Aus dem Institut für Immunologie der

Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. Thomas Brocker

The role of CD83 in T cell development

Dissertation

zum Erwerb des Doktorgrades der Naturwissenschaften

an der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

vorgelegt von

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aus

Bad Aibling

im Jahr

2014

Gedruckt mit Genehmigung der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

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1 SUMMARY

CD83 has been used as maturation marker for dendritic cells (DCs) for a long time, but its actual function is still unresolved. CD83-deficient mice show a drastically reduced $CD4^+$ T cell compartment and, moreover, diminished MHC II levels on antigen presenting cells.

We addressed the question if the reduced MHC II level of cortical thymic epithelial cells (cTEC) is causative for the impaired $CD4^+$ T cell development or if CD83 has additional MHCII-independent functions, such as signalling *in trans*.

To test this hypothesis, we introduced truncated versions of CD83 into TECs. Specifically, we infected embryonic $CD83^{-/-}$ TECs with lentiviral expression vectors encoding mutant CD83 constructs, reaggregated the infected TECs to generate reaggregate thymus organ cultures (RTOCs) and transplanted these under the kidney capsule of recipient mice, where they developed into thymic tissue. This new and fast method enables us to introduce genes of interest into TECs and to study their effects *in vivo*. Using this method, we could demonstrate that reconstitution of the transmembrane domain of CD83 alone is sufficient to rescue normal $CD4^+$ T cell development in $CD83^{-/-}$ RTOCs, whereas the extracellular domain is dispensable for positive selection of thymocytes. Therefore we can exclude cell-cell signalling via CD83-CD83 ligand interaction being crucial for T cell development.

Interestingly, in DCs, the transmembrane domain of CD83 has been shown to inhibit MARCH1-mediated degradation of MHC II. Since we identified also the transmembrane domain to be crucial for $CD4^+$ T cell development, we addressed if this regulatory mechanism also applies for cTECs. We found that cTECs do not express MARCH1 but MARCH8, a close homolog. Both MARCH E3 ligases are known to ubiquitinate MHCII. This finding suggests that CD83 regulates the MHCII level in cTECs via MARCH8 inhibition and therewith contributes to mediate the positive selection of thymocytes. We could confirm this hypothesis using MHCII(K>R)KI mice, which are resistant to MARCH-mediated ubiquitination of MHCII. By crossing the MHCII(K>R)KI alleles to the $CD83^{-/-}$ background, we observed a complete rescue of $CD4^+$ T cell development. Further, in cTECs of these mice the MHCII levels are not altered in the presence or absence of CD83. This rescue of the $CD83^{-/-}$ phenotype demonstrates that CD83 acts upstream/prior to MARCH-mediated MHCII ubiquitination, and is thereby stabilizing MHCII surface expression in cTECs.

In summary, the transmembrane domain of CD83 stabilizes MHCII surface levels of cTECs by inhibiting MHCII ubiquitination, and therewith rendering CD83 expression in cTECs crucial for $CD4^+$ T cell development.

2 ZUSAMMENFASSUNG

CD83 wurde bisher als Aktivierungsmarker von dendritischen Zellen (DZ) genutzt, die Funktion des Moleküls ist jedoch unklar. Erst ein CD83-defizientes Mausmodell offenbarte, dass CD83 eine wichtige Funktion für die Entwicklung von $CD4^+$ T-Zellen hat, da diese Mäuse eine stark verminderte Zahl eben dieser Zellen aufweist. Des Weiteren ist das MHCII-Expressionsniveau diverser Antigen-präsentierenden Zellen (APZ) in der $CD83^{-/-}$ Maus geringer.

Ziel dieser Studie war, herauszufinden was die Ursache für die verminderte $CD4^+$ T-Zellentwicklung war: Beeinflusst CD83 indirekt über ein verringertes MHCII-Expressionsniveau die $CD4^+$ T-Zellentwicklung oder erfüllt CD83 eine MHCII-unabhängige Funktion, wie etwa eine Signaltransduktion *in trans*.

