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Central tolerance induction to the self-antigen PLP

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Abstract

To prevent potential autoimmunity, the thymus instructs developing T cells to discriminate between self and non-self. This instruction is called central tolerance induction and purges the T cell repertoire of self-reacting T cells. Fundamental for central tolerance induction is the expression of otherwise tissue-restricted antigens in the thymus [1, 2]. The two main mechanisms operating in central tolerance are clonal deletion of auto-reactive T cells and deviation of these into the regulatory T cell lineage. While enormous progress has been made in understanding the cellular and molecular basis of central tolerance, the exact parameters that determine whether an auto-reactive T cell is deleted or becomes a regulatory T cell are not yet fully understood.

We have developed a class II-restricted T cell receptor-transgenic model (TCR-PLP1) to study the modes of central tolerance induction to the self-antigen myelin proteolipid protein (PLP) in the context of H-2^{b}. PLP is the main component of the myelin sheath around neurons and of particular interest as it a candidate auto-antigen in *Multiple Sclerosis* in humans. Furthermore, it had been shown that intra-thymic expression of PLP is sufficient to mediate tolerance [3]. Using this novel TCR-PLP1 transgenic mouse, we investigated the modalities of central tolerance induction to a self-antigen expressed in the thymus at physiological levels. We found PLP-specific T cells to be tolerised by clonal deletion and concomitant deviation into the regulatory T cell lineage. Thymic medullary epithelial cell (mTEC)-driven expression and direct presentation of the endogenous antigen PLP mediated tolerance in an autonomous manner, whereas thymic dendritic cells were dispensable for central tolerance induction to PLP. Furthermore, the autoimmune regulator AIRE controlled tolerance induction to PLP by regulating PLP mRNA expression in $mTEC^{hi}$, albeit PLP is not regarded as classical AIRE-dependent antigen. We could also provide evidence, that central tolerance might act in a dedicated time-window. We found negative selection of TCR-$PLP1^{+}$ CD4SP cells to begin at day 3, while PLP-specific regulatory T cells accumulated only after 1 week after birth. Strikingly, we observed a loss of central tolerance induction to PLP at the age of 6 weeks. Age-dependency was not yet considered closely for central tolerance induction and expands its complexity for yet another aspect.

Zusammenfassung

Um der Entwicklung von Autoimmunität entgegenzuwirken, muss der Thymus neu entstehenden T-Zellen die Fähigkeit mitgeben, zwischen körpereigenen und körperfremden Strukturen zu differenzieren. Dieser Vorgang, der als zentrale Toleranzentwicklung bezeichnet wird, identifiziert jene T-Zellen, die auf körpereigene Strukturen reagieren und eliminiert sie. Fundamentale Grundlage ist hierbei die Expression gewebespezifischer Antigene im Thymus [1, 2]. Zentrale Toleranz wird hauptsächlich über zwei Mechanismen vermittelt, einerseits über die Beseitigung autoreaktiver T-Zell-Klone (*clonal deletion*), zum anderen über ein Umleiten der identifizierten Zellen in die regulatorische T-Zelllinie (T_{reg} *induction*). Während zentrale Toleranz auf der zellulären und molekularen Ebene bereits recht gut verstanden wird, ist noch nicht vollständig geklärt, welche Parameter determinieren, ob eine auto-reaktive T-Zelle durch Deletion beseitigt oder ob sie in eine regulatorische T-Zelle umfunktioniert wird.
Um diesen Punkt näher zu untersuchen, entwickelten wir ein T-Zell-Rezeptor transgenes Mausmodell (TCR-PLP1), welches ausschließlich T-Zellen hervorbringt, die das körpereigene Antigen Myelin Proteolipid Protein (PLP) auf dem Haupthistokompatibilitätskomplex H-2^{b} der Klasse II erkennen können. PLP ist Hauptbestandteil der Neuronen umgebenden Myelinscheide und von besonderem Interesse, da das körpereigene Antigen als Kandidat für die Entwicklung Multipler Sklerose beim Menschen gilt. Es konnte gezeigt werden, dass eine intrathymische Expression von PLP bereits ausreichend für die Induktion von zentraler Toleranz ist [3]. Mit Hilfe des neu entwickelten TCR-PLP1 transgenen Mausmodells konnten wir die Stellschrauben zentraler Toleranzinduktion gegenüber einem körpereigenen Antigen untersuchen, welches im Thymus auf physiologischem Niveau exprimiert wird. Dabei fanden wir, dass PLP-spezifische T-Zellen im Thymus sowohl deletiert als auch in die regulatorische T-Zellllinie umgeleitet werden, um Toleranz gegenüber PLP entstehen zu lassen. Sowohl die Expression von PLP in thymischen medullären Epithelzellen (mTECs) als auch die Präsentation des endogenen Antigens PLP durch diese Zellen vermittelt zentrale Toleranz auf autonome Art und Weise. Thymische dendritische Zellen spielten bei der Entstehung von Toleranz gegenüber PLP keine Rolle. Interessanterweise scheint der Autoimmun-Regulator

AIRE von essentieller Bedeutung für die Toleranzinduktion gegenüber PLP zu sein. Obwohl PLP kein typisch AIRE-abhäniges Antigen ist, kontrolliert AIRE die Expression von PLP mRNA in $mTEC^{hi}$ Zellen und reguliert womöglich dadurch die Toleranzinduktion. Weiterhin ergaben sich Hinweise, dass zentrale Toleranz in einem definierten Zeitfenster entsteht. Negative Selektion von TCR-$PLP1^{+}$ $CD4^{+}$ T-Zellen beginnt am Tag 3, während regulatorische T-Zellen erst 1 Woche nach Geburt entstehen. Überraschenderweise konnten wir zudem beobachten, dass in unserem Mausmodell zentrale Toleranz gegenüber PLP nur bis zu einem Alter von 6 Wochen induziert wird und danach verloren geht. Eine Altersabhängigkeit wurde bisher für die Toleranzinduktion nicht genauer beschrieben und erweitert dessen Komplexität um einen weiteren Aspekt.

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1 Introduction

The role of the immune system is to protect the organism from infectious agents and cancer. It is a combination of two fronts: the fast acting innate immune system and the slower but more specific adaptive immune system. When a pathogen infiltrates the first defenses of the body, innate immune mechanisms are brought into action. Defense mechanisms of the innate immune system range from nonspecific chemical defense, such as the complement system and antimicrobial peptides, to the recruitment of effector cells, such as macrophages, natural killer T (NKT) cells, mast cells, and granulocytes to quickly get rid of the pathogenic threat. This system is rapid in action, but limited in the sense that it does not recognise all foreign microbes and substances, especially pathogens that are modified over time.
The adaptive immune system has evolved to target pathogens in a more specific manner, based on its ability to continuously adjust to novel antigenic challenges. This system consists of two branches, (I) humoral immunity mediated by B lymphocytes and antibodies and (II) cellular immunity mediated by $CD4^+$ helper T lymphocytes and $CD8^+$ effector T lymphocytes. A hallmark of the adaptive immune system is (a) the immunological memory which results in a faster and stronger immune response when the pathogens are re-encountered, and (b) the vast diversity and specificity of the antigen receptors expressed on the surface of lymphocytes.

1.1 Central tolerance

Immunological responses mediated by T cells are crucial for providing protective immunity but depend on the acquired ability to discriminate between foreign and self. The T cell receptor (TCR) is composed of two polypeptides ($\alpha\beta$) that contain variable regions and recognise peptide-bound major histocompatibility complexes (MHC). To accomplish the generation of a diversified repertoire of T lymphocytes that can effectively respond to a multitude of potential antigens, thymocytes are continuously generated *de novo* and undergo random gene rearrangements at the TCR loci to produce a different receptor for each

cell. As the antigen-specific receptors of thymocytes are assembled through random somatic DNA rearrangement, the emerging T cell pool inevitably includes TCR specificities reactive to self-antigens. To avoid the potentially pathological state of autoimmunity, it is essential to remove these self-reactive T cells from the repertoire, either by deletion or silencing. Some T cell specificities are dismissed in the thymus soon after generation, because they do not recognise peptides in context of self-MHC, which is one of the prerequisites of further T cell development. For the remaining immature lymphocytes, central tolerance mechanisms assure that they acquire the ability to distinguish between self and non-self, before they are allowed to egress into the periphery. For an effective central tolerance, T cells must be challenged in the thymus with a complex array of different peptides, which present peripheral self-antigens (ubiquitous and tissue-restricted) that the T cells may encounter at any point in their lifetime in the periphery. The major mechanisms of central tolerance are clonal deletion, inactivation, and diversion of self-reacting thymocytes into the regulatory T cell lineage [4]. All these mechanisms occur in the thymus, whereas peripheral tolerance, on the other hand, concerns mature lymphocytes after they have exited the thymus and are circulating in the periphery. Mechanisms for peripheral tolerance also include clonal deletion, anergy, and diversion, but a variety of additional mechanisms come into play, including clonal ignorance and suppression of self-reactive lymphocytes [5–7].
Currently, the relative contributions of dendritic cells, thymic epithelial cells, and other antigen-presenting cells in the thymus to the prevention of autoimmunity are poorly understood [7–9].

1.2 Development of thymocytes

1.2.1 The thymus

T cells, different to other hematopoietic cells, do not develop in the bone marrow but in the thymus. The thymus is the primary lymphatic organ that provides a specialised microenvironment for T cell development. T cells develop in a strictly controlled spatiotemporal manner along different compartments of the thymus. In short, the thymic stroma cells present certain signals to the arriving progenitors to commit to the T cell lineage, guiding them to arrange their antigen receptor, and probe these for functionality and specificity to self.
The thymus is compartmentalised into the outer, darker zone, the cortex, and the inner, lighter zone, the medulla. The cortex is mainly comprised of cortical thymic epithelial

cells (cTECs) and operates in positive selection, while the medulla has been shown to crucially contribute to tolerance induction by expressing otherwise tissue-restricted antigens (TRAs), thereby mirroring the peripheral self. During their guided journey through the thymic compartments, T cells have to pass several checkpoints: Either they die or they pass on to the next developmental stage until eventually being released into the periphery. Selection processes in the thymus ensure that fully differentiated thymocytes fulfill two essential prerequisites: first, that they recognise and respond to foreign peptides presented on self-MHC complexes, and second, that they are tolerant to self-peptides presented.
Three key events mark the developmental progression of these cells:

1. Positive selection
2. CD4/CD8 lineage differentiation
3. Negative selection

1.2.2 Developmental stages

Early T cell development is subdivided into four stages characterised by the cell surface markers CD44 and CD25 and the location of the cell within the cortex [10] (see Fig. 1). The most immature subset of thymocyte precursors (DN1) reside in the perimedullary cortex, lack CD4 and CD8 expression and are characterised by the surface markers $CD44^{+}CD25^{-}$ [11]. Before DN1 cells develop further into the DN2 developmental stage in the inner cortex ($CD44^{+}CD25^{+}$), they expand by proliferation about 1000-fold [12,13]. Further maturation of cells coincides with migration into the outer cortical region and developmental progression to the DN3 ($CD44^{-}CD25^{+}$) stage. Cells reside about 2 days in the subcapsular zone, undergoing proliferative expansion and irreversible commitment to the T cell lineage [14].

1.2.3 Generation of the T cell receptor

A key process of the development of thymocytes is the generation of a T cell receptor (TCR), which also takes place in the cortex. Recombination events start at the DN3 stage and critically depend on the expression of the recombination activation genes (RAG1 and RAG2), presumably regulated by signalling of IL-7 [15]. The RAG genes help to recombine the germ line encoded VDJ segments of the TCRβ locus in two steps [16,17]:
First, rearrangement of the DJ genes take place on both alleles and then, subsequently, the recombined DJ segments rearrange with the V segment forming the TCRβ chain. Productive rearrangement of the TCRβ chain gene on one chromosome, prevents further gene

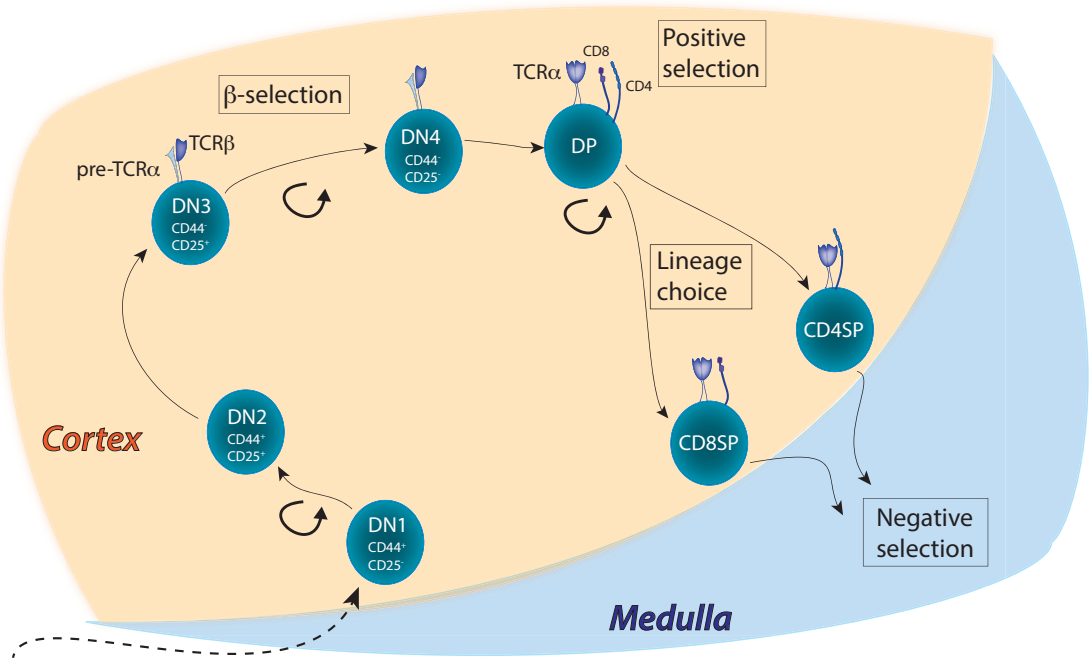

Figure 1: Schematic view of the early stages of thymocyte development. The hematopoietic T cell precursors enter the thymus at the cortico-medullary junction and migrate towards the outer cortex. During that period, the precursors commit to the T cell lineage and undergo several differentiation steps characterised by differential expression of the surface markers CD44 and CD25. During this differentiation, the lymphocytes do not express CD4 nor CD8 and are referred to as double negative cells, namely DN1 to DN4. At the DN3 stage, thymocytes start to rearrange their TCRβ locus, which will be coupled to a pre-TCRα chain in order to test the rearrangement for functionality. When the β-selection is positive, the TCRα loci will also start to rearrange and simultaneously upregulate the expression of the co-receptors CD4 and CD8 (DP). Positive selection by cTECs allows only those thymocytes to progress to the medulla, which have rearranged a functional TCR. During positive selection, thymocytes commit to either the CD4 or the CD8 T cell lineage, depending on the MHC type their TCR is selected by. Positively selected CD4SP or CD8SP thymocytes, will then progress to the medulla, where they are screened for reactivity towards self-antigens, a process called negative selection.

rearrangements at the second TCRβ allele, a process termed "allelic exclusion" [18–20]. This stage marks the first checkpoint during T cell development and is called β-selection. The newly formed TCRβ chain forms a heterodimer with an invariant pre-TCRα chain, CD3d, and this pre-TCR is tested for functionality [21, 22]. Only those T cells that express a fully functional pre-TCR on their surface receive a survival signal and are permitted to progress to the next developmental stage, the CD4/CD8 double positive (DP) stage and will be positively selected [21, 22]. The transition from CD4/CD8 double negative (DN) to double positive (DP) immature T cells is referred to as the fourth stage of early T cell development, the DN4 or pre-DP stage ($CD44^{-}CD25^{-}$ $CD4^{low}CD8^{low}$), and is accompanied by the migration to the outermost cortex, marking an irreversible commitment to the T cell lineage [14, 23]. At the transition from the DN stage to the DP stage, the precursor pool is heavily proliferating in order to guarantee a large pool of cells carrying functional β-chains that can later independently rearrange their α-locus. This will help to enrich the diversity of the TCRαβ T cell repertoire [24, 25]. During the whole expansion phase, the RAG genes are turned off to prevent premature rearrangements of the TCRα locus, which start when the pre-DP cells arrive at the subcapsular zone.

1.3 Positive selection

While the recombination of the TCRα locus is still ongoing, the cells upregulate the co-receptors CD4 and CD8 and are challenged with the next checkpoint, positive selection [23]. Positive selection is characterised by interactions between the DP T cells and cortical epithelial cells. Only those T cells carrying a functional αβT cell receptor that recognises peptides in the context of self-MHC molecules well enough for functional signal transduction are positively selected. This phenomenon is called "recognition of self" and ensures that the randomly generated repertoire is useful to the host, as it is able to recognise peptides presented on self-MHC complexes [26–28]. At this stage, DP T cells can continuously rearrange their TCRα chain until they are positively selected [29]. Further rearrangement of the second TCRα locus occurs when the first receptor combination is not able to interact with thymic self-MHC molecules [30, 31]. As allelic exclusion is not as tightly controlled at the TCRα locus, it is possible that some T cells can express two α chains paired with a common TCRβ chain on the cell surface [32–34].
The binding strength of the interaction between TCR and peptide-loaded MHC complex (pMHC) on cTECs is a critical parameter for survival: If the affinity is "high enough", the T cell will receive a TCR stimulus that leads to downstream expression of survival molecules allowing further progression to the medulla for negative selection [35]. If the

TCR shows “no” or “low ability” to bind to peptide-MHC complexes, the cell does not receive these survival signals and will undergo apoptosis, a process called “death by neglect” [28,36]. Positive selection is a crucial step that enriches for T cell progenitors that are MHC restricted. This step, of course, also enriches for self-reactive cells, thereby requiring the indispensable necessity of negative selection processes in the medulla.

1.4 CD4/CD8 lineage choice.

During positive selection, T cells express both CD4 and CD8 co-receptors. This allows a survival of as many thymocytes as possible in the positive selection process. The next important step immediately following the selection of successfully rearranged and self-restricted TCRs, is the CD4/CD8 lineage choice. Exact modalities for lineage choice are still under debate. Yet, it is widely accepted that DP thymocytes that have been positively selected become $CD4^+$ $CD8^{low}$ intermediate cells, that are still lineage-uncommitted but have the potential to differentiate into either CD4 single positive (CD4SP) or CD8 single positive (CD8SP) thymocytes [37, 38]. Depending on whether the selected cell has a MHCI- or a MHCII-restricted TCR, it will develop into a CD8SP or CD4SP thymocyte, respectively [39–41].

1.5 Negative selection

The process of negative selection is often considered as the third and last critical checkpoint during T cell development. Tolerance in the medulla is established in two ways: recessive tolerance (clonal deletion) and dominant tolerance (the development of regulatory T cells). Both types of tolerance are thymus-dependent processes, based on high-affinity interactions between TCR and peptide-MHC but with two different outcomes for a thymocyte involved in TCR assessment [42].
Subsequent to positive selection and CD4/CD8 lineage commitment, thymocytes translocate to the medulla, where they reside for $4-5$ days before receiving their “exit permit” [43]. Mice with mutations helped to identify the medulla as the place for negative selection as a disorganised or even an absent medullary structure led to severe autoimmunity but unaltered positive selection this these mice [44–47]. Also, mice deficient for the chemokine receptor CCR7, which is important for migration of T cells to the medulla, develop autoimmune pathology [48].
In the medulla, thymocytes with strong reactivity to presented self-antigens will be removed in order to ensure a self-tolerant T cell pool that is of no potential harm to the

organism [49, 50]. It is a coordinated process that depends on the interaction between thymocytes and the major antigen-presenting cell types in the medulla. The two best described antigen-presenting cell types involved in the medulla in negative selection are the thymic dendritic cells (thymic DCs) and the medullary thymic epithelial cells (mTECs) [50–52]. These cells both express higher levels of MHC molecules and in contrast to cTECs, also the co-stimulatory molecules CD40, CD80, and CD86, important for negative selection [53–56]. Recently also B cells were reported to play a role in negative selection, but their full contribution is still be found out [57, 58].

Thymic dendritic cells (thymic DCs). Thymic DCs in the medulla consist of an autochthonous DC population and DCs that arise extra-thymically. The DC population of intra-thymic origin is mostly composed of SIRPα^- (signal regulatory protein alpha-negative) CD11b$^-$ CD8α^{hi} cells [59, 60]. The migratory DC population that originated outside the thymus consists of immature plasmacytoid DCs (pDCs) and the SIRPα^+ CD11b$^+$ CD8α^- subset of conventional DCs (cDCs) [60]. Both migratory DC subtypes can take up and transport circulating antigen to the thymus [61].

Thymic medullary epithelial cells (mTECs). mTECs are unique among thymic antigen-presenting cells (APCs) in that they express an immense number of otherwise tissue-restricted antigens (TRAs) [1, 51, 62]. The mTEC population is mainly composed of two subpopulations: the MHCII^{low} AIRE$^-$ immature, proliferating population (mTEC^{lo}), and the MHCII^{high} CD80^{high} mature population (mTEC^{hi}), which divides further into cells positive or negative for the expression of the autoimmune regulator gene (AIRE) [63–65] (for AIRE see section 1.7) .

Interactions of developing T cells with peptide-MHC complexes on medullary APCs as quality control are indispensable for central tolerance. For negative selection to be productive, self-antigens must be presented as peptide-MHC complexes to developing thymocytes. If the thymocytes react with presented self-antigens too strongly, they are thought to be negatively selected, while if they recognise self-antigens with low affinity, they are allowed to undergo further development. Negative selection of T cells is induced by several mechanisms: Deletion, anergy, and receptor editing. Clonal deletion is the suicide of T cell progenitors that have high affinity for self-antigens, and is considered the hallmark of central tolerance induction [52]. The other processes (anergy and receptor editing) also impair or eliminate high-affinity self-reactive cells but are thought to play a lesser role.

However, even in healthy individuals negative selection is inevitably incomplete, leading to the emergence of T cells that potentially react against “self”. Therefore, there are several additional regulatory mechanisms beyond negative selection to enforce self-tolerance. One of these is the development of regulatory T cells (T_{reg} cells), a process that is strongly

related to negative selection [66]. T_{reg} cells that arise in the thymus and are not induced in the periphery are termed "natural T_{regs}" (nT_{regs}) [67]. FOXP3 (forkhead box protein 3) is currently seen as the master regulator of this regulatory T cell lineage and a major marker of this population [68–71]. Direct evidence for the importance of nT_{regs} comes from experiments showing that depletion of $Foxp3^+$ T_{reg} cells in neonatal mice caused the development of autoimmune disease, which could be cured by the re-introduction of T_{reg} cells [72]. Also, mice with a mutated Foxp3 gene (scurfy mice) develop lethal autoimmunity [73].
Work with the TCR-HA x AIRE-HA mouse model, in which TCR-transgenic T cells recognised their cognate antigen HA on mTECs, provided some crucial evidence that the generation of T_{regs} is based on self-recognition in the thymic medulla and occurs with negative selection [74, 75]. It is now widely accepted that "natural" $Foxp3^+$ $CD25^+$ regulatory T cells originate in the thymus and are the best characterised mediators of dominant tolerance so far [67, 76]. Utilising AIRE-driven CIITA knockdown ($C2TA^{kd}$), to lower the expression of a cognate antigen on MHCII molecules in the medulla, it could be shown that reduced quantity of presented antigen decreased negative selection and fueled T_{reg} development for several tested TCR specificities [77]. This data argued that the instructive phase of T_{reg} development is rather based on the avidity of TCR/peptide-MHC interactions than on the affinity of the TCR interactions.
Thus, not all central tolerance mechanisms cripple self-reactive T cells. The positive selection of regulatory T cell populations in the thymus enables T cells to actively suppress immune responses to structures of self that are recognised in the periphery [52].

1.6 Promiscuous gene expression

While it was easy to understand, how tolerance to ubiquitously expressed or blood-borne antigens is tested and maintained in the thymus, it was far more difficult to understand how thymocytes that are reactive to proteins expressed only in non-thymic parenchymal tissues were tolerised. This was revealed when several studies reported that otherwise tissue-restricted antigens (TRAs) were being ectopically expressed by mTECs in the thymus [1,2]. This so-called promiscuous gene expression (pGE) identified the basis for tolerance induction to a broad array of antigens found in the whole body [78–80]. Strikingly, TRAs were being ectopically expressed despite the tightly controlled lineage-specific gene expression. By pGE, the thymus comprehensively mirrors the immunological self of peripheral tissues and is able to maintain tolerance [2].
Because the expression of a given tissue-restricted antigen is restricted to a very small number of thymic APCs, the proper presentation of such a rare antigen is of critical impor-

tance [1,80]. It is desirable that all thymocytes are exposed to all TRAs during their stay in the medulla, each expressed by a minute number of mTECs, before exiting into the periphery. In order to enlarge the scope of antigen presentation, it was found that mTEC-borne peptides were spread in the thymic medulla uni-directionally from mTECs to neighbouring thymic dendritic cells [80–82]. For presentation of mTEC-derived self antigens by DCs, the peptides may be directly transferred to DCs or involve an intercellular transfer of an entire functional pMHC molecule and other TEC-specific membrane proteins [83,84]. Differences in the quality of tolerance induction when directly or indirectly presented are only slowly emerging and are still under debate. While there is evidence that the direct presentation of antigens by mTECs is sufficient for tolerance induction [77,80,85], it has been shown that some antigens require the transfer to DCs for efficient presentation [86]. Further studies are needed to delineate the impact of indirect vs direct presentation of TRAs.

