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Progressive maturation of CD4 single positive thymocytes

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SUMMARY

T cells play an important role in adaptive immune response and contribute in both cellular and humoral defense against pathogens. The development of T cells takes place in a specialized organ, the thymus. Thymic microenvironment facilitates instruction of hematopoietic progenitor cells into thymocyte lineage, maturation of thymocytes to CD4 and CD8 T cells and generation of self-tolerant T cell receptor repertoire.

In this study, we investigated the thymocyte intrinsic molecular players in the maturation of CD4 T cells and the formation of regulatory T cell (Treg) lineage. We initiated our study from the finding that the inclination toward Treg lineage decreases with the maturation of CD4 T cells. Upon microarray analysis of the gene expression between three CD4 thymocyte maturation stages, we selected nine candidate genes and addressed their roles in lentiviral gain- and loss-of-function bone marrow chimera experiments.

We identified two genes that influenced Treg development in our experimental system, Lztfl1 and Nedd4. We demonstrated that the overexpression of Lztfl1 (leucine zipper transcription factor like-1) abolishes thymocyte entry into the regulatory T cell lineage. We evidenced this in the polyclonal repertoire and observed a tendency that this could hold true for the thymocyte deviation in the presence of its cognate antigen.

Further, we evidenced that the deficiency in the second candidate gene Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4) resulted in increased propensity toward Treg lineage.

In summary, we investigated the roles of intrinsic factors in the T cell development and were able to show that the different CD4 T cell maturation stages carry different molecular signatures. The results of this study add two molecules, Lztfl1 and Nedd4 on the list of factors that influence thymic regulatory T cell development. Altogether, this study shows that the thymocyte intrinsic factors play similarly important role as antigen-presenting cells and cytokine microenvironment in the T cell development.

ZUSAMMENFASSUNG

T-Zellen spielen eine wichtige Rolle in der adaptiven Immunantwort und partizipieren an der zellulären und humoralen Abwehr gegen Krankheitserreger. Die Entwicklung von T-Zellen erfolgt in einem speziellen Organ, dem Thymus. Das thymische Mikromillieu gewährleistet eine Reifung hämatopoetischer Vorläuferzellen in T Zell-Vorstufen und eine weitere Differenzierung dieser Vorstufen zu CD4- und CD8-T-Zellen mit einem selbst-toleranten T-Zell-Rezeptor Repertoire.

In dieser Studie untersuchten wir thymozyten-intrinsische Faktoren bei der Reifung von CD4 T-Zellen und der Bildung von regulatorischen T-Zellen (Treg-Zellen). Grundlage unserer Studie ist die Erkenntnis, dass die Neigung der Vorläuferzellen die Treg-Entwicklung einzuschlagen mit der Reifung von CD4 T-Zellen progredient abnimmt. Nach einer Microarray-Analyse der Genexpression zwischen drei verschiedenen Refungsstadien $CD4^+$ Thymozyten, wählten wir neun Kandidatengene aus und validierten ihre Funktionalität bei der Treg-Entwicklung mithilfe lentiviral transduzierter Knochenmarkschimären.

Wir identifizierten zwei Gene, Lztfl1 und Nedd4, die in lentiviral transduzierten Knochenmarkschimären Treg-Entwicklung maßgeblich beeinflussten. In unseren Experimenten hinderte die Überexpression von Lztfl1 (leucin-zipper-transcriptionsfactor like-1) die Thymozyten am Eintritt zur Differenzierung in regulatorischen T-Zell-Linie. Diese Beobachtung evaluierten wir sowohl innnerhalb eines polyklonalen T Zell Repertoires also auch in Gegenwart eines definierten Modellantigens.
Ferner belegen wir, dass eine Defizienz im zweiten Kandidatgen, Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4), zu einer erhöhten Präferenz der Vorläufer T Zelle zum Eintritt in die Treg-Linie führt.

Zusammenfassend, haben wir die Rollen von intrinsischen Faktoren bei der T-Zellentwicklung analysiert und konnten zeigen, dass die verschiedenen CD4 T-Zell-

Reifungsstufen unterschiedliche molekulare Signaturen tragen. Die Ergebnisse dieser Studie identifizieren zwei weitere Moleküle, Lztfl1 und Nedd4, als wesentliche Komponenten einer suffizienten Entwicklung regulatorischer T-Zellen. Insgesamt zeigt diese Studie, dass die thymozyten-intrinsischen Faktoren ähnlich wichtige Rolle spielen wie die antigen-präsentierende Zellen und Zytokin-Mikroumgebung.

INTRODUCTION

The immune system is an essential part of multicellular organisms. It is a system through which each organism is able to protect itself from infection. It operates on several processes simultaneously in order to provide immune defense. The highly developed immune system is comprised of mechanisms from nonspecific chemical defense, such as the complement system and antimicrobial peptides to the cellular-effector defense, such as the cytotoxic T lymphocytes, most of which are vital for survival. The two major branches of the immune system are the innate and adaptive immune systems. The innate immune system is evolutionary older and based on less diverse and simple recognition and defense mechanisms, while the adaptive immune system uses highly specific and mechanistically further developed processes [1, 2]. The innate immune system recognizes evolutionary conserved, pathogen-associated molecular patterns by utilizing germ-line encoded receptors. Although these receptors offer an important first line of defense and their variety provides recognition of multiple pathogenic structures, for complete immune surveillance in a complex multicellular organism, the innate immune system is not sufficient [3]. As microorganisms constantly improve their ability to infect a host and evade its immune system, the evolutionary pressure has facilitated the development of a further palette of defense strategies, like the adaptive immune system [2].

The major hallmark of the adaptive immune system is its significant variability in terms of pathogen recognition. The adaptive immune system is based on a network of different lymphocyte populations, T and B cells and their major antigen-presenting cells, dendritic cells. Further, the unique ability of B and T cells is the recombination of defined genomic, germ-line encoded elements into the receptors used for pathogen recognition. Through this error-prone, non-homologous, end-joining process, the adoptive immune system generates a vast variability of receptors specific to an enormous palette of potential antigens. Through this diversification of germ-line encoded elements, an enormous number of clones with defined specificity are generated and released in the periphery of the organism for immune surveillance. In addition to the flexibility of the generated repertoire is the ability to improve and increase specificity towards an actual

infecting pathogen upon successful cross talk between involved lymphocytes. Therefore, the adoptive immune system is an orchestrated approach, utilizing highly specific pathogen recognition receptors, based on professional antigen-presentation and multicellular cooperation to fight infection and memorize it for an efficient eradication in case of reoccurring infection [1, 4].

The generation of such a vast repertoire is based on the ability of developing lymphocytes to rearrange V (variable) - D (diversity) - J (joining) immunoglobulin gene segments in a very random fashion. One important caveat in this development is the generation of potentially self-recognizing receptors that would have to be removed from the repertoire. For this scenario, evolution has built several checkpoints at the developmental level to secure the functionality of these rearrangements and the tolerance toward self [5, 6].

From the evolutionary point of view, the origin of somatic diversification may lay in the need to differ between autochthonous parts (resident or 'self' epotopes) and allochthonous parts (passenger, pathogenic, 'nonself') in a mutualistic system between two organisms, e.g. in the gut of a vertebrate which requires bacteria for vitamin production. Additionally, somatic hypermutation offers an efficient way to form a diverse repertoire without vast up-scaling of the genome [1].

One of the major players in the adaptive immune system is the T lymphocyte. T lymphocytes develop from their precursor, coming from the bone marrow into an organ, dedicated for T cell development, the thymus. The thymus is together with the bone marrow, the primary lymphatic organ that provides microenvironment for T cell development. The evolution of the thymus dates most likely to the evolution of VDJ recombination machinery, as it is only found in the species that perform these recombination steps, at earliest in the jawed vertebrates [7, 8].

In the early sixties, general opinion supported by Sir Medawar was that the thymus playes no immunological role and that the presence of lymphocytes in this organ was due to an 'evolutionary accident' [9]. In the experiments with thymectomyzed mice, several researchers were able to demonstrate that this prevented lymphocytic leukemia, and it was further observed that mice thymectomyzed at birth lacked lymphocytes in the blood and all other lymphoid tissues. Furthermore, these mice seemed unhealthy and

showed rapid wasting [10, 11]. The first demonstration of immunological role of thymus was the prevention of the wasting in thymectomyzed mice by thymus transplantation [11]. Additionally, the insight into the tolerogenic role of the thymus was the fact that thymectomyzed mice did not reject the allogeneic skin transplants. Under further examination, it became clear that thymectomy led to only partial loss of lymphocytes and this introduced the idea that the immune system consists of the thymus-dependent and -independent branches [12-14].

Today we know that the thymus arises from the third pharyngeal pouch and that it is essential for the functional immune system as it forms a niche through which signals for T cell lymphopoiesis are available and where non-hematopoietic and hematopoietic cells communicate to keep this process ongoing [8, 15]. The scaffold of thymic stromal cells provide signals to the arriving progenitors to commit to the T cell lineage, guiding them to the rearrangement of their antigen receptors. They probe these for functionality and specificity to self and provide survival signals for those that pass all the checkpoints and are ready to exit into the periphery.

In general, the thymus is composed mostly of developing T lymphocytes. Other hematopoietic cells are a few B cells, macrophages and dendritic cells. The non-hematopoietic compartment is represented by cortical and medullary thymic epithelial cells (TECs) and mesenchymal cells, like fibroblasts and endothelial cells [15]. The morphological compartmentalization shows the outer, darker zone – the cortex and the inner, lighter zone – the medulla. Thymic cortical and medullary epithelial cells play indispensable roles in the T cell development and mirror two major checkpoints, positive and negative selection of developing thymocytes. The thymic stroma is far from a solely structural scaffold but provides signals that drive T cell development.

T cell development

T cells, different to other hematopoietic cells, do not develop in the bone marrow but in the thymus. Common-lymphoid progenitors from the blood enter the thymus and mature to early thymic progenitors that further develop to T cells [16, 17]. The early thymic precursors form a very small population with limited to absent self-renewal potential that gives rise to further mature T lymphocytes. Due to this, T cell lymphopoiesis is dependent on the recruitment of these precursors from the bone marrow [18]. This entry

of precursors is a gated phenomenon, involving a variety of cytokines such as CXCL12, CCL21 and CCL25 and several adhesion molecules P-selectin, VCAM-1, MadCAM-1, ICAM-1, laminin and fibronectin [19-23]. The new arrivals enter the thymus at the cortico-medullary junction through the high endothelial venules and are the earliest T cell progenitors [24, 25]. These cells at the cortico-medulary junction are known as the first of four so-called, double negative populations of developing thymocytes, DN1. They are phenotypically described as $cKit^{+}CD4^{-/low}CD8^{-}CD3^{-}CD25^{-}CD44^{+}$ and are heterogeneous in their expression of CCR9, Flt3 and CD24 [26-28]. Upon their entrance into the thymus, DN1 cells remain at the cortico-medullary junction via CCL19/CCR7 retention and perform up to ten cell divisions, mainly led by cKit/SCF, IL7/IL7R and Hedgehog signaling over Smoothened receptor [29-32]. In this region, cortical stromal cells express high levels of Delta-like molecules 1 and 4, (Dll1 and Dll4) which are essential for the signaling to the Notch1 receptor on the DN1 population and specification to the T cell lineage [33]. This communication is essential for the T cell development as experiments with inactivation of Notch1 or its downstream transcription factor recombination signal binding protein J (RBP-J) leads to complete abrogation of T cell development in favor of ectopic B cell development [34, 35]. The processes happening upon the Notch1/Dll1 and Dll4 interactions in DN1 cells are still unknown.
Continuing their proliferation, T cell lineage specification and moving toward inner cortex DN1 cells acquire their DN2 phenotype, $CD25^{+}CD44^{+}$. This relocation is based on CXCL12 and CXCR4 interaction and leads cells into the region with high IL7 and SCF availability, which in turn further promotes proliferation and survival and most importantly induces the expression of Rag (recombination-activating protein) enzymes [36-39]. The Rag enzymes are responsible for the recombination of VDJ somatic segments and this is the point at which TCR rearrangement starts. Firstly, TCRγ and TCRδ are rearranged [40]. Interestingly, it has been shown that IL7 plays a role in the timey opening of the TCRγ locus upon Stat5 signaling and histone acetylation, as the recombination of this locus is blocked in IL7R deficient mouse [41, 42]. Continuing to the subcapsular zone, DN2 cells transit to the DN3 stage ($CD25^{+}CD44^{-}$), characteristic for ongoing TCRβ rearrangements and irreversible commitment to the T cell lineage. In this stage, rearrangement of TCRβ takes place in two major steps. Firstly, on both

alleles in parallel D-J fragment recombination takes place. Secondly, at only one allele, rearranged DJ fragment recombine to V fragment. The second part of the process is termed ‘allelic exclusion’ and reassures that the cell expresses only one in/frame rearranged TCRβ chain [43-45]. Subsequently, DN3 cells undergo the process of β selection, test of functionality for the newly rearranged TCRβ chain. For this, TCRβ chain couples with the invariant preTCRα chain, CD3δ, ε, γ and its successful ligand-independent signaling results in survival of a cell and acquisition of DN4 ($CD25^{-}CD44^{-}$) / pre-DP stage [46-48].

At the pre-DP stage, cells expand enormously and migrate toward the outermost cortical region. The expression of Rag enzymes is kept relatively low during the expansion phase in order to avoid any further rearrangements of preselected β chains. In this fashion, a pool of cells carrying functional β chains expands and generates a number of cells that will independently rearrange α locus and therefore enrich the TCRαβ repertoire [49, 50]. Upon accomplished TCR α locus recombination, cells acquire DP ($CD4^{+}CD8^{+}$) phenotype and start inward migration through cortex to the medulla, over the next two important checkpoints, the positive and negative selection.

Positive selection

In the process of positive, selection heterodimeric TCRαβ molecules interact with the peptide / MHC molecule complexes on the cortical thymic epithelial cells. Through this process, the thymus ensures that the repertoire generated through primarily random rearrangement is useful to the host, as it is firstly restricted to the self-MHC and will be able to recognize peptides presented within these complexes. The binding strength of this interaction results in signaling over TCR to either allow the cell to survive, or if the newly generated TCR shows no or low ability to bind this complex, the cell will undergo apoptosis, in a process of 'death by neglect' [51, 52]. Receiving a positive signal, the selected cell will downregulate and repress Rag enzymes, express survival molecules Bcl-xL, RORγ, TCF-1 and proceed the migration toward the medulla [53-55].

The process of positive selection was first described in the F1 bone marrow chimeras, in which T cells develop only if the peptide is presented on MHC molecules that are the same as the bone marrow recipient [56]. Further and more direct evidence was provided by TCR transgenic models, in which TCR transgenic T cells developed solely in the thymus of the same haplotype that was used to identify that particular TCR in the first place [57].

Although the CDR1 and CDR2 loops of TCR have inherent affinity for MHC molecules, controlled by germline-encoded amino acids in these regions, the chance of generating a self-MHC-restricted TCR is very low [58-61]. Therefore, DP cells are allowed to rearrange TCRα locus several times in order to pass positive selection. This process is limited by the amount of time between three and four days that each DP cell is given to accomplish multiple rearrangements and pass to the medulla for the next checkpoint [62]. The interaction between TCR and peptide-MHC complex is driven by the contact of conserved amino acid residues between Vα and Vβ TCR chains and MHC helices [63, 64]. In this manner, the CDR3 region of TCR is positioned to interact with the presented peptide.

One of the most fascinating questions in the study of positive selection is the nature of ligands presented on the MHC molecules. As MHC molecules present mostly self-peptides, it is of high interest to understand how the number, origin and similarity between these peptides resemble the real antigen.

Nowadays, it is mainly contended that all positively selecting peptides derived from different models and described as cognate agonists, antagonists, self-antigens or altered peptide ligands activate different molecular networks below TCR or perform this activation with different quality. Therefore, one TCR can in recognition of diverse peptides lead to different T cell response. The TCR signaling has probably evolved to this complexity in order to integrate all signals percepted via single TCR and to separate high-affinity signals from low-affinity signals important for the development and homeostasis [65-70].

The threshold of TCR/MHC interaction in the positive and negative selection has been a longstanding question in immunology. Today, we appreciate that the affinity of the peptide ligand, kinetics and location of Ca^{2+} and Erk signaling play a role [71, 72].

Our understanding of how TCR threshold instructs T cells at the selection point is mainly based on the work using a palette of OVA peptide variants and OTI TCR in FTOC, performed by the Palmer lab [73].

These experiments provided a major picture of TCR signaling involved in positive and negative selection. The investigators designed variants of OTI TCR agonist peptide (OVA variants) to define boundary between positive and negative selection and to study how small changes in affinity translate into cellular fate. They observed how developing thymocytes differ at the levels, rate and localization of CD3ζ, ZAP70, LAT and Erk phosphorylation upon exposure to a palette of variants (negative and positive selectors) with different affinities. They observed that negative selectors induced very rapid Ca^{2+} flux, and faster CD3ζ and ZAP70 phosphorylation compared to the positive selecting ones. Further, negative selectors induced faster LAT phosphorylation, peaking early in the signal transduction, while positive selectors provoked steadier phosphorylation at later points of interaction. Additionally, negative-selecting ligands induced phosphorylation of Erk at the plasma membrane, whereas in positive selection it happened in the cytoplasm [73].

CD4 versus CD8 lineage commitment

CD4 and CD8 molecules are TCR correctors and bind to MHC II and MHC I respectively and exclusively. Their major role is the promotion of TCR signaling via recruiting of the downstream signaling molecules [74].

During the positive selection, cells express both TCR correctors, CD4 and CD8 to allow survival of all thymocyte with successfully rearranged and self-restricted TCRs. Further, depending on whether the selected cell has MHCI – or MHCII – restricted TCR, it will develop into a CD8 or CD4 SP thymocyte. Although heavily investigated, how this process is regulated is still a matter of debate. Firstly, two classical models were described: the stochastic and the instructive model. The stochastic model proposes that the expression of one or other corrector terminates randomly, and that, consequently, through a TCR/driven rescue step, only those cells left with the right corrector will survive [75, 76]. This model is mainly based on the experiments in which corrector expression is enforced or the silencing is ablated. In those models, cells with any of these interferences would appear in the 'false' population. Nevertheless, in none of these experiments, the 'false commitment' rate was about 50% of what would be expected by the model. Additionally, this model proposes that the positively selected thymocyte would be susceptible to cell death in the expectation of the second, TCR-driven rescue step, which was not evidenced, as these corrector 'mismatched' thymocytes develop normally into mature T cells.

Strength-of-signal instructional model provides another explanation to the lineage commitment problem. This model starts from the information that the cytosolic tail of CD4 molecule binds more Lck and would be therefore able to provide a stronger signal than that of the CD8 corrector [77, 78]. According to this model, the strong signal integrated from TCR-corrector engagements would result in CD4 lineage, while signals of lower strength would produce CD8 T cells. Similarly, the duration-of-signal model, proposes that longer TCR interactions lead to downregulation of CD8 expression and result in CD4 lineage commitment, while short ones terminate CD4 expression [79].

Although all classical models provide some insight into the commitment, they have all been contradicted in diverse experimental setups.

Another model, the kinetic signaling model provides probably the best explanation to this phenomenon. This model postulates that the lineage commitment depends on the duration of the TCR signal, as sensors of duration serve the common γ chain cytokines. Further, the kinetic signaling model is based on the observation that positively selected DP thymocytes ceased their expression of CD8 but not CD4 and therefore, $CD4^{+}CD8^{lo}$

cells still remained lineage uncommitted. If the signals provided by engaged TCR would remain, while their intensity in CD8 expression deceases, that cell would commit to the CD4 lineage, as CD8 signaling would obviously have no impact on the ongoing process. On the other hand, if the signaling would lose its intensity, the T cell would re-express CD8 and downregulate CD4 [80, 81].

The network of transcription factors behind this process has been a matter of study for a long time. One of the major components is a molecule that initiates CD8 downregulation, ThPOK. ThPOK is also important for the complete establishment of the CD4 lineage [82-84]. Further, Runx3 has been described as the master regulator of CD8 lineage as it silences CD4 locus [85-87].

Negative selection and immunological tolerance mechanisms

The idea of tolerance 'acquisition' during the immune system development was introduces by the work of the Medawars laboratory in the 1953 [88]. This idea developed through their observations that *in utero* inoculations of mouse embryos with an autologous cell suspension, resulted in a partial or complete tolerance to following skin transplantation from the donor of the cell suspension to the grown up, previously inoculated recipient.

The mechanisms of tolerance induction were thoroughly studied for more than twenty years, when the work of Kappler and Marrack offered strong evidence for the clonal elimination of self-reactive thymocytes [89]. In this work, T cells bearing the Vβ17a TCR chain were eliminated in the presence of superantigens deriving from mouse mammary tumor viruses presented in the context of I-E MHC molecules. Following this work, Palmer's group added further evidence for clonal deletion toward contemporarily known self-antigens, products of Mtv-8 and Mtv-9 for deletion of Vβ5 and Vβ11 TCR clones [90]. Through this work the tolerance idea was based on clonal deletion, but soon it emerged that different mechanisms may be needed to enforce complete tolerance, as clonal deletion is often incomplete.

The general knowledge of tolerance in the immune system is definitely based on the described work, but it has been strengthened by the discovery of two further phenomena – the promiscuous gene expression in the thymus and the development of regulatory T cells as mechanism of instructive tolerance [91-93].

The process of negative selection is often illustrated as the second step in the continuum of T cell repertoire formation. Within this step, thymic microenvironment, mainly by action of mTEC and dendritic cells, sorts out thymocytes with strong reactivity to presented self-antigens. Negative selection ensures generation of T cell repertoire with tolerance to self, meaning that the recognition repertoire of newly produced T cells, should lack all autoreactive T cells and therefore be of no potential harm to the organism [94]. In addition to this layer of tolerance enforcement, there are several regulatory mechanisms beyond the negative selection. One of these is the development of regulatory T cells, a process that is tightly related to the negative selection [95].

According to the majority of the evidence, negative selection takes place mainly in the thymic medulla [96]. This region of thymus contains two major antigen presenting cell types: medullary thymic epithelial cells (mTECs) and dendritic cells. These cells both express higher levels of MHC molecules and in contrast to cTECs, also costimulatory molecules CD40, CD80 and CD86, important for negative selection [97, 98]. Further, it has been shown that negative selection combining strong TCR signals and costimulation affects semi mature T cells ($CD24^{hi}$) residing in the medulla and not, fully developed mature ($CD24^{lo}$) T cells [99, 100]. Nowadays, additional evidence from mice deficient in REL-B, LT-βR or TRAF6 have identified the medulla as the place of negative selection. These mice have defects in cortico-medullary organization, resulting in small or absent medulla and therefore severe autoimmunity but unaltered positive selection [98, 101-103].

From the intrinsic point of view, cortical and medullary tools for generation of presented peptides differ as well. cTECs are characterized by expression of a special β5t proteasome subunit that is incorporated into 20S proteasomes, in place of β5 or β5i subunits and so therefore equipped for generation of different peptide repertoire than in the medulla [104, 105]. Further, APCs in the medulla express high levels of cathepsin S, while cTECs mainly utilize cathepsin L and TSSP (thymus specific serin protease) for formation of MHCII class peptides [106-109]. In addition to the different enzymes involved, cTECs have a higher degree of autophagy than mTECs, which strongly impacts positive selection [110].

Additionally, the antigen presentation spectrum in the medulla is shaped by dendritic cells able to cross-present surrounding antigens and the mTECs with the prominent ability of promiscuous gene expression [111].

The promiscuous gene expression is a unique characteristic of mTECs to reflect peripheral, tissue-restricted antigens and it is based on the expression of a transcription factor AIRE (autoimmune regulator) [112, 113].

Mutations in AIRE lead to autoimmune polyendocrinopathy-candidiases-ectodermal dystrophy (APECED), a severe autoimmunity. AIRE has been intensively studied, yet still a mechanism of action in mTECs still remains unclear. It has been suggested that AIRE promotes ectopic expression of proteins expressed elsewhere, and that it acts as a proapoptotic molecule leading to mTEC death and spread of expressed antigens to the DCs for cross-presentation [114].

In a very thorough study performed by the Mathis laboratory, AIRE has been described as interacting with several molecules from four different process groups. These were firstly proteins involved in nuclear transport, like exportin (XPO)1 and nucleoporin (NUP)93 that would mediate entrance into nucleus and secondly chromatin binding structures like the cohesion complex, that was proposed to be the access point for the initial binding. Further, they evidenced interactions with post initiation RNA polymerase (RNAP)II-mediated transcription machinery, composed of DNA-dependent proteinkinase (DNA-PK), Topoisomerase (TOP)2a and RNA- polymerase II (RNAPII), as well as with molecules involved in pre-mRNA processing [115]. Therefore, we have a picture of AIRE acting at the chromatin opening and transcription modifications levels, leading to noisy gene expression in mTECs and tissue-restricted antigens presentation to the developing thymocytes.

Previously described differences of signaling between positive and negative selection indicated that negative selection provides rapid signal and induces executive mechanisms that lead thymocyte toward apoptosis. The process of apoptosis is the major mechanism to remove negatively selected cells from the thymus and it utilizes prominent molecules of the Bcl-2 family, Bim, Bax and Bak as well as the nuclear orphan steroid receptor Nur77 [116]. The deficiency in Bim or combined deficiencies of Bax and Bak resulted in thymocytes that are refractory to apoptosis [117-120], while

deficiency in Nur77 has no strong impact of the efficiency of negative selection [120]. This is based on the phenomenon that Nur77 binds Bcl-2, an antiapoptotic molecule and turns it into the proapoptotic. However, in the absence of Nur77, the action of Bim and others is not affected. In one recent study, the expression of molecules Ian1 and Lip1 have been described as increasing and decreasing, respectively, with clonal deletion [116, 121]. Still, the exact role of these molecules remains unclear.

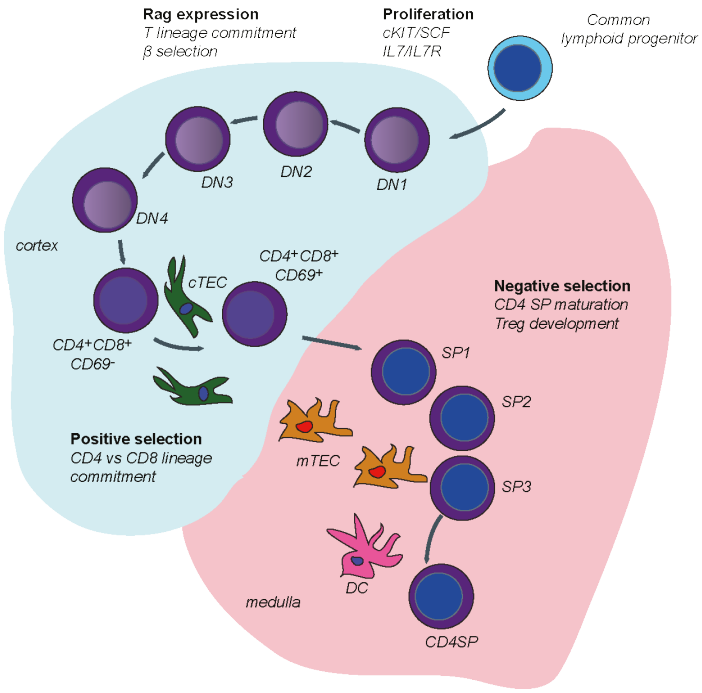


Figure 1: **T cell development in the thymus.** Upon enetering the thymus at the cortico-medullary junction, T cell precursors undergo lineage commtiment, β-selection and positive selection in the cortex. In the medulla, negative selection and final maturation of single positive thymocytes occurs.