Um diese Hypothesen zu testen, haben wir mutierte CD83-Versionen in Thymusepithelzellen (TEZ) eingebracht und deren Effekt auf die T-Zellentwicklung studiert. Dazu infizierten wir murine embryonale TEZ mit Lentiviren, die einen Expressionsvektor dieser CD83-Mutanten enthielten. Nach der Infektion wurden die Zellen reaggregiert und unter die Nierenkapsel von $CD83^{-/-}$ Mäusen transplantiert; dort bildet sich aus den transplantierten Zellen nach 3-5 Wochen ein thymusartiges Gewebe. Mit dieser Methode konnten wir die verschiedenen Domänen des CD83-Moleküls auf ihre Potenz testen, die $CD4^+$ T-Zellentwicklung in $CD83^{-/-}$ Mäusen zu rekonstituieren. Wir konnten zeigen, dass die Transmembrandomäne von CD83 für die $CD4^+$ T-Zellentwicklung wichtig ist, während die Anwesenheit der extrazellulären als auch der intrazellulären Domäne des CD83-Moleküles für die T-Zellentwicklung keine Rolle spielen. Für die T-Zellentwicklung im Thymus können wir somit eine direkte Zellinteraktion bzw. Signaltransduktion über CD83 und dessen Liganden ausschließen.

In DZ wurde gezeigt, dass die Transmembrandomäne von CD83 die MARCH1-vermittelte Endocytose von MHCII inhibieren kann. Daraufhin untersuchten wir, ob ein ähnlicher Regulationsmechanismus in TEZ wirke. Wir haben herausgefunden, dass kortikale TEZ (kTEZ) nicht MARCH1, sondern das nahverwandte Homolog MARCH8 exprimieren. Diese Erkenntnis lässt vermuten, dass CD83 das MHCII-Niveau in kTEZ über die Inhibierung von MARCH8 reguliert. Diese Vermutung konnten wir mittels des MHCII(K>R) Mausmodells bestätigen, dessen MHCII-Moleküle aufgrund eines Aminosäureaustausches resistent gegen MARCH-vermittelte Ubiquitinierung sind. Diese MHCII-Mutante rettete die verminderte $CD4^+$ T-Zellentwicklung der CD83-defizienten Mäuse. Dies deutet daraufhin, dass CD83 eine Funktion innehat, die die Ubiquitinierung von MHCII verhindert, und somit einen stabilisiernden Effekt auf die MHCII Moleküle auf der Zelloberfläche hat.

Zusammenfassend, konnten wir zeigen, dass die Transmembrandomäne von CD83 wichtig ist um das MHCII-Expressionsniveau in kTEZ zu stabilisieren, und somit unabdingbar für die $CD4^+$ T-Zellentwicklung im Thymus ist.

3 INTRODUCTION

Our knowledge regarding immunological signaling networks has greatly expanded over the last decades; many gain-of-function or loss-of-function approaches have contributed to the discovery of new cell subsets, further signaling molecules, or new regulatory and crosstalk mechanisms, thus further unraveling the complexity of signaling networks. CD83, for example, a long-known activation marker in dendritic cells (DCs) whose actual function was not clear, was found to be crucial for $CD4^+$ T cell development. This finding was disclosed in a CD83-knockout mouse model and added another puzzle piece to the selection processes in the thymus. However, the underlying molecular mechanism remained elusive and formed the basis for this study, i.e. to investigate the function of CD83 in the thymus, the organ fostering T cell development. To fully appreciate the aim of the study, a brief introduction on the immune system and, in particular, on the thymus, including the thymic epithelium and T cell development, is given. Thereafter, the current knowledge about the molecule CD83 is outlined. Finally, an overview of the MARCH ubiquitin ligase family is given, which will then lead to the aim of the study.

3.1 The immune system

The biological role of the immune system is to protect the organism from invading pathogens and disease. The highly evolved immune system of mammals is comprised of two branches: the fast non-specific innate immunity, and the slower antigen-specific adaptive immunity.

3.1.1 The adaptive immune system

Adaptive immunity relies on a network of interdependent leukocytes (T cells, B cells, DCs, macrophages: For example, T cells are primarily responsible for cell-mediated immunity, being activated by antigen-presenting cells (APCs) (Macrophages, DCs, B cells). B cells produce antigen-specific antibodies for which they require an activation signal by $CD4^+$ T cells. $CD4^+$ T cells are also referred to as T helper cells, as they help to initiate appropriate immune responses of effector cells. In short, a well-regulated interplay between leukocytes is crucial for an effective adaptive immune response.

The immunological synapse formation between T cells and APCs is the key to an antigen-specific defense against pathogens. It consists of the antigen-specific T cell receptor (TCR) of a T cell interacting with peptide presented in the context of major histocompatibility complex (MHC) molecules, referred to as peptide/MHC complex (pMHC) of APCs.

As a hallmark of the adaptive immunity, T cells and B cells have the unique ability to rearrange their antigen receptor genes, therewith creating a vast variety of rearranged receptors with specificities to a huge array of potential antigens. The generation of such a vast, nearly infinite, repertoire of antigen receptors is accomplished by random rearrangement of V (variable) – D (diversity) – J (joining) immunoglobulin gene segments, which creates the pitfall to develop potentially self-reactive receptors. The development of lymphocytes bearing randomly assembled receptors has to be well-controlled and checked for functionality and self-reactivity. The thymus fosters the appropriate microenvironment to accomplish this substantial task for T cell development.