1.7 AIRE

The importance of the expression of TRAs in the thymus has been emphasised by the study of the rare human immune disorder autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is characterised by a spectrum of autoimmune diseases caused by mutations in the **A**uto**i**mmune **re**gulator (*AIRE*) gene [87–89]. The generation of AIRE-deficient mice was instrumental in identifying the cellular role of AIRE and it became apparent that AIRE is directly linked to central tolerance. Deficiency in the expression of AIRE led to the development of multi-organ autoimmunity with inflammatory infiltrates and autoantibody production, thus recapitulating the pattern of the disease APECED [2,5,89].

The nuclear protein AIRE has a role in promiscuous gene expression of otherwise tissue-restricted antigens [90]. The encoded AIRE protein is expressed primarily in mature medullary thymic epithelial cells ($mTEC^{hi}$) in the thymus [1,2]. AIRE is also expressed in peripheral lymphoid tissues, where its contribution to tolerance is only starting to be unraveled [91]. Analysis of AIRE-deficient mTECs revealed that expression of some but not all TRAs are regulated by AIRE [2,62,92–94]. Although the precise molecular mechanism of AIRE is still largely obscure, its functional domains and several interacting partners have been identified. At first glance, AIRE appears to be a classical transcription factor, however, several findings hint at a broader function of AIRE. Abramson *et al.* [95] showed that AIRE interacts with an unexpectedly large number of binding partners: proteins that are involved in RNA processing, transcription, nuclear transport, DNA damage response, and proteins for chromatin binding and chromatin structure. It was further reported that

AIRE preferentially binds to the unmethylated histone H3 at lysine-4 (H3K4me0), a marker for inactive regions [96]. This indicates that AIRE could function as a transcriptional activator to initiate expression of target genes. Additionally, the interaction of AIRE with the transcriptional co-activator Creb-binding protein and the positive transcription elongation factor b also implies enhancement of gene expression mediated by AIRE [97, 98]. Current models posit that AIRE acts at the chromatin opening, and there, recruits the positive transcription elongation factor b to inactive genes at which arrested RNA polymerase II is already present, leading to noisy gene expression in mTECs [65, 99, 100].

AIRE regulates the expression of many TRAs, but not all [62]. There are also TRAs that are expressed independently from AIRE and may be produced by a distinct subclass of mTECs [64]. It was proposed that some of the AIRE-independent TRAs might be regulated by the LTβR signalling pathway [101]. However, it seems more likely that the autoimmune phenotype in LTβR-deficient mice was caused by the disorganised medullary structure, restraining thymocytes from interacting properly with medullary APCs [93]. Moreover, it was suggested that a subset of TRAs first seemingly dependent on AIRE, might be regulated indirectly by AIRE. As Anderson *et al.* [2] showed that AIRE activates the expression of several transcription factors, these might subsequently mediate the expression of many apparently "AIRE-dependent" TRAs. Thus, the pool of AIRE-dependent antigens might be further enhanced by adding these secondary targets to the direct primary targets [102]. Also, AIRE expression was thought to be proapoptotic, enhancing mTEC death, and therefore, intensifying antigen-spreading to DCs for cross-presentation [5, 103].
It was later found that AIRE might possess even more functions than purely controlling the expression of TRAs. Additionally, it was proposed that AIRE is involved in the regulation of antigen processing and presentation, mTEC differentiation and thymocyte migration [63, 90, 104–106]. *Aire*^{KO} mice were reported to show a disorganised medullary structure with fewer thymic DCs in the medulla [103, 106–108], and delayed migration leading to reduced thymic egress in the early phase after birth [109]. Thus, AIRE-deficiency obviously affects other aspects involved in central tolerance. The contribution of each AIRE-related mechanism in respect to mediating negative selection and T_{reg} induction still needs to be fully unraveled.

1.8 T cell receptor-transgenic animals

T cell receptor (TCR)-transgenic mice have been established as powerful tools in the elucidation of various aspects of central tolerance as well as T cell development. TCR-transgenic animals are generated by the introduction of productively rearranged TCR cDNA into the

genome, and therefore, TCR-transgenic mouse models can be used as a source of naive, almost pure mono-specific T cells. Since sucessfully rearranged T cell receptor chains largely prevent the further rearrangement of TCR loci, the transgene-encoded receptors are subsequently present on the majority of T cells [20]. This made it possible to track the behaviour and fate of T cells with defined specificities. This method was first accomplished with the generation of the HY-transgenic mouse that enabled the study of negative selection *in vivo* [110]. More TCR-transgenic mice followed, providing tools to analyse positive selection and the role of altered peptide ligands in establishing the kinetic signalling model [111, 112]. With the help of TCR-transgenic mouse models expressing a receptor for a self-antigen along with the self-antigen, it was also possible to address questions of central tolerance. These mice provided an excellent tool for studying negative selection, the relevant APC for central tolerance induction, and the molecular signalling events involved. In most of these systems, the genes for model antigens, such as ovalbumin [113] or hen-egg lysozyme [114], are not a natural part of the mouse genome and were rather transgenically introduced. It is clear that the nature of the TCR and of the model antigen (its structure, its localisation, its dose, and the time during which the antigen is available) are all important factors and can have major impacts on the timing and the mechanism by which specific T cells are tolerised [43, 115]. It is therefore important to make use of the most physiological tools possible to avoid studying artefacts.

It has long been quite unclear which flanking sequences exactly are required for proper TCR expression and often heterologous promoter fragments have been used to drive TCR expression. Some combinations have sometimes led to an abnormal timing and regulation of expression of the TCR genes. For this reason, the laboratory of Mathis & Benoist further elaborated TCR cassette vectors, originating from the HY-specific TCR-transgenic mice that carried all essential promoter and enhancer elements for TCR expression [116]. These newly engineered TCR cassette vectors include the natural, TCR-derived regulatory elements and only short segments of rearranged α and β variable regions need to be introduced. These pTα and pTβ cassette vectors are now widely used and were also used for the generation of the TCR-PLP1 transgenic mouse described in this thesis.
However, $\alpha\beta$TCR heterodimers of TCR-transgenic mice are expressed still earlier in development than naturally arising TCRs that have been subject to somatic gene rearrangements (Fig. 2). This could have potential implications for the positive and/or negative selection of the TCR and need to be carefully monitored.

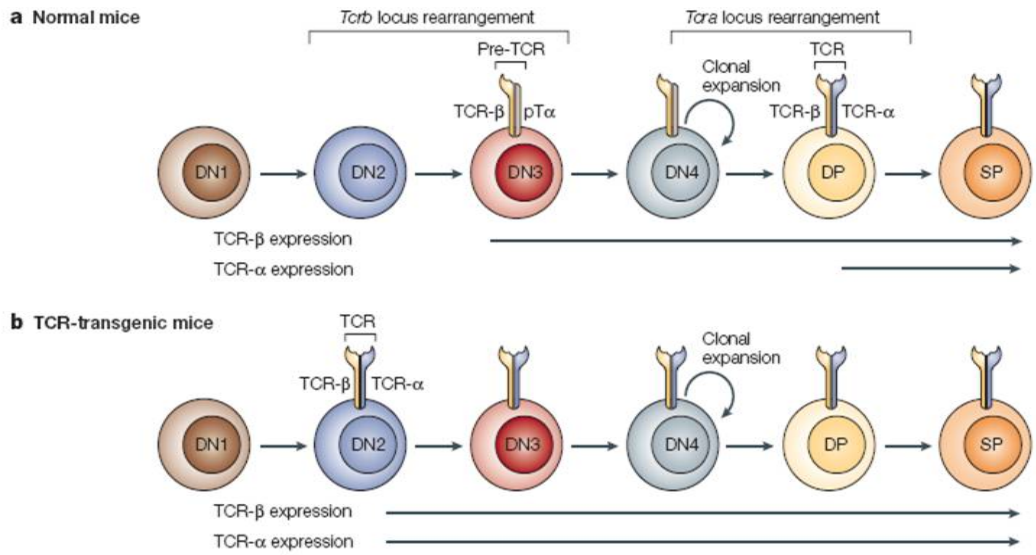

Figure 2: The timing of T cell receptor arrangements in TCR-transgenic mice compared to normal mice. Schematically steps of T cell developmental stages for rearrangement of the TCR in normal mice compared to the early expression of the TCR in TCR-transgenic mice. In TCR-transgenic mice, the TCR is mostly expressed by the DN2 stage. A) In normal T cell development, thymocytes start to rearrange their TCRβ locus at the DN3 stage forming a pre-TCR with the arranged β-chain. Only at the pre-DN4/DP stage, an intact αβTCR heterodimer is produced and brought to the cell surface. B) In TCR-transgenic mice, in contrast, both chains of the αβTCR heterodimer are expressed early in development typically at the DN2 stage. Figure from Hogquist *et al.* (2005) [52].

1.9 Myelin proteolipid protein (PLP)

Myelin is a specialised membrane found exclusively in the nervous system of vertebrates. It is wrapped around the nerve axon and acts as an insulator allowing nerve impulses to pass quickly along the axons by saltatory conduction between the nodes of Ranvier (see Fig. 3 A). Furthermore, it provides stablility, support to the axon, and maintains its integrity. Myelin is composed of lipids and associated membrane proteins. The three main myelin proteins are myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligoglycoprotein (MOG). Myelin is present in both the central nervous system (CNS), where it is produced by oligodendrocytes, and in the peripheral nervous system (PNS), where it is produced by Schwann cells. In the CNS, more than half of the protein found in the myelin sheath is proteolipid protein (PLP) [117]. In the PNS, PLP is present at very low concentrations in Schwann cells, but is not inserted into the PNS myelin membrane [118–121].

PLP is an integral membrane protein with four hydrophobic α-helices as transmembrane domains [122, 123] and is encoded on the X-chromosome (see Fig. 3 B). PLP also exists as isoform DM20 that is generated by alternative mRNA splicing [124]. It differs from full-length PLP by a deletion of an intracellular loop of 35 amino acids but shares the overall 'four-helix-span' topology [125, 126]. It was reported that PLP plays a critical role in oligodendrocyte differentation and survival [127]. Across species, PLP enjoys a strong conservation in its amino acid sequence [121, 128–131] underlining its important role in forming myelin sheaths. For this reason, it is possible to use bovine PLP for the induction of murine experimental autoimmune encephalomyelitis (EAE), a murine model disease for the human *Multiple Sclerosis*.
Substitution or mutations of even single amino acids that lead to a conformational change of PLP, renders PLP incapable of being brought to the cell surface, interfering with oligodendrocyte differentiation and survival [127, 132]. PLP deficient mice have been generated by the introduction of the neomycin resistance gene in exon 1 of the genomic sequence of PLP, resulting in a complete loss of expression for both PLP forms, PLP-full length and the DM20 isoform [133]. Surprisingly, these mice do not suffer from abnormal motor development nor the classical demyelination phenotype, and only develop neurological impairments at the age of 16 months [133, 134].

1.9.1 Experimental autoimmune encepahlomyelitis (EAE)

Experimental autoimmune encepahlomyelitis (EAE) is the murine model disease for *Multiple sclerosis*. *Multiple sclerosis* (MS) is a chronic disorder characterised by the inflammation of

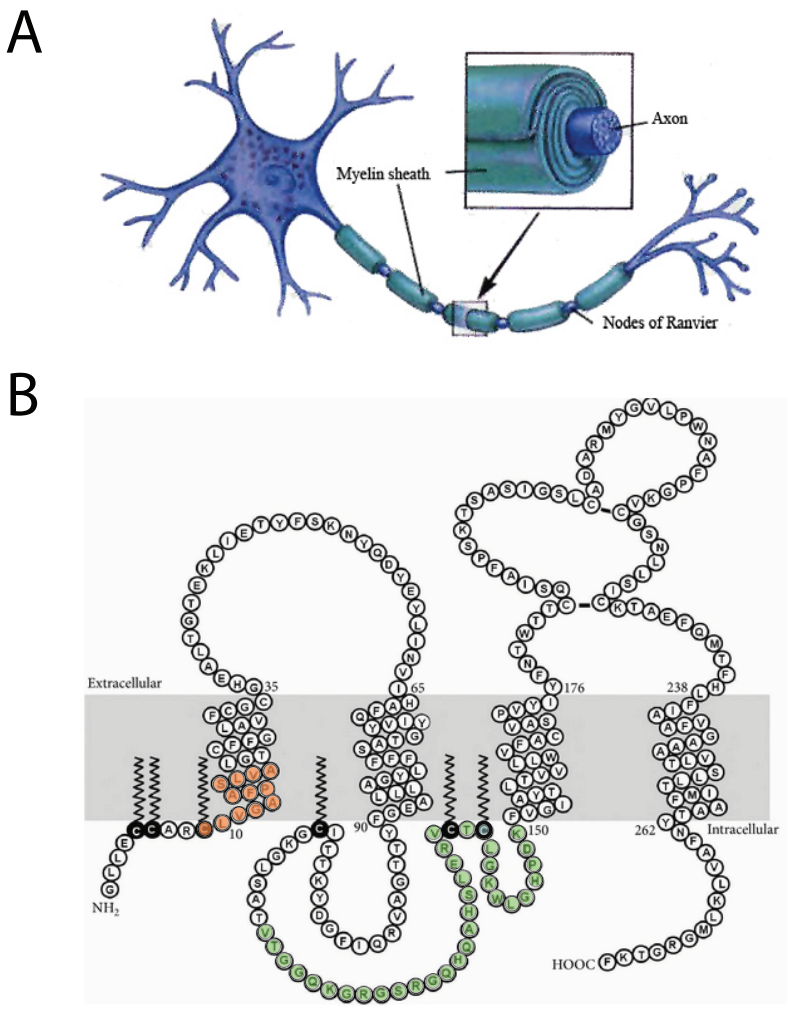

Figure 3: Structure of myelin in the nervous system and the transmembrane protein proteolipid protein (PLP). A) Myelin is an insulating membrane wrapped around axons of the nervous system. It allows nerve impulses to pass quickly along the axons by saltatory conduction between the nodes of Ranvier. In the CNS, more than 50% of all proteins in the myelin sheath are proteolipid proteins (PLP). Illustration from *http://multiple-sclerosis-research.blogspot.com/2013/01/imaging-myelin.html*. B) The protein PLP is depicted schematically with its amino-acid sequence. It is an integral membrane protein with four α-helices spanning the membrane. The position of $PLP_{9\text{-}20}$ is highlighted in red. The deleted stretch of 35 amino acids in the DM20 isoform is indicated with green circles. Illustration adapted from Greer (2013) [118].

the white matter in the central nerve system. The hallmark of the disease is the demyelination of nerve fibers, which then leads to axonal injury, neurological deficits and progressive physical impairment [135]. The precise course of the disease and especially its activation is not yet fully understood. However, it is widely believed that MS is initiated by the infiltration of autoreactive T cells into the CNS and the subsequent immune attack on CNS myelin [136]. Yet in MS, the group of auto-antigens responsible for disease initiation is very heterogeneous [137]. MS is a highly prevalent disease with an estimated incident rate of 1 in 500 in Europe [138].
"Classic EAE" in mice is characterised by ascending paralysis beginning at the tail, followed by hind limb paralysis and forelimb paralysis and is assessed semi-quantitatively using a 5-point scale (see also methods for scale 5 [139]). Upon induction of EAE, inflammatory cells infiltrate the CNS, release inflammatory mediators that will cause inflammation and subsequent tissue destruction, resulting in paralysis of the animal. The nature of the ascending paralysis is due to the fact that the cells begin to infiltrate the CNS in the lumbar region of the spinal cord. The disease progresses in severity as more of the spinal cord becomes inflamed and shares clinical and histopathological similarities to the human *Multiple sclerosis* [117, 140].

1.9.2 Central tolerance to PLP

As *Multiple Sclerosis* is a very severe autoimmune disorder, tolerance mechanisms should ensure the absence of myelin-specific T cells in the organism and PLP has been implicated in the etiology of MS in humans. To tolerise the T cell pool to PLP, the expression of the antigen in the thymus is required. The DM20 form is preferentially expressed in the thymus and secondary lymphoid organs, while the full-length PLP is predominantly expressed in the brain itself [141]. Susceptibility to EAE induction by immunisation with proteins of the CNS depends greatly on the genetic background of the mouse and MHC. In SJL/J mice (H-2^s), immunisation with protein including the immunodominant epitope of this haplotype $PLP_{139\text{-}151}$ leads to the induction of EAE. Immunisation with the same protein/peptide in C57BL/6 mice (short: B6; H-2^b) is without consequences [142, 143] and states the strong tolerance to PLP in this strain. While the B6 strain shows a high resistance to PLP-induced EAE, it can be induced by immunisation with MOG that eventually results in a chronic form of EAE [142, 144]. Klein *et al.* [3] showed with transplantation experiments in $PLP^{-/-}$ animals that the intra-thymic expression of PLP is sufficient for tolerance induction.
The full spectrum of dominant and subdominant immunogenic epitopes of PLP on the B6 background has been identified. Klein *et al.* immunised B6 WT and B6 $PLP^{-/-}$ mice with PLP protein and subsequently re-stimulated the primed lymph node T cells with

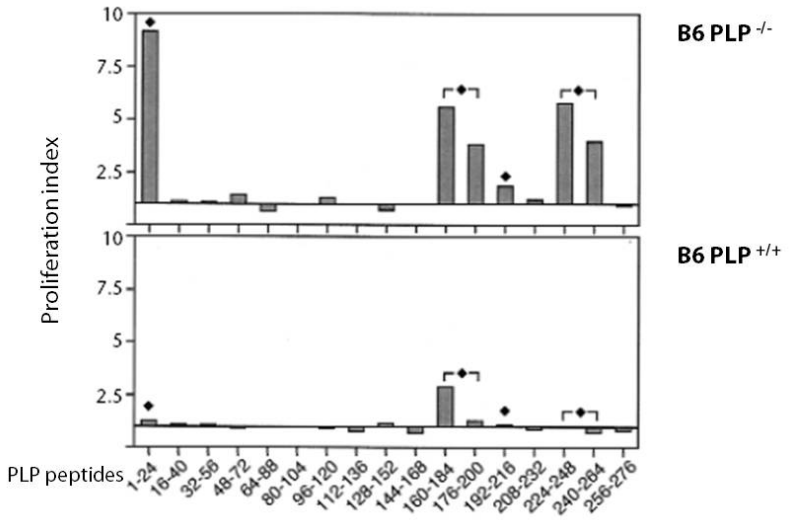

Figure 4: The four immunogenic regions of the PLP protein in the context of H-2^b. Four immunodominant regions in the context of H-2^b could be identified by recall analysis of immunised B6 PLP$^{-/-}$ mice in comparison to B6 WT animals. Reactivities against peptides PLP$_{160\text{-}184}$/PLP$_{176\text{-}200}$ and PLP$_{224\text{-}248}$/PLP$_{240\text{-}264}$ represented overlapping epitopes (marked with a black diamond). Responses to the peptides PLP$_{1\text{-}24}$ and PLP$_{192\text{-}216}$ represent individual epitopes. In B6 wild-type mice (B6 PLP$^{+/+}$), the response to these epitopes was virtually absent. Only a residual reponse to the peptide PLP$_{160\text{-}184}$ was detected. Figure adapted from Klein *et al.* (2000) [3].

sets of overlapping peptides (25 amino-acids in length), spanning the entire sequence of the protein PLP with a shift of 16 amino-acid residues. Specific recall responses to these peptides, revealed four immuno-relevant regions of PLP in the context of H-2^b (Fig. 4). By reduction of the peptide length for restimulation from 24 amino acids to 12 amino acids, it was possible to single out four core-epitopes in the B6 genetic background: PLP$_{11\text{-}18}$, PLP$_{174\text{-}181}$, PLP$_{205\text{-}213}$, and PLP$_{240\text{-}247}$. These achievements set the basis for delineating central tolerance mechanisms to the self-antigen PLP in the following work.

2 Aim of the thesis

With this study, we contribute to understanding central tolerance induction to a tissue-restricted self-antigen of the central nervous system. Specifically, we study the modalities of thymic tolerance induction to the self-antigen myelin proteolipid protein (PLP), a native self-antigen whose intra-thymic expression was shown to be sufficient to mediate tolerance [3].

PLP is one of the major components of the CNS and is considered as candidate antigen in the etiology of the human autoimmune disease *Multiple Sclerosis* [145, 146]. Therefore, functional tolerance induction to PLP is crucial to prevent the development of disease. Also in mice, it was possible to elicit the corresponding disease, experimental autoimmune encephalomyelitis (EAE) by immunisation with PLP protein, yet the severity of the disease depended greatly on the genetic background [117]. While SJL mice, for instance, were highly susceptible to EAE induction by immunisation, the C57BL/6 (B6) mouse strain was strongly resistant to disease development [142]. In B6 mice, the immunogenic regions of PLP in the context of H-2^b were already identified [3] (see also Fig. 4). The core epitopes were namely: $PLP_{11\text{-}18}$, $PLP_{174\text{-}181}$, $PLP_{205\text{-}213}$, and $PLP_{240\text{-}247}$.

We wanted to elucidate central tolerance mechanisms in a physiological setting. Therefore, we chose to study central tolerance induction to the naturally occuring, physiological relevant self-antigen PLP. Since it is difficult to trace individual T cells specific for PLP in a polyclonal system, we generated a PLP-specific TCR-transgenic mouse. Here, we could monitor thymic selection of a single TCR specificity due to the dominant expression of this genetically introduced T cell receptor. It is the first PLP-specific TCR-transgenic mouse model in the B6 background. While there has been a TCR-transgenic mouse model in the B6 background generated for $MOG_{35\text{-}55}$ (2D2 mice) [147], PLP-specific TCR-transgenic mice have only been generated in the SJL/J background [148], and only for the region $PLP_{139\text{-}151}$ (5B6 and 4E3) [148], which is immunodominant in this genetic background. For the B6 background, we focused on the immunogenic epitope $PLP_{11\text{-}18}$, as tolerance induction to this epitope was very efficient. With the generation of the TCR-PLP1 transgenic mouse, we were able to trace the fate of PLP-specific T cells in a thymus with strong tolerance mechanisms to PLP. Furthermore, by crossing the TCR-PLP1 transgenic mouse to

other mouse strains with deficiencies in the expression or presentation capacity of PLP, we were able to delineate the roles and contributions of the different thymic APC subsets for tolerance induction to the antigen PLP.

3 Results

3.1 B6 WT mice show a robust tolerance to PLP

As a starting point, we wanted to recapitulate the tolerogenic response to all four identified immunogenic epitopes in the B6 genetic background that have been published in Klein *et al.* [3] (see also Fig. 4). To this end, WT or $PLP^{-/-}$ mice were immunised with PLP protein and 9 days later, the draining lymph node cells were isolated and *in vitro* restimulated with four different peptides (24-amino acids in length) spanning the identified immunogenic epitopes respectively: peptide $PLP_{1\text{-}24}$ for the core epitope $PLP_{11\text{-}18}$, $PLP_{160\text{-}184}$ for the core epitope $PLP_{174\text{-}181}$, $PLP_{192\text{-}216}$ for the core epitope $PLP_{205\text{-}213}$, and lastly $PLP_{224\text{-}248}$ for the core epitope $PLP_{240\text{-}247}$.
Immunisation of $PLP^{-/-}$ mice showed a primary T cell response to three of the four identified epitopes (Fig. 5). While we detected a stimulation response similar in strength to the peptides $PLP_{1\text{-}24}$, $PLP_{160\text{-}184}$, and $PLP_{224\text{-}248}$, the response to the peptide $PLP_{192\text{-}216}$ was absent or if at all only marginally detectable. In the published pep-scan by Klein *et al.* [3], recall response to the 24-mer peptide $PLP_{192\text{-}216}$ was also only little over background. In WT mice, only the peptide $PLP_{160\text{-}184}$ elicited a weak, but significant recall response of primed lymphocytes. Recall responses to the other peptides were absent in B6 WT mice. Thus, B6 WT mice showed a robust tolerance to PLP, with exception of the peptide $PLP_{160\text{-}184}$. Our data is consistent with the reported observation that tolerance to this epitope was not stringent in B6 WT mice, while tolerance to the other regions of PLP was tightly regulated [3].
The aim of this thesis was to delineate the mechanisms of tolerance induction to the self-antigen PLP in the thymus. As stimulation with the peptide $PLP_{1\text{-}24}$ after immunisation yielded the strongest recall response in $PLP^{-/-}$ mice, but showed a very strict tolerance in B6 WT mice, we focused on the immunogenic region $PLP_{1\text{-}24}$, i.e. the core epitope $PLP_{11\text{-}18}$, for the generation of a PLP-specific TCR-transgenic mouse.