The process of negative selection (recessive tolerance) and development of regulatory T cells (dominant tolerance) are two thymus-dependent processes of tolerance establishment, both based on high-affinity interactions between TCR and peptide-MHC but with two different outcomes for a thymocyte involved in TCR assessment [122].

The importance of this dominant branch of tolerance emerged from the experiments showing that thymectomy between day 2 and 4 upon birth leads to autoimmunity [123, 124]. The link between the $CD4^+CD25^+$ population and the thymus was demonstrated by the work of the Sakaguchi laboratory as they observed suppression of autoimmunity only in the transfer of whole splenocytes or the transfer of $CD4^+CD25^-$ splenocytes accompanied with $CD4^+CD25^+$ thymocytes, additionally distinguishing these cells from the temporarily activated T cells [125]. The generation of these and the recognition of a cognate antigen in the thymus was later linked by experiments using TCR transgenic models and models expressing cognate antigen in the thymus, preferentially in the mTECs [126-128]. One of very prominent models is TCR-HA (hemagglutinin (HA) - specific TCR) crossed to the Aire-HA model. The work on this model provided some crucial evidence that the generation of regulatory T cells (Treg) is based on the recognition of a cognate antigen and goes along with the negative selection [129, 130]. In the following experiments, utilizing AIRE-driven CIITA knockdown, and therefore lower expression of a cognate antigen on MHCII molecules in the medulla, it could be shown that the quantity of the presented antigen decreases negative selection and drives Treg development for several tested TCR specificities. This data show that the instructive phase of Treg development is certainly based on avidity of TCR/peptide MHC interaction and relates this interaction to the proximity of negative selection threshold [131].

According to the ‘two-step-model’ of Treg development, the instructive phase is followed by the consolidation phase, driven by common γ-chain cytokines (IL-2, -7 and -15) and needed to establish a fully mature regulatory phenotype, mainly defined and underpinned by Foxp3 expression [132].

Foxp3, forkhead box protein 3, is contemporarily considered as a master regulator of Treg lineage and a major marker of this population [133-136]. Mutation in the Foxp3 gene in humans results in development of a severe multiorgan autoimmunity, IPEX syndrome (immune-dysregulation, polyendocrionopathy, entheropathy and X-linked syndrome). Also, mice with mutated Foxp3 gene (scurfy mice) develop lethal autoimmunity [137]. The seminal work of Hori and Sakaguchi shows that the development and suppressive function of Tregs is based on Foxp3 expression and that

the retroviral expression of Foxp3 is sufficient to drive naïve cells into the regulatory lineage, transferring their suppressive functions. Further, they characterized these cells for their expression of CTLA4, GITR and CD103 accompanying the CD25 expression and altogether demonstrated that Foxp3 develops a molecular program that is responsible for the suppressive capacity of these cells [133]. The following work on genome-wide actions of Foxp3 puts this molecule in both roles of an activator and repressor of gene expression and therefore, it remains considered as a central molecular leader of regulatory T cell lineage [138]. Therefore the aspects of Treg development should all lead to the stable Foxp3 expression.

The Foxp3 promoter is epigenetically controlled by the SUMO E3 ligase, PIAS1 that recruits DNA methyltransferases and chromatin modeling proteins, like heterochromatin protein 1 (HP1) in order to keep the promoter repressed for transcription initiation [139]. The exact interplay needed to accompany TCR strength involved in displacement of PIAS1 from the promoter is unknown but mice lacking this E3 ligase show increased Treg populations.

Further, the regulatory elements of Foxp3 locus have been extensively studied and the major three sites have been described – conserved non-coding sequence 1, 2 and 3 (CNS1,2 and 3) [140].

The data collected mainly describes occupation of these elements by diverse transcription factors and regulatory molecules in the context of TCR engagement and cytokine instruction. Therefore, upon defined TCR/CD28 occupation, signaling over PKCΘ and CBM complex towards IKK results in recruitment of c-Rel toward promoter region and CNS2 and 3 [141-144]. CNS3 is considered as a pioneer element, which probably coordinates the initial Foxp3 expression. Further, NFAT and AP1 potentially lead to increased cytokine responsiveness and CD25 upregulation. IL-2 and its STAT5 signals further establish, synergize and follow recruitment of CREB, Ets-1 and Runx to an important CNS2 element [140, 145-147]. The demethylation of several CpG motifs in this Treg-specific-demethylated-region (TSDR) results in sustained and stable expression of Foxp3 [148-150]. The partial demethylation of this region is observed in peripherally induced Tregs as well as the occupation of CNS1 via Smad2/3 resulting from the TGFβ presence [151-153]. In sum, a coordinated interplay of signals over TCR

and cytokines provided in the defined environment (‘niche’) determines and influences the development of a regulatory T cell for a potentially harmful, self-reactive thymocyte. This interplay presents one of the most fascinating stages of immune system evolvement, the development of instructive tolerance.

AIM OF THE STUDY

The development and maturation of T cells takes place in the thymus. This educational process includes the commitment of progenitor cells into the T cell lineage and the shaping of a useful and self-tolerant T cell repertoire through positive and negative selection. In addition, this process includes formation of the regulatory T cell population as the mediator of dominant tolerance. At each of these steps, a coordinated interplay between T-cell-receptor signals, antigen peptides presented in the particular thymic microenvironment and cytokines shapes the outcome of thymocyte education.

From previous work on the differentiation of thymocytes, we know that the CD4 single positive compartment represents a diverse population of continually maturing cells. Consecutive stages of maturing CD4 SP thymocytes have been shown to exhibit a decreasing propensity toward entering the regulatory T cell lineage with advancing age.

The aim of this study was to identify intrinsic molecular players that distinguish the consecutive maturation stages of the CD4 SP compartment and thus potentially determine the plasticity of each stage. To do so, we utilized gene expression profiling of these stages accompanied by data verification in real-time polymerase chain reactions. Further, we performed experiments based on lentiviral gain- and loss-of-function specifically in CD4 T cells to functionally validate particular candidate genes. In the final part, we explored the role of one selected candidate in the immune system and particularly in T cells, through conditional knockout.

MATERIAL AND METHODS

Animals

Mice were bred in the animal facility of the Institute for Immunology of the LMU Munich in individually ventilated cages. TCR-HA, AIRE-HA, OTII, RipmOVA, Foxp3-GFP and Foxp3-huCD2 mice have been previously described [129, 134, 154-157]. Nedd4$^{fl/fl}$ mice were kindly provided by Dr. Hitoshi Kawabe [158]. Vav- and CD4-CRE mice were kind gift of Dr. Marc Schmidt-Supprian, Lck-CRE of Prof. Dr. Meinrad Busslinger and Foxp3-CRE of Prof. Dr. Alexander Rudensky. Local law regulation authorities approved all animal experiments.

Antibodies and reagents for flow cytometry

Specificity	Conjugate	Clone	Supplier
B220	PerCP	RA3-6B2	Becton, Dickinson & Co. (BD), Franklin Lakes, NJ, USA
CD3	APC	145-2C11	BD
CD4	V450/ Pacific Blue	GK1.5	BD
CD8	PerCP / APC-Cy7	53-6.7	BD
CD24	Pacific Blue	M1/69	BD
CD25	PE-Cy7	PC61	BD
CD44	APC-Cy7	IM7.8.1	BD
CD45.1	APC / FITC	A20	eBioscience, San Diego, CA, USA
CD45.2	Pacific Blue / APC	53-6.7	eBioscience
CD62L	FITC	Mel-14	BD
CD69	PE-Cy7	H1.2F3	BD
CD177	FITC /APC	2B8	BD
CD127	PE	SB/199	BD
CTLA4	PE	UC10-4B9	BD
GITR	PE / PE-Cy7	DTA-1	BD

Foxp3	PE /APC	FJK-16s	eBioscience
TCRγδ	Biotin	GL3	eBioscience
TCRβ	APC	H57-597	eBioscience
Cd1 Tetramer	PE	PBS-57	Gift from C. Vahl
NK1.1	Biotin	PK136	BD
Qa-2	Biotin	1-1-2	BD
6C10	IgM		Gift from J. Lui
IgM	PE	RMM-1	house
Ki67	PE	B56	BD
Annexin V	PE		BD
7AAD			BD
CD122	PE	TM-b1	eBioscience
TCR-HA	APC	6.5	house
Vα2	FITC/PE	B20.1	eBioscience
Vα3.2	FITC/PE	RR7-16	eBioscience
Vα8.3	FITC/PE	B21.14	eBioscience
Vα11	FITC/PE	RR8-1	eBioscience
Vβ2	FITC/PE	B20.6	eBioscience
Vβ3	FITC/PE	KJ25	eBioscience
Vβ4	FITC/PE	KT4	eBioscience
Vβ5.1	FITC/PE	MR9-4	eBioscience
Vβ6	FITC/PE	RR4-7	eBioscience
Vβ7	FITC/PE	TR310	eBioscience
Vβ8.1	FITC/PE	MR5-2	eBioscience
Vβ8.3	FITC/PE	1B3.3	eBioscience
Vβ10	FITC/PE	B21.5	eBioscience
Vβ11	FITC/PE	RR3-15	eBioscience
Vβ12	FITC/PE	MR11-1	eBioscience
Vβ13	FITC/PE	MR12-3	eBioscience
Vβ14	FITC/PE	14-2	eBioscience
Vβ17	FITC/PE	KJ23	eBioscience
Streptavidin	Pacific Blue/APC		eBioscience

Table 1: **List of antibodies used in this study** – All antibodies and reagents are listed with the respective clone and conjugated fluorophore.

Flow cytometric analysis

FACS staining was accomplished according to standard procedures at a density of 1 × 10^6 to 5 × 10^6 cells per 100µl. Ki67, 7AAD and Annexin-V stainings were performed according to the manufacturer's protocol. Intracellular staining was performed also according to manufacturer's instruction of a Foxp3 staining kit from eBioscience. FACSCanto Cytometer (BD) with FACSDiva software (BD) was used for data acquisition and recording. FACS data analysis was performed using FlowJo software (Tree Star Inc.).

Genotyping

For genotyping, a mouse-tail or ear pieces were digested in 50 µl of digestion buffer for 5 hours at 55°C, followed by Proteinase K heat-inactivation at 95°C for 5 minutes.

Digestion reaction	Gitocher buffer (10x)
3mm mouse tail	670mM Tris pH 8.8
3µl Proteinase K (10mg/ml stock)	166mM ammonium sulfate
2.5µl Triton (10% Stock)	65mM MgCl2
5µl Gitocher Buffer (10x)	0.1% Gelatin
0.5µl β-Mercapto-ethanol	
39µl H2O	

For genotyping polymerase chain reactions 1 µl of 50 µl digestion mix was used as templated DNA

PCR reaction	**PCR Buffer (5x)**
1µl template	250mM KCl
250nM primers (final)	50mM Tris pH 8.3
200µM dNTP (final)	43% Glycerol
PCR Buffer (1x final)	7.5mM MgCl2
Taq Polymerase	2mM Cresol Red
Ad 30µl with H2O	

A PCR programme was designed to be applicable to diverse genotyping reactions and was as following,

Temperature (°C)	duration (s)	cyclcy cles
94	180	
94	45	2
60	**45**	
72	60	
94	45	2
58	**45**	
72	60	
94	45	2
56	**45**	
72	60	
94	45	2
54	**45**	
72	60	
72	300	

Tbl. 2: **PCR protocol used for genotyping reactions.**

For the genotyping reactions following primers were used.

Genotype		Sequence 5' - 3'	Amplicon
TCR-HA	fwd	ACAAGGTGGCAGTAACAGGA	800bp
	rev	ACAGTCAGTCTGGTTCCTGA	
Aire-HA	fwd	ACAGCCACTCCTGTCTTTGC	1300bp
	rev	CTCCGTCAGCCATAGCAAATTTCT	
OTII	fwd	GCTGCTGCACAGACCTACT	160bp
	rev	CAGCTCACCTAACACGAGGA	
RipmOVA	fwd	CAAGCACATCGCAACCA	475bp
	rev	GCAATTGCCTTGTCAGCAT	
			200bp
Foxp3 GFP	fwd	AGACAGACCAGAGGTGTAGT	WT
	rev	TCCTGGGGATGGGCCAAGGGCCAAGG	800bp KI
Foxp3			800 bp
huCD2	fwd	TCTCAGACTCAGGATGACTGT	WT
	int	GGCTTCGGCCAGTAACGTTA	560bp KI
	rev	ATTGCTTGAGGCTGCGTATGA	
			200bp
Nedd4	fwd	GTACATTTTAGTTCATGGTTCTCACAGG	WT
	rev	CAGAGGTCACATGGCTGTGGG	300bp fl
CRE	fwd	GACAGGCAGGCCTTCTCTGAA	500bp
	rev	CTTCTCCACACCAGCTGTGGA	

Tbl. 3: **List of primers used for genotyping PCRs** –Forward and reverse primers with the resulting amplicon size are listed.

Purification of CD4 T cells - Magnetic cell sorting (MACS) and Flourescence-associated cell sorting (FACS)

Magnetic cell sorting (MACS, Miltenyi Biotec) is a technique for isolation of various cell types according to the expression of diverse extracellular markers based on their expression of different antigens on the cell surface.

CD4 SP cells were purified by the staining of thymocyte cell suspensions with biotinylated-anti-CD8 antibody, followed by incubation of cells with streptavidin coated - microbeads (Miltenyi Biotech) and depletion of $CD8^+$ and DP cells using magnetic columns columns, LS-MACS (Miltenyi Biotech). 20µl of MACS Microbeads in total of 200µl of MACS Buffer were routinely used for 10^8 cells. The obtained $CD4^+CD8^-$ fraction was stained for the surface markers of interest, like 6C10, CD69 etc. and further subdivided by FACS on FACSAria cell sorter (BD).

Peripheral CD4 T cells were obtained by the staining of splenocytes and lymph node cells with biotinylated anti-CD4 antibody, followed by incubation of cells with streptavidin Microbeads in order to enrich the suspension for $CD4^+$ fraction. The enriched suspensions were further stained and sorted on FACSAria (cell sorter (BD).

Cloning

Plasmids containing CD4 regulatory elements were kindly provided by Dr. David Klatzmann and Dr. Ishiro Taniuchi. The laboratory of Prof. Dr. Brocker at the Institute for Immunology, LMU provided plasmids containing lentiviral vector backbone and all other plasmids used for intermediate cloning steps. Restriction enzymes were purchased from Roche (Mannheim, Germany) or NEB (Munich, Germany) and used at 10U / ≤1μg DNA at designated temperature for approximate 1h digestion reactions. Cut vectors or inserts were purified on a 1.5 % agarose gel and purified usinga gel extraction kit (Quiagen, Hilden Germany). Ligation was performed using T4 DNA Ligase (Roche, Mannheim Germany) upon vector dephosphorylation by alkaline phosphatase (Roche, Mannheim Germany) using equal stoechiometric amounts of insert and vector. All ligation reactions were heat-shock (20 min on ice followed by 2min at 42°C) transfected in E.coli (DH5α) and plated out on LB-agar plates containing an appropriate antibiotic. Ampicillin (300μg/ml) and kanamycin (50μg/ml) were used as selection markers.

Cloning of lentiviral constructs

Genes of interest were amplified on whole thymocyte genomic DNA by PCR using the following primer pairs:

Gene of interest	**Primers**	
Lztfl1	forw	GGGCCCATGGCAGAGTTGGGC
	rev	CTCGAGGAATTCTTAATCTTCAGATTCATATTTTGC
Slfn1	forw	GGGCCCATGAACATCACCGATGAAG
	rev	CTCGAGGAATTC CTAAGACATGAGGAGCTTGAT
Smpdl3a	forw	GGGCCCGCCACCATGGCGTTGCTGGGCA
	rev	CTCGAGTTATAAATGCTGTTTAAGGCAA
Ms4a6c	forw	GGGCCCATGATTCCACAGGTAGTGACC
	rev	TACTCGAGGAATTCTCACATTCTAAGGGAAGTCAGA
Ifi27a2l	forw	GGGCCCATGTTGGGAACACTGTTTG
	rev	TACTCGAGGAATTC TCAGAGCAAGGCTCCA

Tbl. 4: **List of primers used for amplification of all genes of interest from the thymocyte cDNA** – Forward and reverse primers for each selected candidate are listed.

PCR products were cut using the following enzyme combinations and ligated into fluorescent-protein- and linker(T2a)-carring plasmids. In this way a cassette carring fluorescent protein, linker T2a and gene of interest was created for insertion into the lentiviral vector.

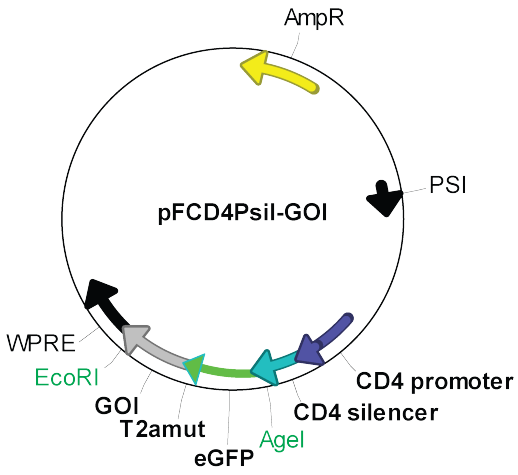


Fig. 2: **Scheme of lentiviral vector** – Important sites: CD4 regulatory elements, eGFP reporter gene bridged to the gene of interest over a T2a linker and inserted into lentiviral backbone over AgeI and EcoRI sites.

Cloning of lentiviral vectors for gene knockdown

Short hairpins were designed using the software RNAi Oligo Retriever (*http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA*) . The murine stem cell virus (MSCV)-based LMP vector was purchased from OpenBiosystems and provided the flanking sequences of the human miR30, endogenously and ubiquitously expressed miRNA. The knock-down of the gene of interest is obtained through the exchange of miR30 sequence with the sequence of artificial short hairpin. We subcloned this regions in the 3' UTR of the eGFP sequence in the lentiviral vectors.

293FT cells in lentivirus production

Transient transfection of 293FT cells was performed for the lentivirus production over three days. $2x10^6$ cells were plated in 10 cm Petri dishes and left to grow up to 75% comfluence. At that point, a mixture of 10µg GOI DNA with 8µg pax2 DNA and 8µg VSVg DNA in 0,5 M $CaCl_2$ in HEBS per Petri dish was added to the cells and left for incubation and uptake over 12-24h. After that, cells were supplied with medium and the supernatant was collected at 24, 48 and 72h upon transfection, pooled and centrifuged at 14000 rpm for 4h to obtain concentrated preparations of virus.

Transduction of NIH3T3 cells for lentivirus titration

NIH3T3 cells were plated out at the concentration of $3x10^4$ cells/well in 24-well plates and left over night. A total of 6 wells were prepared for one virus titration. From each virus stock produced 100µl were used for titration, starting from the first well and titrating down in 1:3 steps over the next 5 wells. Upon 3 days, expression of GFP as the infection marker was used as the parameter on infection efficiency. The amount of virus per µl needed for full infection is then estimated by proportion of infected cells relative to volume of virus used.

Bone marrow infection and chimeras

Bone marrow was obtained from donor mice as femur and tibia were isolated, shortly placed in 80% ethanol and following in PBS. Using a 1 ml syringe bone marrow was flushed out in PBS, filtered and washed. Further, red cell lysis was performed using BD Cell lysis buffer. Donor T cells were depleted by using biotinylated anti-CD4 and anti-CD8 antibodies and streptavidin MACS beads (Miltenyi) according to the manufacturer's protocol.

For further use bone marrow was plated out at $3x10^6$ cells / infection or intra-venously injected into lethally irradiated mice at $1\text{-}3x10^6$ cells / mouse. BALB/c mice were irradiated with 2x450rad and C57-BL6/c with 2x550rad respectively.

Infection was performed in Stemline medium (Sigma) supplemented with IL3, IL7 and SCF (R&D Systems, Wiesbaden, Germany) with 10 µg/ml Polyprene and multiplicity of

infection (MOI) of 1 for 6-8 h. After infection, cells were washed and rested in cytokine-supplemented Stemline medium (Sigma Aldrich, Taufkirchen, Germany) for 2 days. Chimeras were analyzed 6-8 weeks after reconstitution.

RNA Isolation and real time PCR

Total RNA was isolated using the miRNeasy Kit (Roche, Mannheim Germany) and used for cDNA synthesis by iScript™ cDNA Synthesis Kit (BioRad), both steps conducted according to manufacturer's protocol. Quantification of mRNA expression was carried out using SsoFast™ EvaGreen® Supermix on C1000™ BioRad cycler.
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 a gene of interest and the housekeeping gene, β-actin. $\Delta\Delta$Ct values relate the ΔCt differences between the examined populations and the population used for normalization (mainly SP1).

Program used for real time PCR:

Temperature (°C)	Duration (s)	Cycles
94	300	
94	45	40
56	**40**	
72	60	
72	60	

Tbl. 5: **Protocol used for the real time PCR reactions**.

Melting curves for each reaction were analysed between 55° and 95°C with the range on 0.5°C / 30 s.

Following primers were used for the verification of the microarray data obtained:

Gene	sequence fwd	sequence rev	amplicon size (bp)	intron spanning
Rorgt	ACAGGGTCCAGACAGCC	CACCTCCTCCCGTGAAA	181	yes
Eef1a1	GTGCTAATATGCCTTGGTT	CTTGTCAGTTGGACGAGTT	119	yes
Itm2a	AGATCGCCTTCAACACC	TCCTGCGGGACAACTC	127	yes
Lef1	CTTCGCCGAGATCAGTCA	CTGGCCTTGTCGTGGTAG	153	yes
Ube2d3	ATGGCAGCATTTGTCTTG	GGTCGTCTGGGTTTGG	111	yes
Fubp1	TGGTGCTGATAAACCTCTT	CCCTTCATTGCCTCCTA	145	yes
Dub2a	ACAGACAATCAGAACCCAC	CCTGGCAATGGAGACA	96	no
Fyn	GCTCGGAAGGAGACTGG	CTCGCGGATAAGAAAGGTA	191	yes
Tspan13	TGAGGTTTGTTGGTGGCA	GCGAGGGTCTTTCTGGTT	95	yes
Pbrm1	CAGGAGGCTATCCAGTAA	AGGTCCATTGGCTCTAA	177	yes
Samhd1	TTCACCAAGCCACAGG	AACTAACCGCAGATATTCA	140	yes
Ccr9	AGCACAAGGCCCTCAA	TGGCATAAGCGTCAACA	105	no
Cbx3	TGCTGCTGACAAACCA	TTCATGTTCGCCTCCT	150	yes
Cd28	AGGCTGCTGTTCTTGGC	GGCTGACCTCGTTGCTAT	106	yes
Nr4a1	CCTGGCATACCGATCTAAAC	AGGCGGGAACATCAACAC	158	yes
Hcls1	GCATGATGTATCGGTTTCC	CAATGGTCTTGGCTCCC	110	yes
Tm9sf3	TACTTCATCTTCACGTCCTT	TCGTCCACTGCCACCT	157	yes
Lztfl1	CGGTAGATTCCTGCTT	TCGGACTCCACCTCAC	124	yes
Rab1	AGTCCTGCCTTCTCCTT	CTTTCCTGGCCTGCT	145	yes
Wsb1	TCCCAGTGCCGTAAGA	TGCTCAGGAGGCTTGTT	116	yes
Ppp2r5c	AGTCCCTGAGTGTCTACCA	TTGAGAAGTGCCATTACC	100	yes
Lsp1	GCCGTAGCCAGTACCAA	TCCTTTCTGCTCCCACA	129	yes

Zfp445	TCGGTGCCTGACTATTT	AGAGCAGGTTTGGGAGA	197	yes
Foxo3	AAACGGCTCACTTTGTCCCA	TTGCCCGTGCCTTCATTC	164	yes
Bcl2l11	CGGAGACGAGTTCAACGA	ATACCAGACGGAAGATAAAGC	119	yes
Zfp330	GCCTTGCTATGGTGGG	CTGCATCTGTGAGTGGG	105	yes
Egr2	GACCAGATGAACGGAGTG	GAGATGGGAGCGAAGC	104	yes
Olfr767	CTGGATTCGCACCTTC	CTCTTGTTTCCCGTTGT	122	no
Slfn1	CCAGGCGATGATACGC	TGGCAAAGGCAGAAAC	193	no
S100a11	CGGGAAGGATGGAAAC	GGTCAAGGACACCAGGAT	110	yes
Il6ra	CCTTGCTGGTGGATGTTC	CGTTGGTGGTGTTGATTTT	153	yes
Lgals1	CGGGAGCATCACAGAGG	CATGTTGAGGCGGTTGG	100	yes
Pde3b	GAATTGATGGCTTTGTATGTG	AAGGAGGAAGTTGTACTCTGG	189	yes
Ppp1r2	ATCGTGGAAGAGGAACTGA	AAGCCATAGTCTTTGTCAGC	92	yes
Naip3	TGAGCAGCATCATCCA	CAGGTCCGTGACAAGTAG	124	no
Zbtb16	GCCACCTTCGCTCACAT	CCTGTAGTGCGTCTCCAAC	179	yes
Satb1	CATCCAGGTTGGGAAGTG	TGCGACCATTGTTCAGG	165	yes
Rap1a	GAAGATGAACGGGTAGTTG	TCAGAGCTGCTGCTGAC	219	yes
Rac2	TGTGATGGTGGACAGTAAGC	CCACTTGGCACGGACAT	163	yes
Ifi27l2a	GTTGGGAACACTGTTTGG	GCAACTCCACCTCCATT	162	yes
Nedd4	GCTGAACTCTATGGCTCG	GCGATTGAAGCAGGTGT	96	yes
Ms4a6c	TTGGTCTGGCTTTGC	TTCCACATTGGCTTCA	110	yes
Tbrg3	ACCCTTGTTCATCTCA	ATAGTAAAAGGCGAAAG	130	no
Smpdl3a	GCTGTGGCTGACCTCT	GCTGGGTCTGTCTTGTT	173	yes
Dub2a 2	TGCCCAGATCCATAGCAAAT	GCCCAGTGTTCACAAGGT	108	no

Tbl. 6: **List of primers used for RT-PCR analyses** –Forward and reverse primers with the resulting amplicon size are listed. For each gene we tended to design RT-PCR reactions that would cross exon-exon junction and therefore increase the specificity of reaction.

Microarray analysis

Gene expression profiling was performed on Affymetrix Mouse Gene 1.0 ST arrays. RNA from sorted populations, SP1, 2 and SP3 was isolated with the miRNAeasy kit (Qiagen) and labeled with the WT Expression kit (Affymetrix). Raw data obtained were analyzed with the programs R and Bioconductor by collaborating bioinformaticians. Arrays were assessed for quality and were normalized by the robust multi-array average method. Differences in gene expression were extracted with an empirical Bayes moderated *t*-test implemented in the Bioconductor package Linear Models for Microarray Data. False-discovery rate was utilized for sorting the results that were subsequently exported in tab-delimited format [159, 160].

Statistical analysis

Statistical significance was assessed by the two-tailed Student`s *t*-test.

RESULTS

Gene expression profiling of CD4 single positive thymocytes

Upon positive selection, developing thymocytes proceed from the cortex to the medulla and take part in the second step of thymic education by moving through the medullary microenvironment and interacting with diverse antigen-presenting cells.

Instructions received in the medulla have a strong impact on thymocyte fate and are considered to be a major tool of central tolerance to sort out potentially harmful cells from the repertoire. In the most simplistic model, the interactions between antigen - presenting cells and thymocytes result in instruction of thymocyte toward survival, apoptosis or Treg development by the antigen-presenting cell.

Throughout the next five to ten days [161] that a thymocyte spends moving in the medulla, its developmental status may change considerably and influence the decisions that are taken at the moment of interaction with antigen - presenting cells.