3.1.2 MHC expression and regulation

MHC molecules are highly polymorphic glycoproteins encoded by genes in the major histocompatibility complex (MHC). Each molecule has the ability to bind a wide variety of peptides. A TCR interacts directly with both, structures of the peptide and of the polymorphic features of the MHC molecule presenting the peptide. Each TCR is specific for a particular combination of a peptide together with a MHC molecule. The peptide generation and loading onto MHC molecules occurs intracellularly. The two classes, MHC class I (MHCI) and class II (MHCII), present peptides of different subcellular origin (cytosolic and endosomal, respectively), and their non-polymorphic domains are bound by different costimulatory molecules of T cells (CD8 and CD4, respectively).

Two major T cell subsets have evolved which detect and eliminate different pathogens: $CD8^+$ T cells (cytotoxic T cells) are predestinated to detect virus infections via pathogen-derived antigen presentation on MHC class I molecules (pMHCI). The loading of MHCI is specialized to present cytosolic peptides. MHCI molecules are expressed by almost all cells and have the purpose to display cytosolic peptides to $CD8^+$ T cells, which in case of a viral infection, destroy the infected cell. The cytosolic proteins are degraded into peptides by the proteasome. These peptides are then shuttled via the transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum (ER), where they are loaded onto MHCI. After a peptide has bound to a MHC molecule, the pMHC complex leaves the ER and is transported through the Golgi apparatus to the cell surface.

$CD4^+$ T cells are specialized to detect phagocytosed pathogens, as they recognize peptides presented on MHC class II molecules (pMHCII) by APCs. The MHCII loading pathway is specialized

for peptides of extracellular origin. Notwithstanding, there are mechanisms of cross-presentation, meaning the exogenous antigen loaded onto MHCI or endogenous peptides loaded on MHCII (Fig 1).

MHCII molecule expression is restricted to the thymic epithelium and to professional APCs (DCs, Macrophages, B cells). The main purpose of professional APCs is to present foreign antigens and therewith initiate an immune response towards pathogens. For an effective adaptive immune response, the initial step is to display antigens, which have been endocytosed, via MHCII complexes to $CD4^+$ T cells. Extracellular pathogens and proteins are internalized by endocytosis/phagocytosis and delivered into endosomes. The endosomes become increasingly acidic as they progress to the interior of the cell, eventually fusing with lysosomes. These newly formed endolysosomes contain pH-sensitive proteases (e.g. Cathepsins), which degrade the proteins/antigens present in the vesicle. Newly synthesized MHCII molecules or those endocytosed/recycled from the cell surface are shuttled to and fuse with lysosomes, which also contain the generated peptides of the degraded proteins, to form the MHCII loading compartment (MIIC). Upon peptide binding, pMHCII complexes are translocated to the cell surface. The stable binding on MHCII molecules allows a long-term display of the peptide. However, pMHCII complexes can be reinternalized and recycled with a new peptide (MHCII turnover).

Regarding the regulation of MHCII surface expression, it was recently reported, that ubiquitination of it's beta chain targets MHCII for endocytosis (1, 2). In DCs, the ubiquitination is mediated by MARCH1, an E3 ubiquitin ligase, which targets a lysine at position 225 (3). In the MHCII(K>R) mouse model, this $lysine^{225}$ (K) is replaced by an arginine (R), which renders pMHCII complexes resistant to ubiquitination by MARCH ligases (2, 4). Accordingly, the majority of these mutated pMHC complexes were found to be expressed at the surface of immature DCs (cells, which are usually characterized by their high MHCII turnover and substantial intracellular MHCII localization). In line with this, the DCs also showed reduced endocytosis rates. The phenotype of this mouse model suggests that, in DCs, pMHCII endocytosis is mainly regulated via ubiquitination.