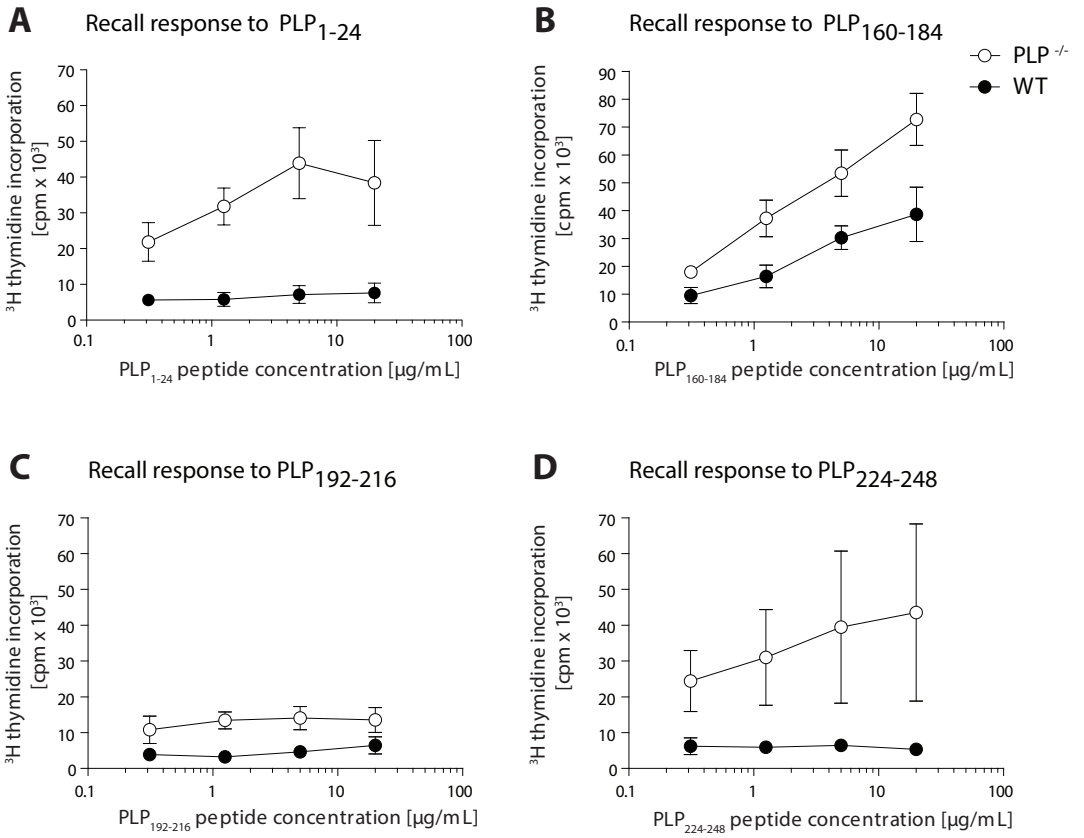

Figure 5: Recall response to four PLP peptides after immunisation with PLP protein. *In vitro* restimulation of draining lymph node cells with titrated concentrations of PLP peptide after immunisation with 50 µg PLP protein. A) Stimulation with $PLP_{1\text{-}24}$ peptide, B) Stimulation with $PLP_{160\text{-}184}$ peptide, C) Stimulation with $PLP_{192\text{-}216}$ peptide, and D) Stimulation with $PLP_{224\text{-}248}$ peptide. In $PLP^{-/-}$ mice, recall responses to the identified immunogenic regions of the PLP protein in the context of H-2^b $PLP_{1\text{-}24}$, $PLP_{160\text{-}184}$, and $PLP_{224\text{-}248}$ were observed. The response to the peptide $PLP_{192\text{-}216}$ was only marginally over background. WT mice showed robust tolerance to all PLP peptides with the exception of the region $PLP_{160\text{-}184}$ (B). Stimulation with this peptides yielded also a recall response in B6 WT mice. Plotted is the mean average (WT: n=3; $PLP^{-/-}$: n=3) with the standard error of the mean (SEM).

Immunisation with PLP protein and PLP_{1-24} peptide yielded the same recall response

PLP is highly conserved between species [121,128–131]. As PLP protein is not commercially available, it needs to be purified from brain extract and we used purified PLP protein extract from cows for our research [117]. As we had access to only a limited amount of protein, we needed to establish that the recall response of PLP-specific T cells after immunisation with a synthetic PLP peptide spanning the relevant core epitope PLP_{11-18}, elicited the same recall response as immunisation with the whole PLP protein.

We compared immunisation with PLP protein with immunisation using PLP_{1-24} peptide and detected by both approaches pronounced proliferation of lymphocytes originating from $PLP^{-/-}$ but not from WT mice (Fig. 6). Both peptides used for the recall response (PLP_{1-24} after immunisation with PLP protein and PLP_{9-20} after immunisation ith PLP_{1-24}) span the immunogenic core epitope PLP_{11-18} that was previously identified by fine-mapping [3]. Immunisation with PLP_{1-24} peptide (Fig. 6 B) yielded a comparable recall response measured by *in vitro* proliferation as immunisation with PLP protein (Fig. 6 A). For this reason, we have performed all following immunisations with the 24-mer peptide PLP_{1-24}.

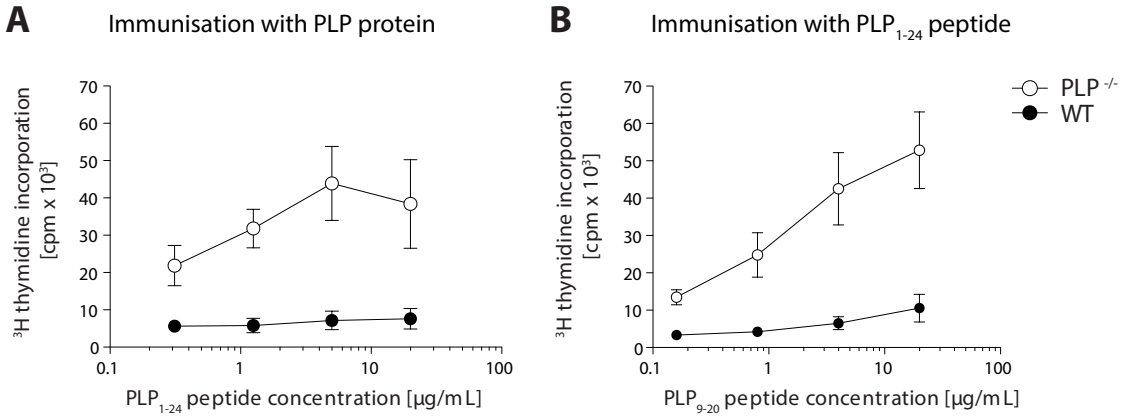

Figure 6: Comparison between immunisation approach with PLP protein and PLP_{1-24} peptide. *In vitro* recall response of lymphocytes of the draining lymph nodes of $PLP^{-/-}$ and WT animals after immunisation. A) Restimulation with PLP_{1-24} peptide after immunisation with PLP protein (n=3 mice each); B) Restimulation with 12-mer PLP_{9-20} peptide after immunisation with 24-mer PLP_{1-24} peptide. Immunisation with PLP_{1-24} peptide yielded comparable recall response in a subsequent *in vitro* stimulation as immunisation with PLP protein. ($PLP^{-/-}$ n=8; WT n=3). Plotted is the mean average of the responses with the standard error of the mean (SEM).

3.2 Generation of the $PLP_{9\text{-}20}$-specific TCR-transgenic mouse

To study central tolerance to PLP, we aimed to generate a TCR-transgenic mouse specific for the core epitope $PLP_{11\text{-}18}$. The basis of our PLP-specific TCR-transgenic mouse (TCR-PLP1) is the transgenic introduction of a rearranged TCR sequence that gives rise to T cells with high specificity towards the peptide $PLP_{9\text{-}20}$, which spans the core epitope $PLP_{11\text{-}18}$.
In order to obtain a TCR specific for $PLP_{9\text{-}20}$, $PLP^{-/-}$ mice were immunised with the 24-mer peptide $PLP_{1\text{-}24}$, which yields an expansion of T cells reactive to $PLP_{1\text{-}24}$. 9 days later, T cells were isolated from the draining lymph nodes and subsequently stimulated with the 12-mer $PLP_{9\text{-}20}$ peptide *in vitro*, which further enriched T cells with specificity towards this more restricted peptide, that spans the core epitope $PLP_{11\text{-}18}$. After 1 restimulation *in vitro*, we fused reactive T cells to the TCRαβ-negative lymphoma reporter cell line BW-NFAT-GFP in order to generate T cell hybridomas. T cell hybridomas are a combination of the reactive T cells and BW5147 cells, which are a thymoma cell line that lacks inherent functional TCR chains. This step was conducted for the sake of easier handling and screening procedures and allowed us to further characterise the T cell hybridomas by staining for TCR Vα and Vβ chains as well as assessing their reactivity to PLP. The early fusion impeded the generation of an *in vitro* false bias but provided a broad panel of reactive T cells for further testing.

Dominant use of the TCR variable region Vβ6 in $PLP_{9\text{-}20}$-specific T cells

In order to select for a representative TCR for the generation of the TCR-transgenic mouse, we first wanted to evaluate if $PLP_{9\text{-}20}$-reactive T cells use a dominant TCR variable Vα and Vβ region. The basis for the generation of the TCR-transgenic mouse was to choose a specific T cell clone that represented T cells that were responsive to the antigen $PLP_{9\text{-}20}$. In order to select for a T cell clone with a TCR that was dominant in its response to $PLP_{9\text{-}20}$, we compared the distribution of TCR variable Vα and Vβ regions before and after immunisation and after *in vitro* restimulation with $PLP_{9\text{-}20}$. During the rearrangements of the TCR loci, numerous variable TCR segments can be recombined with other gene segments: With regards to the variable TCR regions, the TCRα locus consists of 70-80 Vα gene segments, while the TCRβ locus has "only" 52 functional Vβ gene segments [149]. Antibodies for flow cytometry analysis do not exist for all TCR variable regions, therefore, we could compare only the distribution of the TCR variable regions with antibodies available: 4 antibodies

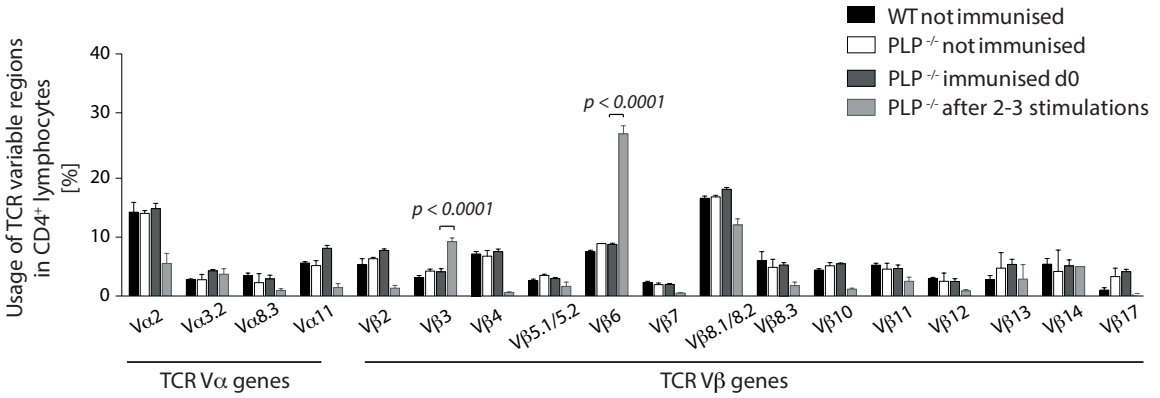

Figure 7: Screen of the distribution of TCR variable Vα and Vβ regions within the CD4SP compartment of lymphocytes before and after immunisation with PLP. Comparison of the TCR variable Vα and Vβ regions in T cells after repetitive stimulation with PLP_{9-20} peptide to T cell pools that were either not immunised or not yet *in vitro* restimulated. After several rounds of stimulation with PLP_{9-20} peptide, an enrichment in T cells expressing a TCR variable region Vβ6 was observed. WT not immunised: n=16, $PLP^{-/-}$ not immunised: n=3, $PLP^{-/-}$ immunised d0 and after $2-3$ rounds of stimulation: n=10. Depicted is the mean average with the standard error of the mean (SEM).

for variable TCRα genes, and 14 antibodies for different variable TCRβ genes. A few restimulations with PLP_{9-20} peptide were sufficient to show an enrichment of T cells using the TCR variable region Vβ6 compared to the T cell pool that was unimmunised or not yet *in vitro* stimulated (Fig. 7). Also an increase in the presence of TCR variable region Vβ3 was observed, but not as high as the level of Vβ6. For the TCR variable α-chains, we could not detect a dominant chain in the T cell repertoire stimulated with PLP_{9-20}.

3.2.1 Selection criteria for a suitable T cell receptor

The T cell clone whose TCR sequence was used for the generation of the transgenic mouse had to fulfill certain criteria:

A) The TCR must be reactive and specific to PLP_{9-20} and PLP protein stimulation.

B) The TCR variable Vα and Vβ regions must be stainable with available antibodies for flow cytometry in order to identify and trace the transgenic T cells later by flow cyotmetric analyses.

A) The TCR reacted highly to PLP_{9-20} peptide and PLP protein

The most fundamental criteria for the selection of the TCR was its reactivity and specificity to PLP. Hybridoma T cells were cloned and screened for their reactivity to PLP_{9-20}. When

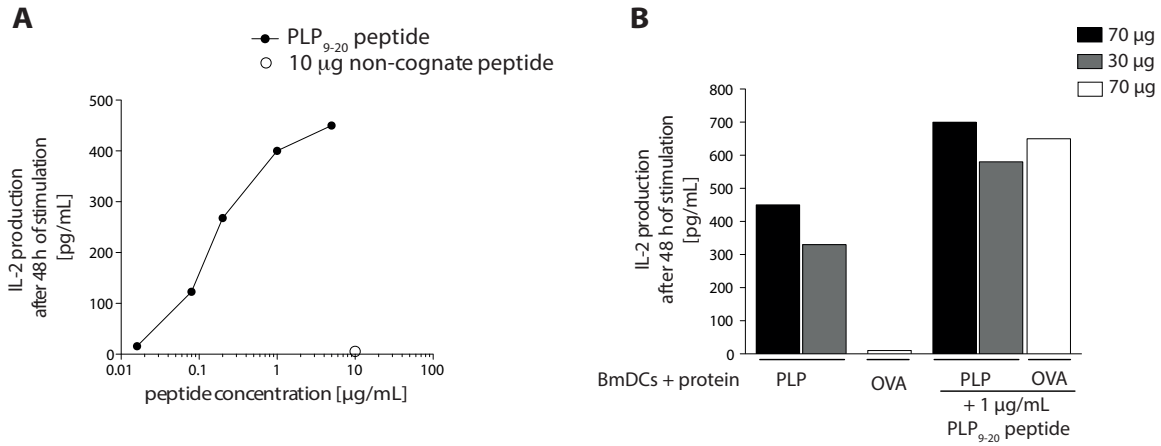

Figure 8: Stimulation of the T cell hybridoma clone D9-119-2 with PLP protein and $PLP_{9\text{-}20}$ peptide. A) *In vitro* stimulation of the T cell hybridoma clone D9-119-2 with bone-marrow derived DCs (BmDCs) pulsed with $PLP_{9\text{-}20}$ peptide or non-cognate peptide ($PLP_{172\text{-}183}$). The T cell hybridoma clone responded highly to stimulation with its cognate antigen in a dose-dependent manner. It did not react to stimulation with non-cognate peptide nor to BmDCs or media alone. B) *In vitro* stimulation of the T cell hybridoma clone D9-119-2 with BmDCs pulsed with either PLP protein or OVA protein. The T cell hybridoma clone responded to stimulation with PLP protein in correlation with the amount of protein that was given, but not to stimulation with OVA protein. All BmDCs were able to stimulate the T cell hybridoma clone when $PLP_{9\text{-}20}$ peptide was added to the culture as a positive control. Proliferation was measured by the production of IL-2 cytokine after 48 hours of stimulation.

we stimulated the T cell hybridoma clone D9-119-2 with titrated concentrations of $PLP_{9\text{-}20}$ peptide, it showed a dose-dependent response with high reactivity to the peptide (Fig. 8 A). Response to stimulation by a non-cognate peptide ($PLP_{172\text{-}183}$) was not observed, showing its specificity to $PLP_{9\text{-}20}$. As we have generated the D9-119-2 clone by immunisation and subsequent stimulation with synthetic PLP peptides, it needed to be verified that the TCR was also reactive to PLP protein. To this end, bone-marrow derived dendritic cells (BmDCs) were *in vitro* pulsed with PLP protein and co-cultured with the T cell hybridoma clone. The reactivity of the hybridoma clone to PLP protein was evaluated by its IL-2 cytokine production after 48 hours of stimulation. The D9-119-2 T cell hybridoma clone reacted to stimulation with PLP protein but not when the BmDCs were pulsed with the control protein OVA (Fig. 8 B). The strength of the stimulation response correlated to the amount of PLP protein given to the BmDC culture.
Thus, the T cell hybridoma clone D9-119-2 was specific to PLP protein and reacted highly to the peptide $PLP_{9\text{-}20}$.

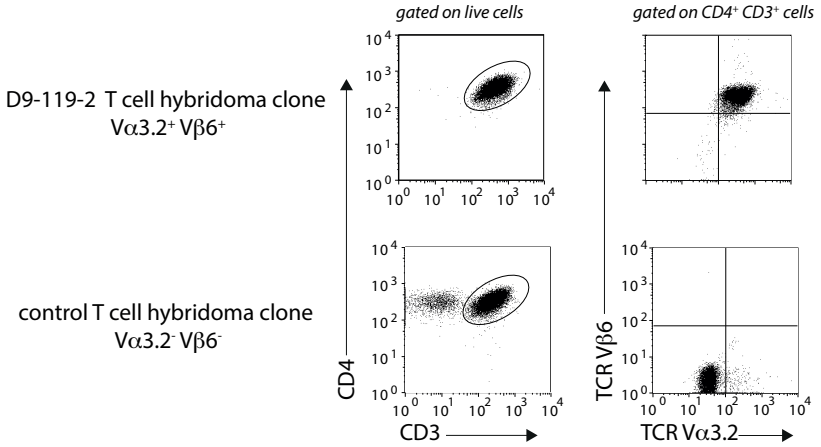

Figure 9: Flow cytometry staining of the TCR of T cell hybridoma clone D9-119-2. Staining of the T cell hybridoma clone D9-119-2 with antibodies for the use of TCR variable region Vα3.2 and Vβ6. The T cell hybridoma clone D9-119-2 was positive for TCR variable regions Vα3.2 and Vβ6.

B) Antibodies were available to trace the TCR by flow cytometry

Having ascertained that the T cell hybdridoma clone D9-119-2 was highly reactive to PLP, the second criteria for a suitable TCR was that antibodies for both TCR variable Vα and Vβ regions were available in order to trace the fate of transgenic T cells in the TCR-PLP1 mouse by flow cytometry at later time points. The TCR variable Vβ6 chain was enriched in PLP$_{9\text{-}20}$-specific T cells, so we tested the T cell hybridoma clone D9-119-2 for the use of the TCR variable region Vβ6 together with available TCR variable Vα antibodies. Indeed, the D9-119-2 hybridoma clone expressed the TCR variable region Vβ6 together with the TCR variable region Vα3.2 (Fig. 9). The ability for staining both TCR variable chains via antibodies, facilitated the monitoring of the development of the PLP-specific T cells in the TCR-transgenic mouse by flow cytometry.

Taken together, the T cell hybridoma clone D9-119-2 was a good candidate for the generation of the PLP-specific TCR-transgenic mouse since it was highly responsive and specific to the relevant PLP$_{9\text{-}20}$ peptide (Fig. 8), and antibodies to stain the TCR were available (Fig. 9). Furthermore, the D9-119-2 clone qualified as a good representative of PLP$_{9\text{-}20}$-specific T cells as it expressed TCR variable Vβ6 region that was shown to be dominant in PLP$_{9\text{-}20}$-responding T cells (Fig. 7).

3.2.2 Cloning of the TCR variable regions into cassette vectors

To introduce the TCR sequence of the hybridoma clone D9-119-2 into the genome and to restrict its expression to T cells, we made use of the TCR expression vector cassettes engineered by the lab of Benoist & Mathis [116]. The advantage of the pTα- and pTβ-vector cassettes is that they included essential promoter and enhancer elements for TCR expression to ensure physiological expression levels of the receptor at the cell surface. Moreover, as the vector cassettes already contained the constant C-region of the TCR, which is identical for all T cells, only the rearranged TCR VαJα- and TCR VβDβJβ-segments needed to be cloned into the vectors.

Cloning the TCR variable regions Vα3.2 and Vβ6 into the pTα- and pTβ-vector cassettes

Before cloning, the RT-PCR products (generated with primers for the TCR Vα3.2 region paired with a Cα primer and Vβ6-Cβ primers, respectively) were sequenced in order to determine the exact usage of TCR VαJα- and TCR VβDβJβ-segments. The TCR segments of the D9-119-2 T cell hybridoma clone were annotated in *ensembl* [*www.ensembl.org*] as the following: the rearranged TCR VαJα-segments were annotated as Trav9d-3.201 and Traj34-201 and the TCR VβDβJβ-segments as Trbv19-201, Trbd1, and Trbj1-1. The designation Vα3.2 and Vβ6 refer to the antibodies that specifically stained the rearranged Vα- and Vβ-regions, respectively, hence, one has to keep in mind that the exact genetic annotations are the ones mentioned above. Here, we will refer to this TCR combination as TCR-PLP1. The sequenced PCR fragments were then sub-cloned into the pTα and pTβ-plasmids, generating PLP-specific cassette vectors (pTα-PLP1 and pTβ-PLP1), respectively.

Verification of the expression and functionality of the TCR-PLP1 cassette vectors

To test the vector plasmids for functionality, we stably transfected A5 T cell hybridomas with both vector cassettes and tested the rearranged TCR-PLP1 for its proper expression on the cell surface as well as its functionality and specificity to $PLP_{9\text{-}20}$. We used A5 T cell hybridomas for transfection, as these cells were in possession of all intracellular components required for correct TCR signalling. The A5 T cell hybridoma clone recognises influenza hemagglutinin peptide ($HA_{107-119}$) in the context of I-E^d [150], and therefore, did not respond intrinsically to peptides presented in the context of I-A^b on APCs of B6 origin.

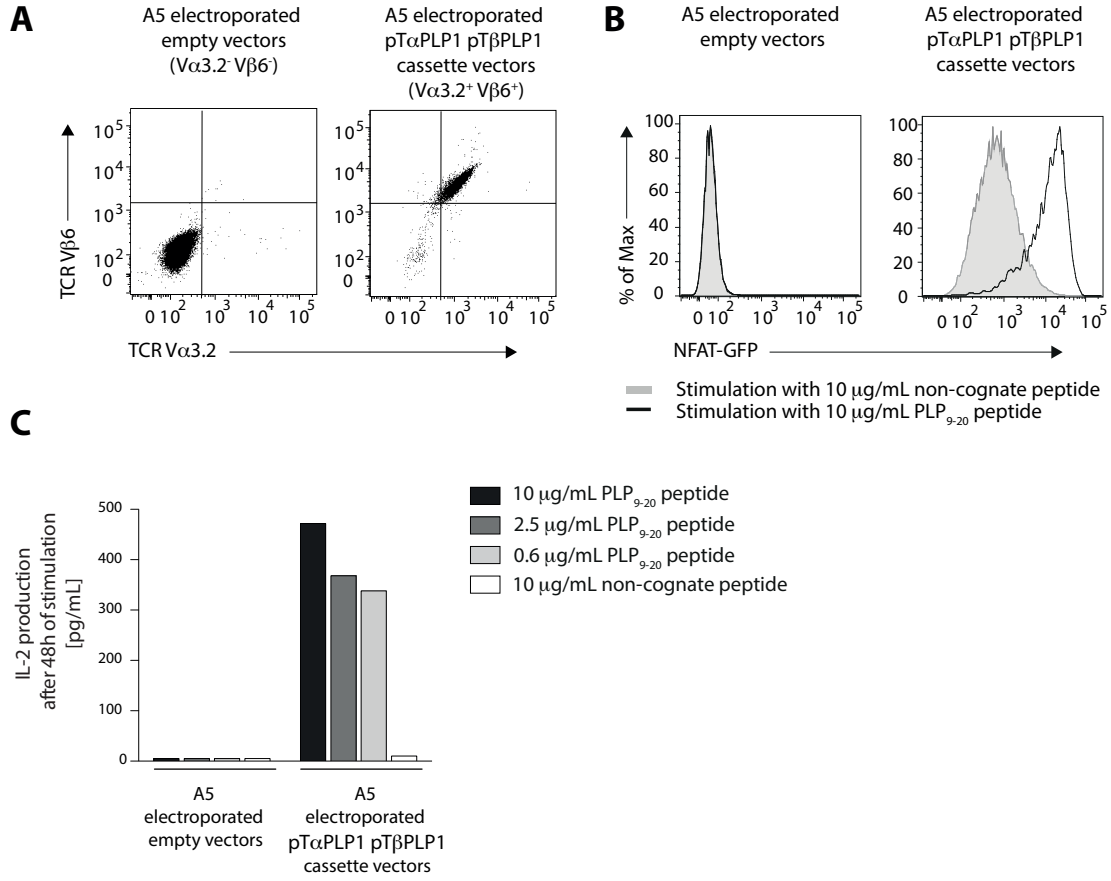

Figure 10: Confirmation of proper surface expression and functionality of the pTα-PLP1 and pTβ-PLP1 cassette vectors. Stable transfection of A5 cells with both expression cassette vectors containing Vα3.2 and Vβ6 segments by electroporation. A) TCR-PLP1 transfected A5 cells showed expression of the TCR-PLP1 TCR receptor ($V\alpha3.2^+$ and $V\beta6^+$) on the cell surface. B and C) When TCR-PLP1 transfected A5 cells were stimulated with PLP_{9-20}, they showed a response measured by the production of GFP (B) and IL-2 (C). The transfected A5 cells did not respond to irrelevant peptide (OVA peptide in (B) and $PLP_{172-183}$ in (C)), confirming the specificity to PLP_{9-20}. Control hybridomas did not respond to any stimulations.