Adding the parameter of time that a thymocyte spends in the medulla for screening many different antigen-presenting cells and by entering multiple interactions, we questioned as to whether the decisions are only made by the antigen-presenting cells or if there are potential thymocyte-intrinsic factors that influences these decisions.

Composition of thymocyte CD4 SP compartment

In order to address the diversity in the CD4 SP compartment, we searched for the earliest thymocyte that leaves the positive selection and for the consecutive maturation stages that compose the total CD4 SP compartment. Through intrathymic transfer of single populations and analysis of emerging progeny, Jin *et al.* provided evidence that these described stages are consecutive and that they emerge from a unidirectional precursor-to-progeny developmental line [162]. We hypothesized that these stages may differ in their gene expression profile and were interested in studying these potentially different molecular signatures in order to understand how thymocyte intrinsic factors influence formation of central tolerance. For these studies, we identified three populations by utilizing maturation markers CD69, CD24, and maturation-dependent glycosylation changes of CD90.

CD69, a TCR-stimulation-dependent activation marker, is highly expressed on the double positive thymocytes undergoing positive selection and on the small population of CD4SP that have recently been positively selected [163]. Its blockade by monoclonal antibodies leads to dysregulation of selection process and dramatic loss of SP compartments [164].

Further, we used the antibody SM6C10 to distinguish between two subpopulations of $CD69^{+}$ SP thymocytes [165]. SM6C10 recognizes a surface carbohydrate epitope of the CD90 molecule. CD90 is ubiquitously expressed on thymocytes and represents their major glycoprotein [166]. The levels of the 6C10 epitope decrease with the 'age' of a thymocyte [165].

Lastly, we used CD24 (HSA, heat-stable antigen) to separate and confirm age of the thymocytes used in the analyses. CD24 is highly expressed on immature thymocytes and declines with maturation [167, 168].

The three SP populations that are the focus of the study were named SP 1, 2 and 3, from the youngest to the oldest ones.

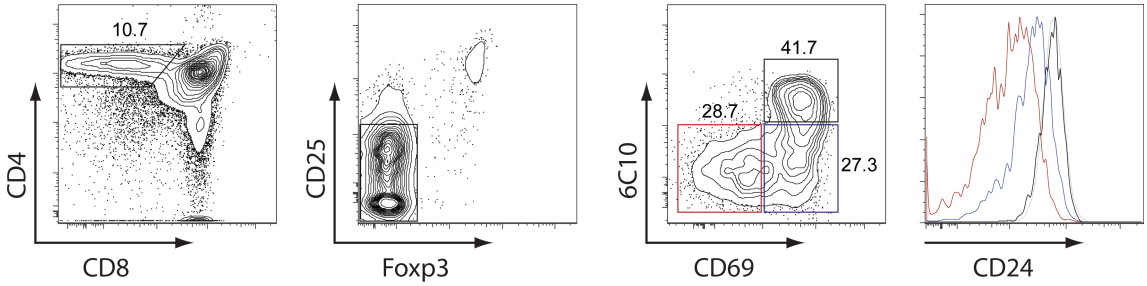


Fig. 3: **Dissection of CD4 SP maturation stages and gating strategy for SP 1, 2 and 3 sorting.** The plots depict stainings of thymus of Foxp3-GFP reporter mouse for markers used for SP1,2 and 3 sorting. Numbers in the plots indicate frequencies of cells within the sorting gates. Histograms show overlays of CD24 expression on SP 1,2 and 3 subpopulations.

Microarray analysis of CD4 SP stages and candidate selection

In order to gain insight into the molecular signature of these different consecutive stages (SP1, 2 and 3), we performed gene expression profiling.

Upon filtering microarray data by defining a 2-fold-change cut-off we obtained a list of 45 differentially expressed genes between three various subsets. Tables 7.1 and 8.1 sum up the results of gene array analysis. In the following Tables 7.2 and 8.2, we listed the differentially expressed genes and their described functions resulting from the general and T cell-related literature research. Further, these tables summarize selection criteria, which we used for each selected candidate. Tables 7.3 and 8.3. present the overview of gain-of-function (GOF) and loss-of-function (LOF) experiments that were undertaken for the selected candidates. For most of the candidates we performed a verification real-time PCR analysis before we narrowed down the candidate selection (Fig 4.).

Interestingly, all the expression differences emerging from the microarray analysis have in common that the gene expression maintains its directionality over the stages. For example, genes are either up- or down-regulated from SP1, 2 to 3, and there are no genes with a relative peak or minimum of expression at the SP2 stage. This observation is consistent with the intrinsic developmental relationship between the stages.

In general, the first selection was based on the genes that showed the highest differential expression (Dub2a and Itm2a). Further, we narrowed the selection down to those that were not extensively described in the thymocyte biology (Slfn1, Il6ra and Nedd4). One molecule was predicted to have transcription factor properties (Lztfl1) and was therefore included in the selection. Finally, we considered those genes interesting that had no described function at all (Smpdl3a, Ms4a6c, Ifi27l2a). In one previous study on the transcriptome of regulatory T cells, two molecules (Smpdl3a and Ms4a6c) were also described. This study by Gavin *et al.*, utilized a model of in-frame insertion of GFP into a stop-codon-disrupted Foxp3 locus to genetically mark cells that were actively transcribing Foxp3 gene but had no Foxp3 protein. Through the comparison of these cells with the regulatory cells carrying the wild type Foxp3 locus they were able to characterize the Foxp3-driven transcriptional program and describe groups of Foxp3-

dependent and Foxp3-amplified genes. According to this study, Smpdl3a was in the group of ‘Foxp3-amplified’ genes, while Ms4a6c was among the ‘Foxp3-dependent’ genes [135].

In sum, we selected four genes from the group with decreasing expression during maturation (Dub2a, Itm2a, Lztfl1 and Nedd4) and five with increasing expression (Il6ra, Ifi27l2a, Ms4a6c, Slfn1 and Smpdl3a) for further analyses.

Gene Accession	Gene	SP1	SP2	SP3	SP1/3	Gene Description
NM_001001559	Dub2a	195,019	87,971	4,018	**48,538**	Deubiquitinating enzyme 2a
NM_009913	Ccr9	139,236	24,873	5,557	**25,055**	Chemokine (C-C motif) receptor 9
NM_008409	Itm2a	474,850	155,932	50,903	**9,329**	Integral membrane protein 2A
NM_033322	Lztfl1	79,732	34,367	9,723	**8,201**	Leucine zipper transcription factor-like 1
NM_007624	Cbx3	125,961	82,897	30,698	**4,103**	Chromobox homolog 3 (Drosophila HP1 gamma)
NM_009122	Satb1	912,677	526,688	258,601	**3,529**	Special AT-rich sequence binding protein 1
NM_010444	Nr4a1	110,153	83,316	31,593	**3,487**	Nuclear receptor subfamily 4, group A, member 1
NM_009741	Bcl2	106,187	52,904	31,479	**3,373**	B-cell leukemia/lymphoma 2
NM_019653	Wsb1	48,569	39,810	15,291	**3,176**	WD repeat and SOCS box-containing 1
BC095996	Tbrg3	56,298	30,368	17,729	**3,176**	Transforming growth factor beta regulated gene 3
NM_057172	Fubp1	215,456	138,576	68,947	**3,125**	Far upstream element (FUSE) binding protein 1
NM_010890	Nedd4	40,331	17,570	13,569	**2,972**	Neural precursor cell expressed, developmentally down-regulated 4
NM_001081251	Pbrm1	173,497	138,198	58,569	**2,962**	Polybromo 1
NM_207680	Bcl2l11	32,328	22,236	11,242	**2,876**	BCL2-like 11 (apoptosis facilitator), Bim
NM_019740	Foxo3	43,928	30,821	16,237	**2,705**	Forkhead box O3
NM_025359	Tspan13	129,215	94,391	49,125	**2,630**	Tetraspanin 13
NM_145541	Rap1a	162,029	119,903	62,101	**2,609**	RAS-related protein-1a
NM_010703	Lef1	347,967	201,569	134,638	**2,584**	Lymphoid enhancer binding factor 1
NM_007642	Cd28	107,729	77,978	45,134	**2,387**	CD28 antigen
NM_018851	Samhd1	129,085	93,684	54,569	**2,366**	SAM domain and HD domain, 1
NM_133352	Tm9sf3	83,251	65,569	36,242	**2,297**	Transmembrane 9 superfamily member 3
NM_145600	Zfp330	36,098	31,903	16,237	**2,223**	Zinc finger protein 330
NM_025356	Ube2d3	269,729	226,569	124,931	**2,159**	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)
NM_008996	Rab1	49,069	39,777	23,070	**2,127**	RAB1, member RAS oncogene family
NM_008225	Hcls1	103,209	61,018	49,621	**2,080**	Hematopoietic cell specific Lyn substrate 1
NM_173364	Zfp445	46,351	45,028	23,164	**2,001**	zinc finger protein 445
NM_001122893	Fyn	148,050	107,163	74,569	**1,985**	Fyn proto-oncogene
NM_010118	Egr2	24,327	12,254	12,254	**1,985**	Early growth response 2

Tbl. 7.1: **List of 28 genes with declining expression between the maturation stages.** Ratio between SP1 and SP3 of ≥ 2 was set as a cut-off and genes are listed according to the decreasing ratio. Full gene names are listed.

Gene	known functions and features	T cell related	selection parameter
Dub2a	induced rapidly upon IL2 stimulation [169]	✓	highest SP1/3 ratio; role in IL2 responsiveness
Ccr9	chemotaxis of precursors to thymus [170]	✓	
Itm2a	induced during positive selection [171]	✓	high SP1/3 ratio; upregulation upon positive selection
Lztfl1	negative regulator of BBSome transport to cilia [172]		high SP1/3 ratio; potential transcription factor; SHH signaling
Cbx3	smooth muscle differentiation [173]		
Satb1	*Il2, Il2ra* silencing, lymphoid lineage commitment [174, 175]	✓	
Nr4a1	TCR signaling [176]	✓	
Bcl2	apoptosis [177-179]	✓	
Wsb1	turnover of IL21R [180]	✓	
Tbrg3	-		
Fubp1	c-myc expression, cell proliferation [181]		
Nedd4	Cbl-b ubiquitination, TCR signaling [182]	✓	TCR threshold regulation; TCR sensitivity in selection
Pbrm1	histone acetylation [183]		
Bcl2l11	apoptosis, negative selection [118]	✓	
Foxo3	Treg development [184]	✓	
Tspan13	potential tumorsupressor [185]		
Rap1a	-		
Lef1	T cell lineage commitment [186]	✓	
CD28	costimulation [187]	✓	
Samhd1	HIV-1 restriction factor in non-cycling cells [188, 189]	✓	
Tm9sf3	-		
Zfp330	-		
Ube2d3	-		
Rab1	member of Ras family		
Hcls1	cytoskeleton remodeling, lymphocyte trafficking [190]		
Zfp445	-		
Fyn	TCR signaling [191]	✓	
Egr2	thymocyte survival at DP stage [192, 193]	✓	

Tbl. 7.2: **List of previously described functions for each gene and considered aspects in the candidate selection**.

Gene Symbol	attempted		GOF		phenotype observed	LOF		phenotype observed
	GOF	LOF	mRNA	Protein		mRNA	Protein	
Dub2a	y	ongoing	-	-	very low infection rates < 1%, levels of overexpression remain to be confirmed	-	-	
Itm2a	y	ongoing	-	-	increased frequency of $Foxp3^+$ cells among Itm2a overexpressing CD4SP (approx. 7% $CD4^+Foxp3^+$), inconsistent	-	-	
Lztfl1	y	y	7.9±3.4x	approx. 2x	reduced frequency of $Foxp3^+$ cells among Lztfl1 overexpressing CD4SP (approx. 1,5 % $CD4^+Foxp3^+$)	knockdown to 25%	-	no differences
Nedd4	y	n	-	-	increased double negative compartment in Nedd4 overexpressing cells, block at DN2/3 stage, very low infection rates < 2%	-	-	

Tbl. 7.3: **Summary of results in GOF and LOF experiments for selected candidates.**

Gene Accession	Gene Symbol	SP1	SP2	SP3	SP1/3 Ratio	Gene Description
NM_019391	Lsp1	61,903	89,302	125,936	0,492	Lymphocyte specific 1
NM_013566	Itgb7	19,178	37,664	40,061	0,479	Integrin beta 7
NM_146318	Olfr767	25,236	35,119	56,477	0,447	Olfactory receptor 767
NM_010559	Il6ra	17,935	29,381	40,614	0,442	Interleukin 6 receptor, alpha
NM_011346	Sell	42,881	98,875	100,830	0,425	L-selectin, lymphocyte
NM_025800	Ppp1r2	7,058	11,272	17,356	0,407	Protein phosphatase 1, regulatory (inhibitor) subunit 2
NM_009008	Rac2	63,470	96,431	184,236	0,345	RAS-related C3 botulinum substrate 2
NM_029803	Ifi27l2a	13,244	26,903	38,680	0,342	Interferon, alpha-inducible protein 27 like 2A, ISG12
NM_028595	Ms4a6c	13,563	40,903	40,650	0,334	Membrane-spanning 4domains subfamily A member 6C
NM_008495	Lgals1	11,543	22,903	37,629	0,307	Lectin, galactose binding, soluble 1
NM_011407	Slfn1	29,075	77,903	96,236	0,302	Schlafen 1
NM_020561	Smpdl3a	3,682	6,697	12,254	0,300	Sphingomyelin phosphodiesterase, acid-like 3A
NM_016740	S100a11	27,882	31,704	99,632	0,280	S100 calcium binding protein A11 (calgizzarin)
NM_001135001	Ppp2r5c	58,597	211,507	211,507	0,277	Protein phosphatase 2 regulatory subunit B gamma
NM_001033324	Zbtb16	11,390	18,237	43,981	0,259	Zinc finger and BTB domain containing 16
NM_011055	Pde3b	11,235	24,933	51,903	0,216	Phosphodiesterase 3B, cGMP-inhibited
NM_007901	S1pr1	6,716	33,678	64,371	0,104	Sphingosine-1-phosphate receptor 1

Tbl. 8.1: **List of 17 genes with increasing expression between the maturation stages.** Ratio between SP1 and SP3 of ≤ 0.5 was set as a cut-off and genes are listed according to the decreasing ratios.

Gene Symbol	known or speculated functions and features	T cell related functions	selection parameter
Lsp1	calcium binding protein [194]		
Itgb7	intestinal homing [195]	✓	
Olfr767	-		
Il6ra	inflammation, Th17 polarization [196]	✓	polarization between tTreg and potentially ‘tTh17’
Sell	lymphocyte migration [197]	✓	
Ppp1r2	-		
Rac2	Rho family GTPase		
Ifi27l2a	IFNα-inducible adipocytokine [198]		unknown function
Ms4a6c	-		predicted 'Foxp3-dependent gene'[135]
Lgals1	T cell survival, TCR tuning [199]	✓	
Slfn1	cell proliferation and growth [200, 201]	✓	role in proliferation
Smpdl3a	-		predicted 'Foxp3-amplified-gene' [135]
S100a11	-		
Ppp2r5c	PP2A regulation, cell proliferation [202]		
Zbtb16	NKT differentiation [203]	✓	
Pde3b	cATP and cGTP metabolism, Treg development [135]	✓	
S1pr1	lymphocyte migration [204]	✓	

Tbl. 8.2: **List of previously described functions for each gene and considered aspects in the candidate selection**.

Gene Symbol	attempted		GOF		phenotype observed	LOF		phenotype observed
	GOF	LOF	mRNA	Protein		mRNA	Protein	
Il6ra	y	n	-	-	no surface expression of IL6Rα by FACS staining, no differences	-	-	
Ifi27l2a	y	n	7.4±6.3x	-	no differences	-	-	
Ms4a6c	y	n	-	-	no differences			
Slfn1	y	y	2.6x	-	no differences	knockdown to 40%	-	no differences
Smpdl3a	y	n	9.2x	-	no differences	-	-	

Tbl. 8.3: **Summary of results in GOF and LOF experiments for selected candidates.**

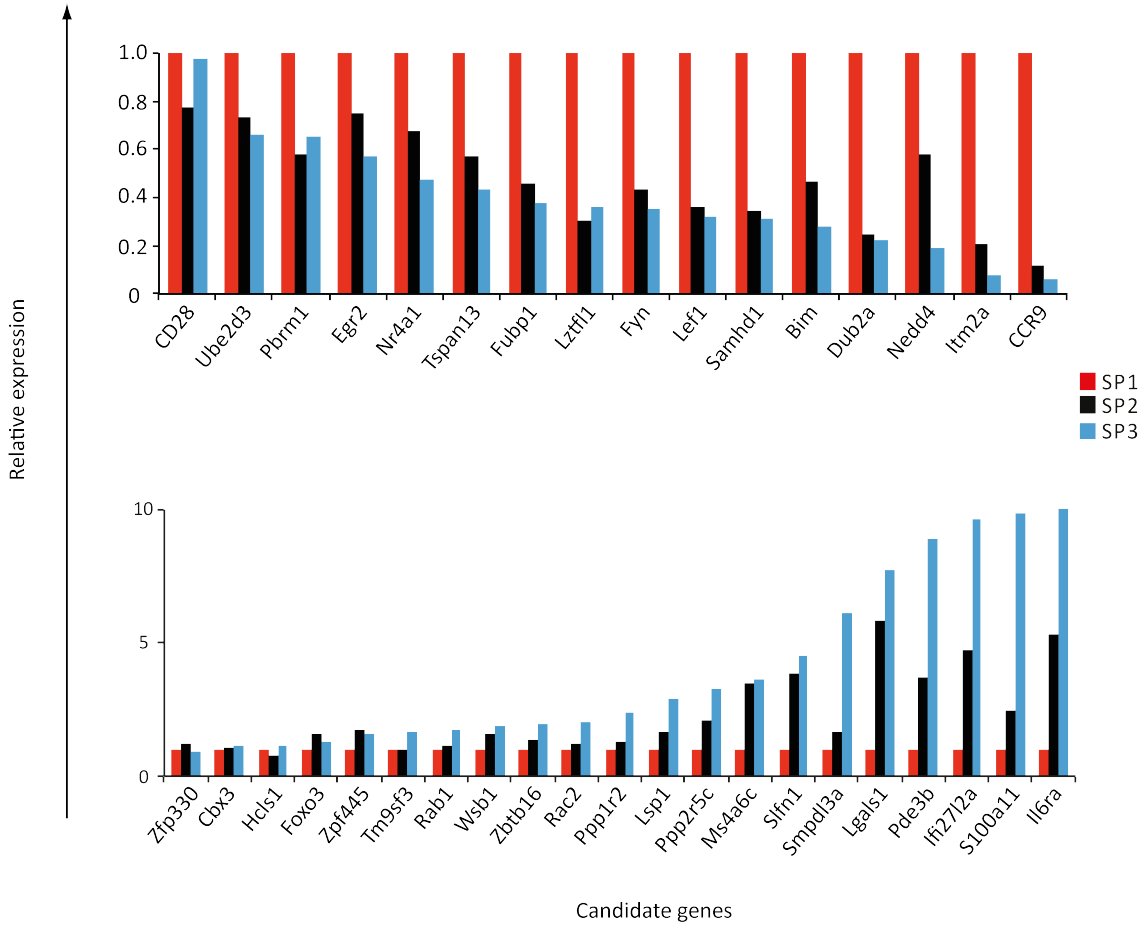


Fig. 4: **Data verification and expression confirmation by real-time PCR for all differentially expressed genes according to microarray analysis.** For all differentially expressed genes confirmatory real-time PCR reactions were designed and performed on sorted SP1,2 and 3. For the comparison, expression level of each transcript at SP1 stage was set as 1. Expressions at SP2 and SP3 were normalized to the level of SP1. Bar diagrams show means of duplicate reactions.

Lentiviral construct design and experimental approach

One possibility to study the function of a gene is through gain- and loss-of-function experiments. We decided to selectively upregulate or knockdown selected genes of interest.

Therefore, we built a system in which we would infect bone marrow with a lentivirus and introduce elements needed for this interference. Further, we would monitor the development of the infected thymocytes and relate it to the uninfected or empty-vector-infected controls.

We built lentiviral vectors composed of following elements (Fig.6):

Expression specificity regulating elements: We used elements derived from the CD4 gene composed of a minimal CD4 promoter and the proximal CD4 enhancer [205]. Further, we inserted an additional regulatory level by introducing a CD4 silencer element, which allows transcriptional repression of CD4 expression in the CD8 cells and therefore limits the gene expression to DP and CD4 SP T cells [206].

2. **Enhanced green fluorescent protein (eGFP)**, utilized as a reporter of infection and construct expression.

3. **T2a linker**: This 54 bp sequence codes for a T2A peptide of *Thosea asigna* virus, a cis-acting-hydrolase element. The bicistronic construct composed of two open reading frames connected via T2A sequence is expressed in one transcript [207]. The 'cleavage' of the two proteins is mediated by 'ribosomal skip' mechanism, induced by terminal proline and penultimate glycine residues within the T2A peptide [208]. In this way, nearly stoichiometric amounts of both parts of construct are produced.

4. **Gene of interest**: This part carries the full coding sequence of each candidate gene.

For the knockdown experiments we used the previously described system (Openbiosystems, Expression Arrest^{TM}). Designed short-hairpin RNAs were cloned in the human miR30 backbone and expressed under the above-described regulatory elements (in detail in *Material and methods*).

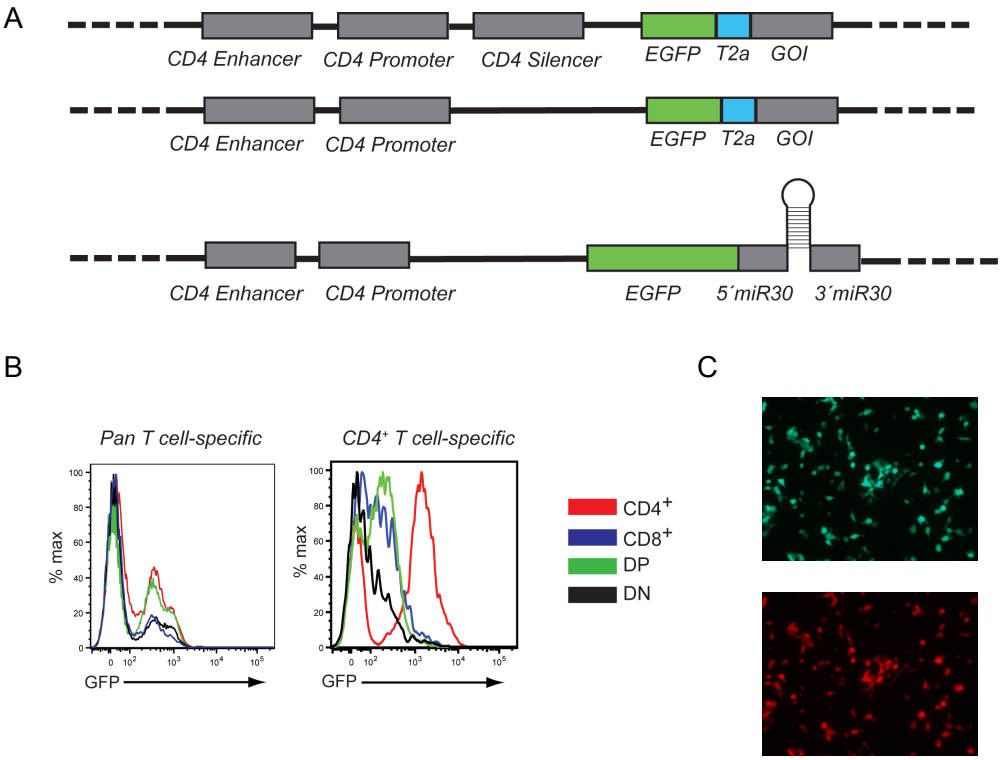
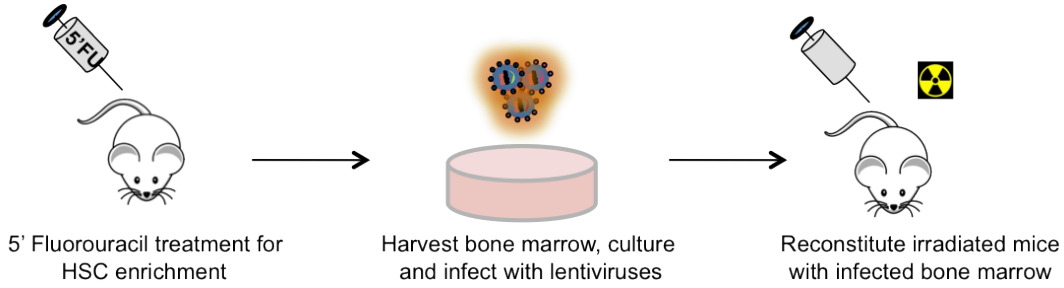


Fig 5: **Lentiviral vectors and expression in T cell lineages.** A – Schematic representation of lentiviral vectors containing described elements for gene expression interference. B - Both vectors guarantee high expression in CD4 SP compartment. In the infections using the pan T cell vector all thymocyte subpopulations express the construct with the highest expression in CD4 SP and DP cells. In the case of CD4-specific vector the expression is mainly restricted to DP and CD4SP T cells. C - Pictures of cells expressing eGFP and mCherry from a bicistronic construct confirm the principle behind the T2A peptide.

The generated vectors provided us with strong tools to study the role of selected genes in the thymocyte development and further whole T cell life. We used these tools to perform bone marrow chimeras in polyclonal and TCR transgenic systems, in order to study general events and TCR / cognate-antigen interaction induced events related to these genes (Fig. 6).

A



B

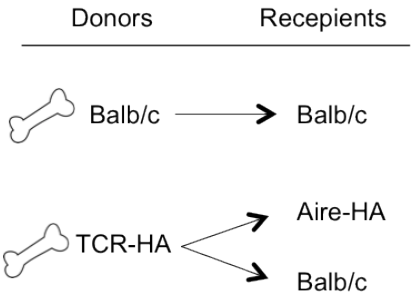


Fig. 6: **Generation of bone marrow chimeras.** A – Donor mice were treated with 5' fluorouracil (i.p.) to enrich hematopoietic stem cells (HSC). Upon four days, harvested bone marrow was cultured in cytokine cocktail containing IL3, IL6 and SCF until infection on the next day. Infected bone marrow cells were injected into lethally irradiated recipients. B – The scheme of bone marrow chimera combinations used for analysis in the polyclonal and TCR transgenic repertoire.

Candidate selection and confirmation of expression levels

In the first set of bone marrow chimeras, we attempted the lentiviral gain-of-function for the selected candidates. Initially, we analyzed the efficiency of infection by percentage of GFP expressing cells.

For three candidates Dub2a, Itm2a and Nedd4, we achieved infection efficiencies between 1-2% (Fig. 7). The infection rates remained low in infections with several different virus preparations and extended infection times. Therefore, we decided that the gain-of-function experiments for these candidates require further optimization. For IL6Rα, we obtained small populations of infected cells but were unable to show that IL6Rα is overexpressed on the surface of the cell. For the remaining candidates (Lztfl1, Slfn1, Ifi27l2a, Smpdl3a and Ms4a6c) we established a robust overexpression system and therefore narrowed down our focus on these molecules and their functions in T cell biology.

List of final candidates	
Gene symbol	Gene description
Lztfl1	leucin zipper transcription-factor-like 1
Slfn1	schlafen 1
Smpdl3a	sphingomyelin-phosphodiesterase acid-like-3a
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C
Ifi27l2a	interferon, alpha-inducible protein 27 like 2A

Tbl. 9: **List of candidates selected upon lentiviral gain-of-function experiments.** One candidate (Lztfl1) was selected from the list of 28 genes with declining expression between the stages, and additional four candidates (Slfn1, Smpdl3a, Ms4a6c and Ifi27l2a) were selected from the list of genes with increasing expression.

