101. Anderson, M.S., E.S. Venanzi, Z. Chen, S.P. Berzins, C. Benoist, and D. Mathis. 2005. The cellular mechanism of Aire control of T cell tolerance. Immunity 23:227-239.
102. Hinterberger, M., M. Aichinger, O. Prazeres da Costa, D. Voehringer, R. Hoffmann, and L. Klein. 2010. Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance. *Nat Immunol* 11:512-519.
103. Ohnmacht, C., A. Pullner, S.B. King, I. Drexler, S. Meier, T. Brocker, and D. Voehringer. 2009. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J Exp Med* 206:549-559.
104. Brocker, T. 1999. The role of dendritic cells in T cell selection and survival. *J Leukoc Biol* 66:331-335.
105. Carlow, D.A., S.J. Teh, N.S. van Oers, R.G. Miller, and H.S. Teh. 1992. Peripheral tolerance through clonal deletion of mature CD4-CD8+ T cells. *Int Immunol* 4:599-610.
106. McCaughtry, T.M., T.A. Baldwin, M.S. Wilken, and K.A. Hogquist. 2008. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J Exp Med* 205:2575-2584.
107. Miyama-Inaba, M., S. Kuma, K. Inaba, H. Ogata, H. Iwai, R. Yasumizu, S.

Muramatsu, R.M. Steinman, and S. Ikehara.1988. Unusual phenotype of B cells in the thymus of normal mice. *J Exp Med* 168: 811-816.
108. Berg, L.J., A.M. Pullen, B. Fazekas de St Groth, D. Mathis, C. Benoist, and M.M. Davis. 1989. Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell* 58:1035-1046.
109. Bensinger, S.J., A. Bandeira, M.S. Jordan, A.J. Caton, and T.M. Laufer. 2001. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4+25+ immunoregulatory T cells. *J Exp Med* 194:427-438.
110. Liston, A., K.M. Nutsch, A.G. Farr, J.M. Lund, J.P. Rasmussen, P.A. Koni, and A.Y. Rudensky 2008. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A* 105:11903-11908.
111. Xing, Y., S.C. Jameson, and K.A. Hogquist. 2013. Thymoproteasome subunit-β5T generates peptide-MHC complexes specialized for positive selection. . *Proc Natl Acad Sci U S A* 110:6979-6984.
112. Sebzda, E. 1994. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science* 263:1615-1618.
113. Alam, S.M. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381:616-620.
114. Viret, C. 2011. Thymus-specific serine protease contributes to the diversification of the functional endogenous CD4 T cell receptor repertoire. *J Exp Med* 208:3-11
115. Honey, K. 2002. Cathepsin L regulates CD4+ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J Exp Med* 195:1349-1358
116. Kasai, M. 1996. Difference in antigen presentation pathways between cortical and medullary thymic epithelial cells. *Eur J Immunol* 26:2101-2107.
117. Klein, L., B. Roettinger, and B. Kyewski. 2001. Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. *Eur J Immunol* 31:2476-2486.
118. Oukka, M. 1997. Selectivity of the major histocompatibility complex class II presentation pathway of cortical thymic epithelial cell lines. *Eur J Immunol* 27:855-859.
119. Munz, C. 2009. Enhancing immunity through autophagy. *Annu Rev Immunol* 27:423-449.
120. Mathis, D., and C. Benoist. 2009. Aire. *Annu Rev Immunol* 27:287-312.
121. Peterson, P., T. Org, and A. Rebane. 2008. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat Rev Immunol* 8:948-957.
122. Surh, C.D. 1992. Two subsets of epithelial cells in the thymic medulla. *J Exp Med* 176:495-505.
123. Nelson, A.J., S. Hosier, W. Brady, P.S. Linsley, and A.G. Farr. 1993. Medullary thymic epithelium expresses a ligand for CTLA4 in situ and in vitro. *J Immunol* 151:2453-2461.
124. Gabler, J., J. Arnold, and B. Kyewski. 2007. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. *Eur J Immunol* 37: 3363-3372.
125. Gray, D., J. Abramson, C. Benoist, and D. Mathis. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med* 204:2521-2528.
126. Consortium, F.G. 1997. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet* 17:399-403.
127. Nagamine, K., P. Peterson, H.S. Scott, J. Kudoh, S. Minoshima, and M. Heino, K.J. Krohn, M.D. Lalioti, P.E. Mullis, S.E. Antonarakis, K. Kawasaki, S. Asakawa, F. Ito, and N. Shimizu. 1997. Positional cloning of the APECED gene. *Nat Genet* 17:393-398.
128. Jiang, W., M.S. Anderson, R. Bronson, D. Mathis, and C. Benoist. 2005. Modifier loci condition autoimmunity provoked by Aire deficiency. *J Exp Med* 202:805-15.
129. Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032-1039.