Furthermore, the A5 hybridoma cells were known to carry a TCR that was not composed of Vα3.2 and Vβ6, but is recognised by a specific antibody called 6.5. Therefore, we could distinguish the introduced TCR-PLP1 from the TCR-HA on the cell surface. For transfection, the two linearised vector cassettes, pTα-PLP1 and pTβ-PLP1, were electroporated with A5 cells together with a linearised plasmid containing a NFAT-GFP reporter with a puromycin resistance gene. Cells that were successfully transfected survived the selection with puromycin and were subsequently screened for the expression of the introduced TCR on the surface. In TCR-PLP1 transfected A5 T cell hybridoma cells, expression of the PLP-specific TCR was detected by staining with antibodies for TCR Vα3.2 and TCR Vβ6 (Fig. 10 A). The expressed PLP-specific TCR was then further tested for functionality and specificity in a stimulation assay. To this end, we co-cultured transfected A5 hybridoma cells together with APCs and cognate or non-cognate peptide. The stimulation was measured by the expression of the NFAT-GFP-reporter as well as by testing the supernatant for the amount of IL-2 cytokine. TCR-PLP1 transfected hybridomas showed antigen-induced NFAT activity in the presence of $PLP_{9\text{-}20}$ peptide as detected by GFP expression (Fig. 10 B) and IL-2 production (Fig. 10 C). The strength of the response was dose-dependent, while control hybridoma cells did not respond to PLP.
In summary, both TCR-PLP1 vector cassettes have been tested sucessfully for functionality of the TCR and reactivity to $PLP_{9\text{-}20}$.

3.2.3 Injection of pTα-PLP1 and pTβ-PLP1 vector cassettes into mouse oocytes

For the DNA injection into mouse oocytes, the prokaryotic parts of the vectors were removed by enzymatic endonuclease digest and the linearised fragments were sent to the transgenic core facility at the Max Planck Institute in Dresden. There, they were injected into fertilised B6N oocytes, which were subsequently transplanted into foster mothers.
A total of nine TCR DNA-positive founder mice were obtained; two founder animals were positive for both TCR Vα3.2 and TCRβ6 transgenes, while another two of the nine founders expressed only the Vα chain, and the remaining five founders expressed only the Vβ chain. The five beta- and two alpha-single transgenic lines showed germ-line transmission of the respective transgene, while both double-transgenic founders were not of further use: one did not show TCR-PLP1-expression on lymphocytes and the other one did not give rise to any progeny. Therefore, we crossed TCR-Vα3.2 single transgenic animals to TCR-Vβ6 single transgenic animals. Preliminary characterisation of different combinations of the founder animals showed identical phenotypes. For all conducted experiments, we made use of TCR Vα3.2 founder N°14 and TCR Vβ6 founder N°10, which will be referred to as TCR-PLP1

transgenic mouse.

3.3 Negative selection and concomitant T_{reg} induction of TCR-PLP1$^+$ T cells in the thymus

In the PLP-specific TCR-transgenic mice, we were now able to closely monitor the fate of TCR-PLP1$^+$ T cells after encountering PLP in the thymus. In order to evaluate the modes and the extent of thymic tolerance induction, we compared TCR-PLP1*Plp*^{WT} mice to TCR-PLP1*Plp*^{KO} animals. TCR-PLP1*Plp*^{KO} mice are deficient for PLP and, hence, TCR-PLP1$^+$ T cells will not be tolerised to PLP in the thymus. In contrast to TCR-PLP1*Plp*^{WT} mice, where PLP is presented in the thymus as tissue-restricted self-antigen and central tolerance will be induced.

The presence (TCR-PLP1*Plp*^{WT}) or absence (TCR-PLP1*Plp*^{KO}) of the self-antigen PLP in the thymus had no impact on total thymic cellularity (Fig. 11 A). Detailed characterisation of the T cell compartments revealed a significant loss of CD4SP T cells in the TCR-PLP1*Plp*^{WT} mice as compared to TCR-PLP1*Plp*^{KO} mice (20.6 ± 3.6 x 10^6 CD4SP cells in TCR-PLP1*Plp*^{KO} thymi vs. 4.9 ± 0.8 x 10^6 CD4SP cells in TCR-PLP1*Plp*^{WT} thymi) (Fig. 11 B). All other compartments in the thymus showed the same cellularity in TCR-PLP1*Plp*^{WT} and TCR-PLP1*Plp*^{KO} mice.

The reduction in the CD4SP compartment of TCR-PLP1*Plp*^{WT} mice suggested strong negative selection of TCR-PLP1$^+$ T cells in the thymus. In 3-week-old TCR-PLP1*Plp*^{KO} mice (no cognate antigen expressed), the percentage of thymic CD4$^+$ T cells was 16.9% ± 1.9% (Fig. 11 C, first column) with the majority of these being TCR-PLP1-specific T cells (85.5% ± 3.2%) (Fig. 11 C, second column). In TCR-PLP1*Plp*^{WT} mice, in contrast, where PLP was presented as endogenous antigen in the thymus, we saw a reduction in the percentage of CD4SP thymocytes (4.02% ± 0.4%) (Fig. 11 C) as well as in absolute cell numbers (see Fig. 11 B). Furthermore, within the CD4SP compartment, we observed a reduction of TCR-PLP1-specific T cells (51.1% ± 4.1% in TCR-PLP1*Plp*^{WT} mice vs. 85.5% ± 3.2% in TCR-PLP1*Plp*^{KO} mice).

It was shown that self-antigen encounter in the thymus can also drive auto-reactive T cells into the regulatory T cell lineage [151, 152]. Therefore, we analysed the abundance of TCR-PLP1-specific T_{reg} cells. While in TCR-PLP1*Plp*^{KO} mice only a minuscule fraction of TCR-PLP1$^+$ T cells was deviated into Foxp3$^+$ T_{reg} cells (0.4% ± 0.1%), the percentage of TCR-PLP1$^+$ T cells selected as regulatory T cells in TCR-PLP1*Plp*^{WT} mice was 10-times higher (4% ± 0.6%) (Fig. 11 C third column). In absolute cell numbers, 10.6 ± 1.8 x 10^4 TCR-PLP1$^+$ Foxp3$^+$ T_{reg} cells were present in TCR-PLP1*Plp*^{WT} mice as compared to only

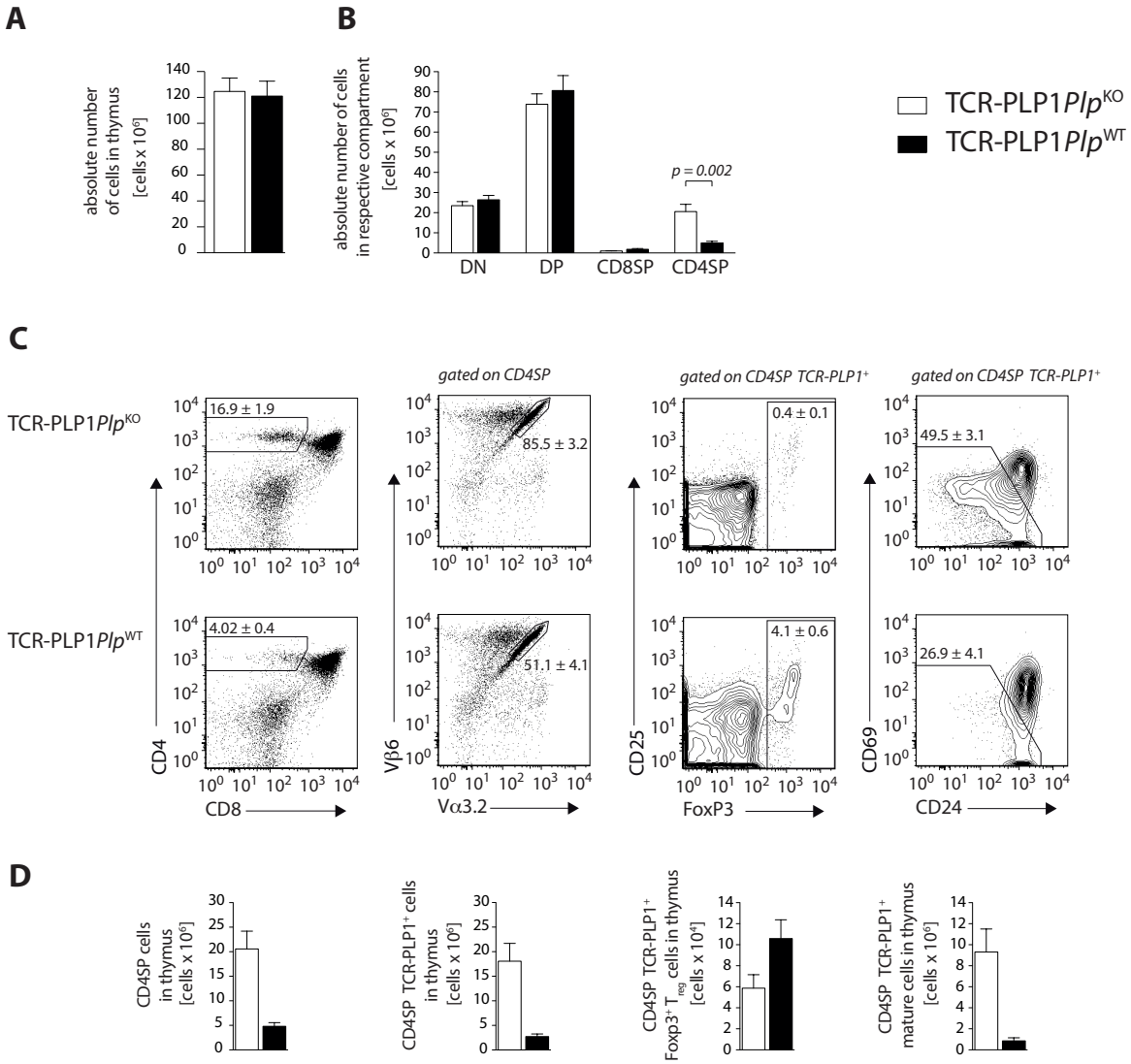

Figure 11: Flow cytometry analysis of the thymi of young TCR-PLP1*Plp*^{WT} and TCR-PLP1*Plp*^{KO} mice. A and B) Absolute cell numbers of the total thymus (A) or within different compartments of the thymus (B). Shown is the average mean in absolute numbers of thymocytes x 10^6 at the age of 3 weeks with the standard error of the mean (SEM). A) TCR-PLP1*Plp*^{KO} and TCR-PLP1*Plp*^{WT} mice demonstrate no difference in total thymic cellularity. B) Absolute cell number of thymocytes in the respective compartment of T cell development: DN, DP, CD8SP and CD4SP cells. In all compartments other than CD4SP, the cell number did not significantly differ between TCR-PLP1*Plp*^{KO} and TCR-PLP1*Plp*^{WT} mice. In the CD4SP compartment, TCR-PLP1*Plp*^{WT} mice showed a significant reduction. C and D) Flow cytometry analysis of the thymic phenotype of TCR-PLP1*Plp*^{WT} mice in comparison to TCR-PLP1*Plp*^{KO} mice at the age of 3 weeks in percentage (C) and in absolute cell numbers x 10^6 (D). Shown is the percentage or absolute cell number of CD4SP cells in the thymus (first column), the percentage of TCR-PLP1$^+$ cells within the CD4SP compartment (second column), the percentage of Foxp3$^+$ regulatory T cell in the TCR-PLP1$^+$ cell population (third column) and the percentage of mature cells within the TCR-PLP1$^+$ cell population in the thymus (fourth column). Depicted are littermates representative for the observed phenotype. The numbers above the gates represent the mean average of all animals of the same cohort ± the standard error of the mean (SEM). Tolerance induction to PLP in the thymus of TCR-PLP1*Plp*^{WT} mice was conducted by negative selection and deviation of TCR-PLP1$^+$ T cells into the regulatory T cell lineage at the CD4SP stage. TCR-PLP1*Plp*^{KO}: n=8, TCR-PLP1*Plp*^{WT}: n=10.

5.9 ± 1.3 x 10^4 in TCR-PLP1*Plp*^{KO} mice (Fig. 11 D).
Taken together, when antigen was present in the thymus, we observed a strong deletion of mature TCR-PLP1$^+$ T cells together with concomitant T_{reg} induction.
As we observed a late deletion of TCR-PLP1-transgenic T cells at the CD4SP stage, we also analysed the population of TCR-PLP1$^+$ T cells in the CD4SP compartment with respect to their maturation status. T cells with a mature phenotype (designated as CD24^{lo}CD69^{lo}) have already passed negative selection and T_{reg} induction and were ready to leave the thymus as naive T cells. In TCR-PLP1*Plp*^{KO} mice, half of the TCR-PLP1$^+$ CD4SP cells were mature (49.55% ± 3.1%) (Fig. 11 C, fourth column). In TCR-PLP1*Plp*^{WT} mice, on the other hand, the mature population of TCR-PLP1$^+$ T cells was considerably diminished (26.9% ± 4.1%).
As we have observed reduced maturation of TCR-PLP1$^+$ CD4SP cells in the thymus of TCR-PLP1*Plp*^{WT} animals, the frequency of CD4$^+$ TCR-PLP1$^+$ T cells arriving in the spleen was correspondingly low (9.1% ± 0.7% in TCR-PLP1*Plp*^{WT} compared to 74.2% ± 4.1% in TCR-PLP1*Plp*^{KO} mice) (Fig. 12 second column). In line with the overall thymic reduction of CD4$^+$ cells, only 1.2% ± 0.2% CD4$^+$ T cells were found in the spleen of these mice. Notably, a large proportion of these TCR-PLP1$^+$ T cells were T_{reg} cells (38.4% ± 1.7%) in contrast to the low percentage of TCR-PLP1$^+$ T_{reg} cells in TCR-PLP1*Plp*^{KO} mice (1.2% ± 0.3%) (Fig. 12 third column).
Taken together, by comparing T cell development of TCR-PLP1 transgenic mice in the presence and absence of the cognate self-antigen PLP, we observed central tolerance induction to PLP in the thymus with an even more pronounced reduction of TCR-PLP1$^+$ T cells and concomitant induction of T_{regs} in the periphery.

3.4 Individual contribution of thymic antigen presenting cell subsets to tolerance induction to PLP

As we observed that central tolerance to PLP was induced via both mechanisms (clonal deletion of TCR-PLP1$^+$ T cells and T_{reg} induction), we next investigated the contributions of the individual thymic antigen presenting cell types to central tolerance to PLP.

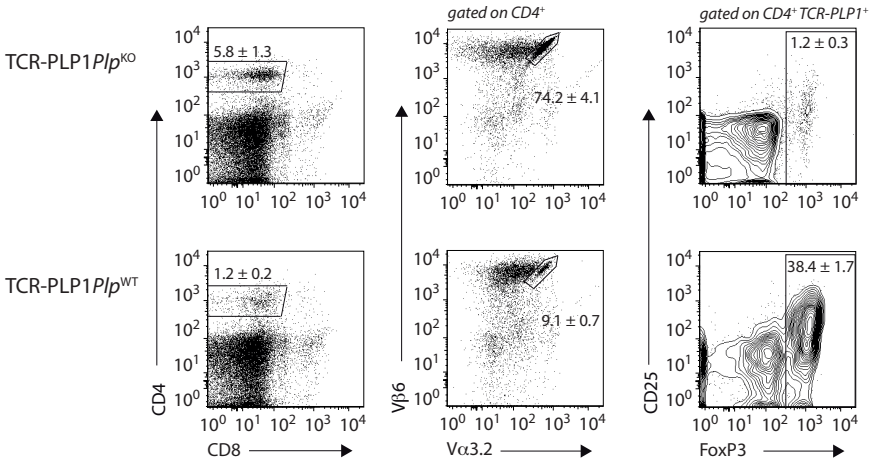

Figure 12: Flow cytometry analysis of peripheral phenotype in young PLP-TCR1 transgenic animals. Flow cytometry analysis of the peripheral phenotype in TCR-PLP1*Plp*^{WT} mice in comparison to TCR-PLP1*Plp*^{KO} mice at the age of 3 weeks. Shown is the percentage of CD4$^+$ cells in the spleen (first column), the percentage of TCR-PLP1$^+$ cells within the CD4$^+$ compartment (middle column), and the percentage of Foxp3$^+$ regulatory T cells in the TCR-PLP1$^+$ cell population (third column). The phenotype of tolerance induced in the thymus of TCR-PLP1*Plp*^{WT} mice translated into the periphery with deletion and deviation of TCR-PLP1$^+$ T cells into the T_{reg} lineage. Depicted are littermates representative of the observed peripheral phenotype. The numbers above the gates represent the mean average of all animals of the same cohort ± the standard error of the mean (SEM). TCR-PLP1*Plp*^{KO}: n=8; TCR-PLP1*Plp*^{WT}: n=10.

3.4.1 Expression of PLP in the thymus

PLP is expressed in TECs and to a lesser extent in thymic DCs

To delineate which APC subset contributed to tolerance induction to PLP, the expression patterns of PLP within different antigen presenting cell types of the thymus were analysed. The amount of PLP mRNA in FACS-sorted thymic cell populations was quantified by qRT-PCR. Care was taken that the amplification spanned the core epitope $PLP_{11\text{-}18}$. We distinguished between the thymic APC subtypes DCs, cTECs, and mTECs, with the mTEC population further subdivided into a mTEC^{hi} and a mTEC^{lo} population with respect to their MHCII-expression level. CD4SP thymocytes were included as a negative control.
Equally high expression of PLP mRNA in both mTEC^{hi} and mTEC^{lo} populations was detected, while thymic DCs expressed PLP to a 4-times lower extent (Fig 13). CD4SP T cells did not show expression of PLP, as expected. cTECs expressed PLP at the highest level, twice as much as the expression level of PLP in mTECs. The expression pattern of PLP mRNA was in line with previous reports using semi-quantitative PCR [1,3], and more recently by a RNA sequencing approach of the entire transcriptome [65].

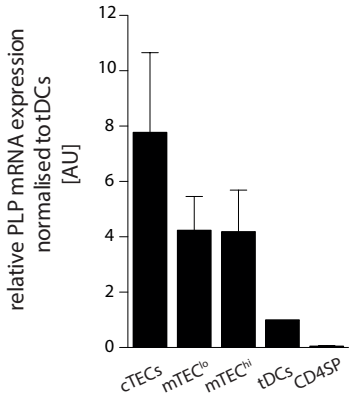

Figure 13: Expression of PLP mRNA in thymic APC subsets. Relative mRNA expression levels of PLP in distinct FACS-sorted thymic subsets normalised to thymic DCs (tDCs). cTECs as well as mTECs expressed high amounts of PLP mRNA, while DCs showed a 4-times lower expression level. CD4SP T cells did not express PLP mRNA. Analysed animals were 10 days of age. The mean average of PLP expression is shown in arbitrary units (AU) ± the standard error of the mean (SEM).

PLP expression by non-hematopoietic cells in the thymus is sufficient for deletion and concomitant T_{reg} induction

Since we have shown that both the non-hematopoietic and the hematopoietic compartment in the thymus expressed PLP at mRNA level, we next investigated which of these compartments mediated tolerance induction to PLP.

For this purpose, we performed thymus transplantation experiments with selective PLP expression in cells of the hematopoietic compartment only, in cells of the non-hematopoietic compartment only, in neither cell type or in both. To do so, we grafted deoxyguanosine-treated thymi from E14 WT or E14 PLP$^{-/-}$ fetuses under the kidney capsule of TCR-PLP1*Plp*^{WT} or TCR-PLP1*Plp*^{KO} mice. By 2-deoxyguanosine (dGuo) treatment, all hematopoietic cells of the fetal thymus have been eliminated prior to transplantation [153]. The transplanted thymi were then seeded, analogues to a normal thymus, with hematopoietic cells of host origin only.

When PLP was expressed only in the dGuo-resistant compartment, i.e. TECs, but not in cells of the hematopoietic system (WT thymi transplanted into TCR-PLP1*Plp*^{KO} mice), it faithfully recapitulated the phenotype of deletion and regulatory T cell induction of TCR-PLP1^{+} cells as seen in TCR-PLP1*Plp*^{WT} mice when compared to TCR-PLP1*Plp*^{KO} mice (Fig. 14 A, B, and C). In contrast, when PLP was expressed only in hematopoietic cells of the host (PLP$^{-/-}$ thymi transplanted into TCR-PLP*plp*^{WT} mice), the phenotype did not show clonal deletion nor T_{reg} induction of TCR-PLP1^{+} T cells but resembled a non-tolerised T cell population like in TCR-PLP1*Plp*^{KO} mice. Thus, we observed tolerance in transplanted thymi when PLP was expressed in the thymic epithelial compartment, but not when it was only expressed by cells of the hematopoietic compartment. Since we have seen

that TCR-PLP1 transgenic T cells were deleted late, in T cell development at the CD4SP stage, the effect of PLP expression only in TECs or only in cells of bone-marrow origin was additionally assessed by examining the abundance of mature cells in the transplanted thymi. In transplants with TECs of WT origin, the frequency of TCR-PLP1$^+$ mature cells in all thymocytes was highly diminished compared to transplanted thymi that were PLP-deficient (Fig. 14 D). This data clearly shows that PLP expression by TECs was sufficient to mediate central tolerance to PLP. Furthermore, the abundance of mature TCR-PLP1$^+$ T cells in transplants with PLP expression only in the hematopoietic compartment (PLP$^{-/-}$ thymi transplanted into TCR-PLP*plp*^{WT} mice) was not different when compared to mice with PLP deficiency in both compartments.
In summary, these results showed that expression of PLP by bone-marrow derived cells did not mediate tolerance induction to PLP, whereas expression of PLP by TECs was sufficient to induce clonal deletion as well as regulatory T cell induction of PLP-specific T cells.

PLP expression in TECs is crucial for central tolerance induction to PLP

To confirm the finding that PLP expression in thymic epithelium cells was sufficient and necessary to induce tolerance, we abrogated PLP expression specifically in TECs by crossing the Foxn1-driven Cre-recombinase together with a loxP-site flanked *Plp* allele to the TCR-PLP1 transgenic mice. This yielded a conditional knock-out of *Plp* in all Foxn1-expressing cells, i.e. cTECs and mTECs.
Our data showed that deletion of PLP expression specifically in TECs, abrogated central tolerance to PLP completely. The TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre mouse, recapitulated precisely the phenotype seen in animals with a deficiency for PLP in all cells. No reduction of CD4$^+$ TCR-PLP1$^+$ T cells or of the overall CD4SP compartment was seen (Fig. 15 A and B). Furthermore, no T_{regs} were induced in the thymi of TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre mouse (Fig. 15 C). This proved an exclusive and crucial role of TECs contributing to central tolerance induction by expression of the antigen PLP.
In sum, PLP expression in TECs was necessary and sufficient for tolerance induction to PLP in the thymus.