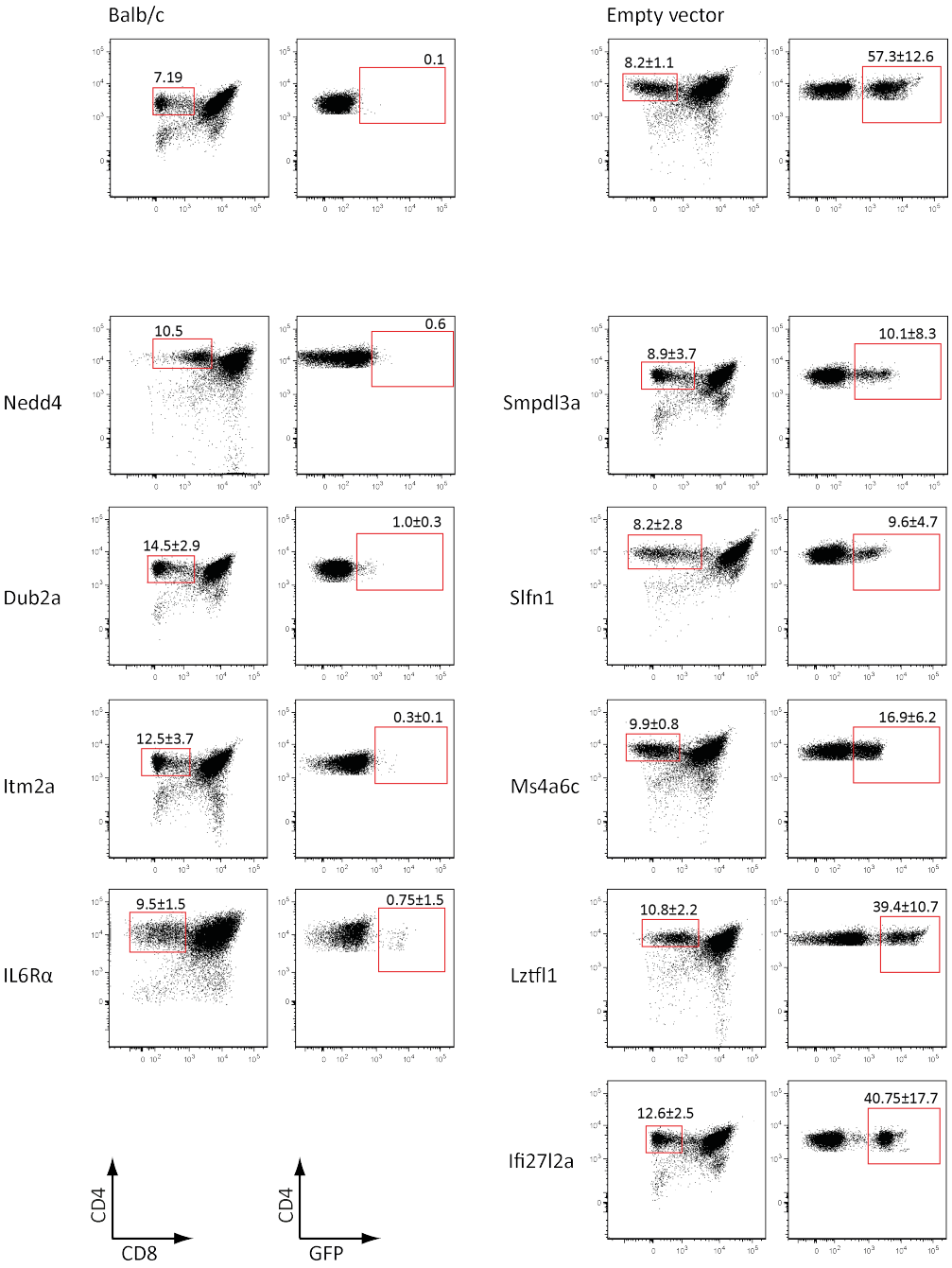


Fig. 7: **Initial screen by gain-of-function experiments.** The depicted plots present CD4 and CD8 staining of thymi in bone marrow chimeras and infection rates of CD4 cells for each candidate. Each plot represents 20 000 events. Numbers above gates present mean frequency ± s.d. of populations within gates. For empty vector controls n=7, for Nedd4 n=2, for Dub2a n=4, for Itm2a n=8, for IL6Rα n=3, for Smpdl3a n=4, for Slfn1 n=8, for Ms4a6c and Lztfl1 n=9 and for Ifi27l2a n=7.

Current knowledge on selected candidates

Lztfl1 (leucine-zipper transcription factor-like 1) was one of the candidates with the strongest downregulation between the 'youngest' and the 'oldest' CD4SP thymocytes, and as a potential transcription factor, we considered this gene to be a promising candidate.

To date, little is known about the functions of Lztfl1. The first description of the Lztfl1 originates from a sequencing of 250 kbp map of short arm on human chromosome 3, 3p21.3 (C3CER1), a segment commonly lost in various tumors [209].

Lztfl1 orthologues were found in all vertebrates but not in invertebrates, plants or fungi. The mouse orthologue of Lztfl1 was mapped to the chromosome 9. The overall similarity between human and mouse cDNA is 86.6 % [172].

Lztfl1 was named due to the presence of the leucine zipper pattern, a helical strech of amino acids in which leucine occurs every seven amino acid residues. This zipper domain is part of the coiled coil at the C terminus while the rest of Lztfl1 seems to have an alpha helical structure according to the bioinformatic predictions [209].

Further, one study has related loss of Lztfl1 to occurrence of gastric cancer in human samples and found that Lztfl1 stabilizes E-cadherin-mediated adhesive junctions and therefore inhibits metastasis of cancer cells via epithelial-mesenchymal transition [210].

Lately, two studies related Lztfl1 to the ciliary transport machinery [172, 211]. The central part of this process is played by a molecular complex, called BBSome, involved in transport of signaling molecules and receptors to the ciliary membrane. This complex has been named after the Bardet-Biedl-Syndrome (BBS), a human genetic disorder related to ciliary disfunction – mainly diagnosed by postaxial polydactily, retinitis pigmentosa, obesity, cognitive impairment and kidney alteration [212, 213].

Seo *et al.*, were able to show that Lztfl1 interacts with Bbs9 and plays a particular role in the BBSome-related transport of SHH (sonic hedgehog) proteins to the cilia [172]. Further, Marion *et al.*, described a first patient with 5bp deletion in Lztfl1 locus leading to a premature stop codon and BBS-like disease with increased levels of sonic hedgehog related proteins – Smo, Ptch1, Gli1, Gli2 and Gli3 [211].

Taken together, Lztfl1 was supposed to negatively regulate the transport of SHH proteins via BBSome in the cilia, while its roles in the immune system are

unexplored. Therefore, we decided to study this protein and its functions in the T cell system primarily.

Slfn1 is a member of the Schlafen family. The upregulation of Schlafen family molecules in thymocytes upon positive selection emerged from a subtractive hybridization of two thymocyte cDNA libraries between non-positively selected AND.4R (AND TCR on H-2^{h4} haplotype) and successfully selected AND.B6 thymocytes (AND TCR on H-2^{b} haplotype) [214]. Via this approach, Slfn1,2, 3/6 and 4/7 were found to be developmentally regulated in the thymocyte maturation. Slfn1 and 2 were shown to be downregulated upon TCR activation, whereas 3/6 and 4/7 were upregulated. The Slfn1 deficient mouse had normal CD4 and CD8 ratios in the thymus and lymph nodes. Later on, Slfn1 molecule was shown to downregulate cyclin D1 and lead to cell cycle arrest. We were interested in exploring how Slfn1 can impact thymic selection and maturation progression in connection to survival at the CD4 SP stage.

Ms4a6c is a member of a novel membrane protein family, MS4A, identified in the hematopoietic cells and structurally related to the cell surface molecules like CD20 and high-affinity IgE receptor β-chain, FcεRIβ. There are at least 12 subgroups (Ms4a1-12) of Ms4a proteins and each member is characterized by the presence of four transmembrane domains [215, 216]. The function of Ms4a6c is unknown and according to the gene expression data bank Immgen, it has the highest expression in naive CD4 T cells.

Smpdl3a, sphingomyelin-phosphodiesterase-acid-like 3a, was first described in a whole-genome screen for the direct liver X receptor (LXR) target genes, as LXR binds to Smpdl3a promoter upon stimulation. Further, a yeast 2-hybrid system described Smpdl3a to be a direct binding partner of a tumor suppressor gene DBCCR1 (deleted in bladder cancer chromosome region 1) in human bladder tumors [217, 218]. To date, there are no described roles of Smpdl3a in the immune system. Its predicted expression is highest in the memory CD4 T cells and Tregs.

Ifi27l2a or ISG12 has been discovered by an oligo-capping signal sequence trap, a technique for screening the cDNA of secretory proteins. This technique was used for

screening in adipocytes upon interferon α exposure and identified Ifi27l2a as a potential IFN α – inducible adipocytokine [198]. According to the Immgen database, Ifi27l2a has the highest expression at the stage of CD4 SP T cells.

Overexpression of selected candidates and the effects on the thymic Treg compartments

The basis of our study is the differential propensity of maturing thymocytes to enter the regulatory T cell lineage and therefore we primarily investigated the formation of Treg compartments upon the overexpression of each candidate gene (Fig. 8). For the four candidates Slfn1, Smpdl3a, Ifi27l2a, Ms4a6c, we did not observe any differences in Treg generation between uninfected and infected compartments. In the infection with the empty vector controls, the GFP^- CD4 SP subpopulation contained 3.2 ± 1.1 %Tregs versus 2.8 ± 0.9 % (p=0.434) in the GFP^+ CD4 SP cells. For the overexpression of Ifi27l2a 2.8 ± 1.2 % versus 2.4 ± 1.2 % (p=0.565) Tregs were observed between GFP^- and GFP^+ populations. In the case of Slfn1, the overexpression did not influence the Treg compartment leading to the similar Treg frequencies with 2.2 ± 0.9 % in the GFP^- and 2.7 ± 1.5 % (p=0.383) Tregs in the GFP^+ cells. Similarly, Ms4a6c gain-of-function did not impact Treg pools with 2.7 ± 0.6 % versus 2.9 ± 1.5 % (p=0.754) Tregs. The overexpression of Smpdl3a induced only slight reduction in thymic Treg compartment with 4.1 ± 1.1 % Tregs in the GFP^- cells versus 2.8 ± 0.9 % (p=0.107) in the GFP^+ cells.
Finally, the overexpression of one candidate, Lztfl1, resulted in a significantly reduced thymic Treg compartment with 3.3 ± 0.8 % Tregs in GFP^- and 1.8 ± 0.5 % (p=0.00001) Tregs in the GFP^+ CD4 SP cells (Fig. 8).

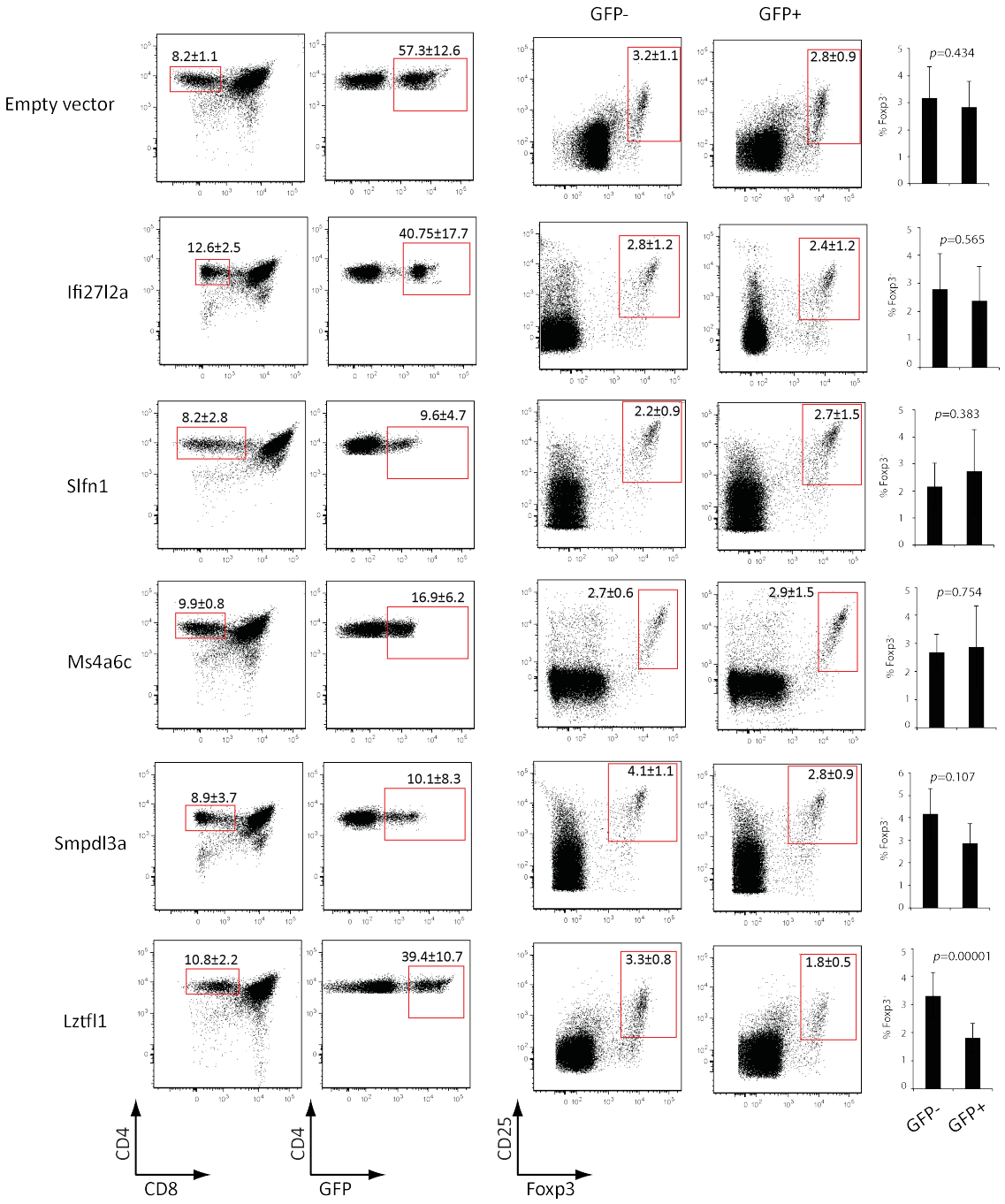


Fig. 8: **Effects of gene overexpression on the thymic regulatory T cell compartment**. The depicted plots present thymic Treg compartments within uninfected and infected CD4SP populations for each candidate. Numbers above and within gates present mean frequency ± s.d.. Bar diagrams present frequency of Foxp3$^+$ cells in each CD4SP GFP$^-$ and CD4SP GFP$^+$ compartment. For empty vector controls n=7, for Smpdl3a n=4, for Slfn1 n=8, for Ms4a6c and Lztfl1 n=9 and for Ifi27l2a n=7.

One potential explanation for the absence of an evident phenotype would be a low level of overexpression. In order to exclude this we performed RT PCR analysis for the majority of candidates (Fig. 9a) and showed certain level of overexpression. The levels of overexpression varied between two and sixteen times relatively to the expression levels of respective candidate in the uninfected subpopulation of CD4SP thymocytes. For Lztfl1 we showed the overexpression also at the protein level by Western blotting (Fig. 9b).

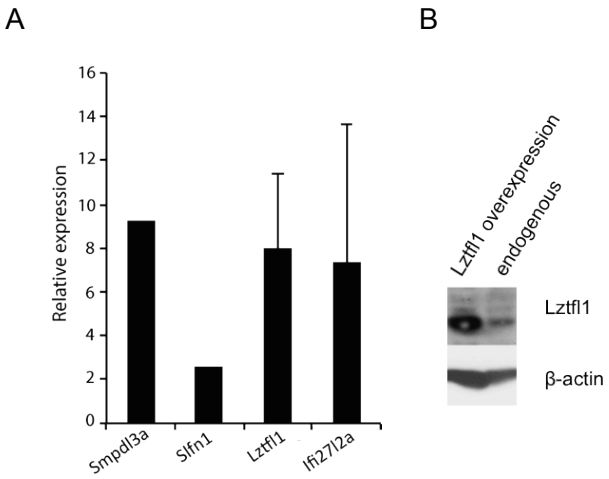


Fig. 9. **Overexpression of selected candidates upon lentiviral infection in bone marrow chimeras.** A – Bar diagram represents the overexpression of candidate mRNA in infected CD4SP thymocytes (GFP^+), normalized and compared to the uninfected CD4 SP thymocytes (GFP^-). The represented bars depict mean ± s.d. of three experiments for Lztfl1 and Ifi27l2a, while for Smpdl3a and Slfn1 means of two experiments are depicted. B – Western blot showing overexpression of Lztfl1 in the total CD4 SP thymocytes of a chimeric and wild type thymus.

Lztfl1 overexpression leads to reduced thymic regulatory T cell compartment

In the initial screen by the lentiviral gain-of-function, we showed that one of the selected candidates, Lztfl1 influences the formation of the thymic Treg compartment. The thymic Treg pool in the Lztfl1-overexpressing population was decreased up to 50% and made only 1.82 ± 0.5 % instead of 3-4 % of all other CD4$^+$SP thymocytes (uninfected and empty-vector-infected) (Fig. 10).

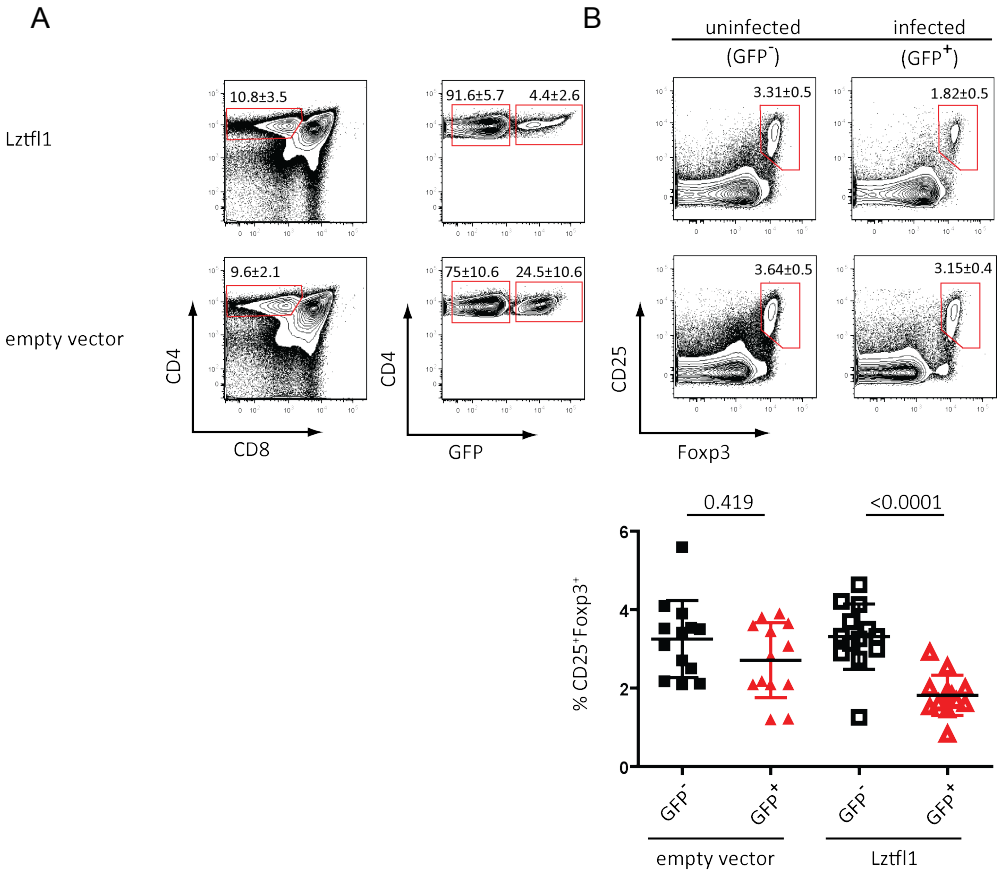


Fig. 10: **Effects of the Lztfl1 overexpression on the thymic Treg compartment.** A – The plots depict CD4 and CD8 staining of thymi in bone marrow chimeras and gating on the uninfected and infected CD4 SP thymocytes to assess the frequency of thymic regulatory T cells in each compartment. B – The plots depict thymic Treg pools in the Lztfl1-overexpressing and the control chimeras. Numbers in the gates represent frequencies ± s.d. of n = 13 for control and n = 12 for overexpression chimeras.

Effects of Lztfl1-overexpression on the peripheral Treg pool

In the analysis of the peripheral Treg pool, the previous observation made in the thymus of the bone marrow chimeras remained evident but the difference had a lower extent and a higher variability, with 11.91 ± 1.2 % in the uninfected control versus 6.16 ± 0.8 % (*p*=0.002) in the Lztfl1-overexpressing population (Fig. 11). Taken together, the overexpression of Lztfl1 in CD4 T cells leads to reduced thymic and splenic regulatory T cell pools.

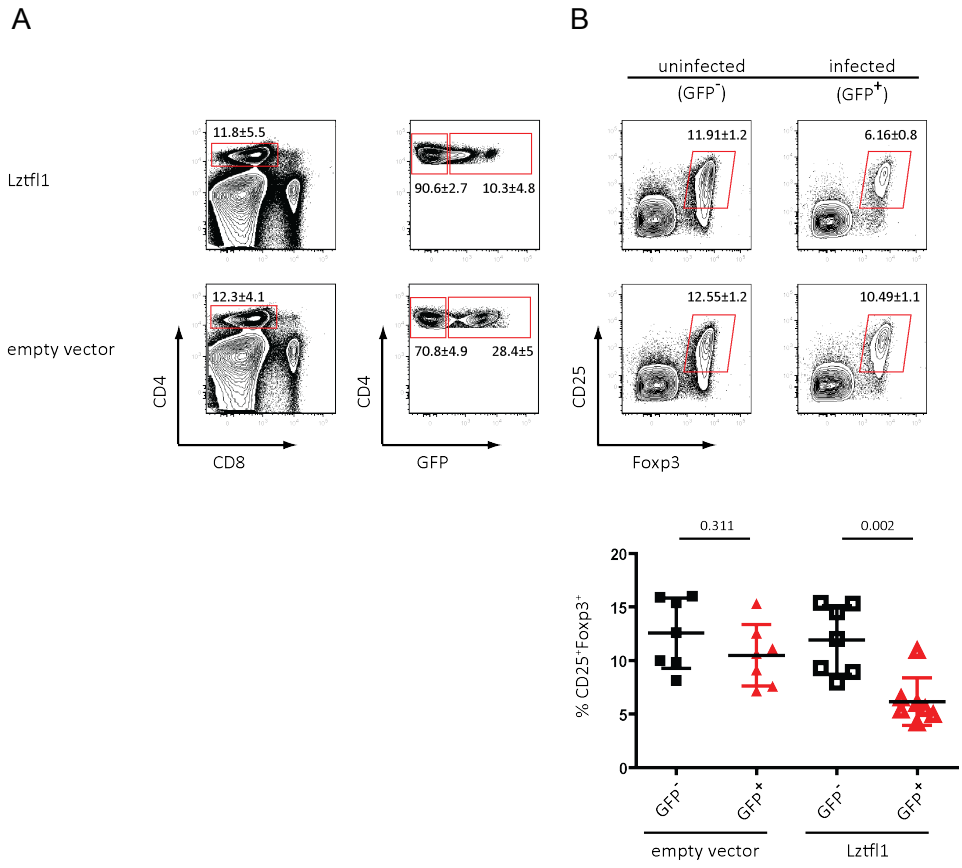


Fig. 11: **Effects of the Lztfl1 overexpression on the splenic Treg compartment.** A – The plots depict CD4 and CD8 staining of splenocytes in bone marrow chimeras and gating on the uninfected and infected $CD4^+$ T cells to assess the frequency of peripheral regulatory T cells in each compartment. B – represents splenic Tregs in the overexpressing and control chimeras, each n = 7. Diagrams below display frequencies in each sample.

Focusing on the potential roles of Lztfl1 in the thymic Treg development, we decided to perform the bone marrow chimeras in a very well established Treg development system utilizing TCR-HA and Aire-HA models.

We overexpressed Lztfl1 in TCR-HA thymocytes and examined the cognate-antigen induced Treg development. We hypothesised that the effects of Lztfl1 overexpression decrease the Treg compartment in TCR-HA x Aire-HA system, confirming and emphasizing the observation from the experiments in the polyclonal repertoire. However, we could not evidence any significant change in the Treg development, although a certain tendency toward the reduction of the Treg compartment by the overexpression of Lztfl1 in the presence of cognate antigen was measurable, with 18.5 ± 6.6 % in GFP^- TCR-HA population versus 13.2 ± 8.1 % (p=0.133) in the GFP^+ population (Fig. 12).

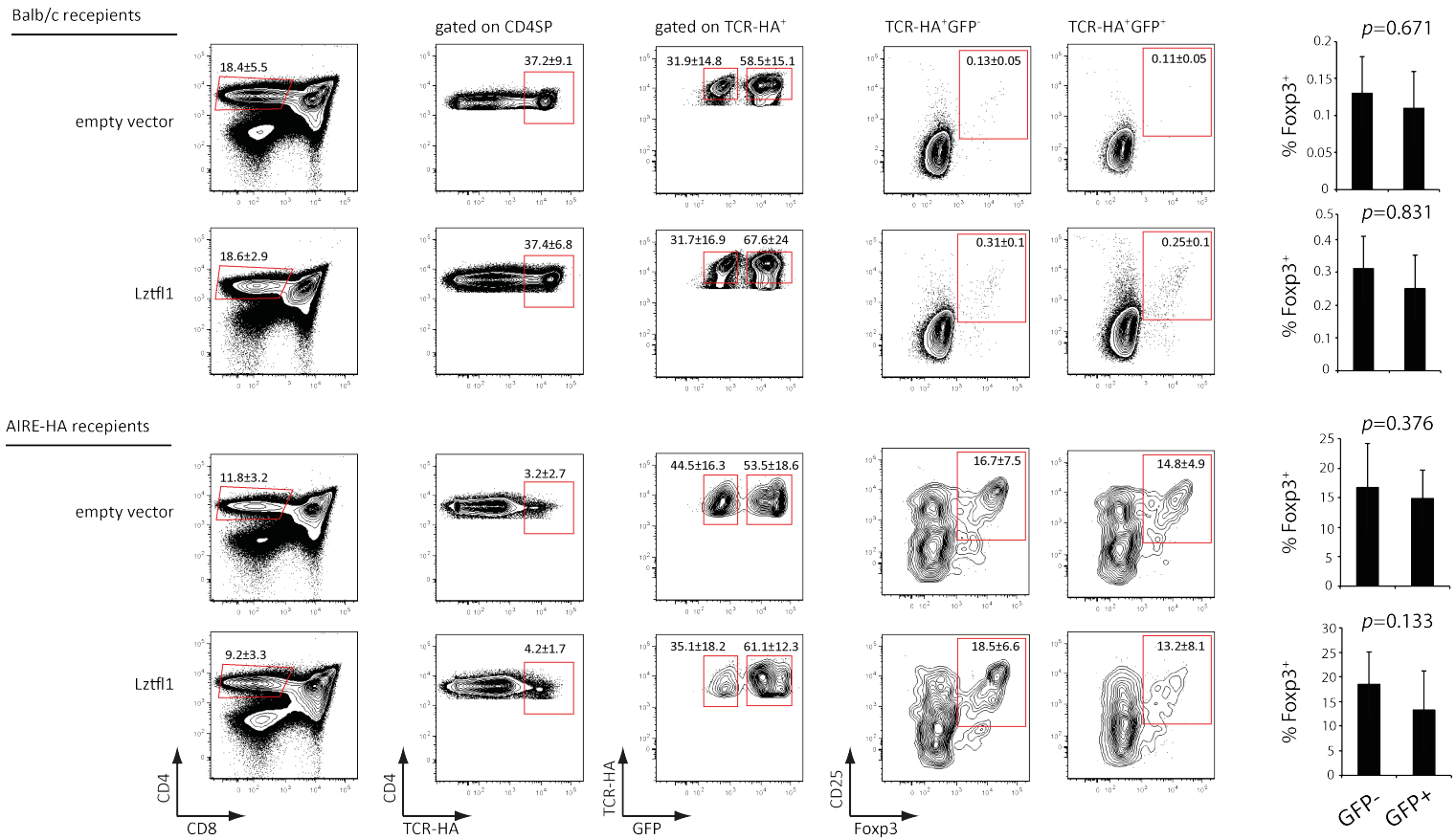


Fig. 12: **Analysis of TCR-HA$^+$ CD4 T cells in Aire-HA recepients upon bone marrow infection with Lztfl1- or control-virus.** The plots depict staining for Treg cells upon gating on TCR-HA$^+$ CD4$^+$ T cells in the thymi of Balb/c or Aire-HA recepients and the numbers within gates represent mean of frequencies ± s.d. Diagrams represent frequencies ± s.d. of n = 8 in each group.