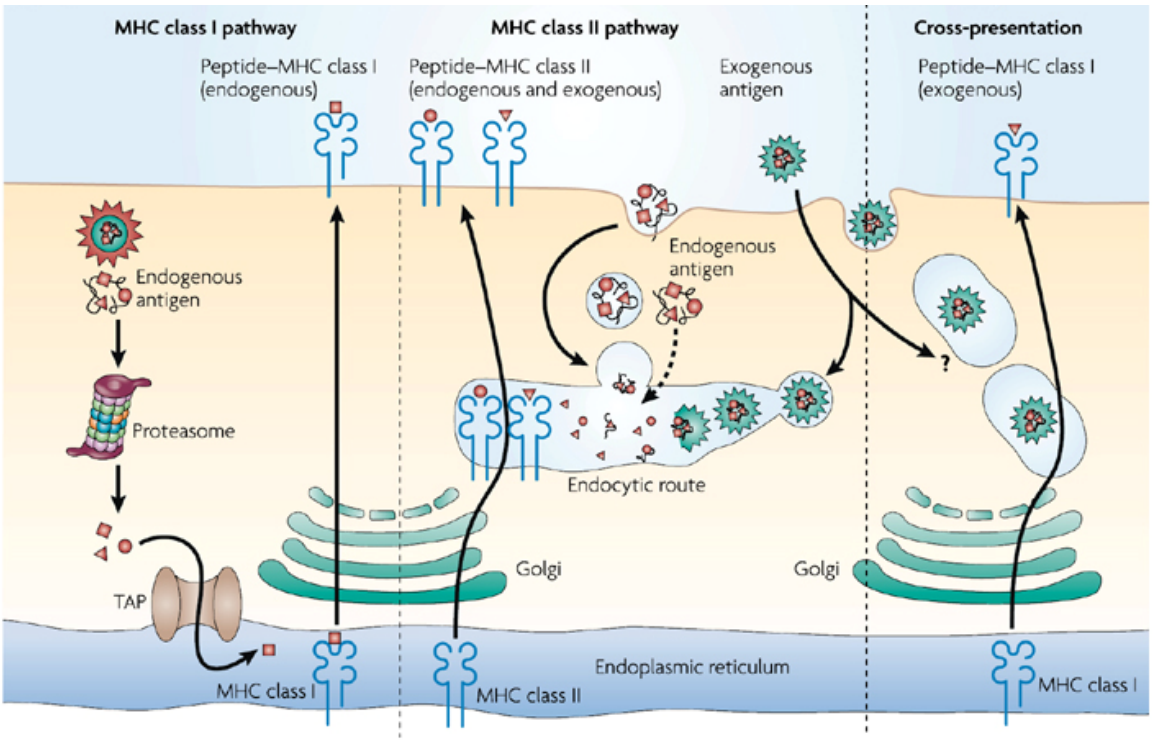

Figure 1: MHCI and MHCII loading pathways.

The MHCI loading pathway is optimized for the presentation of virus-derived peptides via cytosolic protein degradation by the proteasome. pMHCI complexes are recognized by $CD8^+$ T cells, which upon encounter of their cognate antigen, lyse the antigen-presenting/infected cell. The MHCII loading is specialized to present endocytosed/phagocytosed antigen via the destruction of proteins in lysosomes and subsequent fusion with the MHCII loading compartment. There also exist mechanisms of cross-presentation, meaning the presentation of endogenous peptide on MHCII or exogenous antigen on MHCI. For example, macroautophagy enables the shuttling of cytosolic antigen into the lysosomal/MHCII loading compartment (not depicted). pMHCII complexes are recognized by $CD4^+$ T cells which stimulate the APCs to initiate an appropiate immune response (T cell interaction not shown in figure). *Adopted from Villadangos et al. Nature Reviews Immunology, 2007 (5).*

3.1.3 The Thymus

The thymus as a primary lymphoid organ provides the essential niches for the establishment and maintenance of T cell-mediated immunity. The thymus recruits common lymphoid progenitors from the blood stream and mediates their commitment to the T cell lineage. In particular, it establishes a functional and self-MHC restricted T cell repertoire (positive selection), which is tolerant to self-antigens (central tolerance).

3.1.3.1 Thymus development

The murine thymogenesis is initiated with the budding and outgrowth of the endodermal layer from the third pharyngeal pouch at embryonic day 9-11. The outgrowth coincides with the expression of the transcription factor forkhead box N1 (Foxn1) (6). Foxn1 is the earliest and most important factor driving thymus development. In the Foxn1$^{nu/nu}$ ("nude") mouse, a loss-of-function mutation in the Foxn1 gene, abrogates thymic epithelial cell (TEC) development (7). It only forms depauperated thymic tissue (epithelial cysts) that consequently is unable to foster T cell development and thus leads to T cell-deficiency. This mouse model clearly demonstrates the importance of Foxn1 for thymus development and also the importance of the thymic epithelium for T cell development. Additionally, TEC development also depends on neural crest-derived mesenchymal cells, which surround the thymic anlage and provide growth factors (8).