3.4.2 Presentation of PLP in the thymus

Having established that expression of PLP in TECs was sufficient and necessary for the differentiation of TCR-PLP1$^+$ T cells into T_{reg} cells as well as the deletion of TCR-PLP1$^+$ thymocytes, it remained open which antigen presenting cells in the thymus actually presented

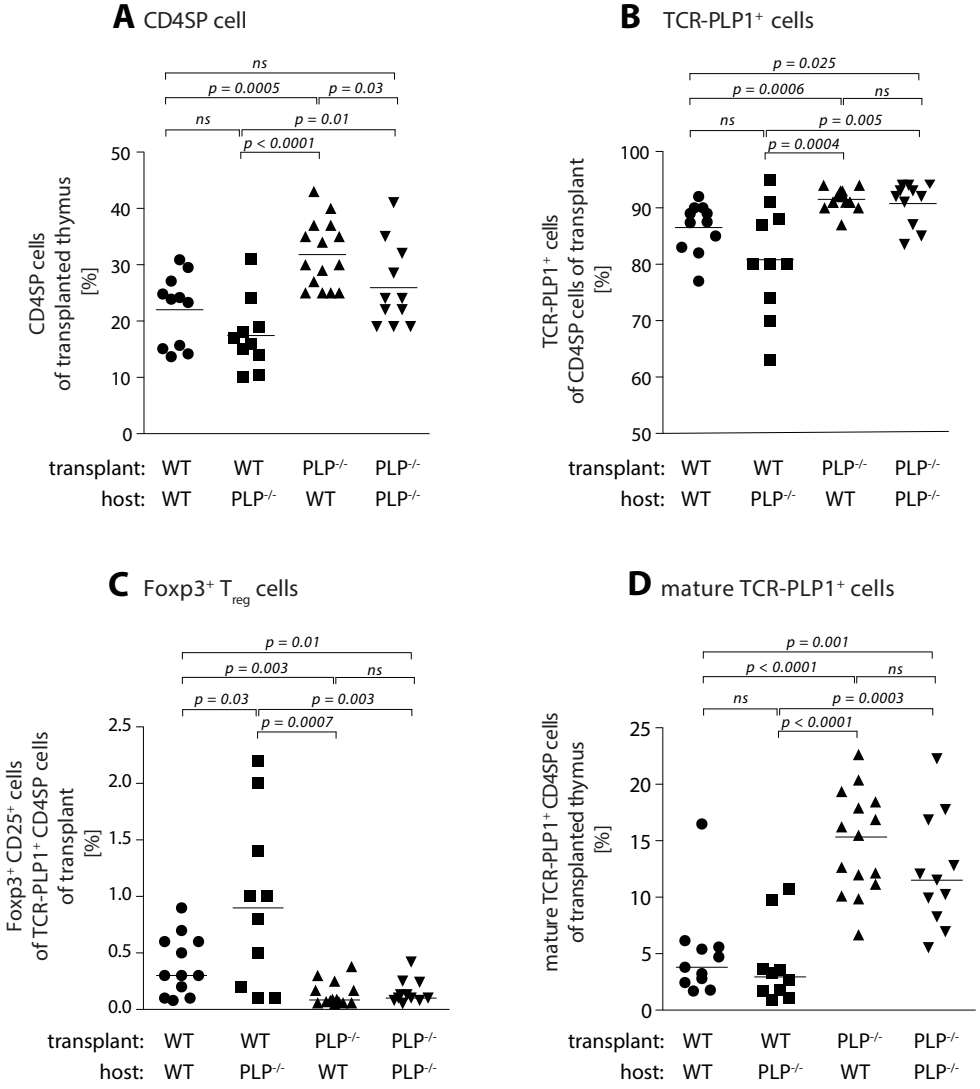

Figure 14: Transplantation experiments to investigate the contribution of PLP expression in the hematopoietic and non-hematopoietic compartments for tolerance induction. Transplantation of WT or PLP$^{-/-}$ thymi (transplants) under the kidney capsule of either TCR-PLP1*Plp*$^{\mathrm{WT}}$ or TCR-PLP1*Plp*$^{\mathrm{KO}}$ mice (hosts). The graph shows (A) the percentage of CD4SP cells within the transplanted thymi, (B) the percentage of TCR-PLP1^{+} cells within the CD4SP compartment of the transplanted thymi, (C) the percentage of Foxp3^{+} $\mathrm{T_{reg}}$ cells in the CD4^{+} TCR-PLP1^{+} T cell population of the transplants, and (D) the percentage of mature TCR-PLP1^{+} T cells of all thymocytes in the transplants. PLP expression by cells of non-hematopoietic origin induced tolerance to PLP as seen in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice. Data were combined from two separate but identical experiments. WT transplants into TCR-PLP1*Plp*$^{\mathrm{WT}}$ hosts: n=11; WT transplants into TCR-PLP1*Plp*$^{\mathrm{KO}}$ hosts: n=10; PLP$^{-/-}$ transplants into TCR-PLP1*Plp*$^{\mathrm{WT}}$ hosts: n=15; PLP$^{-/-}$ transplants into TCR-PLP1*Plp*$^{\mathrm{KO}}$ hosts: n=11.

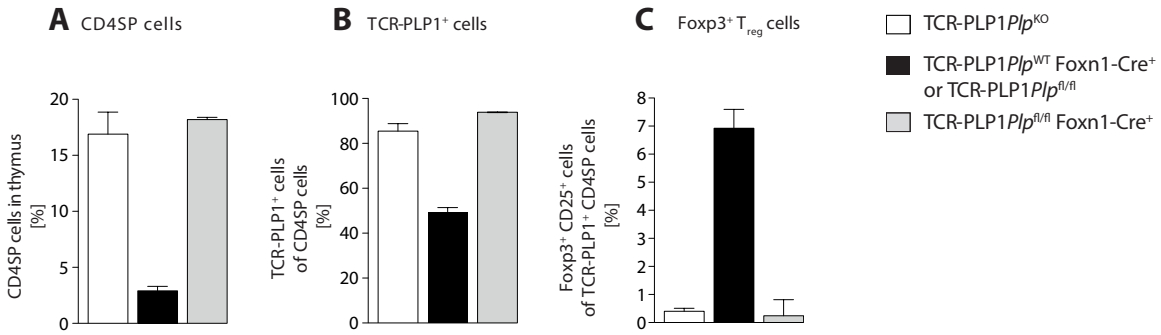

Figure 15: Abrogation of PLP expression exclusively in TECs. The bar diagram shows (A) the percentage of CD4SP cells within the thymus, (B) the percentage of TCR-PLP1$^+$ cells within the thymic CD4SP compartment, and (C) the percentage of Foxp3$^+$ T_{reg} cells in the CD4SP TCR-PLP1$^+$ population. Deletion of PLP expression only in TECs showed a complete abrogation of thymic tolerance induction in TCR-PLP1*Plp*^{WT} mice. Depicted is the mean average of each group with the standard error of the mean (SEM). TCR-PLP1*Plp*^{KO}: n=8; TCR-PLP1*Plp*$^{fl/fl}$: n=2; TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre: n=2.

PLP for tolerance induction. To test this, we selectively manipulated PLP presentation in different APC subsets.

CD11c$^+$ dendritic cells do not present PLP for tolerance induction

It was shown that antigen can be transferred unidirectional from mTECs to thymic dendritic cells, so that DCs can present antigens that they do not express themselves [82, 83]. Even though we have established that the expression of PLP in bone marrow-derived cells was not relevant for tolerance induction, it could still be that thymic DCs receive TRA peptide or even pre-formed TRA-loaded pMHC complexes from TECs [84]. To investigate to what extent thymic DCs (as major antigen presenting cell type of the hematopoietic system) contributed to the presentation of PLP and to tolerance induction in the thymus, we crossed TCR-PLP1 transgenic mice to ΔDC mice. In ΔDC mice, toxic diphteria toxin expression is driven by the Cre-recombinase that is under the control of the CD11c promoter [154]. Consequently in these mice, conventional CD11c$^+$ DCs are constitutively deleted. In the thymus of TCR-PLP1*Plp*^{WT} ΔDC mice, CD11c$^+$ DCs were absent and could therefore not contribute to the presentation of PLP. Thus, we assessed the necessity of PLP presentation by DCs for tolerance induction by comparing the thymic phenotype of TCR-PLP1*Plp*^{WT} ΔDC mice to littermates that did not lack DCs (TCR-PLP1*Plp*^{WT}).
We found that in TCR-PLP1*Plp*^{WT} ΔDC mice, the percentage of CD4SP cells (6.6 ± 1.1), the percentage of CD4SP TCR-PLP1$^+$ T cells (60.8 ± 8.9), and the percentage of PLP1-specific T_{regs} (2.96 ± 0.3) were not significantly different from DC-sufficient TCR-PLP1*Plp*^{WT} control littermates (4.5 ± 0.8 CD4SP cells, 60.0 ± 4.3 CD4SP TCR-PLP1$^+$

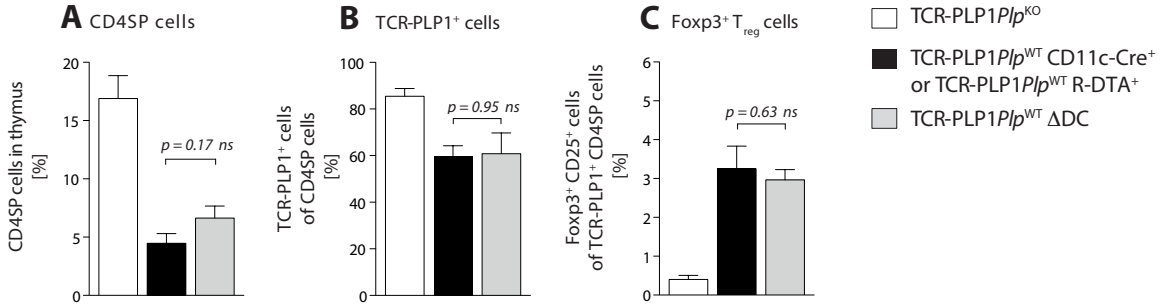

Figure 16: Central tolerance induction to PLP in the absence of DCs. Thymic analysis of TCR-PLP1*Plp*^{WT} ΔDC mice compared to TCR-PLP1*Plp*^{WT} mice. ΔDCs mice are CD11c-Cre/R-DTA mice that lack all CD11c$^+$ dendritic cells due to the toxic expression of diphteria toxin alpha (DTA) that was introduced to the Rosa26-locus. The bar diagrams show (A) the percentages of CD4SP cells within the thymus, (B) the percentage of TCR-PLP1$^+$ cells within the thymic CD4SP compartment, and (C) the percentage of Foxp3$^+$ T_{reg} cells in CD4SP TCR-PLP1$^+$ cell population. CD11c$^+$ DCs did not contribute to the induction of tolerance in the thymus of TCR-PLP1 mice. Depicted is the mean average of each group with the standard error of the mean (SEM). TCR-PLP1*Plp*^{KO}: n=8; TCR-PLP1*Plp*^{WT} CD11c-Cre$^+$ or DTA$^+$: n=4; TCR-PLP1*Plp*^{WT} ΔDC: n=6.

T cells, and 3.2 ± 0.6 TCR-PLP1$^+$ Foxp3$^+$ T_{reg}, respectively) (Fig. 16). Thus, we observed a tolerised CD4$^+$ T cell pool in thymi with PLP sufficient TECs irrespective of the presence or absence of CD11c$^+$ DCs. Presented data clearly indicate that central tolerance induction is mediated by the non-hematopoietic compartment and does not depend on DCs.
We conclude that thymic DCs were dispensable for central tolerance induction to PLP and antigen-transfer from mTECs to DCs for presentation of PLP was not important.
Taken together, thymic DCs did not contribute to the expression nor to the presentation of PLP needed to tolerise PLP-specific autoreactive T cells.

Direct expression and presentation of PLP by mTECs

Since thymic DCs were not involved in central tolerance induction to PLP, we further investigated the roles of cTECs and mTECs to central tolerance induction to PLP. To this end, we wanted to eliminate the capacity for PLP presentation of one or the other cell subset. A complete KO of MHCII expression on mTEC cells was not feasible for two reasons: First, no mTEC nor cTEC lineage-specific Cre-line has been reported, so that the expression of PLP could not be deleted by the loxP-Cre-system in an exclusive manner. Second, as the maturation and homeostasis of mTEC cells is dependent on interactions between mTECs and auto-reactive T cells via the MHCII receptor, a complete MHCII$^{-/-}$ in mTECs would impair the proper development of mTECs and lead to a perturbed medullary architecture [155]. Therefore, we crossed the TCR-PLP1 transgenic mice to major histocompatibility complex class II transactivator (CIITA) knockdown mice (*C2TA*^{kd}). In *C2TA*^{kd} mice, the MHCII

expression level on mTEC^{hi} is reduced to 10% of WT levels [77]. This is due to an AIRE-promoter-driven shRNA which knocks down C2TA expression, which subsequently leads to a diminished expression of MHCII on the cell surface.
We wanted to examine if direct presentation of PLP by mTECs was sufficient for establishing deletion and concomitant T_{reg} induction of TCR-PLP1^{+} T cells. We hypothesised that elimination of MHC class II expression on medullary TECs would demonstrate the loss of PLP presentation by mTECs and, consequently, show the direct contribution of mTECs to tolerance induction to PLP.
When the presentation capacity of mTECs was diminished by *C2TA*^{kd}, we observed less clonal deletion of CD4SP and CD4SP TCR-PLP1^{+} T cells compared to TCR-PLP1*Plp*^{WT} mice: TCR-PLP1*Plp*^{WT} *C2TA*^{kd} showed 9.8% ± 0.8% CD4SP cells compared to 4.02% ± 0.4% in TCR-PLP1*Plp*^{WT} mice and 72.2% ± 1.9% CD4SP TCR-PLP1^{+} T cells compared to 51.1% ± 4.1% in TCR-PLP1*Plp*^{WT} mice (Fig. 17 A and B). In addition, we detected a drastic reduction in the percentage of thymic PLP1-specific T_{regs} in *C2TA*^{kd} mice (1.5% ± 0.3%) compared to TCR-PLP1*Plp*^{WT} mice (4.1% ± 0.6%) (Fig. 17 C). Thus, reducing the presentation capacity in mTEC^{hi} affected central tolerance induction to TCR-PLP1^{+} T cells. Interestingly, the *C2TA*^{kd} genotype did not lead to a complete loss of tolerance to PLP.

In summary, by reducing the presentation capacity of mTEC^{hi}, we could show that mTEC^{hi} contribute to central tolerance. However, this approach does not exclude a potential contribution of cTECs and mTEC^{lo} to tolerance induction.
Taken together, we conclude that not only the expression of PLP by mTECs but also the direct presentation of the antigen by this cell type was indispensable for tolerance induction to PLP.

3.5 The role of AIRE in central tolerance induction to PLP

Through changes in the transcriptional landscape of a large variety of genes, AIRE allows AIRE^{+} mTECs to express an incredible amount of otherwise tissue-restricted antigens [2, 5, 90]. We wanted to see whether tolerance induction to PLP, although being an atypical tissue-restricted antigen due to its expression also in cTECs, DCs and mTEC^{lo}, would also be regulated by AIRE.

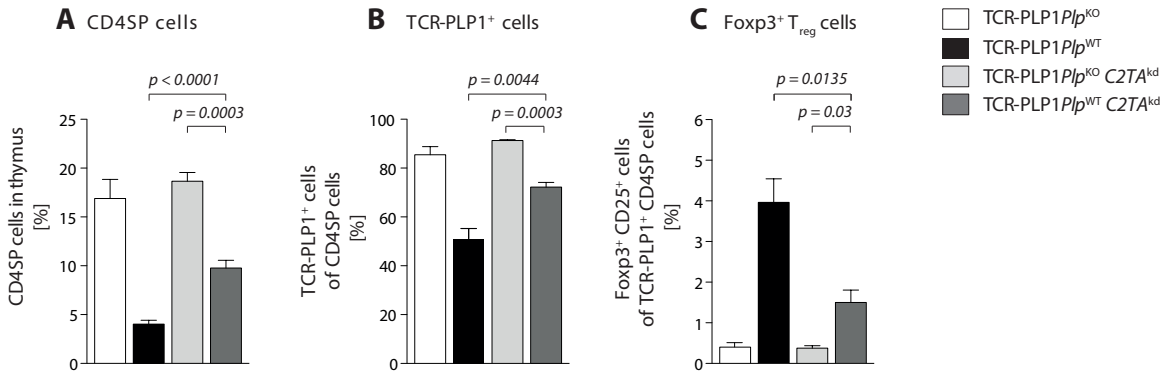

Figure 17: Reduction of the presentation capacity on mTECs by *C2TA*$^{\mathbf{kd}}$. The bar diagrams show (A) the percentages of CD4SP cells within the thymus, (B) the percentage of TCR-PLP1$^+$ cells within the thymic CD4SP compartment, and (C) the percentage of Foxp3$^+$ T_{reg} cells in the CD4SP TCR-PLP1$^+$ cell population. Reduction of the presentation capacities on mTECs by *C2TA*^{kd} led to a diminished tolerance to PLP in TCR-PLP1*Plp*^{WT} *C2TA*^{kd} animals. mTECs mediate clonal deletion and T_{reg} cell induction by direct presentation. Depicted is the mean average of each group with the standard error of the mean (SEM). TCR-PLP1*Plp*^{KO}: n=8; TCR-PLP1*Plp*^{WT}: n=10; TCR-PLP1*Plp*^{KO} *C2TA*^{kd}: n=3; TCR-PLP1*Plp*^{WT} *C2TA*^{kd}: n=5.

3.5.1 AIRE influences PLP expression

To see if AIRE controlled the expression of PLP, we performed qRT-PCR analysis for PLP expression in the thymic mTEC^{hi} population of WT and *Aire*$^{-/-}$ animals. We detected a 6-fold reduction of the PLP mRNA expression in *Aire*^{KO} mTEC^{hi} cells compared to the mTEC^{hi} population of WT mice (Fig. 18). Thus, AIRE regulated the expression of PLP in mTEC^{hi} cells. In future studies, we will address if AIRE also influences the expression of PLP in the other stromal subsets cTEC and mTEC^{lo}, and will also distinguish between mTEC^{hi} AIRE-positive and mTEC^{hi} AIRE-negative cell subsets.

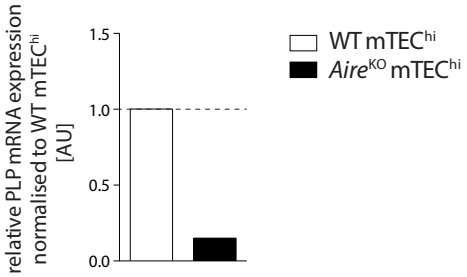

Figure 18: AIRE regulates the expression of PLP in mTEC^{hi}. The relative mRNA expression of PLP in *Aire*^{KO} mTEC^{hi} compared to the expression level of WT mTEC^{hi}. The expression level of PLP was normalised to WT mTEC^{hi}. A deficiency of AIRE reduced the expression of PLP mRNA in the mTEC^{hi} of the thymus. The bar diagram shows the expression levels of one preliminary experiment in arbitrary units (AU).

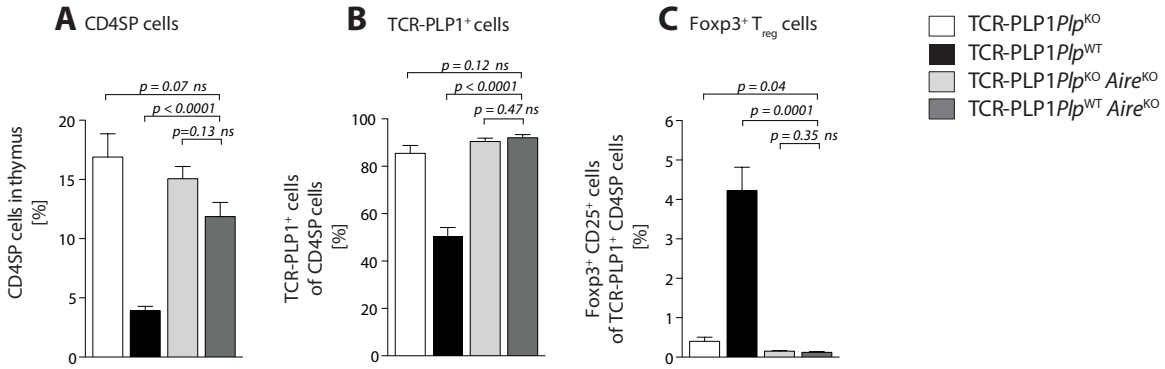

Figure 19: Loss of central tolerance induction when AIRE is absent. The bar diagrams show (A) the percentages of CD4SP cells within the thymus, (B) the percentage of TCR-PLP1$^+$ cells within the thymic CD4SP compartment, and (C) the percentage of Foxp3$^+$ T_{reg} cells within the CD4SP TCR-PLP1$^+$ T cell population. Deficiency of AIRE abolished central tolerance induction to PLP. Depicted is the mean average of each group with the standard error of the mean (SEM) as error bars.
TCR-PLP1*Plp*^{KO}: n=8; TCR-PLP1*Plp*^{WT}: n=11; TCR-PLP1*Plp*^{KO} *Aire*^{KO}: n=3; TCR-PLP1*Plp*^{WT} *Aire*^{KO}: n=6.

3.5.2 AIRE deficiency abrogates central tolerance induction to PLP

Given that AIRE deficiency reduced PLP expression in the mTEC^{hi} population, we further speculated on a role for AIRE in central tolerance induction to PLP.
To address this, we crossed TCR-PLP1 transgenic mice to the *Aire*$^{-/-}$ mice and analysed their thymic phenotype compared to AIRE-sufficient littermates. AIRE deficiency completely abolished the deletion and regulatory T cell induction of TCR-PLP1$^+$ T cells that we had observed before in TCR-PLP1*Plp*^{WT} mice. TCR-PLP1*Plp*^{WT} *Aire*^{KO} mice had a significantly higher frequency of CD4SP cells than TCR-PLP1*Plp*^{WT} mice (12.2% ± 1.4% in TCR-PLP1*Plp*^{WT} *Aire*^{KO} mice compared to 4.02% ± 0.4% in the AIRE-sufficient TCR-PLP1*Plp*^{WT} mice) (Fig. 19 A). Also within the CD4SP compartment, TCR-PLP1$^+$ T cells were no longer deleted (CD4SP TCR-PLP1$^+$ cells: 92.2% ± 1.6% in TCR-PLP1*Plp*^{WT} *Aire*^{KO} mice compared to 51.1% ± 4.1% in the AIRE-sufficient TCR-PLP1*Plp*^{WT} mice) (Fig. 19 B). Moreover, no selection of TCR-PLP1$^+$ regulatory T cells was observed when AIRE was deficient (0.1% ± 0.02% in TCR-PLP1*Plp*^{WT} *Aire*^{KO} mice compared to 4.1% ± 0.6% in the AIRE-sufficient TCR-PLP1*Plp*^{WT} mice) (Fig. 19 C). This data clearly showed that AIRE expression is crucial for T_{reg} induction.
In summary, tolerance induction to PLP was greatly impaired in the absence of AIRE, which indicates mTEC^{hi} (as the exclusive AIRE expressing subset) are the main players in central tolerance to PLP.

3.6 Central tolerance in TCR-PLP1 mice is age-dependent

Central tolerance induction was lost with age

So far, we have analysed central tolerance induction in the TCR-PLP1 mouse model at the age of 3 weeks. In these mice, we observed TCR-PLP1-specific T cells to be deleted as well as deviated into the regulatory T cell lineage. Crucial for tolerance induction was the expression and presentation of PLP by mTECs (see section 3.4).
Next, we wanted to assess the state of tolerance induction in adult mice, as we observed no signs of auto-immunity in aged mice. Analysing animals at the age of 8 weeks, we observed a complete loss of central tolerance induction to PLP also in mice that were sufficient for PLP (Fig. 20). In 8-week-old TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice, the CD4SP compartment was not reduced (34.7% ± 2.5%) compared to TCR-PLP1*Plp*$^{\mathrm{KO}}$ mice (36.8% ± 1.6%) (Fig. 20, first column). Also, the TCR-PLP1^{+} T cells were no longer deleted in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice (92.7% ± 1.6%), nor were TCR-PLP1-specific $\mathrm{T_{reg}}$ cells induced (0.4% ± 0.2%) (Fig. 20, second and third column). Consistently, in the 8-week-old thymi of TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice, mature T cells increased to 55.1% ± 3.2% of TCR-PLP1^{+} T cells, which represented similar levels of mature cells as in TCR-PLP1*Plp*$^{\mathrm{KO}}$ mice (63.2% ± 1.4%) (Fig. 20, fourth column). Thus, adult TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice appear to have lost central tolerance induction in the thymus completely.