Knockdown of Lztfl1 in CD4 SP thymocytes has no impact on Treg pools

In the attempt to further validate the results of lentiviral gain-of-function experiments, we performed knockdown for Lztfl1. Therefore we generated three different designer miRNAs, assembled of miR30 backbone and predicted, Lztfl1-targeting shRNAs (microRNA-adapted shRNA expression vectors). We tested the efficiency of three different miR constructs by RT PCR for Lztfl1, normalizing the results to the empty vector (miR30) infections. From the obtained results of two independent experiments we decided to focus on Lztfl1 miR1, which reduced the expression of Lztfl1 to around 20% of the level in the control (Fig. 13).

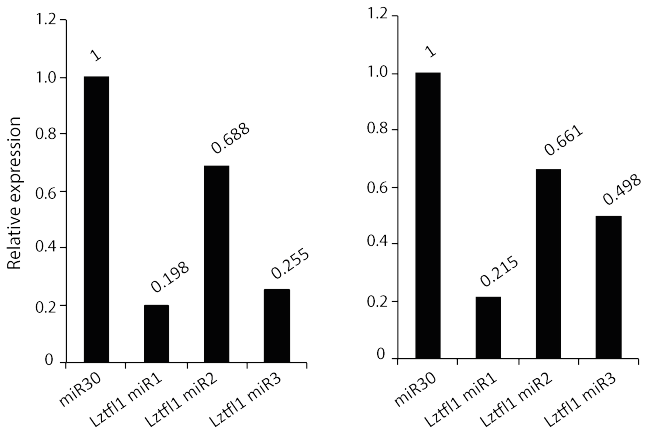


Fig. 13: **Expression of Lztfl1 in CD4SP thymocytes in bone marrow chimeras using three different designer miRNA knockdown constructs.** The bar diagrams depict RT PCR measurements of Lztfl1 expression in CD4 SP with constructs carrying different shRNAs agains Lztfl1 or miR30 (empty vector control) in two different experiments. The expression level in the infection with miR30 control was set as one and all the other infections were normalized to it.

We infected bone marrow with either constructs containing Lztfl1 miR1 or only miR30 (empty vector) and analyzed thymic development in the knockdown of Lztfl1. The frequencies of $CD25^{+}Foxp3^{+}$ CD4 thymocytes were very similar between all uninfected and infected groups. This result shows that the reduction in Lztfl1 levels does not impact the formation of Treg compartment, with 2.4 ± 0.4 % Tregs in the uninfected compared to similar frequency of 2.7 ± 0.3 % (p=0.631) Lztfl1miR1 infected CD4SP. Nevertheless, it remains unclear whether the residual amounts of Lztfl1 may be sufficient to maintain the normal functions of Lztfl1.

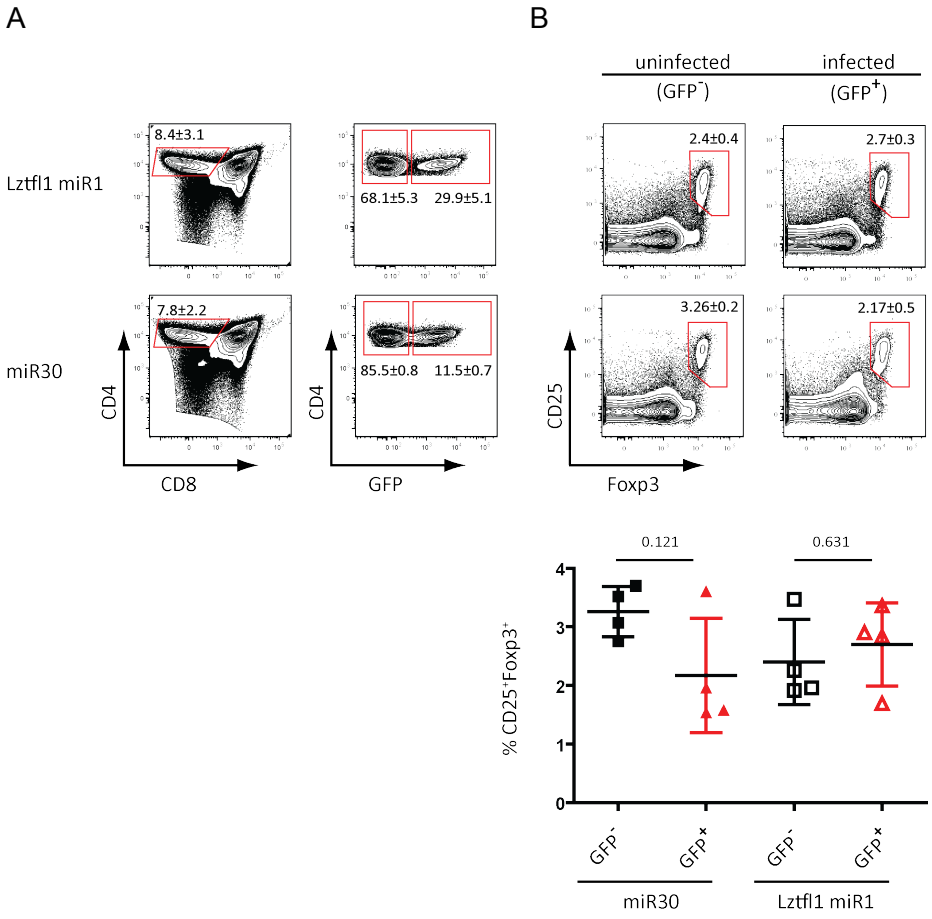


Fig. 14: **The effects of Lztfl1 knockdown on the thymic Treg pool.** Presented plots depict thymic Treg compartments in bone marrow chimeras in infections with Lztfl1 miR1 virus and miR30 virus (empty control) upon gating on CD4 SP and then on uninfected and infected populations. Numbers in the gates present mean frequencies ± s.d..The diagram presents Treg frequency in each set of chimera for n = 4.

From these observations and experiments, we concluded that the most efficient and conclusive way to study the roles of Lztfl1 would be to generate a mouse model, carrying Lztfl1 alleles with conditional knockout potential (floxed) and to breed it to diverse CRE models. Therefore, we generated ES cell clones containing a targeting construct for conditional knockout of Lztfl1 (Fig. 15).

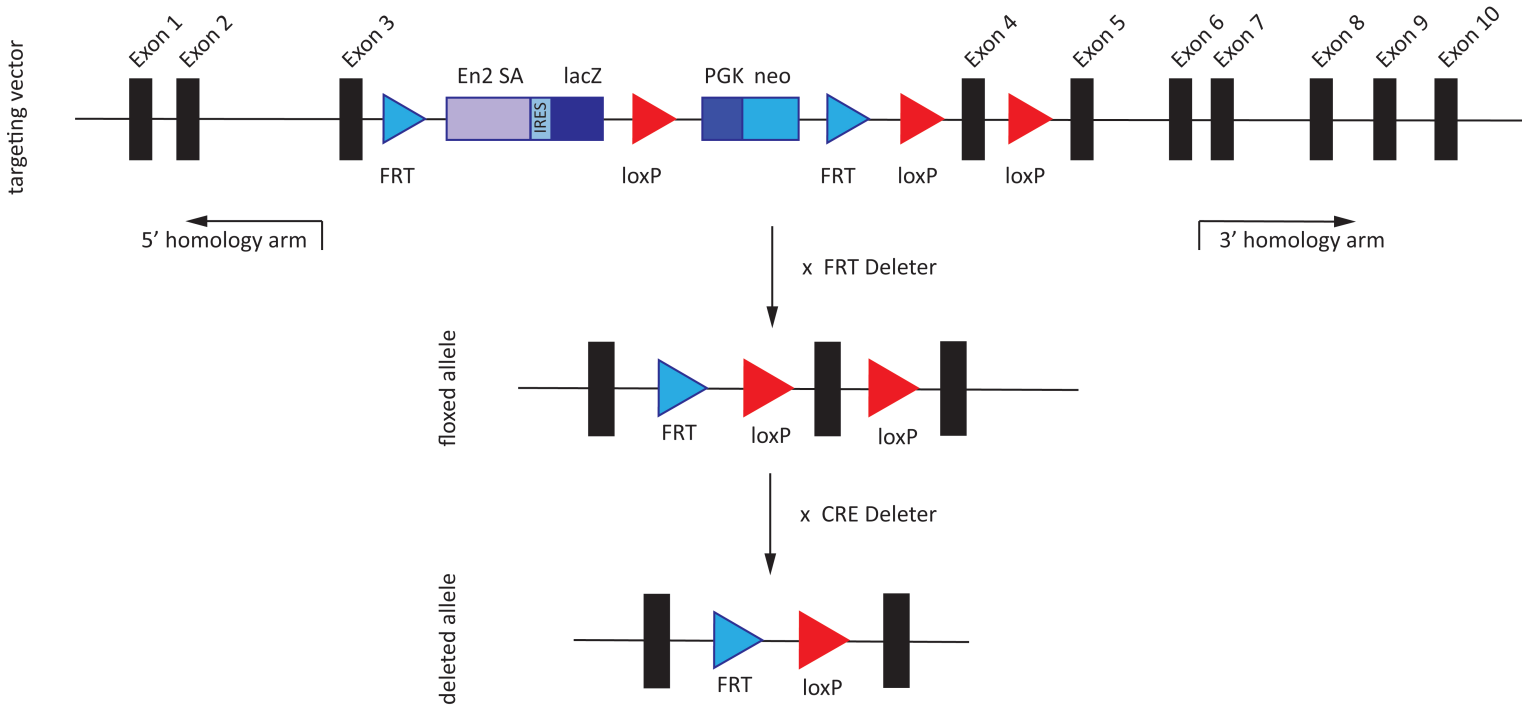


Fig. 15: **Scheme of the Lztfl1 targeting construct**. This scheme represents the targeting construct created by KOMP targeting facility (www.komp.org, Knockout Mouse Project). The major features of the construct are the floxed exon four, surrounding loxP sites, LacZ reporter and neomycin resistance elements flanked by FRT sites. Through the utilization of FRT deleter one can remove the reporter and resistance elements and end with the conditional allele of Lztfl1. Further any CRE deleter can be used to conditionally and specifically remove Lztfl1.

Further selection and focus on the remaining candidates

Upon the initial screen via the described lentiviral overexpression system, we extended our analyses and included the availability of the knockout mouse models in the candidate selection. For one of the previously selected candidates, Nedd4, a conditional knockout mouse model was available and we approached the study of Nedd4 in the thymocyte development.

Current knowledge about Nedd4

The discovery of Nedd4 (**n**eural precursor cell **e**xpressed, **d**evelopmentally **d**ownregulated protein **4)** is based on the subtraction cloning of developmentally downregulated genes between embryonic and adult mouse brain [219, 220]. Later, Nedd4s role in the regulation of axonal sensitivity in *Drosophila* and neural crest closure in higher organisms have emerged from its interactions with developmentally important molecules like Commissureless, roundabout and Rap2A [158, 221]. The complete deficiency on Nedd4 is due to this lethality at the embryonic day 12.
Nedd4 is one of several E3 ubiquitin ligases described in the immunological context. It is a HECT (homologous to the E6-AP carboxyl terminus) family E3 ubiquitin ligase, meaning that it is uniquely responsible for the substrate specificity and performs ubiquitin transfer onto a targeted protein in association with an E2 ligase. The protein structure of Nedd4 is characterized by the presence of C2 domain, three WW domains and HECT domain. The C2 domain brings the Nedd4 molecule into the proximity of diverse adaptor proteins, while WW domains typically determine substrate specificity. The HECT domain carries intrinsic catalytic activity. Interestingly, Nedd4 has been described to mainly perform K63 ubiquitination instead of classical K48, which indicates proteosomal degradation. K63-linked ubiquitination can have different meanings than degradation, like transport or activation [222, 223].
The major work that relates Nedd4 to T cells has been performed on peripheral T cells in the context of activation [182, 224]. At the same time, no data on roles in thymocyte biology has been gathered. From the experiments on Nedd4 deficient fetal liver chimeras, we know that Nedd4 deficient T cells proliferate poorly and have

increased levels of Cbl-b due to the lack of Nedd4-mediated targeting for degradation [182]. Nedd4 deficient T cells in this system maintain their naïve phenotype and produce less IL2 upon activation, leading to the conclusion that this deficiency leads to hyporesponsiveness. Further, B cells undergo class switching with lower frequency, which might result from moderate T cell activation and unsuccessful help to the B cells, or it might be B cell intrinsic. According to this model, there are no impacts on the thymic T cell development and selection processes or further Treg generation [182]. As this model describes a phenotype based on the regulation of TCR responsiveness, we reasoned that the thymocyte development might need reconsideration in a more clear approach, using conditional ablation of Nedd4.

The data provided from the work of Yang *et al.* based the rationale to ask how these potentially hyporesponsive thymocytes perform in the selection processes. Moreover, the relatively slight effects of Nedd4 on the peripheral TCR signal fine-tuning proposed a very interesting tool, in the context of TCR signal strength studies of the negative selection versus regulatory T cell development.

Expression profile of Nedd4 among T cell subsets

We firstly confirmed the expression pattern by real-time PCR over a range of relevant T cell populations (Fig. 16). Consistent with the microarray results, the expression level of Nedd4 decreases from the CD4 SP1 stage (rel. expression 1,00) over the SP2 stage (rel. expression 0.599) to SP3 stage (rel. expression 0.196). The lowest expression level of Nedd4 was found to be at the stage of a naive T cell from peripheral lymphatic organs, spleen and pooled lymph nodes. These observations indicate that the level of Nedd4 decreases with the developmental stage from selected thymocytes to the fully developed T cells in the periphery.

When we analyzed the expression of Nedd4 at the stages prior to T cell selection process, we observed an interesting expression pattern in the double negative compartment. Prior to the T cell lineage commitment, at the stages of early thymic progenitor and double negative stage 1, we detected relatively low Nedd4 expression compared to the expression at the following stages, double negative 2,3 and 4. According to these results, we assumed that Nedd4 might play an important role at the double negative stage 2-3.

As the stimuli through the TCR play one of the most important roles in the education and the lifetime of a T cell, we compared the expression of Nedd4 between the pre- and post-positive selection double positives. In this comparison the level of Nedd4 decreases with the ongoing positive selection.

In addition, in the periphery we observed ten-fold increase in mRNA expression levels of Nedd4 between the naive (rel. expression 0.003) and memory T cell (rel. expression 0.037).

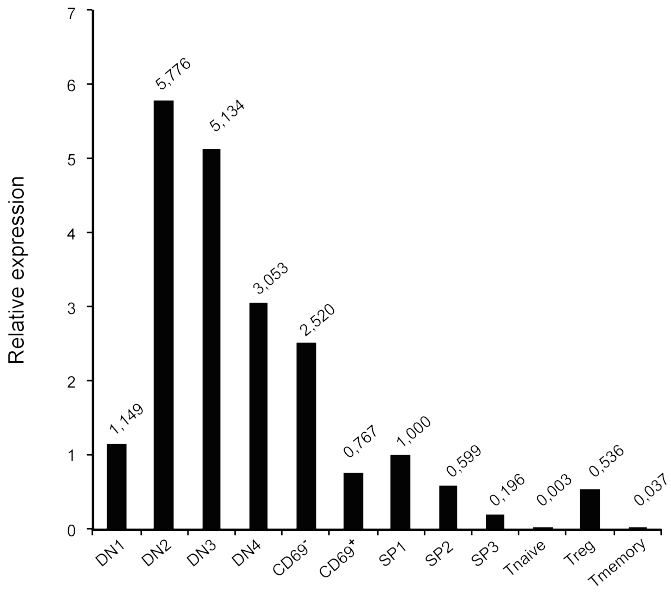


Fig. 16: **Relative expression of Nedd4.** Bar diagram represents means of two real-time PCR reactions for each T cell population. Expression of Nedd4 at the SP1 stage was set to one and all further expressions were normalized to that level.

We compared our expression data to the expression profiles of Nedd4 in the gene expression data base, Immgen (http://www.immgen.org/) and could confirm the expression levels for the majority of populations. In the case of DN1 subset we observed lower levels as those reported by Immgen. This discrepancy might result from different gating strategies, as we did not differentiate the between early thymic precursors (ETP) and ETP to DN2a transitional stages, in order to maximize our sorting yield and efficiency. Nevertheless, the expression profile generated allows us an overview of Nedd4 expression in the thymus.

Conditional deletion as experimental approach to study Nedd4 in T cell biology

The study of Nedd4 expression pattern over the T cell lineage subsets and its dynamics founded a basis to analyse the effects of Nedd4 deficiency in the immune system and T cells, in particular.

The major advantage in this experimental intention was the availability of a conditionally deficient Nedd4 mouse model [158].

This model was designed to carry an exon flanked by the loxP sites between the C2 and the first WW domain of Nedd4. Via crossing to any possible CRE deleter model, this would allow the ablation of Nedd4 expression at the particular level.

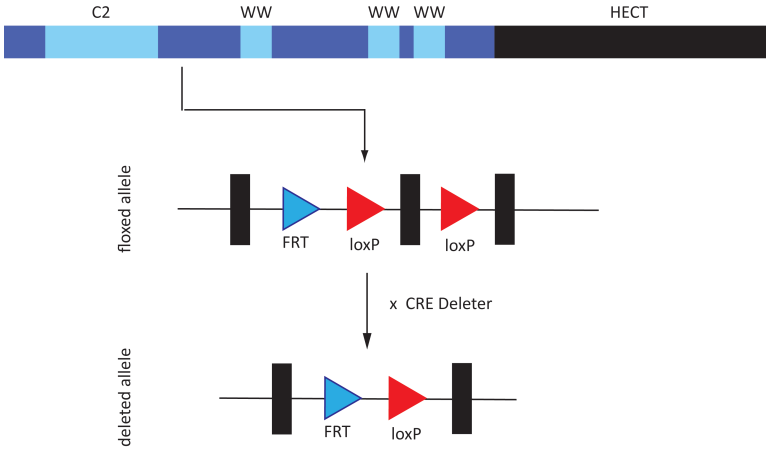


Fig. 17: **Construct of Nedd4 conditionally deficient allele.** The targeting region is between C2 and the first WW domain of Nedd4. Upon crossing with a FRT deleter, elements like LacZ reporter and neomycin resistance have been removed and the construct was left with a loxP flanked exon ready for conditional ablation.

In order to investigate the roles of Nedd4 in the T cell development, we decided to utilize four different CRE deleters: Vav-, Lck-, CD4- and Foxp3-CRE. Undertaking this approach, we wanted to dissect the study of Nedd4 at the different developmental stages and relate potential phenotypes to the stage-specific functions of Nedd4.

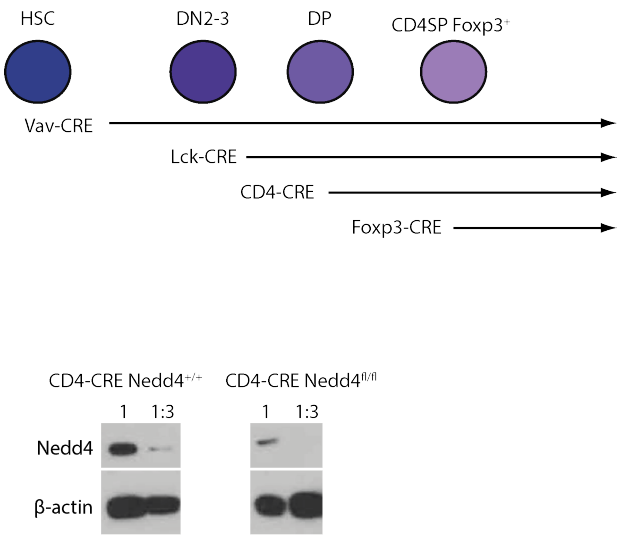


Fig. 18: **Four different CRE deleters for stagewise Nedd4 deletion.** A – In order to address stage-specific roles of Nedd4, we utilized four different CRE deleters, starting with the earliest Vav-CRE (deletion at the stage of hematopoietic stem cells, HSC). Over the Lck-CRE (deletion at the DN2/3 stage), to the CD4-CRE (deletes at the DP stage). The Foxp3-CRE delete starts at the stage of $CD4^{+}Foxp3^{+}$ thymocytes. B- We assessed the efficiency of CD4-CRE deletion by Western blotting using the total thymi of CD4-CRE $Nedd4^{+/+}$ and CD4-CRE $Nedd4^{fl/fl}$ mice. We performed 1:3 dilutions of the cell lysates in order to reliably quantify Nedd4 amounts at the protein level. The deletion efficiency of Nedd4 was considered to be adequate, as the estimation of Western blot shows reduction in Nedd4 protein below 60%.

Thymocyte subsets and phenotypical characteristics upon Nedd4 ablation

In the primary experiments, we analyzed the thymi of the four described Nedd4 deletion models (Vav-, Lck-, CD4-, or Foxp3-CRE x Nedd4$^{fl/fl}$) and compared it to the respective controls carrying the wild type loci of Nedd4 (Vav-, Lck-, CD4-, or Foxp3-CRE x Nedd4$^{+/+}$). The overall thymic morphology and cellularity was very similar between the examined genotypes of each model.

We firstly investigated the CD4 SP developmental stages using the CD4-CRE deleter (Fig. 19 and 20). Determined by CD4 and CD8 staining, double positive compartments of both genotypes did not vary significantly; 86.4 ± 2.5 % in the WT versus 83.6 ± 3.9 % (p=0.157) in the conditional knockout (cKO), followed by changes of similar extent in CD4 SP: 6.6 ± 1.4 % in WT and 7.8 ± 2.4 % (p=0.208) in the cKO and CD8 SP: 1.6 ± 0.2 % in the WT and 2.0 ± 0.2 % (p=0.124) in the cKO (Fig 19A).

Further, the ablation of Nedd4 did not show any effect on the distribution of the three CD4 SP subpopulations: SP1 (6C10^{+}CD69^{+}) with 32.2 ± 1.4 % in WT and 34.7 ± 0.4 % (p=0.123) in the cKO, SP2 (6C10^{-}CD69^{+}) with 19.1 ± 0.6 % in WT and 17.1 ± 1.1 % (p=0.166) in the cKO, SP3 (6C10^{-}CD69^{-}) with 30.9 ± 1.6 % in WT and 29.2 ± 1.0 % (p=0.405) in the cKO (Fig. 19B).

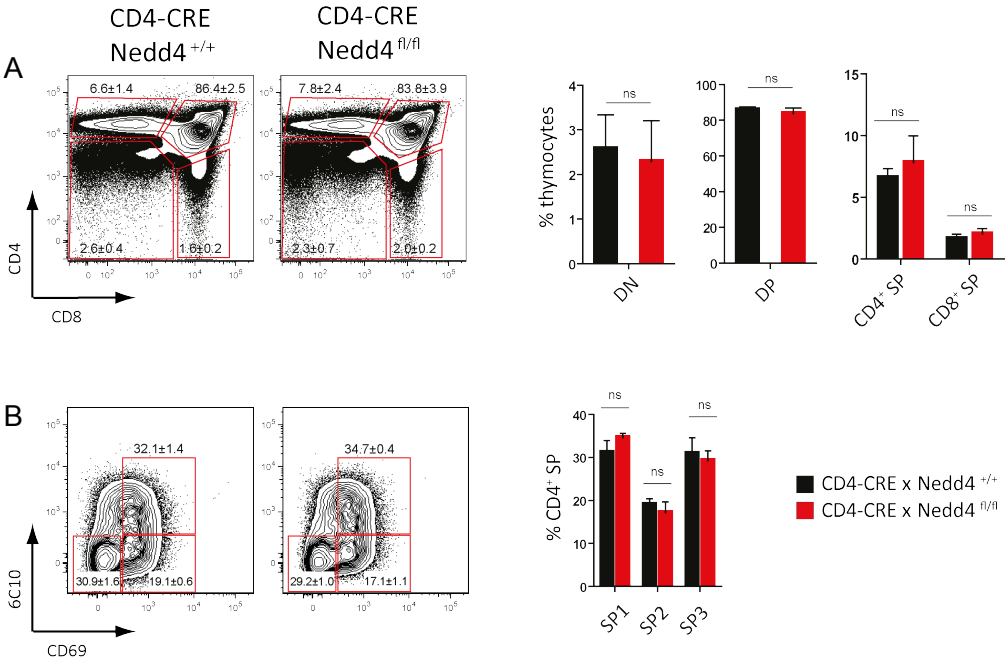


Fig. 19: **Analysis of the thymi and CD4 SP compartments of the Nedd4 deficient mice.** Depicted plots represent thymi of CD4 CRE mice (CD4-CRE Nedd4$^{+/+}$) and mice carrying CD4-CRE-mediated Nedd4 deletion (CD4-CRE Nedd4$^{fl/fl}$). A - General distribution of thymocytes in both genotype combinations (n=6). B - Distribution CD4 SP subsets, SP1,2 and 3 in both genotypes. Diagrams on the right show frequency means ± s. d. of subpopulations for both genotypes CD4-CRE Nedd4$^{+/+}$ (black bars) and CD4-CRE Nedd4$^{fl/fl}$ (red bars).

Next, we analyzed the expression of CD3 molecule between the DP and CD4 SP stages in both genotypes. As our hypothesis would assign Nedd4 to be a TCR positive regulator, we speculated that the effects of positive selection in the absence of Nedd4 would affect CD3 upregulation at the single positive stages. However, both Nedd4 deficient and sufficient DP thymocytes were able to enhance CD3 expression upon positive selection with equal quality (Fig. 20A).

Further, we wanted to investigate the proliferative capacity of thymocytes in both genotypes. We used Vav-CRE model in order to include DN compartments in the analysis. We measured the Ki67 expression in diverse thymocytes populations. Nevertheless, in this aspect both groups compared showed indistinguishable results throughout all subpopulations (Fig. 20B).