130. Klein, L., B. Kyewski, P.M. Allen, K.A. Hogquist. 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nat Rev Immunol* 14:377-91.
131. Aschenbrenner, K., L.M. D'Cruz, E.H. Vollmann, M. Hinterberger, J. Emmerich, L. K Swee, A. Rolink, and L. Klein. 2007. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol* 8:351-358.
132. Gallegos, A.M. and M.J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med* 200:1039-1049.
133. Wu, L., and K. Shortman. 2005. Heterogeneity of thymic dendritic cells. *Semin immunol* 17:304-312.
134. Li, J., J. Park, D. Foss, and I. Goldschneider. 2009. Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus. *J Exp Med* 206:607-622.
135. Joffre, O.P., E. Segura, A. Savina, and S. Amigorena. 2012. Cross-presentation by dendritic cells. *Nat Rev Immunol* 12:557-569.
136. Proietto, A.I., M.H. Lahoud, and L. Wu. 2008. Distinct functional capacities of mouse thymic and splenic dendritic cell populations. *Immunol Cell Biol* 86:700-708.
137. Hadeiba, H., K. Lahl, A. Edalati, C. Oderup, A. Habtezion, R. Pachynski, L. Nguyen, A. Ghodsi, S. Adler, and E.C Butcher. 2012. Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance. *Immunity* 36:438-450.
138. Atibalentja, D.F., C.A. Byersdorfer, and E.R. Unanue, 2009. Thymus-blood protein interactions are highly effective in negative selection and regulatory T cell induction. *J Immunol* 183:7909-7918.
139. Villadangos, J.A., and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29:352-361.
140. van Meerwijk, J.P., S. Marguerat, R.K. Lees, R.N. Germain, B.J. Fowlkes, and H.R. MacDonald. 1997. Quantitative impact of thymic clonal deletion on the T cell repertoire. *J Exp Med* 185:377-383.
141. Proietto, A.I., D.S. van, P. Zhou, A. Rizzitelli, A. D'Amico, R.J. Steptoe, S.H. Naik, M. H. Lahoud, Y. Liu, P. Zheng, K, Shortman, and L. Wu. 2008. Dendritic cells in the thymus contribute to T-regulatory cell *induction. Proc Natl Acad Sci U S A* 105:19869-19874.
142. Wirnsberger, G., F. Mair, and L. Klein. 2009. Regulatory T cell differentiation of thymocytes does not require a dedicated antigen-presenting cell but is under T cell-intrinsic developmental control. *Proc Natl Acad Sci U S A* 106:10278-10283.
143. Li, M.O., and A.Y. Rudensky. 2016. T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat Rev Immunol* 16:220-233.
144. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273-280.
145. Pircher, H., K. Burki, R. Lang, H. Hengartner, and R.M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 342:559-561.
146. Josefowicz, S.Z, L.F. Lu, and A.Y. Rudensky. 2012. Regulatory T cells: mechanisms of dif- ferentiation and function. *Annu Rev Immunol* 30:531-564.
147. Hodgkin, P.D., W.R. Heath, and A.G. Baxter. 2007. The clonal selection theory: 50 years since the revolution. *Nat Immunol* 8:1019-1026.
148. Modigliani, Y., A. Coutinho, P. Pereira, N. Le Douarin, V. Thomas-Vaslin, O. Burlen-Defranoux, J. Salaun, and A. Bandeira. 1996. Establishment of tissue- specific tolerance is driven by regulatory T cells selected by thymic epithelium. *Eur J Immunol* 26:1807-1815.
149. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 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.
150. Modigliani, Y., P. Pereira, V. Thomas-Vaslin, J. Salaun, O. Burlen-Defranoux, A.