Time-window for negative selection and $\mathbf{T_{reg}}$ induction

Due to this unexpected observation, we addressed central tolerance induction in relation to age in more detail. To see if there is a certain time-window for central tolerance, we analysed the thymic phenotype at time-points ranging from the first day after birth up to 12 weeks of age. In the first 2 weeks of life, we assessed the phenotype of central tolerance induction every second day, while after 4 weeks it was evaluated only every other week. We observed central tolerance to PLP to be age-dependent (Fig. 21): In the first 3 days after birth, the thymi of both TCR-PLP1*Plp*$^{\mathrm{WT}}$ and TCR-PLP1*Plp*$^{\mathrm{KO}}$ mice showed comparable percentage of CD4SP and TCR-PLP1^{+} T cells. After that, TCR-PLP1^{+} T cells in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice were subject to central tolerance induction: in the thymi of these mice we observed strong deletion of CD4SP and TCR-PLP1^{+} T cells that peaked at $2-3$ weeks of age (Fig. 21 A and B). Then, central tolerance induction gradually diminished until it was completely lost at the age of 6 weeks.
Inversely correlated to the deletion of TCR-PLP1^{+} T cells, we observed the induction of

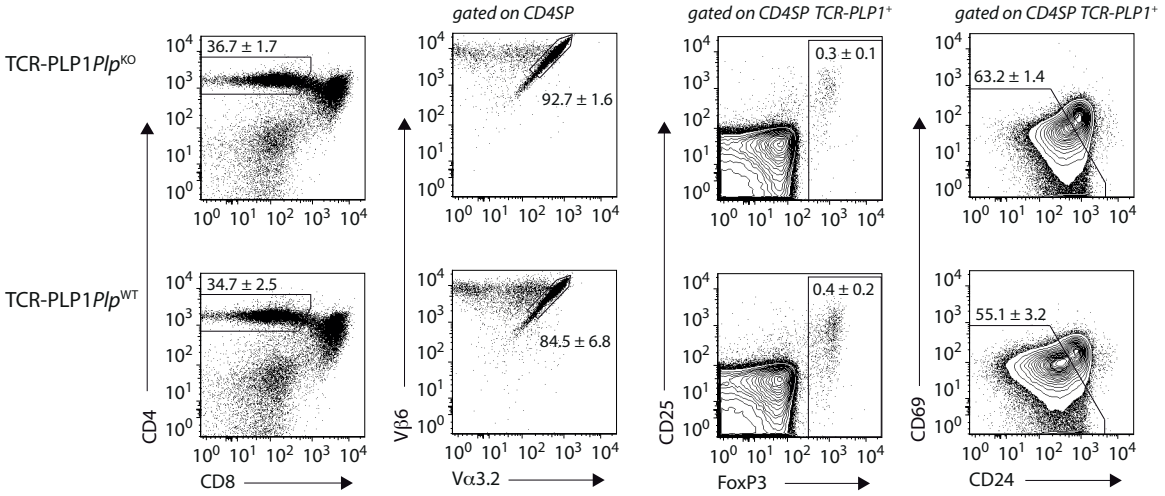

Figure 20: Thymic phenotype of adult TCR-PLP1*Plp*^{KO} and TCR-PLP1*Plp*^{WT} mice. Representative littermates of TCR-PLP1*Plp*^{KO} and TCR-PLP1*Plp*^{WT} mice were analysed for their thymic phenotype at the age of 8 weeks. Shown is the percentage of CD4SP cells in the thymus (first column), the percentage of TCR-PLP1$^+$ cells within the CD4SP compartment (second column), the percentage of Foxp3$^+$ regulatory T cell in the CD4SP TCR-PLP1$^+$ T cell population (third column), and the percentage of mature cells within the TCR-PLP1$^+$ T cell population in the thymus (fourth column). Adult TCR-PLP1*Plp*^{WT} mice have completely lost tolerance to PLP in the thymus. The numbers above the gates represent the mean average of all animals of the same cohort with the standard error of the mean (SEM). TCR-PLP1*Plp*^{KO}: n=4; TCR-PLP1*Plp*^{WT}: n=7.

TCR-PLP1$^+$ T_{reg} cells (Fig. 21 C): In the first few days, emergence of TCR-PLP1$^+$ Foxp3$^+$ T_{reg} cells was neglegible. At 1 week, PLP-specific T_{regs} started to appear and at the age of 3 weeks they composed around 4% of the CD4SP TCR-PLP1$^+$ T cell population. These observations are consistent with a recent study, which also reported thymic T_{reg} cells to appear in a polyclonal CD4SP compartment from day 4 on [156].
In conclusion, we observed a time-window for central tolerance induction to PLP in TCR-PLP1*Plp*^{WT} mice. Negative selection of TCR-PLP1$^+$ T cells was operating in the thymus from day 3 until 6 weeks of age, whereas PLP-specific T_{regs} started to accumulate only after the first week in TCR-PLP1*Plp*^{WT} mice and induction lasted until 6 weeks of age. In adult mice older than 6 weeks, central tolerance was completely lost.

No differential expression of PLP in the aged thymus

We hypothesised that the loss of tolerance in TCR-PLP1*Plp*^{WT} mice with age was a consequence of a potential loss or decrease in PLP expression in the thymus. Therefore, we compared the expression level of PLP mRNA in thymi of young (10 days of age) and adult WT animals (10 weeks of age). Due to technical limitations to isolate cTECs from adult thymi, we analysed only mTEC^{lo}, mTEC^{hi}, and thymic DCs.

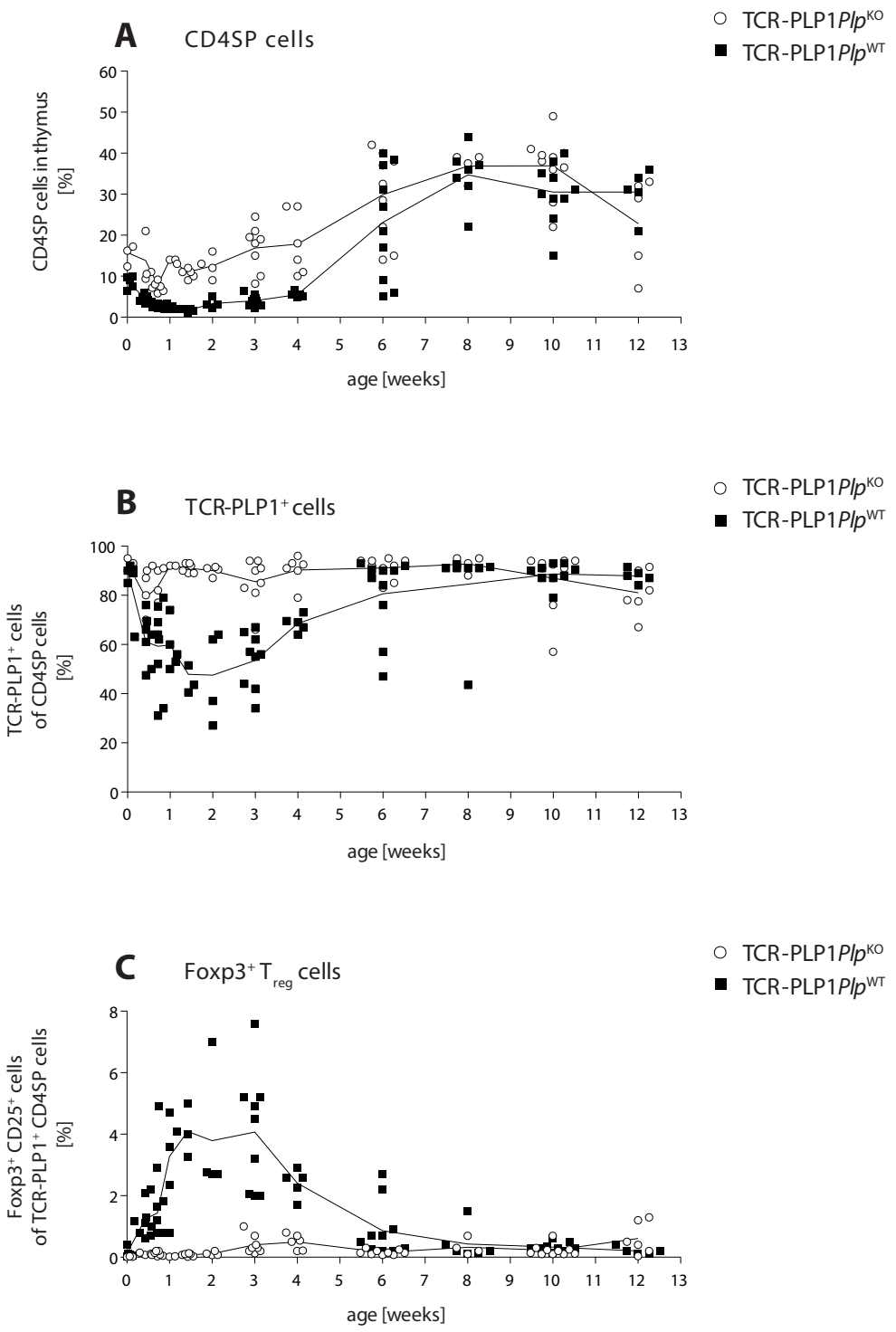

Figure 21: Central tolerance induction to PLP is confined to a time-window. The thymic phenotype of TCR-PLP1*Plp*^{KO} and TCR-PLP1*Plp*^{WT} mice was assessesd at different time points. A gradual loss of thymic tolerance was observed in TCR-PLP1*Plp*^{WT} mice. Shown is (A) the percentages of CD4SP cells in the thymus plotted against the age of the mice, (B) the percentages of TCR-PLP1^{+} cells within the CD4SP compartment as a function of the animal's age, and (C) the percentages of Foxp3^{+} regulatory T cells in the CD4SP TCR-PLP1^{+} T cell population plotted against the age of the mice. Time points analysed ranged from 1 day after birth to 12 weeks after birth.

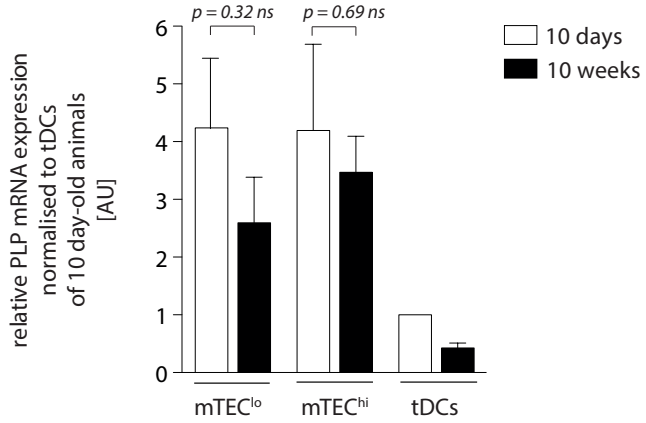

Figure 22: Expression of PLP mRNA in thymic APC subsets at different ages. Relative mRNA expression of PLP in the thymic APC subsets mTEC^{lo}, mTEC^{hi}, and thymic DCs (tDCs), normalised to the expression levels of DCs in 10-day-old animals. Young mice were 10 days of age, old mice were 10 weeks of age. PLP was not differentially expressed in young versus old thymic APC subsets. The bar diagram shows the mean average of 3 individual experiments in arbitrary units (AU). Error bars are depicted as the standard error of the mean (SEM).

We did not detect a significant difference in PLP mRNA expression in old versus young thymic APC subsets (Fig. 22). Hence, the change in central tolerance between young and adult TCR-PLP1*Plp*^{WT} mice cannot be based on a differential expression of the antigen in the thymus. Further possibilities, like a differential translation of PLP protein, will be addressed in future studies.

4 Discussion

Negative selection operates in the thymic medulla via two mechanisms in order to maintain immunological self-tolerance: clonal deletion of potentially hazardous self-reactive T cells, and the production of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (T_{reg} cells), which act in the periphery to control self-reactive T cells that escape thymic selection. The contribution of the different cellular subsets and the exact modalities for the choice of either negative selection or T_{reg} induction are not yet fully deciphered.
Much progress in understanding the mechanisms of central tolerance has been made by studying fate decisions in TCR-transgenic animals. In many studies, TCR-transgenic mice have been used which express a TCR-specificity against a transgenic gene such as chicken ovalbumin (OVA), influenza hemagglutinin (HA) or human C-reactive protein (hCRP) [74, 85, 157]. The expression of these introduced neo-self-antigens mostly exceeded physiological expression levels of thymic self-antigens and added yet another concern to the already artificial system.
We have generated a model system for the study of central tolerance induction towards an endogenous tissue-restricted antigen. In the novel TCR-PLP1 transgenic mouse, T cells are reactive to an immuno-dominant epitope of myelin proteolipid protein (PLP) in the context of $I\text{-}A^b$. This mouse model allowed us to study the fate of thymocytes which see their cognate antigen $PLP_{9\text{-}20}$ at physiologic levels. Furthermore, as the B6 background has strong tolerance mechanisms against PLP [3, 142], we were able to investigate what type of APC and further factors contributed to tolerance induction to PLP.
Presented data of the model system TCR-PLP1 indicate the following implications for tolerance induction to the self-antigen PLP:

1. Central tolerance to PLP is induced by clonal deletion and concomitant T_{reg} induction of TCR-$PLP1^+$ T cells.

2. Thymic medullary epithelial cells (mTECs) express and present the antigen PLP autonomously. Tolerance to PLP is mediated by mTECs exclusively, while thymic DCs do not contribute to tolerance induction.

3. The expression of PLP mRNA in mTEC^{hi} is controlled by AIRE. AIRE deficiency abrogates central tolerance in TCR-PLP1*Plp*^{WT} mice completely.

4. Central tolerance induction to PLP is restricted to a distinct time window starting a few days after birth. After 6 weeks, central tolerance to PLP is lost.

4.1 Central tolerance to PLP is induced by clonal deletion and concomitant T_{reg} induction of TCR-PLP1$^+$ T cells

Characterisation of the phenotype of TCR-PLP1*Plp*^{WT} mice in the thymus revealed that the presence of the antigen PLP induced tolerance to TCR-PLP1-specific T cells. We observed TCR-PLP1$^+$ T-cells to be partly deleted and a fraction concomitantly redirected into the T_{reg} lineage.
In some TCR transgenic models, a dominant mode of tolerance induction was observed. Either T cells were predominantly deleted or in addition to deletion also deviated into the T_{reg} lineage. In the Dep x hCRP mouse model, for instance, clonal deletion of the specific transgenic T cells dominated the thymic phenotype of tolerance induction [85]. In other models, such as the TCR-HA x AIRE-HA model [74], deviation of antigen-specific T cells into the T_{reg} lineage concomitant to the deletion was often observed. Likewise, in the TCR-PLP1 model, we observed clonal deletion concomitant to deviation of regulatory T cells as mode of tolerance induction to PLP. Furthermore, T cells specific to $PLP_{9\text{-}20}$ were deleted at the CD4SP stage of T cell development, unlike, for instance, hCRP-specific T cells which were deleted at an early DP stage [85].

4.2 Medullary thymic epithelial cells express and present the antigen PLP autonomously

We detected PLP transcripts in all thymic epithelium subsets, namely cTECs, immature and mature mTECs (mTECs^{lo} and mTEC^{hi}, respectively), as well as in thymic DCs, albeit to a smaller degree (Fig. 13). This expression pattern was in line with previous reports [1, 3]. Due to its expression also in cTECs and DCs, PLP is referred to as an atypical tissue-restricted antigen (TRA), since a typical TRA would be expressed only by AIRE$^+$ mTEC^{hi} cells [1]. Therefore, we investigated if the expression of PLP in the individual thymic APC subsets was crucial for tolerance induction to TCR-PLP1$^+$ T cells.

EXPRESSION OF PLP. In order to delineate the role of PLP expression by APCs of the hematopoietic (DCs, B cells) or non-hematopoietic compartment (TECs), we performed transplantation experiments, in which selectively cells of the hematopoietic or non-hematopoietic compartment lacked PLP expression. When cells of non-hematopoietic origin were sufficient for PLP, deletion and T_{reg} induction of TCR-PLP1$^+$ T cells was detected. In contrast, when PLP was only expressed by cells of the hematopoietic compartment, TCR-PLP1$^+$ T cells were not tolerised (Fig. 14). This indicated that PLP expression by hematopoietic cells was not crucial for tolerance induction to PLP.
To prove a crucial role for TECs in PLP expression needed for tolerance induction, we employed an additional system where PLP expression was absent only in thymic epithelial cells due to the Foxn1-Cre-driven excision of the floxed *Plp* gene. In TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre mice, we observed a complete loss of tolerance towards TCR-PLP1$^+$ T cells in the thymus when PLP expression was abolished in TECs (Fig. 15). Thus, expression of PLP by the remaining thymic dendritic cells and/or B cells did not lead to tolerance induction in the thymus and revealed a crucial role for TECs in PLP-expression for tolerance induction. As PLP expression was ablated in all Foxn1-expressing cells (TECs), we could not differentiate between cTECs and mTECs and decipher their individual contribution to PLP tolerance induction. Some studies have suggested that cTECs cannot induce tolerance to self-antigens [158–160], whereas others have stated the opposite [161–164]. It has previously been reported that cTECs can also mediate clonal deletion of auto-reactive T cells, although rather for T cells specific for "ubiquitous" self-antigens [165]. Furthermore, the group of Kristin Hogquist has stated that cTECs were not the major player in the induction of apoptosis in the cortex, but rather depended on a contribution from cortical dendritic cells [43]. Even though we cannot formally exclude a role of cTECs for tolerance induction to PLP, we think it is rather unlikely, as TCR-PLP1*Plp*^{WT} and TCR-PLP1*Plp*^{KO} animals showed equal numbers of DP cells in the thymus, indicating no deletion in the cortex. Recent work revealed that in the cortex, more than 50% of all signalling thymocytes at the DP stage are deleted by strong signals of high-affinity ligands that induce rapid apoptosis [166–169]. If PLP-specific T cells were not deleted in the cortex at the DP stage, it is likely that the expression of PLP in cTECs does not result in the expression of exactly the same epitope as in thymic DCs or in mTECs. Through the use of the unique lysosomal proteases cathepsin L and thymus-specific serine protease (TSSP), cTECs possess a unique processing machinery for the generation of "private peptides" [7, 170–172]. Therefore, cTECs might display different epitopes of PLP for cortical selection and therefore, selection of $PLP_{9\text{-}20}$-reactive T cells will only occur at the CD4SP stage in the medulla. It remains interesting, though, why cTECs and DCs express $PLP_{9\text{-}20}$ mRNA, although they seem not to contribute to tolerance induction.

PRESENTATION OF PLP. Antigens expressed by TECs may be transferred to and presented by thymic DCs [82, 83]. Albeit we have seen the expression of PLP by DCs not to be crucial for tolerance induction, DCs might still play a role in the presentation of PLP. To address the contribution of intercellular PLP transfer from mTECs to DCs, we analysed the T cell development in TCR-PLP1Plp^{WT} mice deficient for dendritic cells (TCR-PLP1$Plp^{\mathrm{WT}}\Delta$DC): No difference in tolerance induction was observed in the presence or absence of DCs (Fig. 16). This indicated that tolerance induction to PLP did not require the physical presence of DCs, nor an antigen transfer of PLP to DCs.
Migratory DCs (such as plasmacytoid DCs and Sirpα^{+} DCs) are capable of taking up and transporting circulating antigen to the thymus [60, 173]. In TCR-PLP1$Plp^{\mathrm{fl/fl}}$ Foxn1-Cre animals, in which only TECs are deficient for PLP expression, no contribution of DCs in PLP presentation was observed (Fig. 15). Therefore, we also excluded a contribution of migratory DCs which could have taken up PLP in the periphery and presented the imported PLP in the thymus [8]. However, we cannot differentiate whether migratory DCs did not transport peripheral PLP to the thymus, or if all DC subsets are unable to present PLP for tolerance induction. The necessity of antigen transfer of a particular mTEC-derived antigen to dendritic cells for presentation may depend on the frequency or thymic localisation of mTECs that express the antigen, or the amount of antigen expressed. PLP is a tissue-restricted antigen and it is believed that only 1-3% of mTECs express the respective antigen [1,80]. Therefore, it is somewhat surprising that PLP presentation is not maximised by antigen-spreading to DCs in order to increase the likelihood of cognate antigen-encounter for thymocytes. Yet the necessity for cross-presentation seems to be very individual for each antigen and cannot be generalised.
Formally, we still need to show that the conditional deletion of PLP expression in TCR-PLP1$Plp^{\mathrm{fl/fl}}$ Foxn1-Cre mice, was truly deleting all PLP expression in TECs. *In situ* hybridisation for PLP would represent a good tool to visualise the lack of PLP expression in TECs. However we are confident of a high deletion rate of PLP as residual expression of PLP in TECs would promote tolerance induction, which was completely absent in these animals.

Having excluded cross-presentation of PLP by dendritic cells, we next addressed direct presentation of PLP by mTECs. In $C2TA^{\mathrm{kd}}$ mice, the presentation capacity of mTECs is reduced by an AIRE-promoter-driven knockdown of the MHC class II transactivator (C2TA) [77]. Interference with the MHCII expression on mTECs, led to a reduced tolerance induction to PLP in TCR-PLP1Plp^{WT} $C2TA^{\mathrm{kd}}$ mice (Fig. 17). This showed that mTECs present the antigen PLP autonomously to T cells and induce clonal deletion as well as deviation of PLP-specific T cells into the $\mathrm{T_{reg}}$ lineage. We did not observe an absolute loss of tolerance in TCR-PLP1Plp^{WT} $C2TA^{\mathrm{kd}}$ mice, but only a reduction of the phenotype. As

the *C2TA*^{kd} approach did not entirely eliminate MHCII from mTECs but only diminished its surface density to approximately 10% of WT levels, the residual MHCII molecules in TCR-PLP1*Plp*^{WT} *C2TA*^{kd} mice might be still sufficient to censor part of the TCR-PLP1^{+} T cells. It was speculated that in *C2TA*^{kd} mTECs with diminished MHCII expression, preferably CD4^{+} T cells at the very high end of the affinity spectrum were tolerised by the remaining 10% of pMHCII molecules [77]. In favour of this, the TCR-PLP1 T cell receptor seems to fall into the category of high-affinity receptors, as we have observed that already $PLP_{9\text{-}20}$ peptide in the femtogram-range triggered a stimulation of CD4^{+} TCR-PLP1^{+} peripheral T cells in an *in vitro* stimulation assay (data not shown). The other possibility is that cTECs do have an influence on tolerance induction to PLP, which will only become apparent when the contribution of mTECs is diminished.
Collectively, we have shown that mTECs express and present the antigen PLP directly to auto-reactive T cells and that DCs do not contribute to tolerance induction to PLP.

Also in other models, such as the AIRE-OVA x DO11.10 system [77] or the AIRE-HA x TCR-HA system [74], in which mTECs had an autonomous role in antigen presentation, DCs were found to be dispensable for tolerance induction. These models have confirmed the ability of mTECs to efficiently present antigens themselves and had previously given rise to the hypothesis that the type of APC will ultimately determine the mode of tolerance induction. Currently, two main theories want to explain differences in fate choices of auto-reactive T cells: One hypothesis is that the fate decision of an auto-reactive T cell is determined by the affinity with which the TCR binds to pMHC [151]. The other model postulates that the avidity of the TCR:pMHC interactions is relevant [174, 175]. The *C2TA*^{kd} model developed by Hinterberger *et al.* argued in favour of the avidity hypothesis. In a TCR-transgenic model system with diminished MHCII-restricted antigen presentation by mTECs, the efficacy of negative selection shifted more to the induction of T_{reg} cells, when all other parameters such as the amount and tissue specificity of antigen expression and the responding thymocyte population were kept identical and potential cross-presentation by DCs was excluded [77]. In our TCR-PLP1 transgenic model, we have observed that PLP-specific T cells carrying the same TCR were deleted as well as redirected into the T_{reg} lineage, both mediated by mTECs (Fig. 16). Diminishing the presentation of PLP on mTECs by crossing the *C2TA*^{kd} mouse to our TCR-PLP1 model, resulted in a pronounced escape of auto-reactive T cells into the periphery (Fig. 17). This break down of central tolerance was in this case due to reduced pMHCII on mTEC^{hi} as all other parameters were kept constant. This observation in the TCR-PLP1 model also argues towards the avidity model (number of pMHC-TCR interactions) and against the affinity model as the quality of the interactions was not altered.

In other TCR-transgenic models, the antigen presentation by DCs was dispensable but not

absent. In the AIRE-GCL x Dep transgenic system, for instance, indirect presentation by DCs was not essential for negative selection *in vivo*, yet operated in parallel with the direct presentation by mTECs [176]. This redundancy was then suggested to give central tolerance a certain robustness [66]. With the TCR-PLP1 mouse model, we show for the first time that mTECs are not only capable of directly present antigen for ensuring both modes of tolerance induction, but that the expression and presentation of an antigen by mTECs is crucial for tolerance induction and that DCs did not play a role in induction of tolerance to this antigen. Thus, we have shown with our model an example for an exclusive role of mTECs in inducing tolerance to a self-antigen of the periphery. In the polyclonal system, Perry *et al.* [86] demonstrated that DCs and mTECs play non-redundant roles in deletion and T_{reg} cell selection. The notion for redundancy between mTECs and DCs in tolerance induction was mostly formed by fate decision analysis in TCR-transgenic models with an introduced neo-self-antigen. By the genetic introduction of neo-self-antigens, it is likely that the expression levels were higher than physiological conditions. Therefore, the suggested redundant roles for mTECs and DCs in inducing central tolerance to these antigens may have been caused by atypical expression levels. High expression of an antigen on mTECs might favour an additional cross-presentation by DCs, while antigen expression at physiological levels would potential limit tolerance induction to mTECs only. In favour of this explanation is the observation of an essential role of mTECs in the AIRE-GCL^{lo} x Dep system. Here, the antigen GCL (**G**FP-**C**-reactive protein-**L**ight chain 3-fusion protein) was expressed at roughly fourfold lower mRNA levels when compared with AIRE-GCL mice, and in contrast to the latter, mTECs were shown to be crucial for tolerance due to the autophagy-dependent mode of direct presentation [176].
Thus, our findings have implications for the previously suggested redundancy in the roles of thymic APCs for central tolerance induction. It is possible to think about a demarcation between a crucial role of mTECs versus thymic dendritic cells dependent on the extent of antigen expression. Perry *et al.* [86] suggested that tolerance to rare tissue-restricted antigens are preferentially mediated by mTECs, while DCs mediate tolerance to more frequent antigens. PLP is assumed to be expressed at low levels [65], and hence, is in line with this suggestion.