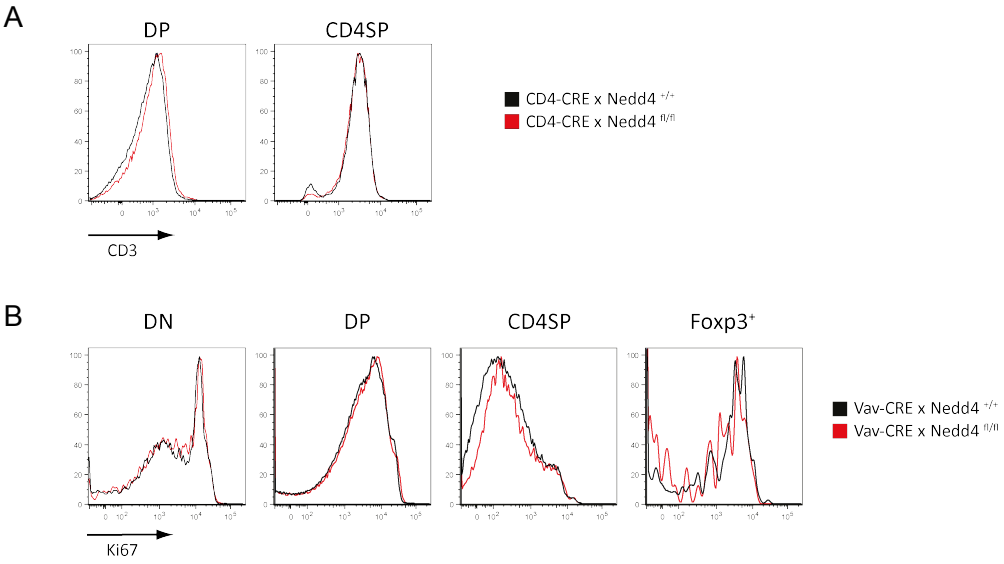


Fig. 20: **Expression of CD3 ζ chain and proliferation marker, Ki67.** A - Histogram overlays of CD3 ζ chain between the CD4-CRE x Nedd4$^{+/+}$ (black lines) and CD4-CRE x Nedd4$^{fl/fl}$ (red lines) DP and CD4 SP cells. B – Expression of the proliferation market Ki67 on the DN, DP, CD4SP and thymic Tregs between the Vav-CRE x Nedd4$^{+/+}$ (black lines) and Vav-CRE x Nedd4$^{fl/fl}$ (red lines)

Due to the Nedd4 expression profile, we were interested to see potential changes in the double negative thymocyte compartment. We utilised Vav-CRE deleter for this analysis, to allow sufficiently early Nedd4 ablation.

The double negative (lineage negative) compartment of Nedd4 sufficient and deficient thymi seemed to segregate normally into all double negative stages 1 to 4 (Fig. 21A). Double negative population 1 contributed with 9.7 ± 1.5 % in WT and 8.2 ± 1.4 % (p=0.498) in cKO, followed by the small population of double negatives 2 with 2.6 ± 0.4 % in WT and 2.1 ± 0.5 % (p=0.427) in cKO. The two larger populations of double negative 3 and double negative 4 were similarly contributing with 45.6 ± 5.9 % in WT and 57.6 ± 3.9 % (p=0.120) in cKO for the former and with 33.1 ± 5.4 % in WT and 31.9 ± 3.6 % (p=0.859) in cKO for the latter population. Additionally, the frequences of early thymic progenitor were also very similar with 4.3 ± 0.2 % in WT and 4.1 ± 1.1 % (p=0.844) in cKO (Fig. 21B).

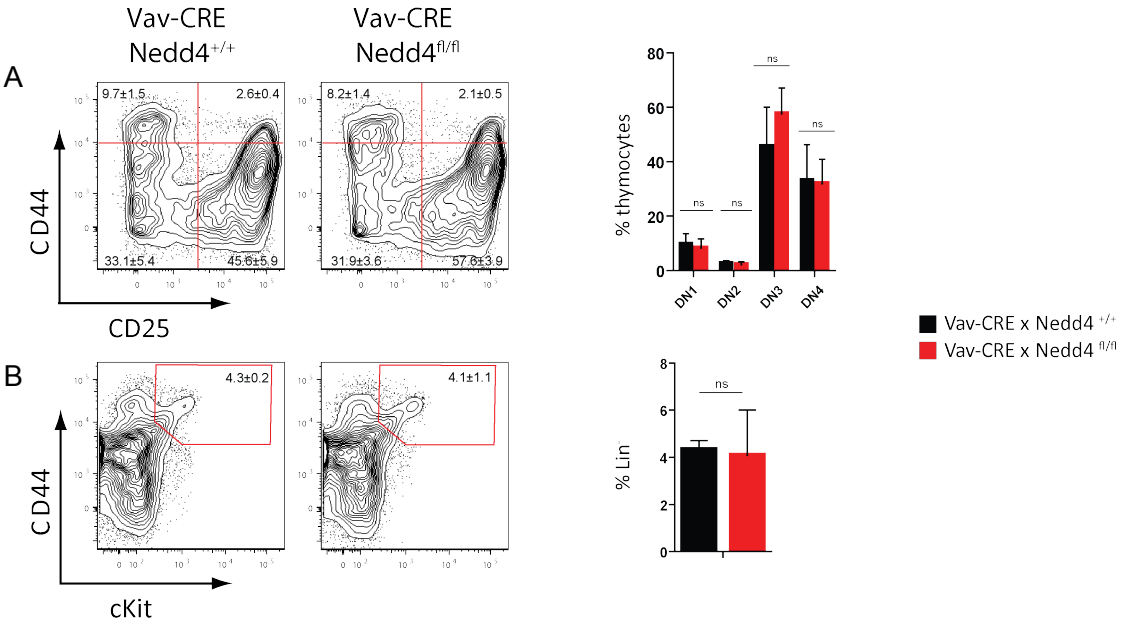


Fig. 21: **Early thymocyte developmental stages in the absence of Nedd4.** A - Depicts double negative compartments of Vav-CRE Nedd4$^{+/+}$and Vav-CRE Nedd4$^{fl/fl}$ thymi and the diagram represents frequency means ± s.d. of each double negative population 1-4. B – Staining for ETP (early thymic precursor) and the frequency for each genotype are represented upon gating on lineage negative cells and CD44 and c-Kit positive cells.

The events at the double negative stage involve mainly commitment to the T cell lineage and the β selection, leading to the formation of preselection pool of αβ T cells. The major role in this process is played by preTCR signaling that is essential for the lineage commitment between the αβ versus γδ T cells. Therefore, we investigated the presence of γδ T cells in the Nedd4 deficient thymi. The frequency of γδ T cells in the cKO was 0.09 ± 0.02 % and therefore very similar to that of WT, 0.12 ± 0.01 % (p=0.285) (Fig. 22A). Furthermore, we investigated the generation of NKT cells in Vav- and CD4-CRE constellations. As for the γδ T cells, the ablation of Nedd4 did not have any effect on the NKT cell pool in both genotypes, with 0.2 ± 0.05 % in WT and 0.27 ± 0.06 % (p=0.479) in cKO (Fig. 22B).

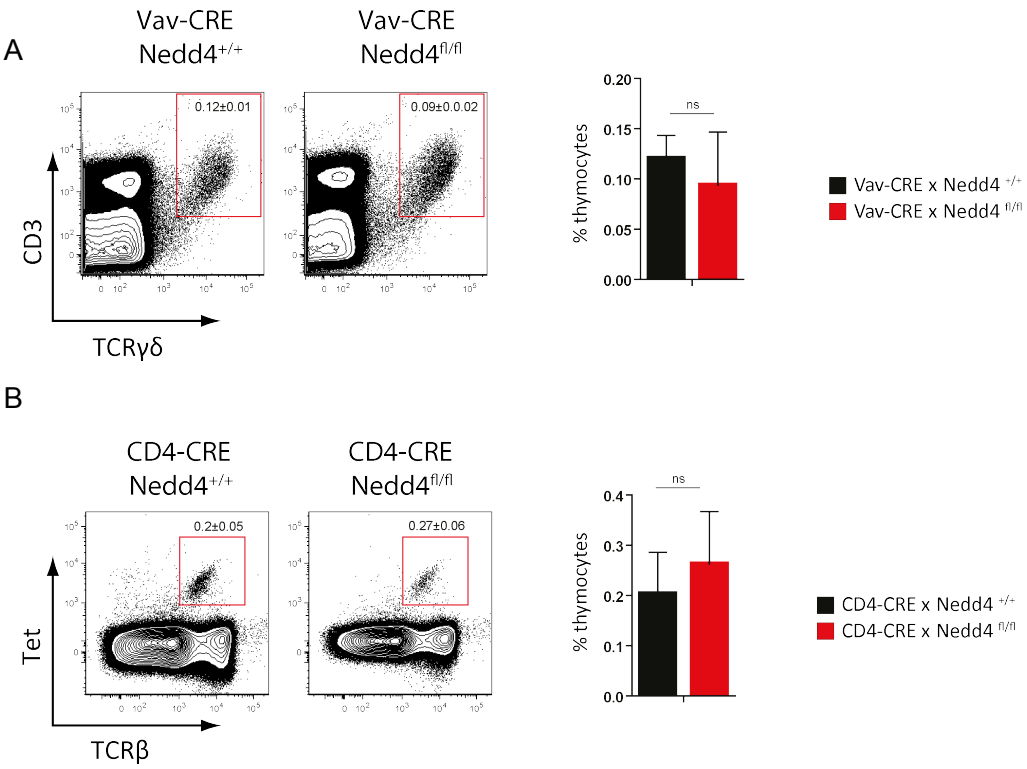


Fig. 22: **γδ and NKT cell development in Nedd4 sufficient and deficient thymocytes.** A – The plots depict percentages of γδ T cells upon ablation of Nedd4. Numbers in gates represent means of frequencies of gated population upon staining for CD3 and TCRγ on total thymocytes. B – NKT cells upon staining for the major markers TCRβ and CD1d tetramer PBS-57 (Tet) and gating on whole thymocytes. On the right frequency means ± s.d. are represented. For γδ T cells n=6 and NKT cells n=3.

Taken together, we were unable to identify any changes or defects in T cell development by the absence of Nedd4 at the thymocyte stage and moved on toward the search for potential differences in the peripheral immune organs.

Nedd4's potential roles in the peripheral T cell immunity

For the investigation of the events ongoing in the periphery, we screened three major sites: spleen, pooled peripheral lymph nodes and mesenteric lymph nodes. At the first glance, mice conditionally deficient in Nedd4 by CD4-CRE showed normal CD4 / CD8 ratios and frequencies in the periphery. CD4 T cell frequencies were 13.3 ± 0.9 % in the CD4-CRE Nedd4$^{+/+}$ and 10.4 ± 2.3 in the CD4-CRE Nedd4$^{fl/fl}$ (p=0.316) (Fig. 23A). Utilising other CRE models like Vav- and Lck-CRE we observed similar results and also no changes in CD8 frequencies.

The activation status of the CD4 T cells, measured by the expression of CD62L and CD44 was very similar between both genotypes; frequency of naïve CD4 T cells was 74.6 ± 2.4 % in WT and 75.8 ± 2.2 % (p=0.705) in cKO. The memory pools were also very similar with 14.4 ± 1.3 % in WT and 13.0 ± 1.6 % (p=0.531) in cKO (Fig. 23B).

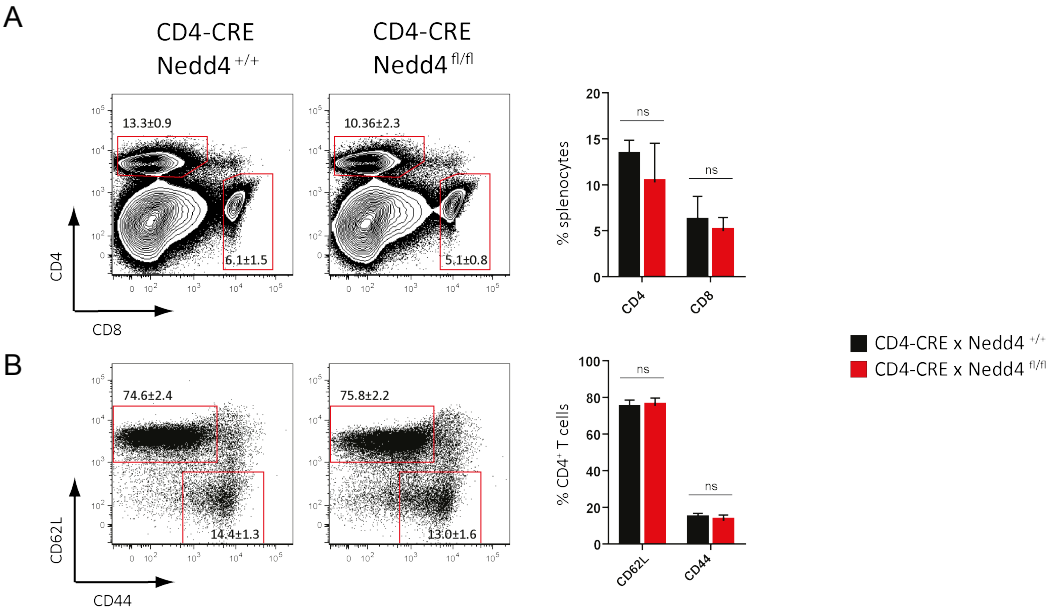


Fig. 23: **CD4 and CD8 staining of splenocytes.** A - CD4 and CD8 T cells in the spleens of CD4-CRE x Nedd4$^{+/+}$ and CD4-CRE x Nedd4$^{fl/fl}$ mice. The numbers within gates represent frequencies of of each cell population. B – CD62L and CD44 staining for the activation status of CD4 T cells; CD4-CRE Nedd4$^{+/+}$ (black bars) CD4-CRE Nedd4$^{fl/fl}$ (red bars).

The expression of Nedd4 in the peripheral Tregs was shown to be higher than the expression in the naive cells (Fig. 16). We examined thymic and peripheral Treg pools for the frequency of the $CD25^{+}Foxp3^{+}$ T cells and expression of their common markers (Fig. 24).

The frequencies in the thymus, spleen, peripheral and mesenteric lymph nodes were very similar between the CD4-CRE $Nedd4^{+/+}$ and CD4-CRE $Nedd4^{fl/fl}$. Very same result was obtained in the experiments using Foxp3-CRE deleter. In the thymi of CD4-CRE $Nedd4^{+/+}$ mice 2.7 ± 0.1 % $Foxp3^{+}$ T cells were found, which resembles the frequency of $Foxp3^{+}$ cells in the CD4-CRE $Nedd4^{fl/fl}$ situation, 2.8 ± 0.3 % (p=0.823) (Fig. 24A). The levels of the Treg marker expression like CTLA4, GITR, CD28 and CD122 (IL2R-β chain) remained unaffected both in the thymus and the peripheral organs with the absence of Nedd4 (Fig. 24B). In the pools of the peripheral lymph nodes for both genotypes, we found similar frequencies of Tregs with 13.2 ± 0.6 % and 13.6 ± 0.7 % (p=0.700). Consistent with this observation, frequencies in the spleen did not differ between both genotypes with 7.9 ± 0.2 % in WT and 7.6 ± 0.3 % (p=0.349) in the cKO.

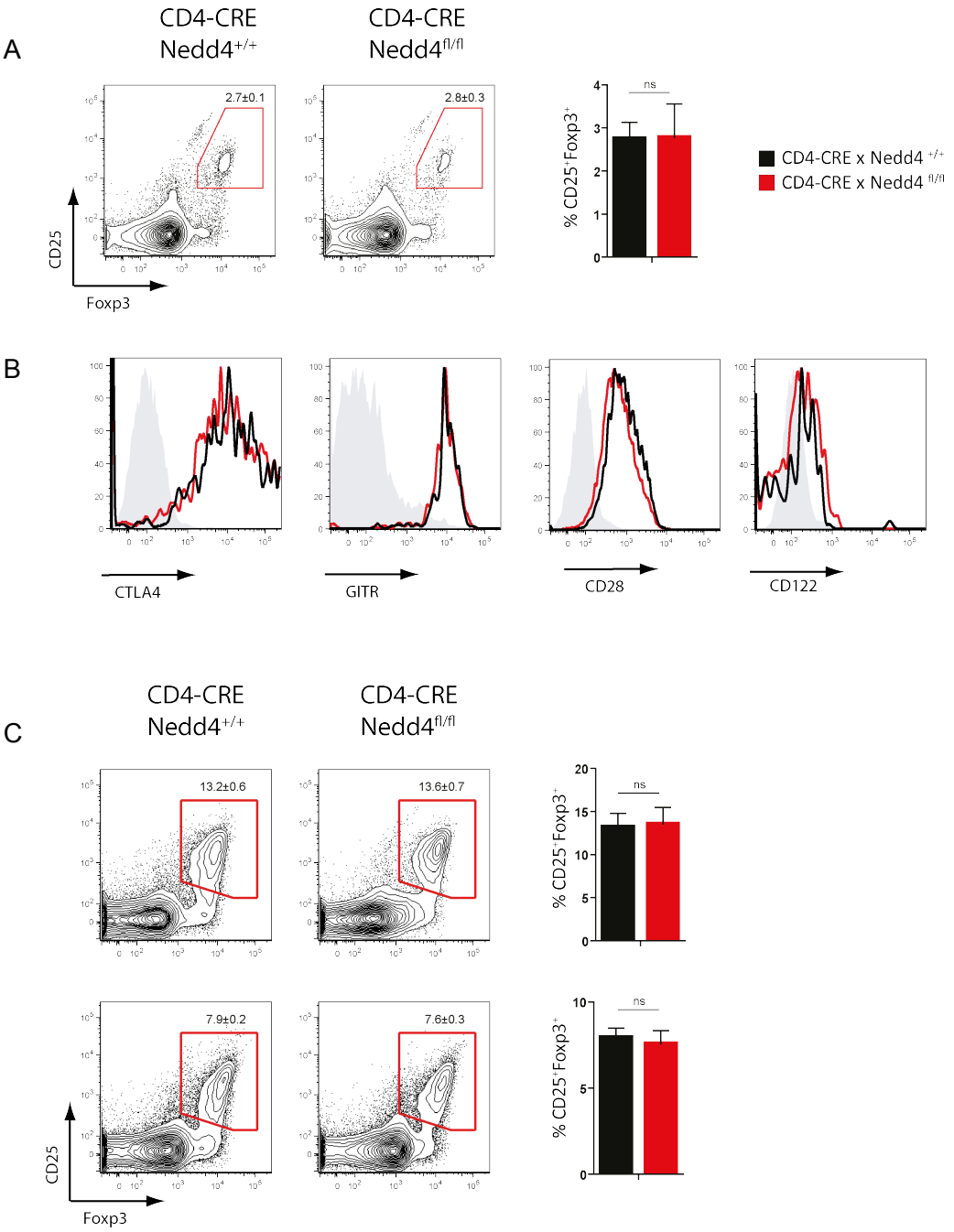


Fig. 24: **Regulatory T cell compartments.** A – The frequency of thymic Treg ccells in both genotypes CD4-CRE Nedd4$^{+/+}$ and CD4-CRE Nedd4$^{fl/fl}$ is presented. B - The histogram overlays show the expression of common Treg markers between two genotypes (black lines represent CD4-CRE Nedd4$^{+/+}$ and red lines represent CD4-CRE Nedd4$^{fl/fl}$). C – The staining of regulatory T cells in the pooled lymph nodes and in the spleen for each genotype.

TCR repertoire in Nedd4 sufficient and deficient T cells

During the process of negative selection the TCR signal strength has an essential role in T cell life and lineage determination. We considered that Nedd4 deficiency and disruption of TCR signal fine-tuning might influence the V α and V β chain repertoire. Therefore, we investigated the frequency distribution of diverse V α and V β chains in the CD4 T cells between the two genotypes in the thymus and the spleen. Nevertheless, were did not identify any particular specificities that were affected by the lack of Nedd4 (Fig. 25).

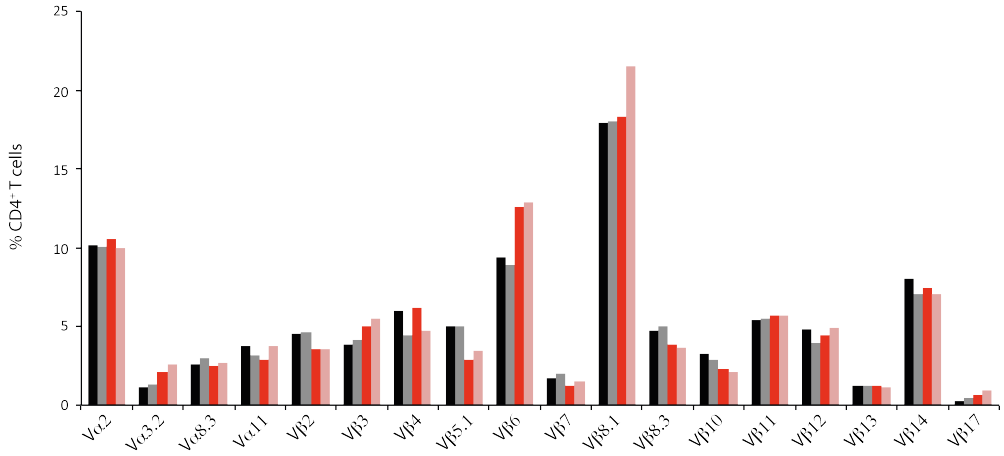


Fig. 25: **Distribution of T cell receptor V α and β chains in the thymus and spleen.** In black bars, CD4SP thymocytes of CD4-CRE Nedd4$^{+/+}$ and in grey those of CD4-CRE Nedd4$^{fl/fl}$ are depicted. For the frequencies in the spleen, red bars represent CD4-CRE Nedd4$^{+/+}$ and light red CD4-CRE Nedd4$^{fl/fl}$ (n=2).

In summary, Nedd4 deficient and sufficient thymic and mature T cells, as well as Tregs, did not differ in the frequency or any examined phenotypic parameters. Next, we extended our analysis to the functional properties and challenged the cells in several functional assays to compare their responses to diverse stimuli.

Proliferation and IL2 production in the absence of Nedd4

Firstly, we examined the proliferative capacity of Nedd4 sufficient and deficient CD4SP thymocytes upon TCR stimulation. Thymocytes from both mice proliferated poorly upon αCD3 and αCD28 stimulation, as expected. Interestingly, Nedd4 deficient thymocytes diluted CFSE less than wild type thymocytes indicating even lower degree of proliferation in the absence of Nedd4 (light blue and red, filled histograms) (Fig. 26).

By the addition of 10U IL2, the proliferative capacity of Nedd4 deficient cells was increased to the initial level of Nedd4 sufficient cells (light blue line and red filled histogram). The addition of 100U IL2 could not overcome the defects of Nedd4 deficient cells and further increase the degree of proliferation (light and dark blue lines). In the case of Nedd4 sufficient cells (brown and red lines) the addition of extrinsic IL2 enhanced the proliferation, as well.

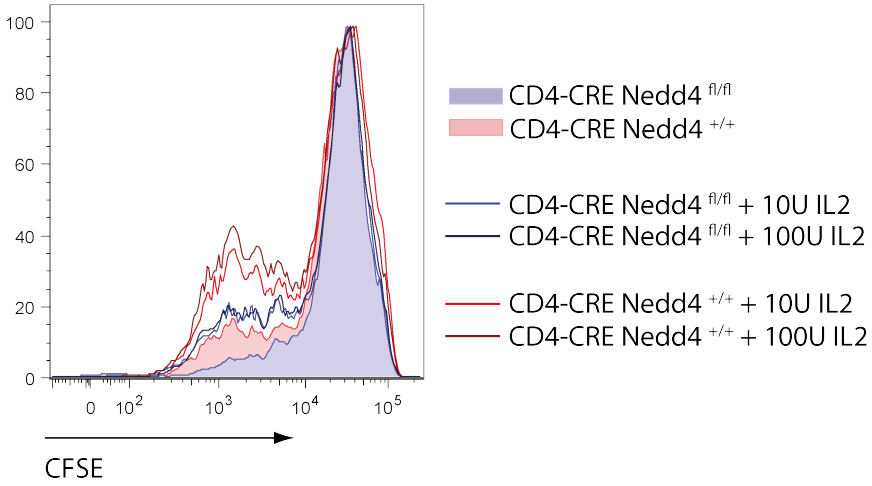


Fig. 26: **CFSE dilution of αCD3/αCD28 stimulated CD4 SP thymocytes.** The proliferation of CD4 SP thymocytes of each genotype was assessed in the absence and presence of IL2. Without the addition of IL2 Nedd4 deficient thymocytes proliferated less but this impairment was reconstituted upon addition of 10U IL2. Additional IL2 could not increase the level of proliferation in Nedd4 deficient thymocytes to the level of Nedd4 sufficient once.

In order to confirm this result we compared two populations in an IL-2 ELISA for a range of αCD3 antibody concentrations. We could confirm that Nedd4 deficient

thymocytes produce less IL2 and this finding is prominent at the αCD3 levels that are sufficient for the full TCR activation (Fig. 27).

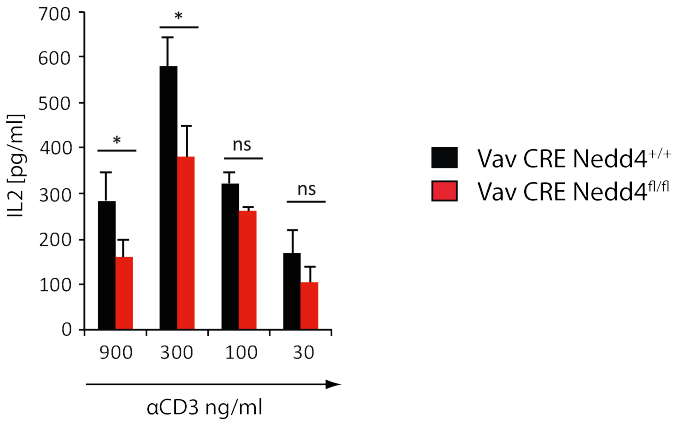


Fig. 27**: IL2 production of Nedd4 sufficient and deficient CD4 SP thymocytes.** The production of IL2 assessed by ELISA at different concentration of αCD3 antibody. At the high concetrations of 900 and 300 ng/ml Nedd4 deficient cells produced less IL2, while at the lower concentrations cells most likely did not experience full activation.

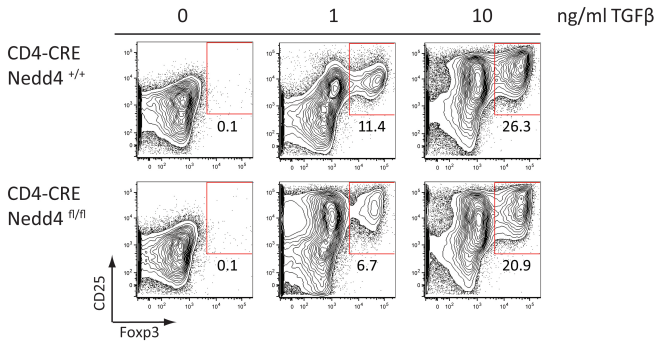
We reasoned that Nedd4 deficient cells interpret TCR (αCD3/αCD28) stimulus differently than Nedd4 sufficient cells, namely as of lower intensity and this triggers only lower activation and IL2 production. Similar result was observed by Yang *et. al* [182] using peripheral $CD4^+$ T cells and we could confirm this also for the CD4SP thymocytes.

Nedd4 and TGFβ signaling

From the research of protein database of European Bioinformatics Institute (http://www.ebi.ac.uk/pdbe/), we identified a Nedd4-like molecule (Nedd4L) with 65.4 % similarity with Nedd4 at the protein level. Nedd4L was described to terminate TGFβ-induced signaling by ubiquitination and degradation of Smad2/3 [225]. Due to the similar ubiquitin ligase properties of these two molecules, we examined the involvement of Nedd4 in TGFβ signaling. The assay was set up to examine the TGFβ signals in the context of the polarization capacity of naive cells in a Treg / Th17 *in vitro* polarization assay [225]. Therefore, we stimulated naive T cells from

both Nedd4 deficient and sufficient genotypes in culture under polarizing conditions. However, we observed that deficient cells maintain their plasticity within the range of different TGFβ concentrations and perform once again, as similar as sufficient counterparts (Fig. 28), reaching very similar frequencies of polarized cells at all different cytokine concentrations.