The thymic stroma is composed of DCs, macrophages, fibroblasts, endothelial cells, and thymic epithelium and is forming a specialized cellular matrix that mediates the unique function of the thymus. The thymic architecture is directly linked to its function: structural disorganization of the thymic stroma comes along with impaired T cell development (6).

The postnatal thymus is compartmentalized into cortex and medulla. The outer morphologically darker zone, the cortex comprises mainly of cortical thymic epithelial cells (cTECs) and macrophages. The inner morphologically lighter zone, the medulla contains medullary thymic epithelial cells (mTECs), DCs, B cells and macrophages.

3.1.3.2 Thymic epithelial cells

Unlike most epithelia, the epithelial cells of the thymus express both MHC class I and class II antigens and are therefore regarded as APCs. Cortical TECs (cTECs) and medullary TECs (mTECs) differ in localization, morphology and function. They can be distinguished phenotypically by the expression of several markers: cTECs can be identified by the expression of EpCAM, MHCII, Ly51, CD205, Cytokeratin 8 and Cytokeratin 18. mTECs are defined as EpCAM^{+}, Cytokeratin 5^{+} and Cytokeratin 14^{+} (6); they can be further fractionated into immature and mature cells by the expression level of MHCII and the co-stimulatory molecule CD80: CD80^{lo} MHCII^{lo} and CD80^{hi} MHCII^{hi}, respectively. The expression of the autoimmune regulator (Aire) is thought to be the terminal differentiation step of mTECs (9).

TECs play an essential role in managing intrathymic T cell development: They operate positive and negative selection of thymocytes, Treg induction, and also release thymopoietic factors such as

chemokines and cytokines that provide survival, proliferation, and differentiation signals to thymocytes (10). In other words, TECs are essential for T cell development. In particular, cTECs mediate positive selection of thymocytes in the cortex, and mTECs are crucial for tolerance induction in the medulla (see section 3.1.4).

The importance of TECs becomes evident in mice mutant for TEC-specific proteins: In Foxn1-mutant mice, the mutation of this TEC-specific transcription factor, causes the complete loss of the T cell arm. Another example for the importance of TECs for the adaptive immunity is demonstrated in the Aire-deficient mice model, which suffers from multi-organ autoimmune disease (11). The mTEC-specific gene Aire mediates the expression of a large array of self-antigens (promiscuous gene expression), including those that are tissue-restricted (TRA), and is therewith essential to accomplish central tolerance. In combination with the extensive expression of self-antigens, TEC use a special mechanism to present self-antigens on MHCII: macroautophagy, a mechanism allowing endogenous peptide loading on MHC class II molecules, has a central role in both positive and negative selection. Disturbed macroautophagy in TECs causes autoimmune disease and multi-organ inflammation as mice lacking Atg5 (a molecule essential for autophagy) specifically in TECs show multi-organ inflammation due to an insufficient self-presentation during negative selection (12).

In sum, these examples of genetic modifications of TECs causing T cell abnormalities and autoimmune diseases clearly demonstrate the importance of TECs for the establishment of a functional and self-tolerating T cell repertoire.

On the other hand, TEC development is also dependent on thymocyte interactions. Mutants in which T cell development is intrinsically arrested at different stages of development show impaired TEC differentiation. For example, $Rag2^{-/-}$ mice lacking double positive thymocytes (DP) display a disturbed medullary architecture whereas the cortical compartment forms normally. Furthermore, in $Rag2^{-/-}$ $\gamma_c^{-/-}$ mice thymocyte development is arrested earlier, which is then accompanied with abnormal cortical and medullary architecture (6).

Taken together, T cell development and TEC development require bi-directional signaling, which is referred to as TEC-thymocyte crosstalk. Only few receptor-ligand pairs (lymphotoxin β receptor/ligand, RANK/RANKL, CD40/CD40L, and Notch/Notch ligands) involved in cross-talking have been identified so far (13-17). Although the mechanisms/interactions driving positive and negative selection have a huge impact on functional adaptive immunity, the understanding of the molecular processes involved is incomplete.

The limited knowledge of TECs and of their functions is partly due to the fact that Foxn1 expression is lost in cell culture. Foxn1 is the primary lineage-defining transcription factor, which

has great impact on the physiology of TECs. Its loss upon culture renders most *in vitro* studies pointless. An additional limitation is the low TEC number that can be obtained per thymus (only 0.05 percent of total thymic cells). These hurdles may contribute to the limited knowledge in TEC development and thymocyte selection processes.