Coutinho, N. Le Douarin, and A. Bandeira. 1995. Regulatory T cells in thymic epithelium-induced tolerance. I. Suppression of mature peripheral non-tolerant T cells. *Eur J Immunol* 25:2563-2571.

151. Baldwin, T.A., M.M. Sandau, S.C. Jameson, and K.A. Hogquist. 2005. The timing of TCRa expression critically influences T cell development and selection. *J Exp Med* 202:111-121.

152. Punt, J.A., B.A. Osborne, Y. Takahama, S.O. Sharrow, and A. Singer. 1994. Negative selection of CD4+CD8+ thymocytes by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD28. *J Exp Med* 179:709-713.

153. Kishimoto, H., and J. Sprent. 1999. Several different cell surface molecules control negative selection of medullary thymocytes. *J Exp Med* 190:65-73.

154. Pinon, J.D., V. Labi, A. Egle, and A. Villunger. 2008. Bim and Bmf in tissue homeostasis and malignant disease. *Oncogene* 1:41-52.

155. Bouillet, P. 2002. BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* 415:922-926.

156. McCarty, N. 2005. Signaling by the kinase MINK is essential in the negative selection of autoreactive thymocytes. *Nat Immunol* 6:65-72.

157. Thompson, J. and A. Winoto. 2008. During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. *J Exp Med* 205:1029-1036.

158. Buhlmann, J.E., S.K. Elkin, and A.H. Sharpe. 2003. A role for the B7-1/B7-2: CD28/CTLA-4 pathway during negative selection. *J Immunol* 170:5421-5428.

159. Takahashi, S., H. Kataoka, S. Hara, T. Yokosuka, K. Takase, S. Yamasaki, W. Kobayashi, Y. Saito, and T. Saito. 2005. In vivo overexpression of CTLA-4 suppresses lymphoproliferative diseases and thymic negative selection. *Eur J Immunol* 35: 399-407.

160. Ohki, H., C. Martin, M. Coltey, and N.M. Le Douarin. 1988. Implants of quail thymic epithelium generate permanent tolerance in embryonically constructed quail/chick chimeras. *Development* 104:619-630.

161. Ohki, H., C. Martin, C. Corbel, M. Coltey, and N.M. Le Douarin. 1987. Tolerance induced by thymic epithelial grafts in birds. *Science* 237:1032-1035.

162. Salaun, J. 1990. Thymic epithelium tolerizes for histocompatibility antigens. *Science* 247:1471-1474.

163. Sakaguchi, S., K. Fukuma, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med* 161:72-87.

164. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

165. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

166. Williams, L.M., and A.Y. Rudensky. 2007. Maintenance of the Foxp3- dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8:277-284.

167. Lin, W., D. Haribhai, L.M. Relland, N. Truong, M.R. Carlson, C.B. Williams, and T.A. Chatila. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8:359-368.

168. Liston, A. and A.Y. Rudensky. 2007. Thymic development and peripheral homeostasis of regulatory T cells. *Curr Opin Immunol* 19:176-185.

169. Tang, Q., K.J. Henriksen, E.K. Boden, A.J. Tooley, J. Ye, S.K. Subudhi, X.X. Zheng, T.B. Strom, and J.A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171:3348-3352.

170. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12:431-440.