Klein *et al.* [3] have reported that tolerance to PLP was established equally well in bone marrow chimerae with PLP sufficiency in both the TEC compartment or in cells of the hematopoietic compartment. As they immunised mice with whole PLP protein and measured the recall response of peripheral T cells, they did not have the possibility to distinguish between central and peripheral tolerance nor between tolerance to individual epitopes. We, in contrast, have the ability to a) delineate the contributions of APC types to tolerance induction specifically to the epitope $PLP_{11\text{-}18}$, and to b) trace the fate of PLP-specific T cells

first in the thymus, and then, to follow these cells into the periphery in order to further assess the influence of tolerance mechanisms there. In the thymus, we have seen TCR-PLP1$^+$ T cells to be deleted and deviated into the T_{reg} lineage. In the periphery of TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre animals, we have observed clonal deletion acting on TCR-PLP1$^+$ T cells that have previously not been tolerised in the thymus due to the absence of PLP expression in TECs (data not shown). Thus, additional negative selection in the periphery has shaped the TCR-PLP1$^+$ T cell pool. Furthermore, preliminary data have indicated that beside negative selection, peripheral TCR-PLP1$^+$ T cells, which have escaped thymic selection in TCR-PLP1*Plp*^{WT} mice, adopt an anergic phenotype and no longer responded to PLP stimulation (data not shown). Moreover, we have been able to determine that the cell type presenting PLP in the periphery is of hematopoietic origin (data not shown). Thus, it is feasible that DCs or other APCs of bone marrow origin present PLP to peripheral TCR-PLP1$^+$ T cells and induce anergy as an additional dominant tolerance mechanism. However, further investigations are necessary to characterise the putative peripheral tolerance mechanism in more detail.

Notwithstanding, we may conclude that tolerance induction to PLP is a combination of central tolerance in the thymus (clonal deletion and deviation into the regulatory T cell lineage) with additional tolerance mechanisms in the periphery, such as further clonal deletion and anergy [7].

In a situation where peripheral tolerance accounts for the only tolerance mechanism to PLP, the dimension of these mechanisms will become apparent. If it is not possible to elicit EAE in TCR-PLP1*Plp*$^{fl/fl}$ Foxn1-Cre animals, that did not show central tolerance, peripheral tolerance mechanisms must be very potent and represent a second layer of tolerance induction.

Furthermore, tolerance mechanisms to the other epitopes of PLP may very well depend on cells of the hematopoietic compartment. The proposition by Klein *et al.* [3] was that cells of the non-hematopoietic compartment as well as cells of bone marrow origin mediated tolerance to PLP. This conclusion may come from the combination of central and peripheral tolerance together or reflect the fact that the APC type mediating tolerance to a given PLP epitope is highly individual. While we have conclusively shown in this study that dendritic cells do not mediate central tolerance to epitope $PLP_{11\text{-}18}$, tolerance induction to the other PLP epitopes in the context of I-A^b might very well depend on the expression and/or presentation by hematopoietic cells and would need to be assessed on an individual basis.

4.3 AIRE deficiency abrogates central tolerance in TCR-PLP1*Plp*^{WT} mice

Lack of AIRE selectively abolishes the antigen reservoir function of mTECs for AIRE-dependent TRAs [2,90]. We have seen that central tolerance induction to $PLP_{9\text{-}20}$ is greatly impaired in the absence of AIRE. As we have shown that PLP was not expressed only in mTEC^{hi} but also in cTECs, thymic DCs, and mTEC^{lo}, it was not entirely expected to see a complete loss of tolerance induction to PLP in TCR-PLP1*Plp*^{WT} *Aire*^{KO} animals (Fig. 19). When we quantified the mRNA expression of PLP in mTEC^{hi}, we detected a 6-fold reduction of PLP mRNA expression in *Aire*^{KO} mTEC^{hi} compared to WT mTEC^{hi} (Fig. 18). Therefore, we concluded that AIRE controlled tolerance to PLP, although we cannot base this result on the simple explanation of an AIRE-dependent control of expression, as PLP is also expressed AIRE-independently in cTECs and mTEC^{lo} (Fig. 13). Future quantification of PLP mRNA expression with respect to the presence of AIRE should also include cTEC and mTEC^{lo}, and further discriminate between AIRE-positive and AIRE-negative mTEC^{hi}.

Even though the expression of PLP might not be totally dependent on AIRE, we do see a reduction of PLP mRNA in *Aire*^{KO} mTEC^{hi}. Thus, while mTEC^{lo} also express PLP mRNA, only mTEC^{hi} may be relevant for tolerance induction. We propose three hypotheses that are not mutually exclusive to explain an influence of AIRE on the tolerance induction to PLP.

First, there might be a critical threshold of antigen expression necessary for tolerance induction. Thus, the expression of PLP by mTECs possibly lies slightly above this critical threshold and in the case of AIRE deficiency, the amount of transcribed PLP mRNA by mTEC^{hi} falls below this threshold, subsequently, leading to a breakdown of central tolerance to PLP.

Second, AIRE might have more influence than purely regulating the transcriptional landscape of TRAs. There is evidence that AIRE is also involved in the translation and/or presentation of antigens. Kuroda *et al.* [104] described in their study, that the transcription of alpha-foldrin retained the same levels in *Aire*^{WT} and in *Aire*^{KO} mice, while the protein translation was affected by AIRE deficiency. Also Hubert *et al.* [177] stated that AIRE regulates aspects of antigen presentation, namely the antigen transfer from mTECs to dendritic cells. Although we have shown for the antigen $PLP_{9\text{-}20}$ that presentation by thymic DCs did not play a role, AIRE might affect the synthesis of PLP protein or the presentation of the antigen by mTEC^{hi}. The next step to decipher the influence of AIRE in tolerance induction to PLP would be to compare the amount of PLP protein in *Aire*^{KO} versus WT TEC populations. To see a direct effect of AIRE on PLP presentation, we would

need to establish a sensitive *ex vivo* presentation assay to measure even small changes in the presentation of PLP by mTECs.
Third, loss of tolerance with AIRE deficiency could possibly also reflect a problem of T cells to scan efficiently the rare mTECs that present PLP. It was reported that the chemokine landscape changes with the absence of AIRE [90, 108]. Thus, a change in chemokines could potentially influence the interaction of thymocytes with mTECs, modifying their migration behaviour or the length of their contacts with stromal cells, leading to a loss of tolerance induction [178, 179].

Presented data are summarised in a graphical scheme in Figure 23.

4.4 Central tolerance induction to PLP is restricted to a distinct time-window

Remarkably, we found tolerance to PLP in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice to be dependent on the age of the thymus. We have identified a time window for negative selection and deviation of auto-reactive T cells into the $\mathrm{T_{reg}}$ lineage. Only a few days after birth, we detected clonal deletion of PLP-specific T cells in the thymic compartment of CD4SP cells in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice. Furthermore, we demonstrated that induction of regulatory T cells does not occur before the end of the first week. At day 7, $\mathrm{T_{regs}}$ started to accumulate in TCR-PLP1*Plp*$^{\mathrm{WT}}$ mice, consistent with other publications reporting the emergence of $\mathrm{T_{regs}}$ between day 4 and day 5 [156, 180]. For the development of $\mathrm{T_{regs}}$, a two-step model was proposed. First, an "instructive" phase up-regulates CD25 on the cell surface, which is dependent on the TCR and favours self-reactive TCR specificities. Then, a second phase, the "consolidation" phase, occurs in which expression of Foxp3 starts driven by cytokines independently of the TCR [181]. The time required to complete this two-step process may be reflected in the later emergence of Foxp3^{+} regulatory T cells.
We detected maximal clonal deletion and selection of regulatory T cells at 2 weeks. Strikingly, tolerance induction was then lost completely at 6 weeks, regardless of whether the antigen was present or absent in the thymus. Whether this is a peculiarity of the TCR-PLP1 transgenic mouse or a phenomenon also observed in other model systems, has to be further investigated.
A first possible explanation for a loss of tolerance was reduced amounts of PLP: either a reduction of PLP expression on the mRNA level, or a diminished PLP synthesis rate on the protein level. Since a tissue-restricted antigen, such as PLP, is only presented by 1-3% of mTECs [1, 80], a further reduction of PLP could then lead to insufficient antigen encounters to induce tolerance. The quantification of PLP mRNA revealed no significant differences

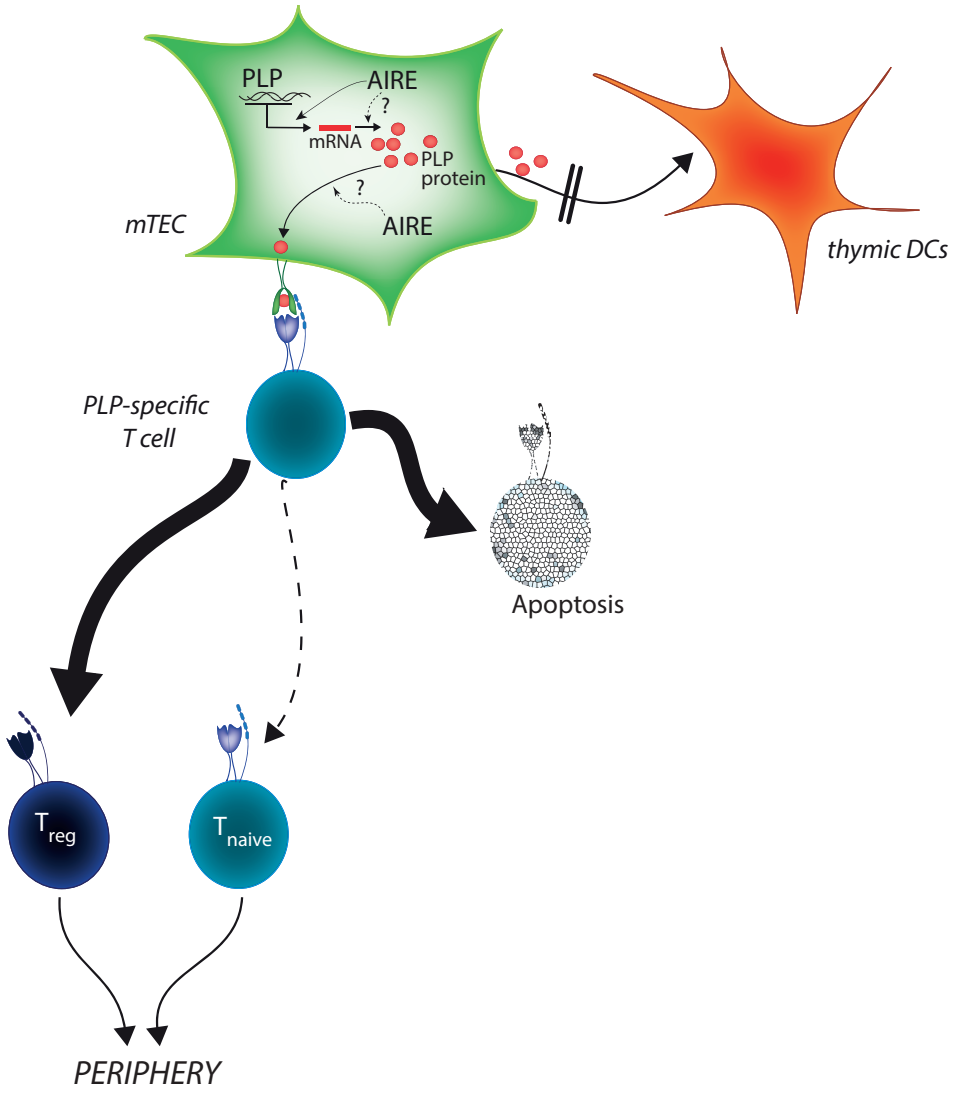

Figure 23: Graphical summary of tolerance induction to PLP in the young thymus. In the young thymus, expression of the antigen PLP only by mTECs is relevant for tolerance induction. Thymic DCs do not take part in central tolerance induction to PLP and antigen transfer from mTECs to DCs does not happen. The expression of PLP mRNA in mTEC^{hi} cells is dependent on AIRE, whether the translation of PLP protein also depends on AIRE is under investigation. To what extent the presentation of PLP relies on AIRE still needs to be elucidated but might have also an effect. In TCR-PLP1*Plp*^{WT} animals, the main portion of TCR-PLP1^{+} T cells that have seen PLP presented by mTECs are either redirected into the regulatory T cell lineage or are deleted by apoptosis. Only very few TCR-PLP1^{+} T cells escape thymic selection and egress into the periphery as naive T cells.

between young and old thymi, therefore, we conclude that changes in expression would not account for the loss of tolerance in adult TCR-PLP1*Plp*^{WT} mice (Fig. 22). Alternatively, an age-dependent decline of PLP protein synthesis could also be responsible for the loss of tolerance. At the moment, we are in the process of collecting material of thymic APC subsets mTEC^{lo}, mTEC^{hi}, cTECs, and thymic DCs from young and old animals to assess the amount of PLP protein by Western blotting.
We considered additional factors that may change with time causing a loss of tolerance induction to PLP with age: (1) Differences in the presentation of PLP in old thymi, (2) a different hormone status with adulthood , or (3) a change in the composition of thymic APCs and the size of niches.

(1) Besides a possible reduction of the PLP protein, the presentation capacity of mTECs could also be gradually reduced, eventually falling under a certain threshold where central tolerance induction fails. As we have observed a diminished but still present tolerance induction in TCR-PLP1*Plp*^{WT} *C2TA*^{kd} mice (Fig. 17), where MHCII surface molecules had been reduced to 10%, a gradual reduction of the presentation capacity of mTECs is unlikely to be the only reason for the entire loss of central tolerance to PLP. It is more likely that a combination of several effects abrogates the tolerance induction to PLP with age. To address reduced presentation of PLP, again, we would need to develop an *ex vivo* presentation assay with high sensitivity. The amount of PLP presented on *ex vivo* isolated mTECs may be under the detection threshold, as the expression of a given TRA seems to be confined to only a small fraction (only 1-3%) of mTECs [1, 80, 85, 182]. A sensitive assay that detects quantitative differences in the nature of TCR:pMHC contacts would therefore be needed. It might be possible to take advantage of measuring the Ca^{2+}-influx downstream of the TCR when TCR-PLP1^{+} T cells see their cognate antigen on APCs. Whether such an assay would be feasible for detection of PLP presentation on rare mTECs, or even the gradual loss of it, remains undetermined.

(2) Thymic involution is closely associated with immunosenescence, a degeneration of the immune system. It is widely accepted that with age the thymus undergoes changes called involution, which are characterised by a progressive decline in thymus size and structure, and eventually leads to the reduction in *de novo* generation of T cells [183]. It was published that sexual hormones, that rise with the entry into puberty, partly drive the decrease of thymic elements, eventually leading to age-related involution [184–188]. Especially a critical role of testosterone on thymic involution has been reported [185]. As tolerance to PLP in TCR-PLP1*Plp*^{WT} mice vanished at the age of 6 weeks, which is marked as the entrance to adulthood, we speculated whether modified levels of sexual hormones had evoked the change in tolerance induction. To counteract changes in the hormone levels by puberty, we castrated male TCR-PLP1*Plp*^{WT} mice, since it was described that various defects in stromal

turnover that accompanied involution were rapidly reversed following the ablation of the sex steroids [189]. However, we did not observe any change in the phenotype nor a delay in the loss of tolerance (data not shown). Also, no differences in tolerance induction to PLP between males and females were obvious. For this reason, we dismissed the explanation of a hormonal influence on thymic composition or cell interactions for the loss of tolerance.

(3) As third possibility, we considered that changes in the thymic composition could cause a loss of tolerance to PLP. These changes could be a) a change in thymic niches relevant for PLP, or b) a differential composition of AIRE-expressing cells. Developing thymocytes compose the largest cell population in the thymus. If T cell progenitors continue to ingress into the thymus while the thymus is not growing proportionally, thymocytes will then find themselves in a competitive situation for scanning all antigens displayed on mTECs and thymic DCs. Here, it is to be noted that the presentation of PLP is not enlarged by antigen hand-over to thymic DCs and, additionally, may further be restricted to a very limited number of mTECs due to stochastic fluctuations in promiscuous gene expression [99, 190]. It was found that the turnover of thymic epithelium happened quickly and frequently, but later diminished upon thymic involution [189]. Thus, it is possible that with a higher ratio of T cells : TECs, TCR-PLP1^{+} T cells start to compete for interaction with mTECs for negative selection in the medulla [191, 192]. Because of limited access to PLP on mTECs, intraclonal competition might then restrict the extent of induced tolerance to PLP.
Furthermore, the composition within the thymic epithelial cell population might be changing with time. The expression level of PLP on a per cell basis might be constant, but the abundance of cells able to induce tolerance might decrease with time in the thymus, causing the loss of phenotype in aged TCR-PLP1 mice. To test this hypothesis, we need a) to further analyse the composition of the thymic subsets in young versus old TCR-PLP1 transgenic animals discriminating between *Aire*$^{+/+}$ and *Aire*$^{-/-}$ subsets, and b) to measure the abundance of AIRE protein and PLP protein over time. In preliminary data, we have seen that the proportion of AIRE-expressing cells within the mTEC population diminished significantly within the first 2 weeks (data not shown). Thus, it is feasible to speculate that AIRE-expressing mTEC^{hi} become less numerous within an aging thymus. Moreover, it was described that AIRE expression has a pro-apoptotic effect, as AIRE^{+} mTECs in particular have a relatively fast turnover with an estimated half-life of 2 weeks [63, 193]. Therefore, we wondered if the loss of thymic tolerance after 6 weeks might be linked to a progressive loss of mTEC^{hi} cells or even only AIRE-expressing mTEC^{hi}. As we have seen that PLP expression in mTEC^{hi} was controlled by AIRE, a progressive loss of AIRE-expressing mTEC^{hi} would then also lead to a reduced PLP expression in the total thymus (see Fig. 24). Further investigations will help to clarify if the change in tolerance was due to less PLP presented or to fewer cells capable of presenting PLP efficiently. To formally test the crucial involvement of

mature AIRE-expressing mTEC^{hi}, we could block the development of mature mTEC^{hi}, as it was recently published that *in vivo* administration of an anti-RANKL antibody selectively blocked the generation of AIRE^{+} mTEC^{hi} [194]. Alternatively, TNF receptor-associated factor 6 (TRAF6)-deficient mice were shown to lack AIRE^{+} mTECs [46] and could represent a good tool to study the contribution of AIRE^{+} mTEC^{hi} for tolerance induction to PLP.

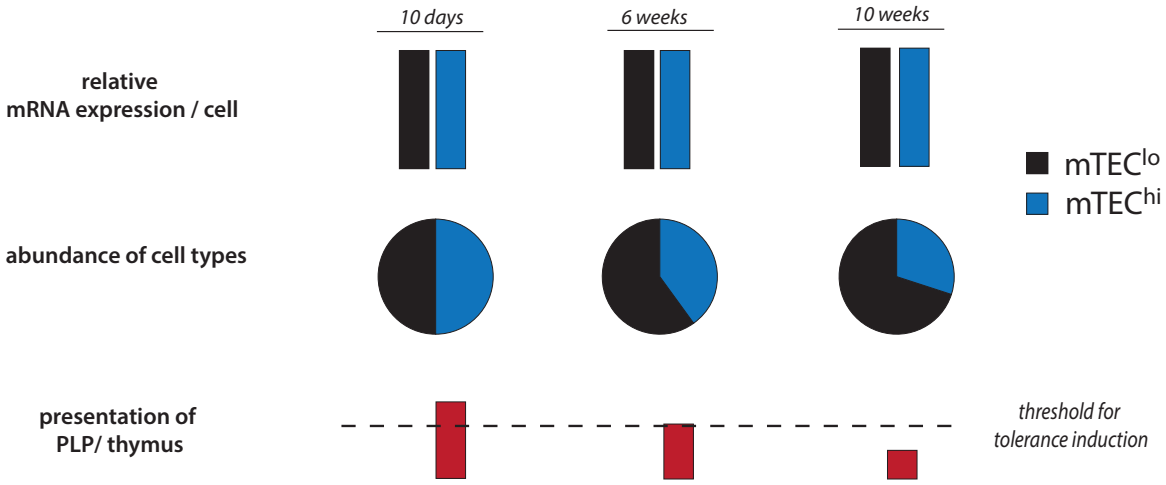

Figure 24: Hypothesised link between the composition of the thymic compartment in regards to a change in the ratio of mTEC^{lo} to mTEC^{hi} populations and PLP presented per thymus inducing tolerance, while PLP expression on a per cell basis remained constant.
First row: The relative mRNA expression of PLP/cell is depicted here for mTEC^{lo} cells in black and mTEC^{hi} cells in blue. The PLP expression did not change in these thymic subsets between young and old mice significantly, represented here as even bars.
Second row: Change of the ratio mTEC^{lo} : mTEC^{hi}. With aging, the relative abundance of the mTEC^{hi} population in all TECs is decreasing.
Third row: Hypothesised amount of PLP being presented in the thymus. With a decrease in mTEC^{hi}, the amount of presented PLP is reduced. Further assuming that there is a critical threshold of PLP presentation for efficient tolerance induction, the loss of tolerance with age in TCR-PLP1*Plp*^{WT} mice could be explained by a reduction of mTEC^{hi} cells able to present PLP to TCR-PLP1^{+} T cells.

4.5 Summary

We generated a TCR-transgenic mouse model which allowed us to visualise central tolerance mechanisms towards the endogenous tissue-restricted antigen PLP, which is expressed at physiological levels in the thymus. We found $PLP_{9\text{-}20}$-specific T cells to be tolerised by clonal deletion and concomitant deviation into the regulatory T cell lineage. Furthermore, presented data show that mTEC-driven expression of the endogenous antigen PLP mediates tolerance induction in an autonomous manner, whereas thymic DCs were dispensable for central tolerance induction to PLP. We could also provide the first evidence, that central tolerance might act in a certain time window: In the TCR-PLP1 mouse model, deletion of T cells started from day 3, while regulatory T cells accumulated not before 1 week after birth. At the age of 6 weeks we observed a breakdown of central tolerance induction. It remains to be determined if this loss of tolerance is linked to changes in thymic composition, and if there are additional tolerance mechanisms acting in the periphery to maintain tolerance.

5 Methods

5.1 Genotyping

For genotyping, mouse tails were digested in 50 µL digestion buffer for 5 hours at 55°C, followed by proteinase K heat inactivation at 95°C for 5 minutes. Subsequently, 1 µL of the digested tail DNA was used for genotyping. Genotyping reactions for the TCR-PLP1 α- and β-transgenes as well as for the PLP-gene were carried out using the TD58x30 program (see table 1). PCR reactions for genotyping CD11c-Cre, DTA, $Aire^{KO}$, $PLP^{fl/fl}$, Foxn1-Cre, and $C2TA^{kd}$ followed the PCR program TD54x30.

5.2 Immunisation

Mice were immunised with 50 µg to 100 µg peptide or protein emulsified in complete Freund's adjuvant (cFA). The amount of peptide was taken up in 50 µL PBS and mixed with 50 µL of cFA by sonification. 50 µL of this emulsion was subsequently injected subcutaneously into the footpad of the hindleg of the mouse. To analyse the T cell response in a proliferation assay, the immunised mouse was sacrificed 8-9 days post immunisation and the draining lymph nodes (poplietal and inguinal) were removed for analysis.

5.3 *In vitro* restimulation

After immunisation, the inguinal and poplietal lymph nodes were removed, pooled and single cell suspensions were cultured in 24-well plates at a concentration of 4 x 10^6/mL in the presence of 5 µg/mL $PLP_{9\text{-}20}$ peptide in cIMDM medium (see table 8). At day 3 and day 7 of culture, 20 U/mL recominbant human IL-2 (hIL2) were added and the culture volume was increased to 2 mL/well. T cells were restimulated every 10 days with 50.000 T cells/well

Table 1: PCR program used for genotyping.

Table 2: Genotyping program TD58x30.

Temperature	Time (min:sec)	
94℃	3:00	
94℃	0:45	|
64℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
62℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
60℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
58℃	0:45	|30x
72℃	1:00	|
72℃	5:00	
15℃	forever	

Table 3: Genotyping program TD54x30.