3.1.3.3 Reaggregate Thymus Organ Cultures

An innovative approach to study thymocyte selection processes *in vitro* was taken by the Graham Anderson lab. They established the reaggregate thymus organ culture (RTOC) technique, which allows single-cell suspensions of embryonic thymic stromal cells to be reaggregated to form thymic 3D structures. The technique was established to manipulate the thymic composition of cells and therewith investigating thymic selection processes (18). In brief, single-cell suspensions of embryonic (E14.5-E16.5) non-hematopoietic thymic stromal cells can reaggregate when put at high density onto a nylon membrane floating on culture medium for 2-7 days (19). When the resulting RTOCs were transplanted under the kidney capsule of mice, it was shown that they develop further into properly compartimentalized and functional thymic tissue within a few weeks. The fact that the RTOCs can grow for weeks or even months under the kidney capsule indicates that the embryonic stromal cells contain progenitor cells, which are capable to replenish the thymic epithelial microenvironment (20).

3.1.3.4 Dendritic cells

Dendritic cells are the most potent APCs to induce primary immune responses. Upon exposure to inflammatory stimuli associated with pathogens or tissue damage, immature conventional DCs (cDCs) undergo complex phenotypic changes, collectively known as maturation. These changes include rapid expression of CD83 at the surface, transiently increased phagocytosis and macropinocytosis, changes in proteasomal activity, increased co-stimulatory molecule expression, higher MHCII surface levels due to higher synthesis rates and decreased turnover rates of MHCII (21, 22). The MHCII turnover in cDCs is regulated by ubiquitination by MARCH1 (2, 23). These coordinated changes enable mature cDCs to present antigens, which were captured at the site and time of activation, for extended periods. After migration to peripheral lymph nodes, the extended presentation of potentially pathogen-derived peptides allows the stimulation of specific T cells (22).

Thymic dendritic cells are part of the thymic stroma and participate in antigen presentation to thymocytes mainly in the medulla. In the thymus, DCs adapt a rather mature phenotype with high MHCII surface levels, despite the absence of inflammatory stimuli. Furthermore, the MHCII turnover rate of thymic DCs is also decelerated compared to splenic DCs, which renders them efficient antigen presenters (24). Thymic DCs consist of three major subsets: and $CD8^+$ conventional DCs (cDCs), $Sirpa^+$ cDCs, and plasmacytoid DCs (pDCs). $CD8^+$ cDCs are thymus-derived and are therefore named resident DCs. $Sirpa^+$ cDCs are also referred to as migratory DCs which can take up antigen in the periphery and present it in the thymic medulla. Regarding negative selection, there are no functional differences known between $CD8^+$ and $Sirpa^+$ cDCs. Both receive antigen from mTECs and they are equally efficient in antigen presentation for negative selection ((25), own observations). Plasmacytoid DCs (pDCs) are known to be poor presenters of mTEC-derived peptides ((26), own observations). However, recent studies indicate a role of pDCs in sampling self-antigens in the periphery and presenting them in the thymus, therewith contributing to central tolerance (27). Unlike cDCs, in pDCs the ubiquitination of MHCII is not reduced by maturation stimuli (28). This difference in MHCII regulation was found being determined by differential MARCH1 regulation. Notably, the activation of toll-like receptors prevents both cDCs and pDCs from migrating to the thymus, hence conceivably preventing tolerance induction towards pathogens (29).

3.1.4 T cell development

This study investigates the αβ-T cell development, therefore, we focus on those T cells in the introduction.

As αβ-T cells mature, they migrate through the thymus according to a defined pattern. Each area defines a discrete microenvironment promoting distinct stages of development: after entering the thymus through high endothelial venules at the cortico-medullary junction, the common lymphoid progenitors mature to early thymic progenitors, which lose self-renewal capacity, and then upon further development commit to the T cell lineage. Therefore, T cell lymphopoiesis is dependent on the constant import of progenitors from the bone marrow (30).

Of note, this aspect is exploited for the generation of bone marrow chimera. In chimera, the recipient's radio-sensitive hematopoietic system can be depleted via lethal irradiation and reconstituted with bone marrow of a genetically different donor. After 5 weeks the T cells (of bone marrow's genotype) that developed in the recipient's radio-resistant thymic stroma can be analyzed.