171. Yao, Z. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109:4368-4375.
172. Antov, A., L. Yang, M. Vig, D. Baltimore, and L. Van Parijs. 2003. Essential role for STAT5 signaling in CD25+CD4+ regulatory T cell homeostasis and the maintenance of self-tolerance. *J Immunol* 171:3435-3441.
173. Vang, K.B., J. Yang, S.A. Mahmud, M.A. Burchill, A.L. Vegoe, and M.A. Farrar. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J Immunol* 181:3285-3290.
174. Fontenot, J.D., J.P. Rasmussen, M.A. Gavin, and A.Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat immunol* 6:1142-1151.
175. Liu, Y. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat immunol* 9:632-640.
176. Walker, L.S., and A.K. Abbas. 2002. The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat Rev Immunol* 2:11-19.
177. Xing, Y., and K.A. Hogquist. 2012. T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol*, 4:a006957.
178. Alegre, M.L., K.A. Frauwirth, C.B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1:220-228.
179. Mueller, D.L. 2010. Mechanisms maintaining peripheral tolerance. *Nat Immunol* 11:21-27.
180. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 7:445-480.
181. Krummel, M.F., and J.P. Allison. 1996. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 183:2533-2540.
182. Walunas, T.L., D.J. Lenschow, C.Y. Bakker, P.S. Linsley, G.J. Freeman, J.M. Green, C.B. Thompson, and J.A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405-413.
183. Tivol, E.A., F. Borriello, A.N. Schweitzer, W.P. Lynch, J.A. Bluestone, and A.H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA- 4. *Immunity* 3:541-547.
184. Waterhouse, P., J.M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K.P. Lee, C.B. Thompson, H. Griesser, and T.W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270:985-988.
185. Perez, V.L., L. Van Parijs, A. Biuckians, X.X. Zheng, T.B. Strom, and A.K. Abbas. 1997. Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* 6:411- 417.
186. Kurtz, J., F. Raval, C. Vallot, J. Der, and M. Sykes. 2009. CTLA-4 on alloreactive CD4 T cells interacts with recipient CD80/86 to promote tolerance. *Blood* 113:3475-3484.
187. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 Control over Foxp3+ Regulatory T Cell Function. *Science* 322:271-275.
188. Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141-151.
189. Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203:883-895.
190. Probst, H.C., K. McCoy, T. Okazaki, T. Honjo, M. van den Broek. 2005. Resting dendritic cells induce peripheral CD8þ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 6:280-286.
191. Chambers, C. A. 2001. The expanding world of co-stimulation: the two-signal model

revisited. *Trends Immunol* 22:217–223.
192. Morelli, A.E., and A.W. Thomson. 2007. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* 7:610 – 621.
193. Mahnke, K., E. Schmitt, L. Bonifaz, A.H. Enk, and H. Jonuleit. 2002. Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol Cell Biol* 80:477-483.
194. Mellor, A.L., and D.H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762-774.
195. Brunner, T., R.J. Mogil, D. LaFace, N.J. Yoo, A. Mahboubi, F. Echeverri, S.J. Martin, W.R. Force, D.H. Lynch, C.F. Ware, and D.R. Green. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* 373:441-444.
196. Combadière, B., M. Freedman, L. Chen, E.W. Shores, P. Love, and M.J. Lenardo. 1996. Qualitative and quantitative contributions of the T cell receptor zeta chain to mature T cell apoptosis. *J Exp Med* 183:2109-17.
197. Lenardo, M.J. 1991. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* 353:858-861.
198. Sytwu, H.K., R.S. Liblau, and H.O. McDevitt. 1996. The roles of Fas/APO-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. *Immunity* 5:17-30.
199. Hildeman, D.A. 2002. Activated T cell death in vivo mediated by proapoptotic bcl-2 family member bim. *Immunity* 16:759-767.
200. Pellegrini, M. 2003. Shutdown of an acute T cell immune response to viral infection is mediated by the proapoptotic Bcl-2 homology 3-only protein Bim. *Proc Natl Acad Sci U S A* 100:14175-14180.
201. Bouillet, P. 1999. Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 286: 1735-1738.
202. Hoglund, P. 1999. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J Exp Med* 189:331-339.
203. Andre, I. 1996. Checkpoints in the progression of autoimmune disease: lessons from diabetes models. *Proc Natl Acad Sci U S A* 93:2260-2263.
204. Compston, A. and A. Coles. 2002. Multiple sclerosis. *Lancet* 359:1221-1231.
205. Lafaille, J.J., K. Nagashima, M. Katsuki, and S. Tonegawa. 1994. High incidence of spontaneous autoimmune encephalomyelitis in immunodeficient anti-myelin basic protein T cell receptor transgenic mice. *Cell* 78:399-408.
206. Waldner, H., M.J. Whitters, R.A. Sobel, M. Collins, and V.K. Kuchroo. 2000. Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic for the myelin proteolipid protein-specific T cell receptor. *Proc Natl Acad Sci U S A* 97:3412-3417.
207. Bettelli, E., M. Pagany, H.L. Weiner, C. Linington, R.A. Sobel, and V.K. Kuchroo. 2003. Myelin oligodendrocyte glycoprotein-specific t cell receptor transgenic mice develop spontaneous autoimmune optic neuritis, *J Exp Med* 197:1073-1081.
208. Greer, J.M., and M.B. Lees. 2002. Myelin proteolipid protein-the first 50 years. *Int J Biochem Cell Biol* 34:211-215.
209. Anderson, T.J., P. Montague, N. Nadon, K.A. Nave, and I.R. Griffiths. 1997. Modification of Schwann cell phenotype with Plp transgenes: evidence that the PLP and DM20 isoproteins are targeted to different cellular domains. *J Neurosci Res* 50:13-22.
210. Kuchroo, V.K., A.C. Anderson, H. Waldner, M. Munder, E. Bettelli, and L.B. Nicholson. 2002. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. *Annu Rev Immunol* 20:101-123.
211. Knapp, P.E., R.P. Skoff, and D.W. Redstone. 1986. Oligodendroglial cell death in jimpy mice: an explanation for the myelin deficit. *J Neurosci* 6:2813–2822.
212. Hodes, M.E., V.M. Pratt, and S.R. Dlouhy. 1994. Genetics of Pelizaeus-Merzbacher