A



B

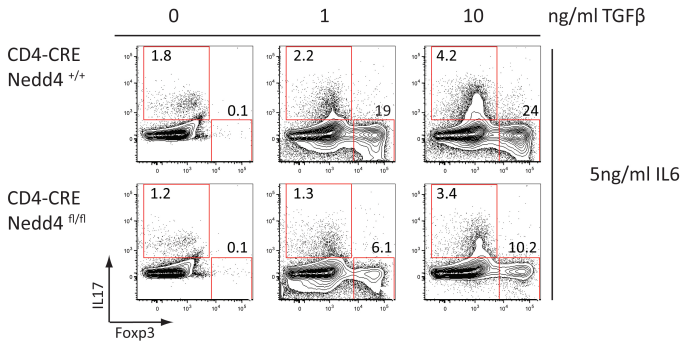


Fig. 28: **iTreg and Th17 polarization experiments.** A – Assesment of propensity of Nedd4 deficient and sufficient CD4 SP thymocytes to enter iTreg lineage in the presence of TCR stimulus and TGFβ over a range of concentrations between 0 and 10 ng/ml. B - Polarization toward Th17 vs iTreg in the presence of IL6 over a range of TGFβ accompanied with TCR stimulus. The numbers in the plots present means of two wells per condition.

Summary of the analyses in the conditional deletion of Nedd4

In summary, we employed the model of conditional deletion of Nedd4 at different stages of T cell life and were unable to demonstrate any major differences in T cell phenotype and functionality over the range of maturation stages, processes and functional assays. We therefore conclude that Nedd4 is not playing any central role in T cell development, education in the thymus and performance in the periphery.

Nevertheless, as some intrinsic cell defects become prominent primarily in a competitive environment between deficient and sufficient cells [184], we assessed the performance of Nedd4 deficient cells in the mixed bone marrow chimera setup.

Analysis of Nedd4 deficient mixed bone marrow chimeras

The experimental setup of the mixed bone marrow chimeras was to generate a mixture of equal composition between Nedd4 sufficient (CRE x $Nedd4^{+/+}$) and deficient (CRE x $Nedd4^{fl/fl}$) bone marrow. This system assesses the performance of both cell types in parallel at every stage and in every process.

The bone marrow composition was assessed by examining the presence of $CD45.1^+$ Nedd4 sufficient and $CD45.1^-$ ($CD45.2^+$) Nedd4 deficient cells in the hematopoietic stem cells, common lymphoid progenitors and in lineage positive cells (Fig. 29.). For the measurements within these compartments we used Vav-CRE deleter, to secure ablation of Nedd4 at the very early time point. The assessed populations showed mainly equal ratios in the presence of both cell types and therefore provided a basis for further experiments. The total bone marrow was composed almost equal amounts of Nedd4 deficient and Nedd4 sufficient cells. The ratios were consistent within $Lin^-c\text{-}KIT^+$ population (enriched for hematopoietic stem cells) with 48.6 ± 5.9 % of deficient and 51.9 ± 5.9 % sufficient cells (p=0.648). Further, all lineage-defined cells in the bone marrow represented 47.4 ± 6.9 % of deficient and 52.6 ± 4.9 % sufficient cells (p=0.462). In addition, the specification to the lymphoid lineage is not affected by Nedd4 ablation as the composition of common lymphoid progenitor cells ($Lin^-c\text{-}KIT^{int}CD127^+$) also mirrors ratios from the total bone marrow with 51.2 ± 1.7 % of WT cells versus 48.8 ± 1.7 % cKO cells (p=0.338).

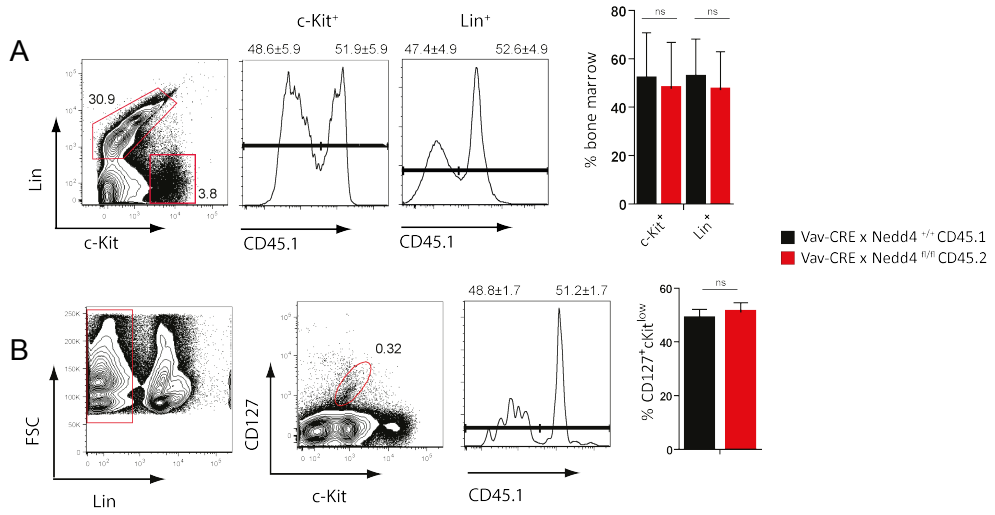


Fig. 29: **Analysis of total bone marrow in Vav-CRE mixed bone marrow chimeras.** A – The chimerism in total bone marrow as well as in lineage positive cells and hematopoietic precursor cells. B – The chimerism of common lymphoid progenitors (CLP) (n=10 and n=4 for CLP staining).

Composition of thymic compartments in the mixed bone marrow chimeras

In the thymus of Vav-CRE Nedd4$^{+/+}$ and Vav-CRE Nedd4$^{fl/fl}$ chimeras, we observed some differences in the composition of the thymocyte subsets by measuring the contribution of each genotype to the total chimerism. The DN1 compartment containing the early thymic progenitors was equally composed of both genotypes with 53.6 ± 8.9 % Nedd4 deficient and 46.3 ± 8.9% sufficient cells (p=0.585), however further stages of double negative compartment, DN2 to DN4 showed smaller population of Nedd4 deficient cells with the contribution dropping between 16.7 ± 3.6 % in DN2, to 20.2 ± 5.9 % in DN3 and further to 29.4 ± 16.1 % in DN4 (Fig. 30). These compartments were filled by Nedd4 sufficient cells, with 83.2 ± 3.7 % (p<0.0001) in DN2, 79.8 ± 5.9 (p=0.0004) in DN3 and 70.6 ± 16.5 % (p=0.121) in DN4. We assumed that at this stage cells go through changes in which Nedd4 may play a role and therefore deficient cells experience a disadvantage, measurable by their chimerism contribution. This observation is supported by the evidence that in the chimeras using CD4-CRE instead of Vav-CRE, showed no differences in the contribution to the double negative compartment (Fig. 32).

Further in the thymocyte development, the chimerism ratios equalized to the starting ≈50:50 ratio, suggesting that the deficient cells have an advantage at the preceding

selection steps. At the DP stage deficient cells contributed more than in the DN compartment with 36.9 ± 12 % versus 63.1 ±12.5 % of sufficient cells (p=0.194) and by the SP stage these cells reached the equal contribution as the sufficient cells with 56.4 ± 9.2 % (p=0.361) in CD4 SP and 57.8 ± 6.5 % (p=0.137) in CD8 SP (Fig. 30). The thymic Treg population was composed of 63.8 ± 3.9 % deficient and 36.1 ± 3.9 % sufficient cells (p<0.0001), which indicates that although the total CD4 SP stage comprises equally of both genotypes, Treg compartment seems to be preferentially filled by Nedd4 deficient cells.

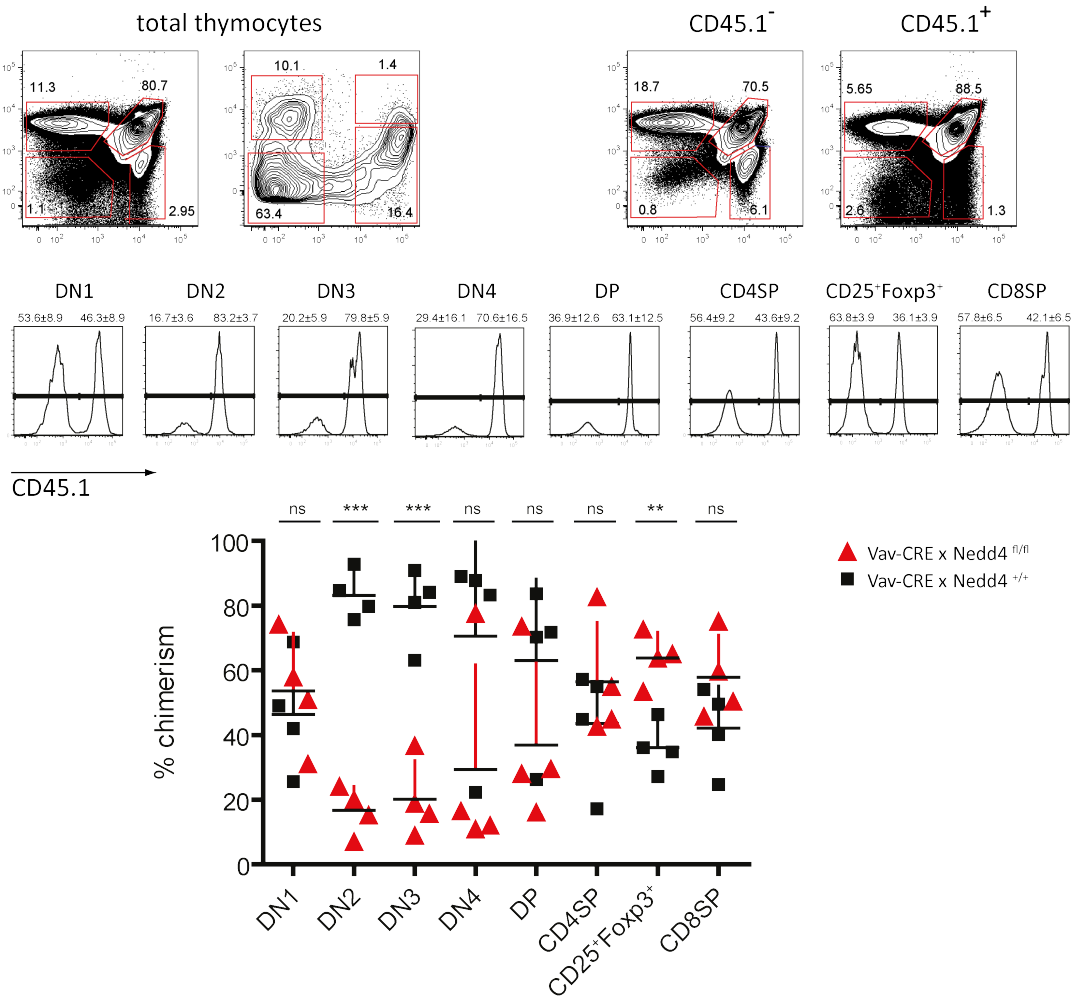


Fig. 30: **Composition of thymic subpopulations in mixed bone marrow chimeras.** A - Thymocyte subpopulations of Vav-CRE mixed bone marrow chimeras segregated by CD4 and CD8 staining. Histograms show CD45.1 expression of different thymocyte subsets and numbers indicate means ± s.d. of frequencies within the gates. B - Diagram presents means ± s.d. of chimerism contribution for Vav-CRE x Nedd4$^{+/+}$ (black squares) and Vav-CRE x Nedd4$^{fl/fl}$ (red triangles).

Disadvantages and the DN2/3 stage and the γδ T cell lineage contribution

At the stage of DN2/3, one of the major decisions, apart from T cell lineage commitment is the αβ versus γδ lineage decision. Therefore, we were interested whether Nedd4 impacts this process. Despite the fact that we were unable to observe any change in γδ T cell frequencies in our first set of analysis, in the mixed bone marrow chimeras, Nedd4 deficient cells contributed slightly but significantly more to the γδ lineage than the sufficient cells did, with 0.192 ± 0.01 % compared to 0.127 ± 0.01 % (*p*=0.0018).

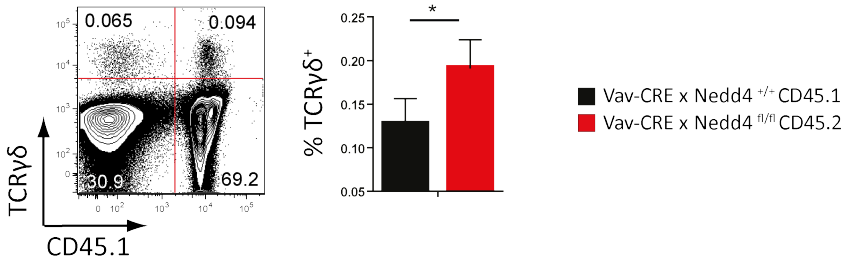


Fig. 31: **Contribution to the γδ T cell lineage.** The panel shows higher contribution of Nedd4 deficient T cells to the γδ T cell lineage. Plot presents staining gated on whole thymocytes.

If one would assume that the preTCR and TCR signaling pathways utilize the same downstream molecules, this observation might be explained by the fact that the Nedd4 deficient cells interpret preTCR at the β selection stage as of lower strength and therefore preferentially enter the γδ T cell lineage. This hypothetical scenario provides just one possibility to explain the observed differences, as the role of signal strength of preTCR in the lineage commitment is still controversial. Nevertheless, we evidenced the higher propensity toward the γδ lineage in the absence of Nedd4.

Nedd4 deficiency promotes entry into Treg lineage

In the analysis of CD4 SP compartment of mixed bone marrow chimera, utilizing CD4 CRE deleter a difference between the contribution of deficient and sufficient cells was evident at the $CD4^{+}CD25^{+}Foxp3^{+}$ level. The thymic Treg pool consisted of 68.78 ± 3.2 % Nedd4 deficient cells and 30.06 ± 4.0 % (p<0.0001) sufficient ones. The $CD25^{+}Foxp3^{-}$ as well as CD4 SP stage in general was not affected and consisted of both genotypes equally (Fig. 32). This segregation was evident also in the peripheral lymphoid organs (Fig. 33).

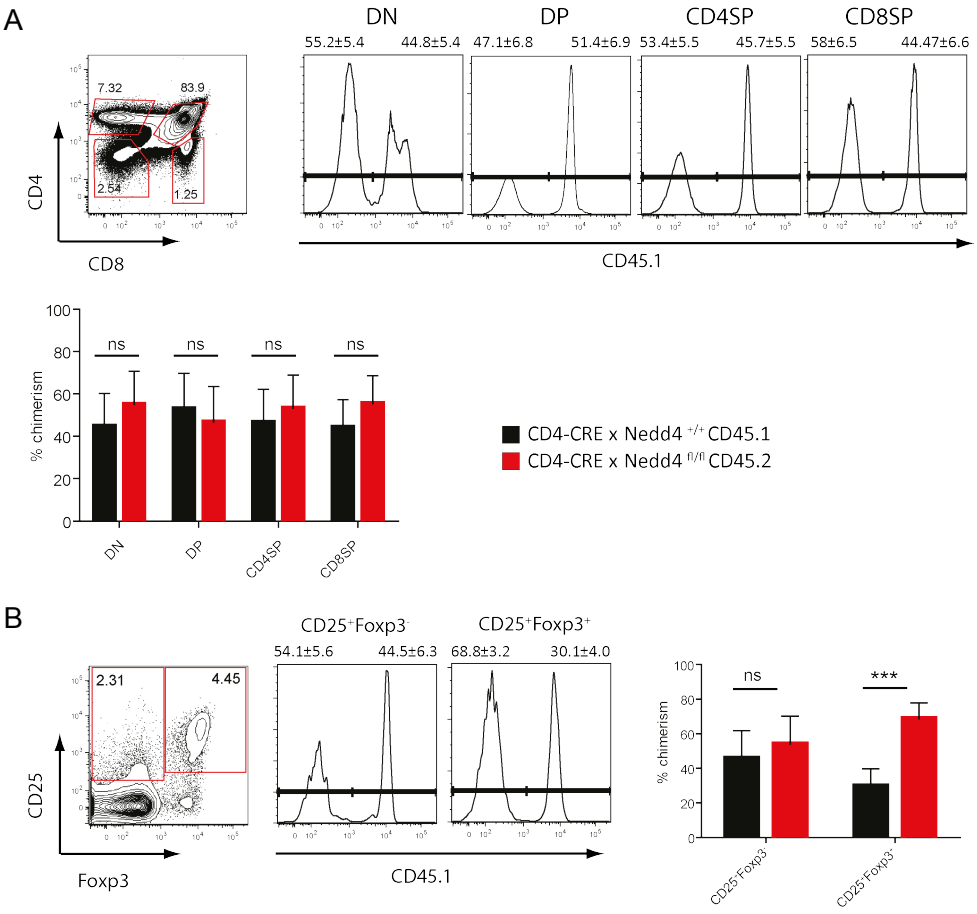


Fig. 32: **Thymic Treg compartment in the mixed bone marrow chimera.** A – The plots present chimerism between CD4 CRE $Nedd4^{+/+}$ ($CD45.1^{+}$) and CD4 CRE $Nedd4^{fl/fl}$ ($CD45.1^{-}$) cells in the major thymic compartments, including $CD25^{+}Foxp3^{-}$ and $CD25^{+}Foxp3^{+}$ populations. B – Diagrams present frequencies measured in each sample.

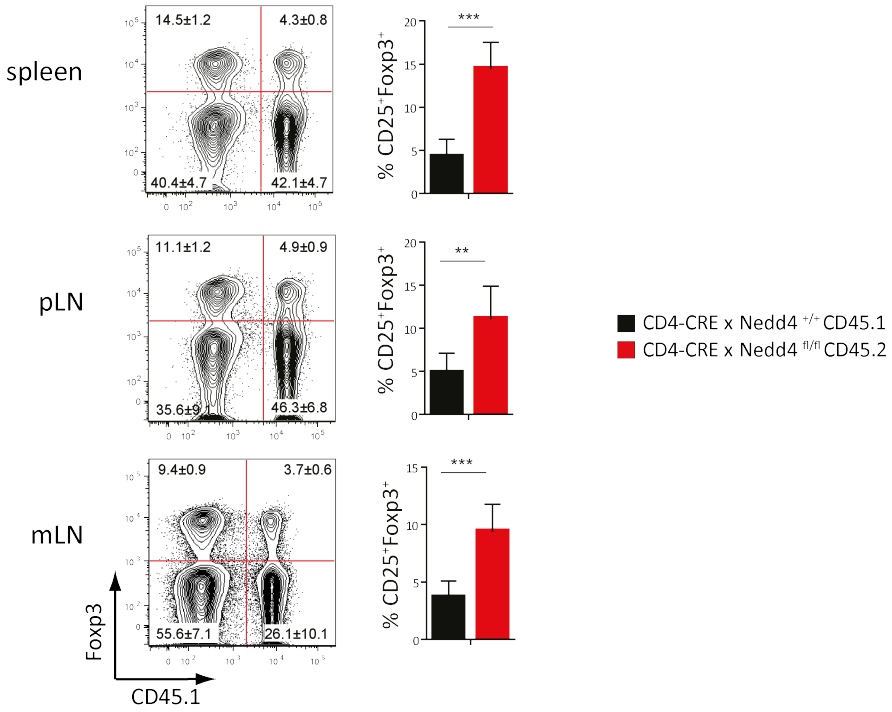


Fig. 33: **Contribution of Nedd4 deficient cells to the CD25^{+}Foxp3^{+} CD4 T cells in the peripheral lymphoid organs.** Panel represents data for the spleen, pooled lymph nodes and the mesenteric lymph nodes

In sum, we analyzed the composition of the cellular compartments in the Vav-CRE and CD4-CRE Nedd4 deficient models and were able to show that Nedd4 has roles in the DN2/3 stage and potentially the γδ lineage commitment. Further, we evidenced the advantage of Nedd4 deficiency in the propensity toward Treg lineage, compared to the Nedd4 sufficient counterparts.

Next, we extended our analysis to the periphery and examined the activation status of peripheral CD4 T cells. In the spleen as well as in the pooled lymph nodes, some minor difference in frequency of naive and activated / memory cells was evident (Fig. 34). The Nedd4 deficient compartment contained more naïve; 77.1 ± 3.1 % versus 65. 8 ±3.6 (p=0.046) and tendentially less activated cells; 14.1 ± 2.6 % versus 22.6 ± 2.5 % (p=0.061), which is in line with the publication of Yang *et. al* [182] and the idea of the T cell hyporesponsiveness due to the Nedd4 deficiency. This result is however evident only at the 16 weeks post reconstitution. At the earlier time points the differences are not so prominent and do not reach significance.

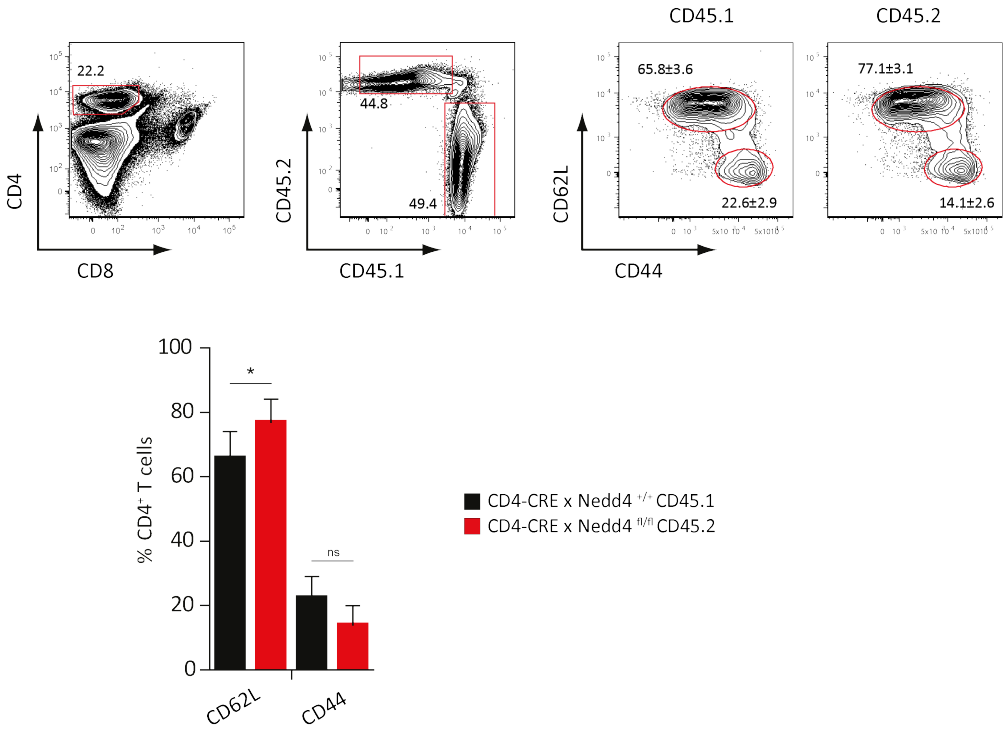


Fig. 34: **Activation status of CD4 T cells in the CD4 CRE mixed bone marrow chimeras.** As depicted CD4 cells were distinguished for CD45.1 Nedd4 sufficient and CD45.2 Nedd4 deficient T cells. Further, the segregation of these cells in CD62L and CD44 was examined and diagrams show the frequencies of naïve and memory CD4 T cell population within each genotype.

To additionally support the hypothesis that Nedd4 deficient T cells are intrinsically hyporesponsive and show defects in TCR signal regulation and adaptation we transferred mixtures of equal numbers of deficient and sufficient cells into Rag1$^{-/-}$ animals and assessed the degree of activation 5 days upon injection.

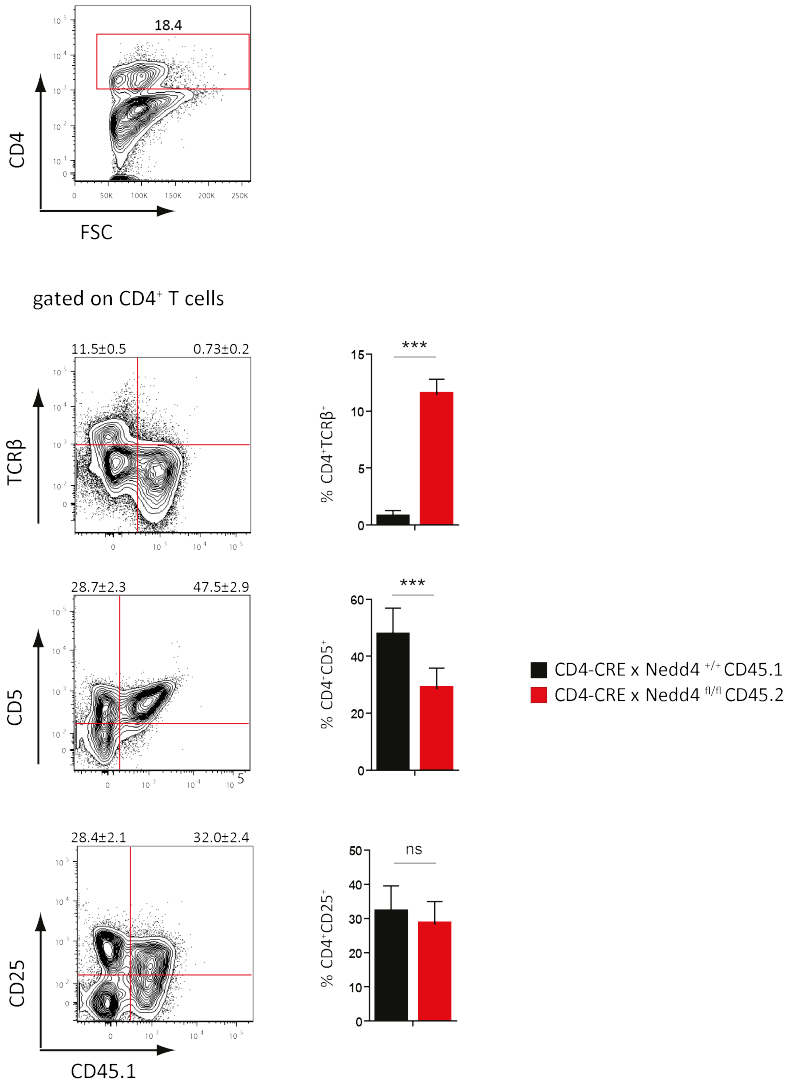


Fig. 35: **Adoptive transfer of mixed CD4 T cells from CD4 CRE Nedd4$^{+/+}$ (CD45.1^{+}) and CD4 CRE Nedd4$^{fl/fl}$ (CD45.1^{-}) into Rag$^{-/-}$ animals.** Transferred cells were gated on CD4 and distinguished by expression of CD45.1. The expression of TCRβ, CD5 and CD25 was determined on each population. Mean of frequencies for examined parameters ± s.d. are depicted in the diagrams.

The Nedd4 deficient cells did not downregulate their TCRβ as highly as sufficient cells. 11.49 ± 0.5 % Nedd4 deficient cells compared to 0.73 ± 0.2 % (p<0.0001) kept their TCR expression upon transfer and likely TCR stimulation. The CD5 expression,

directly correlating to the TCR signal a cell has received [226], was significantly lower in deficient cells with 28.67 ± 2.3 % compared to 47.5 ± 2.9 % (p<0.0001) in sufficient population. The amount of CD25 on the surface was similar between the two groups.

Therefore, we concluded that Nedd4 deficient cells are intrinsically hyporesponsive and do not depend on extrinsic signals of other CD4 T cells nor any surrounding.