The scaffold of thymic stromal cells provides the signals for the arriving progenitors to commit to the T cell lineage as DN1 cells (double negative for the markers CD4 and CD8) and guides them through further developmental stages. During the DN2 stage the thymocytes migrate towards the subcapsular region. DN3 cells rearrange their TCR β-chain locus. The rearranged genes are tested for functionality (β selection). For β selection, the successful coupling of the rearranged TCR β-chain with an invariant pre-TCR α-chain and CD3 subunits results in a ligand-independent survival signal that leads to massive proliferation and further progression to the DN4 stage. At the DN4 stage, the V and J regions of the TCR α-chain rearrange to each other. This rearrangement continues sequentially and on both chromosomes, until a TCR α-chain is formed that can associate with the already formed β-chain to make a mature TCR. After concomitant upregulation of CD4 and CD8, Double positive (DP for CD4 and CD8) thymocytes migrate back inward through the cortex for positive selection. Of note, prior to positive selection the thymocyte development is completely TCR-pMHC-independent but requires other stromal supplies (Fig 2) (30).

3.1.4.1 Positive selection of thymocytes

In the cortical region, the interaction between the DP thymocytes bearing a randomly rearranged αβ-TCR with pMHC complexes displayed by cTECs determines a thymocyte's destiny: The process of positive selection ensures the survival of only those thymocytes whose rearranged αβ-TCRs recognizes self-peptide in the context of MHC complexes well enough to transduce a signal. In particular, the binding strength of the TCR-pMHC interaction determines which thymocytes are positively selected: Thymocyte with "high enough" affinity for pMHC receive a TCR stimulus. This translates into downstream signaling, which leads to the expression of survival molecules allowing further progression to the medulla for negative selection. Thymocytes' TCRs that do not interact with sufficient affinity with cTECs' pMHC, are not triggered to signal downstream, thus, do produce survival signals and therefore undergo apoptosis (death by neglect) (30).

The positively selected thymocyte will only be able to recognize its specific peptide within the context of the MHC haplotype it has been selected on (MHC restriction). The MHC restriction was first described in bone marrow chimera, whose T cells recognize only peptide that is presented by the same MHC haplotype as that of the bone marrow recipient (31). Further evidence was found in TCR-transgenic mice (T cells expressing a transgenic rearranged TCR), whose T cells were only positively selected in a thymus of the same MHC haplotype as the haplotype the TCR originated from (32).

Besides the haplotype, also the MHC class is restricting: Being selected by a pMHCI or pMHCII complex determines a thymocyte's fate in respect to becoming either a $CD4^+$ T helper cells or a $CD8^+$ cytotoxic T cell. CD4 and CD8 molecules are TCR co-receptors that bind exclusively to conserved regions of MHCII and MHCI respectively. Their major role is the promotion of the TCR signaling by recruiting and bringing downstream signaling molecules in close vicinity (33).

Several models have been proposed to explain the CD4/CD8-lineage choice; the kinetic signaling model, a favored model, suggests that upon positive selection, all DP thymocytes slightly downregulate the co-receptor CD8. This would not influence the TCR signaling of a CD4- and MHCII-dependent interaction and therefore the cell will be deviated into the CD4 lineage. However, if the thymocyte is MHCI-restricted and consequently dependent on interactions of CD8 with pMHC, the TCR signal will decrease upon CD8 downregulation and therewith, lead to an irreversible CD8 lineage commitment. In conclusion, the CD4/CD8 lineage choice of thymocytes is determined by the quality and duration of the TCR signaling during the TCR-pMHC interaction of positive selection (30, 34).