disease. *Dev Neurosci* 15:383-394.
213. Nave, K.A., and O. Boespflug-Tanguy. 1996. Developmental defects of myelin formation: from X-linked mutations to human dysmyelinating diseases. *Neuroscientist* 2:33-38.
214. Klugmann, M., M.H. Schwab, A, Pühlhofer, A. Schneider, F. Zimmermann, I.R. Griffiths, and K.A. Nave. 1997. Assembly of CNS myelin in the absence of proteolipid protein. *Neuron* 18:59-70.
215. Greer, J.M. 2013. Autoimmune t-cell reactivity to myelin proteolipids and glycolipids inmultiple sclerosis. *Mult Scler Int* 2013:151427.
216. Voskuhl, R.R. 1998. Myelin protein expression in lymphoid tissues: implications for peripheral tolerance. *Immunol Rev* 164:81-92.
217. Tuohy, V.K., R.A. Sobel, and M.B. Lees. 1988. Myelin proteolipid protein-induced experimental allergic encephalomyelitis. Variations of disease expression in different strains of mice. *J Immunol* 140:1868-1873.
218. Whitham, R.H., D.N. Bourdette, G.A. Hashim, R.M. Herndon, R.C. Ilg, A.A. Vandenbark, and H. Offner. 1991. Lymphocytes from SJL/J mice immunized with spinal cord respond selectively to a peptide of proteolipid protein and transfer relapsing demyelinating experimental autoimmune encephalomyelitis. *J Immunol* 146:101-107.
219. Klein, L., M. Klugmann, K.A. Nave, V.K. Tuohy, and B. Kyewski. 2000. Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat Med* 6:56-61.
220. Thompson, S.D., J. Pelkonen, J.L. Hurwitz. 1990. Concomitant T-cell receptor alpha and delta gene rearrangements in individual T-cell precursors. *Proc Natl Acad Sci U S A* 87:5583-5586.
221. Kouskoff, V., K Signorelli, C Benoist, and D Mathis. 1995. Cassette vectors directing expression of t cell receptor genes in transgenic mice. *J Immunol Methods* 180:273-280.
222. Brocker, T., M. Riedinger, and K. Karjalainen. 1996. Redirecting the complete T cell receptor/CD3 signaling machinery towards native antigen via modified T cell receptor. *Eur J Immunol* 26:1770–1774.
223. Andersen, P.S., C. Geisler, S. Buus, R.A. Mariuzza, and K. Karjalainen. 2001. Role of the T cell receptor ligand affinity in T cell activation by bacterial superantigens. *J Biol Chem* 276: 33452–33457.
224. Akkaraju, S., W.Y. Ho, D. Leong, K. Canaan, M.M. Davis, and C.C. Goodnow. 1997. A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity* 7: 255–271.
225. Kurts, C., W.R. Heath, F.R. Carbone, J. Allison, J.F. Miller, and H. Kosaka. 1996. Constitutive class I-restricted exogenous presentation of self antigens in vivo. *J Exp Med* 184: 923–930.
226. Goverman, J., A. Woods, L. Larson, L.P. Weiner, L. Hood, and D.M. Zaller 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* 72:551-560
227. Lafaille, J.J. 2004. T-cell receptor transgenic mice in the study of autoimmune diseases. *J Autoimmun* 22:95-106.
228. Kohm, A.P., P.A. Carpentier, H.A. Anger, and S.D. Miller. 2002. Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol* 169:4712-4716.
229. Reddy, J., H. Waldner, X. Zhang, Z. Illes, K.W. Wucherpfennig, and R.A. Sobel. 2005. Cutting edge: CD4+CD25+ regulatoryT cells contribute to gender differences in susceptibility to experimental autoimmune encephalomyelitis. *J Immunol* 175:5591–5595
230. Reddy, J., Z. Illes, X. Zhang, J. Encinas, J. Pyrdol, L. Nicholson, R. Sobel, K.W. Wucherpfennig, and V.K. Kuchroo. 2004. Myelin proteolipid protein-specific CD4+CD25+ regulatory cells mediate genetic resistance to experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 101:15434–15439.