Nedd4 and the superantigen-induced negative selection

Finally, if we hypothesize that the absence of Nedd4 rescues T cell from the negative selection and instructs it into the Treg lineage, the strong TCR signals would be interpreted as of lower strength. One of the possibilities to test this is the superantigen mediated selection [227]. We therefore generated F1 animals between the BL/6 and BALB/c mice as donors of one part for the mixed bone marrow chimera to introduce the I-E molecule, needed for the superantigen recognition. We postulated that the Nedd4 deficient CD4 SP compartment shows enrichment for SAg-specific Vβ chains (Vβ3, 5.1, 6, 11 and 12) and that T cells bearing these chains show enriched Foxp3 expression. We evidenced equal deletion of specific Vβ chains between the two genotypes (Fig. 36) and similar contributions of Nedd4 deficient and sufficient cells in the Foxp3$^+$ compartment (Fig. 37).

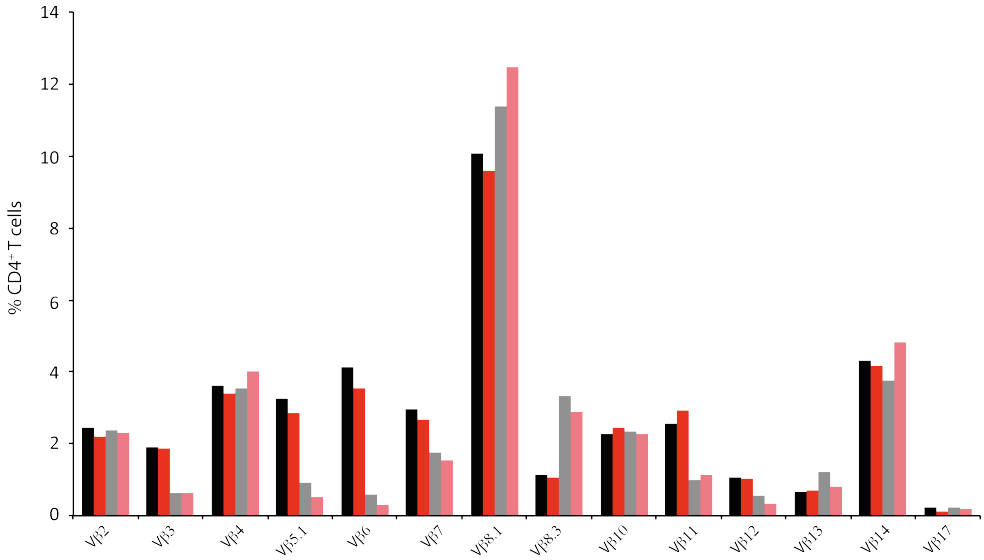


Fig 36. **Superantigen mediated deletion.** Bar diagram presents the frequency of Vβ chains in the BL/6 mixed bone marrow chimera (black - Vav-CRE Nedd4$^{+/+}$, red - Vav-CRE Nedd4$^{fl/fl}$) and in the F1 setting (gray – F1 Vav-CRE Nedd4$^{+/+}$, light red - Vav-CRE Nedd4$^{fl/fl}$).

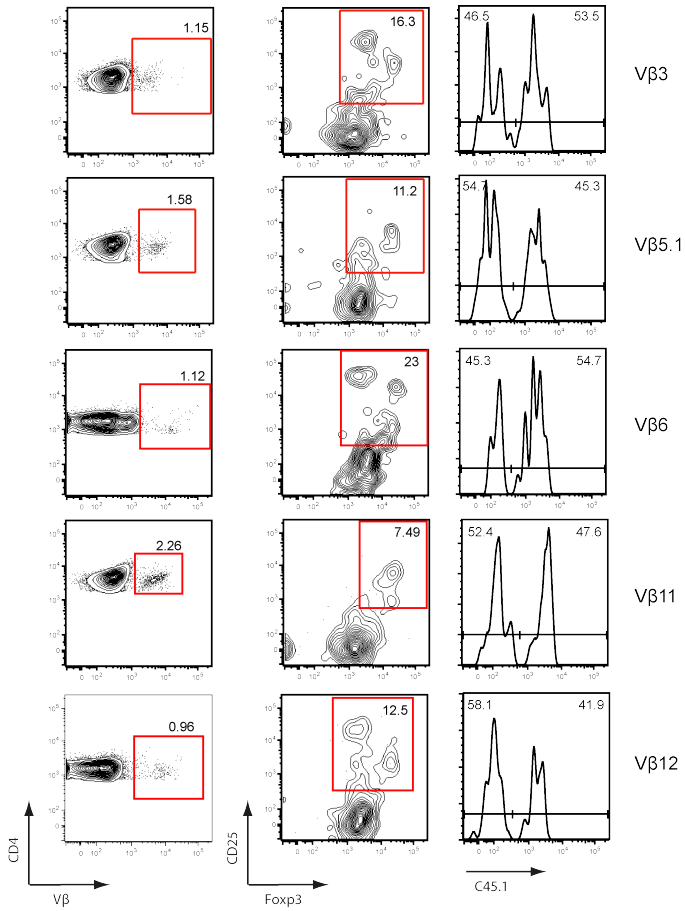


Fig 37. **Foxp3 populations in the deleted Vβ specificities.** The plots present frequency of the SAg-deleted Vβ species in the CD4 compartment. Further, the contribution of the each genotype to the Treg compartment of each Vβ population is depicted.

The observation from this experiment indicate that the process of SAg-mediated deletion is not affected by the deficiency of Nedd4 and that the Nedd4 plays potentially a minor role impacting only the TCR fine tuning.

Taken together, we showed that Nedd4 deficient cells are intrinsically hyporesponsive, produce less IL2 and proliferate less compared to the wild type cells. In the competitive situations Nedd4 deficient cells have higher propensity to enter γδ and Treg lineages. The data collected during this project and presented here forms a basis for a study of the Nedd4's TCR fine tuning properties in the negative selection versus Treg deviation that is certainly to be examined in the single-specificity TCR / cognate-antigen models.

DISCUSSION

Gene expression profile of CD4 SP thymocytes as basis

The data obtained through microarray analysis indicated that the three analyzed populations SP1, 2 and 3 are very similar to each other and suggest the differences in maturation, plasticity and developmental status results from only small number of differentially expressed genes. In the search for the molecules that might be the basis for these developmental differences, we focused on two candidates – Lztfl1 and Nedd4. Lztfl1 had no reported involvement in the immune system, while Nedd4 was already reported to be involved in TCR signaling in the periphery. From that perspective, we had one candidate with completely unknown functions and one candidate which functions we applied to the thymic T cell development. What one can conclude from the overall microarray analysis result is that the examined CD4 SP stages do not exceptionally differ between each other. The directionality of observed changes in the gene expressions adds to the previous work of Jin *et al.*, that SP 1, 2 and 3 stages represent continual and consecutive developmental points of the total CD4 compartment and that regulation of expression for each gene has already taken its direction. Apart from this conclusion and in the relationship to the work performed by Wirnsberger *et al.* it becomes obvious that the propensity toward Treg development and possibly many other decisions does not need major changes in the gene expression. These observations support the notion that cell fate dependens on several issues but definitely minor changes in the expression of some intrinsic factors add to that. Lastly, the palette of differentially expressed genes included not only molecules from the TCR signaling but also targets like cytokine-inducible proteins, nucleoside metabolism regulators and cell cycle players.

Lztfl1 – Ciliary transport, immunological synapse and T cell fate

Leucine zipper transcription factor - like 1 is downregulated with the maturation in CD4 SP cells and was selected as one of the candidates.
In the initial screen, we showed that overexpression of Lztfl1 at the CD4 SP stage prevented the developing thymocytes to enter regulatory T cell pool. Besides from this, we were unable to describe any other difference in the T cell system due to Lztfl1 overexpression or partial knockdown. Therefore, this is the first and only evidence that Lztfl1 has any implications in the T cell biology.

To date, Lztfl1 has been mainly described in the context of the ciliary molecular transport machinery, BBSome, as previously mentioned and the human orthologue was recently renamed to BBS17 (*Ensemble June 2013*). Although the idea of ciliary system in the immune system and particularly in T cells has almost no appreciation, we identified some potential similarities between current knowledge of BBSome biology and the immune system.

The BBSome is a protein complex involved in the ciliogenesis [228]. Primary cilia are cellular projections or microtubule-based organells that emerge from the surface of almost all cells. The first connection of BBSome to the cilia biogenesis was based on the observation that *bbs* mutated *C.elegans* has shorter sensory cilia. Further work showed that the BBSome is present in the cilia and its subunits are conserved throughout all ciliated organisms [213, 229-231]. BBSome is a complex of seven core proteins that have been initially discovered in patients with Bardet Biedl syndrome. This syndrom is a genetic ciliary defect characterized by a combination of severe developmental abnormalities, like polydactyly, retinal degeneration, renal abnormalities, hypogenitalism and obesity [232].

BBSome is required for functionality of cilia by stabilizing intraflagellar trafficking (IFT) proteins complexes (Fig. 38). IFT particles are composed of multiple IFT molecules that in turn transport newly produced proteins in the anterograde direction from the Golgi apparatus to the tip of the cilium and in the retrograde direction from the cell membrane, as the part of the recycling process back for the degradation.

This transport with all its players, BBS and IFT proteins is central to communication and sensing of the surrounding to the cell [233].

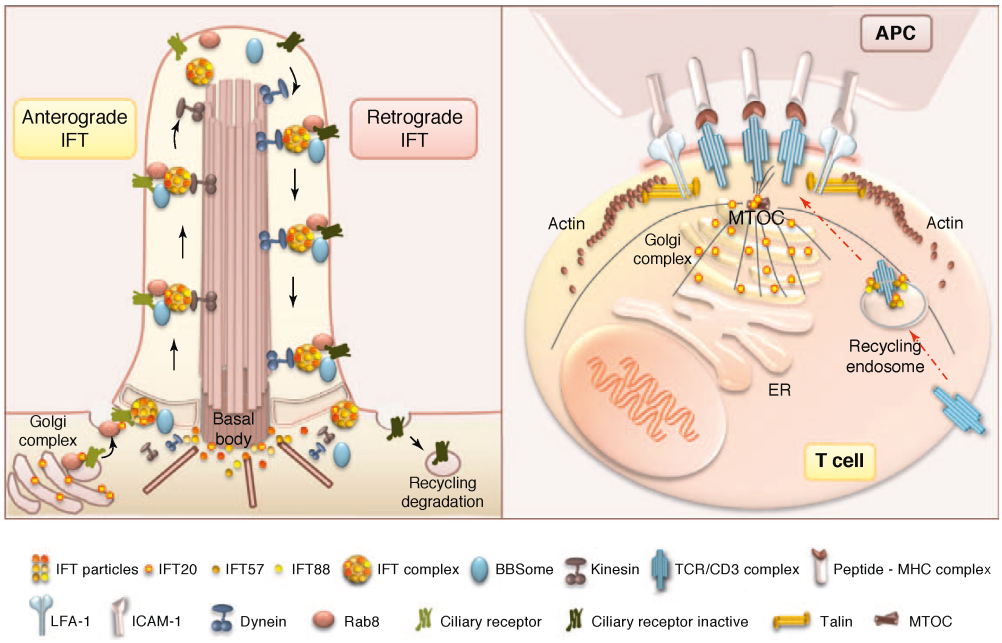


Fig.38: **Scheme of ciliary transport involving IFT and BBS molecules and counterpart of this process in the context of immunological synapse.** (Adapted from Finetti *et al.* Trends in Immunology 2011)

The current position of Lztfl1 as the negative regulator of BBSome recruitment to the ciliary membrane is based on the work of Seo *et al.*. Here, Lztfl1 has been found to interact with BBSome subunit Bbs9 and inhibits ciliar entry of the BBSome, scattering it in the cytoplasm away from usual position near centrosomes and ciliary base. The assembly of BBSome did not seem to be impaired by lack or overexpression of Lztfl1 in this work, but ablation of Lztfl1 lead to increased levels of Smo protein in the ciliary membrane [172].

In order to merge this observation with our interest in the Lztfl1's roles in the immune system, we broadened the idea that cilium, although not observed in the lymphocytes, might have a pendant in the immunological synapse. There are some important similarities between the cilium and the immunological synapse. Firstly, both can serve as a place of exchange and communication between two cells.

Secondly, the centriole and Golgi apparatus are placed in the proximity of both structures and can provide needed material [234]. Additional insight in the importance of IFT molecules was provided by work of Finetti *et al.*, who showed that IFT20 was essential for sustained TCR signaling, as knockdown of IFT20 resulted in impaired tyrosin phosphorylation and impaired TCR activation. Upon TCR stimulation, IFT20 has been shown to interact with IFT57 and IFT88 in addition to direct TCR binding and so initiates the TCR recruitment to the synapse [235]. Whether these IFT molecules interact with BBSome in T cells is still unknown.

One possible postulation is that IFT complexes need to form and assemble with BBSome for the transport of TCR and accessory molecules to the immune synapse to initiate the signaling and secondly, it might be that IFT/BBSome mechanism is important in ceasing the signal via retracting TCR molecules from the membrane.
If we assume that Lztfl1 interacts with Bbs9 in the T cells as well and therefore prevents accessibility of BBSome for IFT-mediated TCR transport to the membrane, a Lztfl1 overexpressing thymocyte would have difficulties to pass the check point of positive selection. In our experiments this was not the case. We observed normal frequencies of CD4 single positive cells in the thymus upon both selection steps. The fact that Treg compartment is smaller, one could explain with the scenario that Lztfl1 overexpression keeps BBSome away from the immunological synapse and prevents recycling of TCR molecules, leading to higher number of TCRs available and potentially longer interactions. If the decision between apoptosis and Treg commitment is influenced by avidity of the signal and the timing, Lztfl1 could have a role in this aspect. According to these simple scenarios Lztfl1 mice could have interesting defects in TCR selection process. However, this remains to be studied. At present, our main goals next to the generation of the conditional knock out model are exploration of Lztfl1s interactome in T cells via protein co-immunoprecipitation and studies of its localization in context of different T cell subsets and upon T cell stimulation.

Nedd4 and TCR fine-tuning

Nedd4 was one of the molecules with decreasing expression between the SP1, 2 and 3 populations. Despite the difficulties in the initial screen by gain-of-function experiments and with the advantage of the availability of conditional knockout model, we explored the roles of Nedd4 in the T cell biology. In the immunologically relevant literature, Nedd4 has been described to target Cbl-b for ubiquitination and so promotes T cell activation [182] (Fig. 39.). In contrast to the Cbl-b deficiency, characteristic for the spontaneous autoimmunity, Nedd4 deficiency shows less systemic effects. In the Cbl-b deficiency the activation of T cells proceeds in the absence of CD28 signaling and despite normal Treg development, mice develop strong autoimmunity [236, 237]. As Nedd4 is part of the TCR signaling machinery, but does not interfere enormously with the full T cell activation cascade, we considered it as a good tool to assess the importance of TCR fine-tuning in the thymic selection processes.

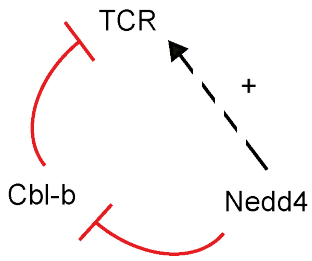


Fig. 39: **Nedd4 targets Cbl-b for ubiquitination and degradation.** A model of Nedd4 action in T cell signaling, proposed by Yang *et. al.*. Nedd4 ubiquitinates Cbl-b and promotes its degradation by proteasome. The absence of Cbl-b, as one of the important inhibitory mechanisms, results in lower TCR threshold as prompt T cell activation. If there is an additional effect of Nedd4 on the promotion of TCR signal and T cell activation remains unclear.

In contrast to previously published work on Nedd4, we designed a T- cell specific ablation of Nedd4 via four different, stage-consecutive CRE deleters, Vav-, Lck-, CD4- and Foxp3 - CRE. Further, we examined the performance of Nedd4 conditionally deficient cells in direct comparison to the Nedd4 sufficient cells in the mixed bone marrow chimeras.

The studies performed on the conditional deletion of Nedd4 did not reveal any additional differences than those already described by Yang *et al.*, like lower proliferation capacity and IL2 production, as well as intrinsic hyporesponsiveness.

Further, thymic T cell development did not seem to be affected by the loss of Nedd4 at any stage in these experiments. This data provided us with the information that the effect of Nedd4 at the TCR signaling level does not massively impact generation of a T cell population but most likely, this fine-tuning solely shifts the whole repertoire of generated T cells (Fig. 40). Therefore, newly generated T cells do not differ in their numbers, frequencies or phenotypical markers but probably in the affinity of their TCRs, compared to the theoretically possible repertoire generated in the presence of Nedd4.

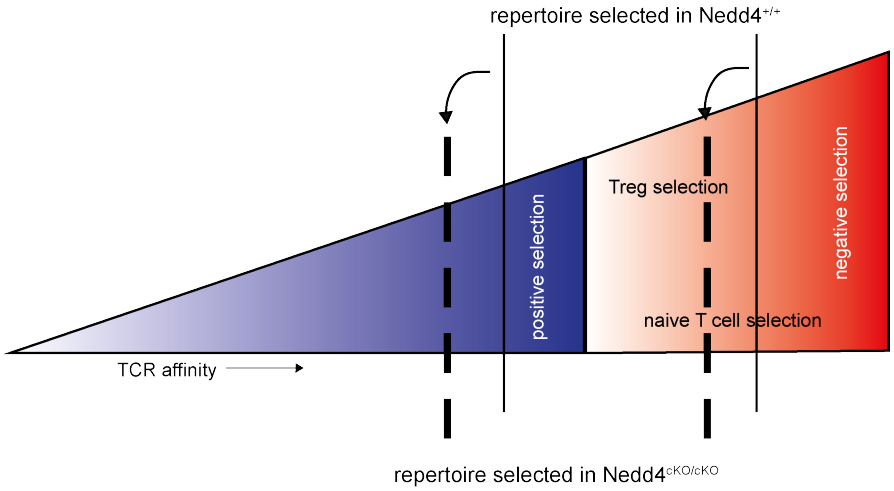


Fig. 40: **Thymocyte development and fate in context of TCR affinity.** In the Nedd4 sufficient cells ($Nedd4^{+/+}$) a defined repertoire of naïve and Treg T cells is generated. In the absence of Nedd4 ($Nedd4^{cKO/cKO}$), developing thymocytes are hyporesponsive and interpret received TCR signals as of lower strength. This leads to the shift in the generated repertoire.

In order to test this, one would have to have several defined specificities and compare their selection progression with or without Nedd4. With this defined spectrum of specificities one could ask whether certain specificities pass the selection steps differently in the absence of Nedd4. This might then define a particular TCR strength 'window' for which the described TCR fine-tuning by Nedd4 might play a role. A tool that has been described by Lee *et. al* [238], a series of retrogenic TCRs with different affinities for OVA could be used to assess the impact of Nedd4 in the selection of each specificity and to simultaneously track the potential shift in the repertoire.

In the search for some general defects, we performed mixed bone marrow chimera in order to compare the fitness of deficient and sufficient cells at all levels of T cell life. This approach has a vast advantage to the analysis of the 'straight' deficiencies, as it applies clonal pressure to the cells and assesses their ability to compete, while crystalizing the differences based on the Nedd4 deficiency. Therefore, one gains information about the intrinsic properties of cells.

In this experimental setup, we observed the disadvantage of Nedd4 conditionally deficient T cells at the double negative stage in the early thymic development. If one would assume that molecules accompanying TCR signaling also play a role in preTCR signals, Nedd4 might be of importance. In this hypothetical preTCR signaling, Nedd4 absence would lead to the increased Cbl-b levels and inhibition of signals via preTCR, meaning that Nedd4 deficient cell would be hyporesponsive at the stage of β selection (DN2 - 3). As the signals provided from the newly rearranged Vβ chain accompanied by invariant pre-TCRα chain are the major parameter for the cell to assess the functionality of Vβ chain and enter the next cycle of rearrangements to become an αβ T cell, this hyporesponsiveness might be a great disadvantage.

The options for a cell failing in the β selection are either cell death or entrance into the γδ T cell lineage. The work on γδ T cells development still has some debatable issues concerning the preTCR signal quality and strength needed to enter this lineage, therefore further correlations to our observations would be highly speculative [239-241]. It has been proposed that DN2 population independently of ongoing TCR rearrangements has the ability to enter one or the other lineage according to the signals received from the IL7 and Notch1, and that further expression of the TCR leads to confirmation of the decision or cell death. It has been shown that IL7Rα^{hi} DN2 cells preferentially enter γδ lineage, than the IL7Rα^{lo} counterparts [242]. Onward, additional factor in this precommitment is played by Notch1 [243, 244]. The absolute requirement for Notch1 has been shown only for αβ T cells, as γδ T cells can compensate effects of Notch1 via Id3, it is generally accepted that Notch1 is essential for proliferation and survival of cells at the DN3 stage. Interestingly, at this point we evidenced loss of Nedd4 conditionally deficient cells and speculate that Nedd4 may play role in Notch1 endocytosis in developing T cells, as it was shown to do in *D. melanogaster* [245]. The loss of Nedd4 leads to accumulation of Notch1 on the surface due to absence of endocytosis and loss of

Notch1 signal. This correlation might explain the absence of DN3 cells in the Nedd4 deficient compartment in our mixed bone marrow chimeras. We performed flow cytometric analysis of Notch1 expression on Nedd4 sufficient and deficient cells but could not evidence any differences. Additionally, CD25 as marker that correlates with the received Notch1 signals remained also unchanged. Nevertheless, it might be that Notch1 amounts or expression levels do not change with Nedd4 deficiency but that solely trafficking becomes affected, delayed or disturbed.

Next observation in our experiments was that the Treg compartments are preferentially composed of Nedd4 deficient cells. On top of that, we observed that deficient cells despite their lower frequency at the double negative stage, manage to catch up with the sufficient cells and fill in the single positive compartments. This suggested that Nedd4 deficient cells cross the selection checkpoints faster than sufficient cells or experience some promotion at these steps. We proposed that the Nedd4 deficient cells interpret signals of high strength at the stage of negative selection as moderate due to the hyporesponsiveness and therefore escape apoptosis, fill up the CD4 SP compartment and become converted into Treg lineage preferentially (Fig. 41). The fact that the Treg pool is not enlarged argues for the hypothesis that there must be a selecting niche with limited capacity and potential saturation that does not allow boost of regulatory T cells out of all potentially autoreactive Nedd4 deficient T cells. This argument would also explain why we could not observe general increase in the regulatory population in the non-mixed situation of conditional knockout mice.

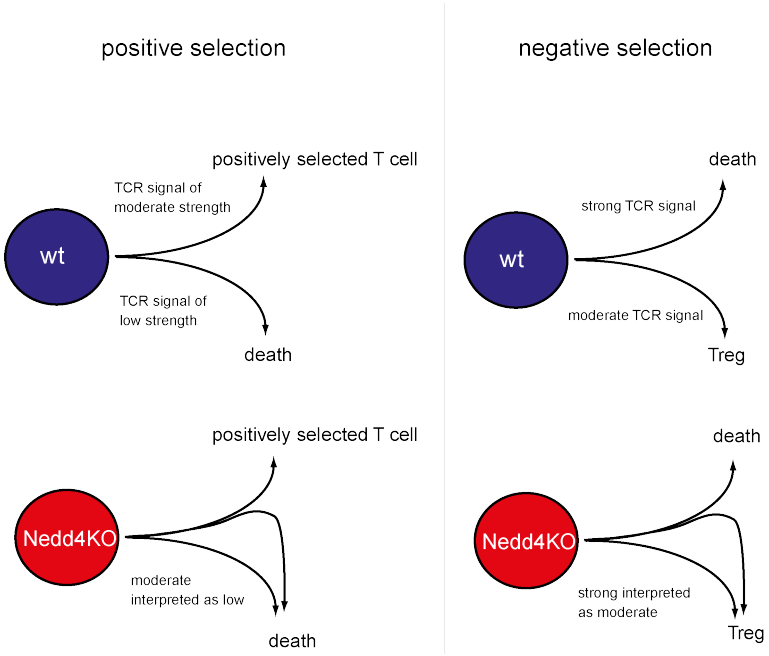


Fig. 41: **Effects of differential TCR signal interpretation of the thymic selection of Nedd4 deficient cells.** At the step of positive selection Nedd4 deficient (Nedd4KO, red) cells experience disadvantage, as their TCR signal does not result in rescue, while at the step of negative selection Nedd4 deficient cells interpret strong signals as moderate and escape the negative selection, potentially as Tregs.

This hypothetical model of Nedd4's role in thymic selection has some overlapping points with the results of work of Hwang and Love, in which the attenuation of TCR signal (utilizing signaling deficient mutant of TCRζ chain) leads to the impaired negative selection and promotion of Treg development [246].

In the experiment of superantigen-mediated selection we failed to show that the proposed model applies. The fact that this particular system of negative selection induces very strong TCR signals, which even overcome the need of ITAM units in the induction of deletion [247], argues that this negative result might not dismiss the whole model. Further approaches involving the TCR transgenic models and their cognate antigen models (like TCR-HA and Aire-HA) should be used for future experiments.

OUTLOOK

In order to better understand the roles of Lztfl1 and Nedd4 in the thymocyte biology several open questions need to be answered in the further work.

For the part related to Lztfl1, primary aim would be to generate the conditional knockout mouse. Secondly, the characterization of this mouse model in polyclonal or TCR transgenic context should add to the knowledge on Lztfl1.

Other open questions, like the importance of the BBsome and the intraflagellar transfer (IFT) molecules in T cells can be addressed by quantitative PCR analyses of T cell subpopulations and further confirmed by Western blotting. In addition, the results generated by Seo *et. al* showing the interaction between Lztfl1 and Bbs4 need to be recapitulated in T cells. For this one could generate a construct of tagged Lztfl1 and express it in T cell lines or over the lentiviral infections in T cells of bone marrow chimeras. This tag would then allow the immunoprecipitation of Lztfl1 and its interaction partners.

Next, for the part of Nedd4 analyses, major task would be to investigate available TCR transgenic models and their selection in the presence of a cognate antigen on the Nedd4 sufficient and deficient background. Further, one could investigate if Nedd4 utilizes the same mechanism of TCR fine tuning over Cbl-b ubiquitination in the thymocytes as well as in the mature T cells. In consideration of the high Nedd4 expression in the double negative compartment, one could investigate the roles of Nedd4 in the Notch transport and signaling, as some data on this has been generated in *D. melanogaster*.

In sum, for both candidates several open questions remain. Nevertheless, the tools for the investigation of these questions are generally available and some answers could soon complement our knowledge on these molecules.

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Publikationen

Regulatory T cell differentiation: turning harmful into useful.
Klein L, Jovanovic K., *Immunity*. Sep 2012

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Konferenzen

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Viral functions and immunomodulaton, SFB455 meeting, München, Deutschland 2010 / *poster presentation*

European Thymus Workshop, Noordwijkerhout, Niederlande 2011

Munich Minisymposium in Immunology, München 2011 / *talk*

German Meeting on Immune Regulation, Berlin 2012 / *talk*

	Transcription and Chromatin, EMBL Heidelberg 2012
Preise	Richard Batchelor Preis in Immunologie, vergeben in 2010 von der Medizinischen Fakultät an Imperial College London

Sprachen	Serbisch (Muttersprache) Deutsch und Englisch (fließend) Französisch (Grundkenntnisse) Bulgarisch (Grundkenntnisse)
EDV Kenntnisse	MS Office, iWork, Adobe Illustrator and Photoshop PubMed, FlowJo, Cluster/TreeView, GEPAT, ChemOffice, SciFinder, EndNote, Papers
Hobbys	Aerobic Step und Wandern Fremdsprachen lernen und lehren TED Vorträge

München, 12. März 2014

Ksenija Jovanovic