Temperature	Time (min:sec)	
94℃	3:00	
94℃	0:45	|
60℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
58℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
56℃	0:45	|2x
72℃	1:00	|
94℃	0:45	|
54℃	0:45	|30x
72℃	1:00	|
72℃	5:00	
15℃	forever	

with either 400.000 lethally irradiated (3000 rad) erythrocyte-depleted, syngenic splenocytes or 30.000 lethally irradiated bone marrow-derived dendritic cells together with cognate peptide. Again, at day 3 and day 7, 20 U/mL hIL2 were added.

5.4 ^{3}H-thymidine T cell proliferation assay

Proliferation of peripheral T cells was measured by incorporation of ^{3}H-thymidine. To this end, 4 x 10^{5} pooled lymph node cells were cultured together with 3 x 10^{4} irradiated BmDCs per well in cHL-1 medium with titrated amounts of peptide. T cells were cultured in the presence of cognate or non-cognate antigen for 4 days at 37℃ in round-bottom 96-well plates. As a control for the efficacy of immunisation, tuberculin purified protein derivate (PPD) was used since immunisations were carried out using cFA which contains part of *mycobacterium tuberculosis* in order to boost the immune response. T cell proliferation was quantified after 48 h of culture by pulsing cells with 1 µCi ^{3}H-thymidine/well for 20 h before harvesting cells onto filters and determining the amount of incorporated radioactive-

labelled thymidine using a BetaPlate liquid scintillation counter (Wallac, Gaithersburg, MD). Results shown are expressed as mean counts per minute of duplicates or triplicates.

5.5 Culture of bone marrow-derived dendritic cells (BmDCs)

Mice were anaesthetised and euthanised by cervical dislocation. Both hind legs were surgically removed, muscles were peeled off from tibia and femur and bones were transported in PBS. After sterilisation of the bones in 70% ethanol for 1 min, both ends of the bone were cut open and the bone marrow (Bm) was flushed out with the help of a syringe under sterile conditions. The Bm cell suspension was passed through a nylon cell strainer and red blood cells were lysed in 10 mL ammonium chloride solution. After 2 washing steps, cells were incubated in Petri dishes of 10 cm diameter in a concentration of 0.2 x 10^6 cells/mL and a starting volume of 10 mL of cIMDM. Of note, the Petri-dishes were not coated for cell culture use. For a GM-CSF culture, cIMDM was complemented with 10 ng/mL recombinant murine granulocyte/macrophages-colony stimulating factor (GM-CSF) (PeproTech). The primary cells were cultured for 8 days in complete IMDM medium replenishing the medium and cytokines after 3 and 6 days. At day 3, the culture volume was increased to 20 mL.

At day 6, it was possible to pulse BmDCs with protein, by adding the protein to the cell culture in the desired concentration for a minimum of 6 hours. Before harvesting the next day, BmDCs were matured by the addition of 300 ng/mL *E.coli*-derived LPS (Sigma). BmDCs were then either directly used in a proliferation assay or frozen for later use. To control the quality of the APCs, maturation markers as MHCII and CD80 can be monitored by flow cytometry.

5.6 Fusion of activated T cells to BW-NFAT-GFP

T cell hybridomas can be obtained by fusing activated T cells with the thymoma cell line BW5147 that lacks inherent functional TCR chains. BW-NFAT-GFP cells were used as fusion partner, which are a BW cell line that additionally carry a GFP reporter under the NFAT promoter (kind gift of Dr. Dominic van Essen, Institute de Recherche sur le Cancer et le Vieillissement, Nice). 3 days prior to fusion, T cells needed to be stimulated in order to be activated and highly proliferating at the time for fusion. T cells were mixed with the BW fusion partner in a ratio of 1 : 3. Then, 0.5 mL of pre-warmed polyethylene glycol 1500

(PEG1500, Roche) was added slowly in order to initiate the two cell types to fuse. The cell suspension was stirred gently for another minute before pre-warmed medium was added drop-wise to gradually dilute the PEG solution. After diluting out the PEG, the cells were taken up in fresh cIMDM and gently distributed onto flat-bottom 96-well plates (roughly 100 µL cell suspension/well). After 1 day of incubation, 2x HAT medium was added to start the selection process. Due to the addition of aminopterin, the DNA *de novo* synthesis in BW cells is blocked while fused BW cells have acquired the ability to synthesise DNA via the "salvage pathway". T cells alone cannot survive more than 5 days without continuous supply of IL-2 and stimulus, and subsequently, die as well.
On day $3-5$, most non-hybrid cells were dead, while hybrids start to grow in the selection medium. After 10 days of culture, T cell hybridomas can be grown first in cIMDM with HT only and after weaning them of the supplements hypoxanthine/thymidine in normal cIMDM.

5.7 Cloning

Genomic DNA was prepared from 1 x 10^6 D9-119-2 T hybridoma cells by digestion with proteinase K (Sigma), followed by phenol extraction and precipitation with ethanol. The specific primers that were used to amplify the genomic DNA encoding the TCR chains were designed to start 70 base pairs upstream from the start codon for the TCR Vα3.2 chain and 30 base pairs upstream for the TCR Vβ6 chain in order to include essential splice signals. Both primer pairs also introduced specific restriction sites for later cloning.

The genomic DNA encoding the VJα- and VDJβ-regions of the clone D9-119-2 TCR were then cloned into the unique XmaI/SacI restriction site of the pTα or the unique XhoI/SacII restriction site of the pTβ cassette vector, generating the pTαPLP1 and pTβPLP1 transgene expression constructs, respectively (Fig. 25). Cloning was verified by sequencing with primer pairs for the constant C-region together with region in the TCR variable Vα3.2 or TCR variable Vβ6, respectively. Vector sequences were removed by enzymatic digestion with restriction enzymes and these constructs were purified and sent for injection.

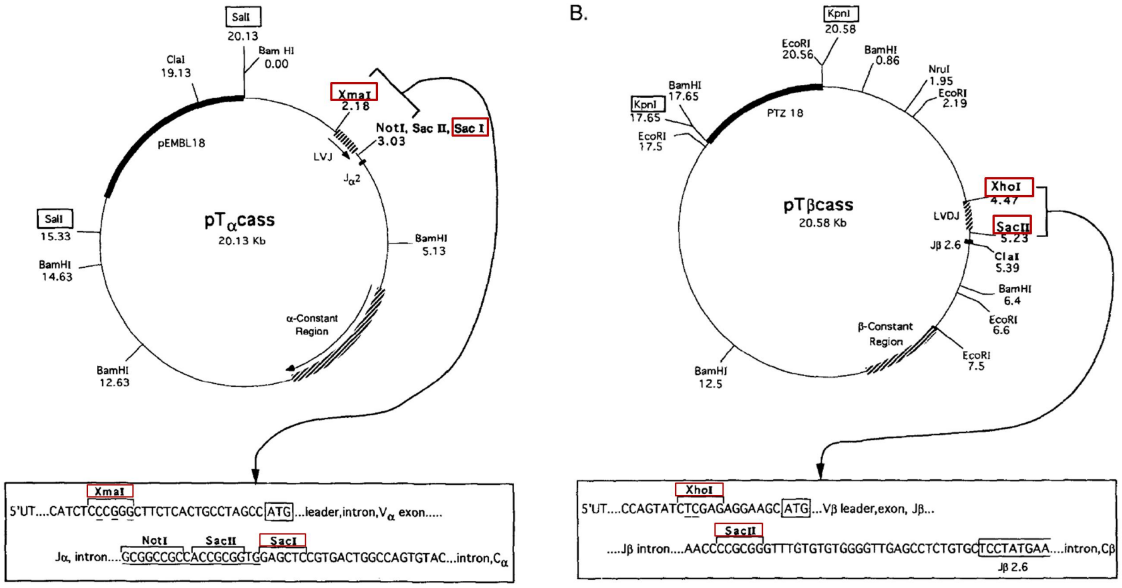

Figure 25: Scheme of pT cassette vectors with designated restriction sites for cloning. pTα cassette vector and pTβ cassette vector are shown schematically with designated restriction sites to clone the desired genomic variable TCR region. For pTα cassette, the restriction enzymes XmaI and SacI, for the pTβ cassette, the restriction enzymes XhoI and SacII were used. Scheme adapted from Kouskoff *et al.* (1995) [116].

5.8 Electroporation of transgenic constructs for verification into A5 cells

To stably express the transgenic pT cassette vectors in A5 or Dep cells, the cells were electroporated at 250 mV with 25 µg linearised pTβ cassette vector, 25 µg linearised pTα cassette vector, together with 5 µg linearised NFAT-GFP vector that contained a puromycin resistance gene. The electroporated cells were plated in a 10 mL dish over night and plated the next day into 96-well containing cDMEM medium supplemented with puromycin for selection. The cells that were restistant to puromycin treatment, were selected and analysed for TCR expression and stimulation.

5.9 IL-2 ELISA

The concentration of the cytokine IL-2 is often taken as measurement for stimulation and T cell activation. IL-2 concentration was determined in culture supernatants of T cell hybridoma cells stimulated by antigen primed BmDCs or splenocytes. The supernatant was taken for analysis 72 hours after stimulation with peptide by quantitative capture

ELISA according to the manufacturers guidelines (BD OptEIA™, BD Bioscience). Assays were developed with TMB Microwell Peroxidase Substrate and read at 450 nm.

5.10 Phenotypic analysis

For phenotypic analysis of TCR-PLP1 transgenic mice, thymus and spleen were comminuted between two glass slides, filtered with FACS buffer through a nylon mesh to remove cell clusters, and cell numbers were determined by counting the cell suspension using a CasyCounter machine (Innovatis).

5.11 Flow cytometric analysis

Surface staining was performed according to standard procedures at a density of 1-2 x 10^6 cells/ 50 µL and volumes were scaled up accordingly. Cells were labelled with the respective antibodies (see table 10) for 20 min on ice in the dark. Intracellular Foxp3 staining was performed according to manufacturers instruction of the staining kit (eBioscience). Flow cytometry measurements were performed on a FACS Canto II machine (Becton Dickinson) using FACS DIVA software (BD) and FlowJo software (Treestar inc. USA) for analysis.

5.12 Deoxyguanosine treatment

E14 to E16 thymic lobes were placed on 0.45-mm nylon membrane (Millipore) swimming in a 6-well culture plate filled with 6 mL of fresh 8% FCS supplemented cIMDM medium supplemented with 1.35 mM 2-deoxyguanosine for the removal of all bone marrow derived cells. The fetal thymic organ cultures (FTOCs) were incubated for 5 days prior to transplantation under the kidney capsule of mice.

5.13 Transplantations

Recipient mice, $4-6$ weeks of age, were narcotized with 200 µL/10 g body weight of a combination of ketamine (0.8 mg/mL, Ketavet, Pfizer) and xylazine (5 mg/mL, Rompun, Bayer, Germany) in PBS. Their eyes were protected from light and from drying out with

Bepanthen cream (Bayer, Germany). The site of incision was shaved and disinfected. A small incision was made through skin and peritoneum. The kidney was lifted out and fixed at an exposed position. The fragile kidney capsule was carefully scratched with a scalpel. Then, the capsule is lifted up with fine forceps and the fetal thymic lobe was slipped underneath the capsule. Then, the kidney is pushed back to its normal position, the sticky peritoneum pulled back together, and the skin is stapled with $2-3$ staples. Mice were sacrificed 3.5 weeks later, and the grown transplanted thymi were analysed by FACS.

5.14 Isolation of thymic antigen presenting cells

Thymi were harvested and cleared from connective tissue and fat, cut into very small pieces using scissors and resuspended in pre-warmed digestion medium. 1 mL digestion medium was used per thymus. Digestion was performed in a round-bottom tube at 37℃. The cell suspension was also pipetted up and down softly every 5 min to apply additionally some mechanical force for better and faster digestion. After 35-35 min the cell suspension was transferred to 4℃ and 0.01 volume of 0.5 M EDTA was added. After 5 min incubation time, the cell suspension was filtered and washed in FACS buffer and resuspended in Percoll™(ρ 1.115; GE Healthcare). A second layer of Percoll (ρ 1.055) and a third layer of FACS buffer was carefully added on top. After the layering, the gradient was centrifuged at 4℃ and 1350 g with slow acceleration and no break for 30 min. The upper interface, containing the desired low density cell fraction, was harvested and washed in FACS buffer. Cells were now ready for staining.

The Percoll density gradient solutions were prepared as follows:

Table 4: Percoll density gradient solution.

ρ 1.115 (High density Percoll)	ρ 1.055 (Low density Percoll)
9 vol. Percoll stock (ρ 1.134)	1 vol. Percoll (ρ 1.115)
1 vol. PBS (10x)	1.09 vol PBS (1x)
25 mM HEPES pH=7.2 (final)	

5.15 Assessment of EAE

Mice were examined for signs of EAE. The score of EAE was graded as follows:

Table 5: Classification of assessment of EAE score.

Score	appearance
0.5	tip of tail was flaccid
1	complete tail was flaccid
1.5	tail flaccid and one foot is weak
2	1 foot is weak and effect on the other leg can be seen.
2.5	one leg is completely limb
3	complete paralysis of both hind legs
3.5	mouse is moving on front legs only or even partially front leg paralysis; when mouse is on its side, it has problems getting up
4	no moving but eating
after score 4	the mouse was saccrificed.

5.16 RNA isolation and quantitative real time PCR (qRT-PCR)

Total RNA was isolated using the miRNeasy Kit (Qiagen). The concentration and quality of the isolated RNA was determined by NanoDrop ND1000 (Thermo Scientific) accepting a A260/A280 ratio of 2 ± 0.2. The total RNA was subsequently used for cDNA synthesis by iScript™cDNA Synthesis Kit (BioRad) in order to perform subsequently quantitative real-time PCR (qRT-PCR). Both steps were conducted according to manufacturers protocol. Quantification of mRNA expression was carried out using a C1000™ Thermal cycler from BioRad (CFX96 Real-Time System). Biorads CFX Manager software was used for analysis. Calculation of gene expression levels was carried out by using following formula:
Relative expression $= 2^{\Delta\Delta Ct}$
Ct values represent the threshold cycle for each transcript detected and ΔCt represents the difference between the threshold cycle for the housekeeping gene and the gene of interest ($housekeepinggene - geneofinterest$). As housekeeping gene, hypoxanthine guanine phosphoribosyltransferase, *HPRT*, was used. ΔΔCt values relate the ΔCt differences between the examined populations and the population used for normalisation (e.g. thymic DCs of 10-day old animals or $mTEC^{hi}$ of WT animals).

Program used for real time PCR:

Table 6: Protocol used for the real time PCR reactions.

Temperature	**Time** (min:s)	
95℃	0:30	
95℃	0:05	|
56℃	0:10	|40x
65℃	0:05	
95℃	decreasing the temperature in steps of 0.5℃ gradually	
15℃	0:20	
4℃	*forever*	

5.17 Statistical analysis

Data were first tested for normality using the Kolmogorov-Smirnow (KS) and Shapiro-Wilk test (Graphpad Prism Version 5.0c). All data with a group size large enough have passed the normality test positively. Some groups were too small in size to perform an assessement for normality, but were expected to distribute normally with a higher group size. Statistical significance for normally distributed data sets was then assessed by the two-tailed Students t-test. Significance was given when $p<0.05$.

6 Material

6.1 Mice

Mice were bred in the animal facility of the Institute of Immunology at the LMU Munich in individually ventilated cages under specific pathogen free (SPF) conditions. C57BL/6 (B6) is an inbred strain of the Institute of Immunology. *PLP*$^{-/-}$ mice were obtained from Klaus Nave from the Max-Planck-Institute for Experimental Medicine, Göttingen and are described in Klugmann *et al.* [133]. Δ DC mice were a kind gift of David Voehringer and are described in Ohnmacht *et al.* [154]; AIRE$^{-/-}$ mice have been described in Ramsey *et al.* (2002) [195]; *C2TA*$^{\mathrm{kd}}$ mice were generated by Hinterberger *et al.* (2010) and are described in [77]; Foxn1-Cre mice were described in Gordon (2007) [196]; PLP$^{\mathrm{fl/fl}}$ were kindly provided by Hauke Werner, Max Planck Institute for Experimental Medicine, Göttingen.
The TCR-PLP1 transgenic mice were generated during this work. Pro-nuclear injections for generating the TCR-PLP transgenic mouse, were performed by the transgenic animal facility of the Max-Planck-Institute of Molecular Cell Biology and Genetics in Dresden by Ronald Naumann. Local law regulation authorities approved all animal experiments.

6.2 PLP peptides

PLP peptides were purchased at BioTrend in a quantity of 20 mg and a purity of >80% (HPLC).

Table 7: Sequences of synthetic PLP peptides.

PLP peptide	Sequence
$PLP_{1\text{-}24}$	GLLECCARCLVGAPFASLVATGLC
$PLP_{160\text{-}184}$	VVWLLVFACSAVPVYIYFNTWTTCQ
$PLP_{192\text{-}216}$	TSASIGSLCADARMYGVLPWNAFPG
$PLP_{224\text{-}248}$	LSICKTAEFQMTFHLFIAAFVGAAA
$PLP_{9\text{-}20}$	CLVGAPFASLVA
$PLP_{172\text{-}183}$	PVYIYFNTWTTC

6.3 Cell culture media with supplements

For all primary cell cultures, IMDM medium supplemented with the following reagents was used and is in the following referred to as complete IMDM (cIMDM) medium. HL-1 medium resembling cIMDM medium without the addition of FCS was used for proliferation assays. Digestion medium including a mix of collagenase/dispase was crucial for TEC isolation. cIMDM was supplemented with HAT as selection medium for freshly generated T cell hybridoma.

Table 8: Ingredients for cell culture media complemented with the listed supplements.

Medium	supplements
cIMDM	500 mL IMDM medium containing L-glutamine (GE Healthcare) 8% FCS (BioChrome) 1% L-glutamine (200 mM) with penicillin/streptomycine (100x) (PAA) 1% MEM non-essential amino acids (100x) (PAA) 1 mM sodium pyruvate (Gibco) 50 µM β-Mercaptoethanol (Gibco)

Medium	supplements
cHL-1	500 mL HL-1 medium (Whittaker) 1% L-glutamine (200 mM) with penicillin/streptomycine (100x) (PAA) 1% MEM non-essential amino acids (100x) (PAA) 1 mM sodium pyruvate (Gibco) 50 µM β-Mercaptoethanol (Gibco)
Digestion medium for TEC isolation	500 mL RPMI medium 0.2 mg/mL Collagenase (Roche) 0.2 mg/mL Dispase I (Roche) 2% FCS 25 mM HEPES (pH7.2) 25 µg/mL DNase I (Roche)
HAT-selection medium	500 mL cIMDM medium 12 mL hypoxanthine/thymidine (HT) 0.6 mL aminopterine

6.4 Buffers and solutions

All buffers and solutions were made with water purified in a Millipore filter system (pore size 0.22 μm) (Biocel). All chemicals met the highest purity level. Established chemicals and laboratory equipment, which are not listed in the following, were obtained from Roth, Sigma-Aldrich, Merck, Boerhinger Mannheim, Invitrogen and new England Biolabs inc.

Table 9: Composition of buffers and solutions.

Name	Composition
Gitocher digestion buffer (10x)	670 mM Tris pH 8.8 166 mM ammonium sulfate 65 mM $MgCl_2$ 0.1% gelatin
Digestion buffer for tails	3 µL proteinase K (10 mg/mL stock) 2.5 µL Triton (10% stock) 5 µL Gitocher Buffer (10x) 0.5 µL β-Mercaptoethanol 39 µL H_2O

Name	Composition
PCR Red-buffer (5x)	250 mM KCl 50 mM Tris pH 8.3 43% glycerol 7.5 mM $MgCl_2$ 2 mM Cresol Red
PBS (10x)	800 g NaCl 20 g KCl 115 g $Na_2HPO_4 \cdot 2\ H_2O$ 20 g KH_2PO_4 pH adjusted to $7.2 - 7.4$
FACS Buffer	1x PBS 2% FCS 2 mM EDTA
Aminopterin (1000x)	17.6 mg aminopterine 5 mL 0.1 N NaOH in 90 mL *Aqua bidest* neutralise with 0.1 N HCl keep at -20℃ in the dark
HT	272.2 mg hypoxanthine (2 mM) 77.5 mg thymidine (3.2 mM) in 100 mL *Aqua bidest* heat up to 50℃ add NaOH until powder dissolves

6.5 Antibodies

Table 10: List of all antibodies used in this study. All antibodies are listed with the respective clone and conjugated fluorophore.

Specificity	Conjugate	Clone	Supplier
CD4	APCCy7	GK1.5	Biolegend
CD4	V500	RM-4-5	BD
CD8	PE/Cy5	$53 - 6.7$	Biolegend
CD24	Pacific Blue	M1/69	eBioscience
CD25	PE/Cy7	PC61	Biolegend
CD44	APC-Cy7 and APC	IM7	Biolegend
CD62L	APC	MEL-14	Biolegend

Specificity	**Conjugate**	**Clone**	**Supplier**
CD69	PE-Cy7	H1.2F3	Biolegend
Foxp3	APC	FJK-16s	eBioscience
TCR Vα2	PE and Biotin	B20.1	BD
TCR Vα3.2	FITC and Biotin	RR3-16	BD
TCR Vα8.3	FITC	B21.14	BD
TCR Vα11.1/11.2	PE	RR8-1	BD
TCR Vβ2	FITC and PE	H57-597	BD
TCR Vβ3	FITC	KJ25	BD
TCR Vβ4	FITC and PE	KT4	BD
TCR Vβ5.1	FITC and Biotin	MR9-4	BD
TCR Vβ6	FITC and PE	RR4-7	Biolegend and BD
TCR Vβ7	PE	TR310	BD
TCR Vβ8.1/8.2	FITC	KJ16-133	eBioscience
TCR Vβ8.3	PE	IB3.3	BD
TCR Vβ10	PE	B21.5	BD
TCR Vβ11	FITC	RR3-15	BD
TCR Vβ11	PE	KT11	Biolegend
TCR Vβ13	FITC	MR12-3	BD
TCR Vβ12	Biotin	MR11-1	BD
TCR Vβ14	FITC and Biotin	14-2	BD
TCR Vβ17	FITC	KJ23	BD

6.6 Primer list

Primers used for genotyping:

Table 11: List of all primers used for genotyping. Sequence of forward and reverse primers are indicated as fwd and rev, respectively.

Genotype		**Sequence** $5' \rightarrow 3'$
PLP^{WT}	fwd	GAAAGGTTCCATGGTCAAGG
PLP^{WT}	rev	CTGTTTTGCGGCTGACTTTG
PLP^{KO}	rev	CTTGCCGAATATCATGGTGG
TCR-PLP1 Vα3.2	fwd	ACAACAGAGCTGCAGCCTTC
TCR-PLP1 Vα3.2	rev	GCAGTGCTAGGAAGGGCGGC
TCR-PLP1 Vβ6	fwd	CCCAGAGCCAAAGAAAGTC
TCR-PLP1 Vβ6	rev	AGCCTGGTCCCTGAGCCGAA
PLP-floxed	fwd	GACATAGCCCTCAGTGTTCAGG
PLP-floxed	rev	GAATCCTGCATGGACAGACAG
$C2TA^{kd}$	fwd	TAAATTCTGGCTGGCGTGG
$C2TA^{kd}$	rev	ACCGGACTAGTGGAAAAGCGCCTCCCCTACC
CD11c-Cre	fwd	CGATGCAACGAGTGATGAGG
CD11c-Cre	rev	GCATTGCTGTCACTTGGTCGT
DTA	fwd	TACATCGCATCTTGGCCACG
DTA	rev	CCGACAATAAATACGACGCTG
Foxn1-Cre	fwd	CTCTCCTCCGAGTATCCAATCTG
Foxn1-Cre	rev	CCCTCACATCCTCAGGTTCAG
AIRE-KO	fwd	AAGCCGTCCAGGATGCTAT
AIRE-KO	int	GTCATGTTGACGGATCCAGGGTA
AIRE-KO	rev	AGACTAGGTGTTCCCTCCCAACC

Primers used for real-time PCR, sequencing and cloning into the pT cassette vectors:

Table 12: List of all primers used for quantitative Real-time PCR and cloning. Sequence of forward and reverse primers are indicated as fwd and rev, respectively.

Genotype		**Sequence** $5' \rightarrow 3'$
PLP qRT-PCR	fwd	GGGCTTGTTAGAGTGTTGTGC
PLP qRT-PCR	rev	GAAGAAGAAAGAGGCAGTTCCA
HPRT qRT-PCR	fwd	TGAAGAGCTACTGTAATGATCAGTCAAC
HPRT qRT-PCR	rev	AGCAAGCTTGCAACCTTACCA
Vα3.2	fwd	TCCTACTTTGGGACACCT
sequencing	rev	TTTCGGCACATTGATTT
Vβ6	fwd	TGCTGGGTAACCCTTTG
sequencing	rev	ACCTCCTTGCCATTCAC
pTα cassette	fwd	ATCCCGGGCTTTCCTGCCTGTCCTGTTCCA
cloning	rev	ATGAGCTCGCAGTGCTAGGAAGGGCGGC
pTβ cassette	fwd	AAGTCGACCCAGAGCCAAAGAAAGTC
cloning	rev	TACCGCGGAGCCTGGTCCCTGAGCCGAA

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