231. Hogquist, K.A., T.A. Baldwin, and S.C. Jameson. 2005. Central tolerance: Learning self-control in the thymus. *Nat Rev Immunol* 5:772-782.
232. Anderson, G., K. Partington, and E. Jenkinson. 1998. Differential effects of peptide diversity and stromal cell type in positive and negative selection in the thymus. *J Immunol* 161:6599–6603.
233. Klein, L., and B. Kyewski. 2000. Self-antigen presentation by thymic stromal cells: a subtle division of labor. *Curr Opin Immunol.* 12:179–186.
234. Matzinger, P., and S. Guerder. 1989. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature* 338:74–76.
235. Brocker, T., M. Riedinger, and K. Karjalainen. 1997. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo*. J Exp Med* 185:541–550.
236. Merkenschlager M., M.O. Power, H. Pircher, and A.G. Fisher. 1999. Intrathymic deletion of MHC class I-restricted cytotoxic T cell precursors by constitutive cross-presentation of exogenous antigen. *Eur J Immunol* 29:1477–1486.
237. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science 248:1349.*
238. Martinez, R.J., N. Zhang, S.R. Thomas, S.L. Nandiwada, M.K. Jenkins, B.A. Binstadt, and D.L. Mueller. 2012. Arthritogenic self-reactive CD4+ T cells acquire an FR4hiCD73hi anergic state in the presence of Foxp3+ regulatory T cells. *J Immunol* 188:170-181.
239. Tuettenberg, A., E. Huter, M. Hubo, J. Horn, J. Knop, B. Grimbacher, R.A. Kroczek, S. Stoll, and H. Jonuleit. 2009. The role of ICOS in directing T cell responses: ICOS-dependent induction of T cell anergy by tolerogenic dendritic cells. *J Immunol* 182:3349-3356.
240. Sakaguchi, S., K. Wing, Y. Onishi, P. Prieto-Martin, and T. Yamaguchi. 2009. Regulatory T cells: how do they suppress immune responses? *Int Immunol* 21:1105-1111.
241. Smith, A.J., Y. Liu, H. Peng, R. Beers, M.K. Racke, and A.E. Lovett-Racke. 2011. Comparison of a classical Th1 bacteria versus a Th17 bacteria as adjuvant in the induction of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 237:33-38.
242. Kyburz, D., and M. Corr. 2003. The KRN mouse model of inflammatory arthritis. *Springer Semin Immunopathol* 25:79-90.
243. Gordon, J., S. Xiao, B. 3rd. Hughes, D.M. Su, S.P. Navarre, B.G. Condie, and N.R. Manley. 2007. Specific expression of lacz and cre recombinase in fetal thymic epithelial cells by multiplex gene targeting at the foxn1 locus. *BMC Dev Biol* 7:69.
244. Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* 166:557-580.

6. Appendix

6.1 Abbreviations

APC	Antigen presenting cell
BM	Bone marrow
bp	Base pairs
CD	Cluster of differentiation
cDNA	complementary DNA
CFA	Complete Freund's adjuvant
CFSE	Carboxy-fluorescein diacetate succinimidyl ester
CMJ	Cortico-medullary junction
CNS	Central nervous system
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DN	Double negative
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
GFP	Green fluorescent protein
ELISA	Enzyme linked immunosorbent assa
ETPs	Early thymic progenitors
FACS	Fluorescent activated cell scanning
i.p	Intraperitoneal
i.v	Intravenous
kDa	Kilodalton
KO	Knockout
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte protein
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cells
NK cells	Natural killer cells

NOD	Non-obese diabetic
OVA	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PSG1	Platelet-selectin glycoprotein ligand 1
RAG	Recombination-activating gene
SD	Standard deviation
SEM	Standard error of the mean
SP	Single positive
SPF	Specific pathogen free
TBE	Tris buffer EDTA
TBS	Tris buffer saline
TCR	T cell receptor
Tg	Transgenic
Treg	Regulatory T cell
WT	Wild-type

7. Acknowledgments

It is my great pleasure to thank those who helped me complete my thesis works. You made my life and this thesis possible with your collaborations, advices and support.

Firstly, I would like to express my deep gratitude to Prof. Dr. **Ludger Klein** for giving me the opportunity to perform this PhD project in your group on such an inspiring research topic. I thank you for your supervision, critical comments and continuous support during the past years. While instilling a tough scientific attitude in me, you were also able to maintain a fantastic working atmosphere in the lab, which made it not only stimulating to work there but also fun. I did discover my true scientific passion for the thymus during my time in your lab.

My thanks go to the past and present Klein lab members who supported me in various respects of science. My special thanks goes to **Maria** for your guidance throughout my thesis. When I first joined our lab, you taught me experimental techniques, but even more importantly you trained my as a scientist and encouraged me to think critically and independently. You have always taken your time to discuss science as well as share your good mood and enthusiasm with me. Your constructive comments and constant encouragement brought out the best in me. I would also like to thank **Julia**, for taking me 'under your wings' during my initial project work, your sense of humor and cheerful personality made my work more pleasant. **Christine,** thank you very much for teaching me the secrets of molecular biology and for your continual support throughout my time in the lab. **Tobi**, as one of our “PLP people”, I am very happy to have been surrounded and inspired by your positive relaxed attitude and cheerful nature! **Elisabetta,** I thank you for your positive energy and precious friendship. Your kindly personality really impressed me a lot! It is so good to know you! **Tomo,** thank you for your helpful suggestions and your willingness to share with us! I am deeply grateful for your guidance through many problems**. Madlen,** I really enjoy to talk with you, thank you for the nice moments we shared during the past years**. Sonja,** thank you for your patience and your help when I face difficulties during my study. **Lena**, thank you for critically reading some parts of my thesis and gave me valuable comments.

I would also like to thank **all present and former members of the Institute for Immunology of LMU** for creating a scientifically stimulating working environment.

I would also like to thank my friends **Qingqing, Wei, Xilong, Quan** for your friendship supported me to go through the happy days when we are in Munich.

Last, I would like to thank to my boyfriend, **Huichao**, for being there every step of my way. Without you, I should be far away from this final exciting day! I would like to express my deepest respect and love to **my parents** for your support and constant encouragement throughout my whole life.

.