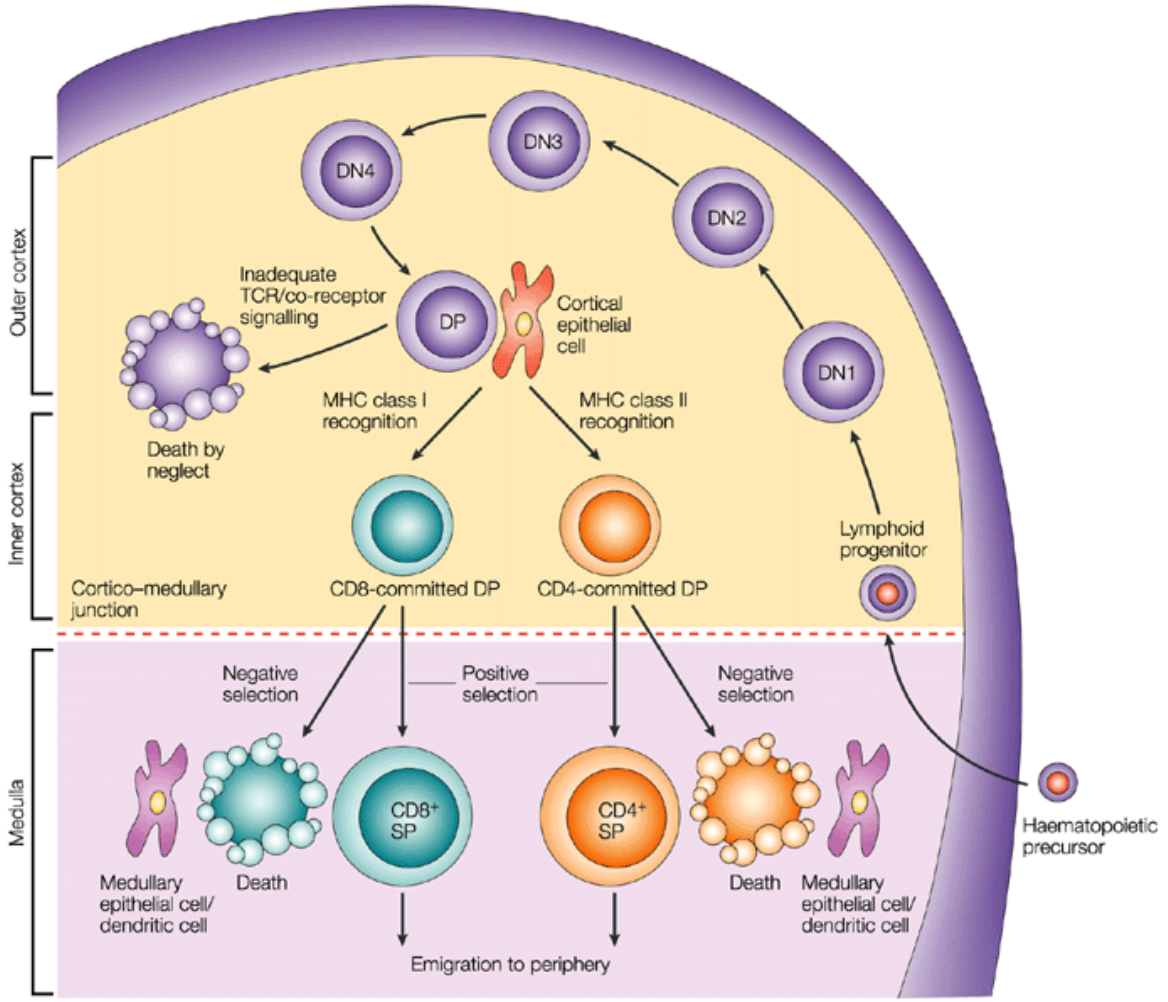

Figure 2: T cell development in the thymus.

The hematopoietic precursors enter the thymus at the cortico-medullary junction and migrate towards the outer cortex. During that period, the precursors commit to the T cell lineage and undergo several differentiation steps as double negative cells (respective to CD4 and CD8 expression), namely DN1 to DN4, during which the thymocytes rearrange their TCR gene segments. Positive selection by cTECs allows only those thymocytes to progress to the medulla, which have rearranged a functional TCR. During positive selection, thymocytes also commit to either the $CD4^+$ or the $CD8^+$ T cell lineage, depending on the MHC type their TCR is selected by. In the medulla, the $CD4^+$ or $CD8^+$ thymocytes are screened for reactivity towards self-antigens, which is mediated mainly by mTECs and DCs. One mechanism to induce central tolerance is clonal deletion: Upon encountering their specific antigen with high affinity in the medulla, these auto-reactive thymocytes are negatively selected, meaning driven into apoptosis (Treg induction is not depicted). Thymocytes not activated by self-antigens are allowed to egress the thymus and seed the periphery. *Adopted from Ronald N. Germain, Nature Reviews Immunology, 2002 (35).*

3.1.4.2 Central tolerance

The next developmental checkpoint occurs in the thymic medulla, where the interactions between thymocytes and self-antigen-presenting APCs (mainly mTECs and DCs) induce the defusing of potentially hazardous-to-self thymocytes (Fig 2). mTECs have the special feature of expressing a huge array of genes, including tissue-restricted genes, whose peptides can then be presented on MHC molecules of mTECs themselves or transferred to DCs to amplify the surface of presentation (36, 37). This feature is called promiscuous gene expression and allows the testing of the thymocytes' reactivity against any possible antigen the cell may encounter throughout the body. Thymocytes are migrating through the medulla for a period of 4-5 days, which allows them to scan the medullary environment for self-antigen encounter (38). Thymocytes interacting with a pMHC with high affinity receive a death-inducing signal. The elimination of thymocytes, which react with "too high" affinity to self-peptide/MHC complexes, is termed negative selection/clonal deletion, hence, induces central tolerance. The affinity threshold of the TCR-pMHC interaction scrutinizing between positive and negative selection has been investigated by the Palmer lab: They designed variants of the OTI TCR agonist peptide OVA with different affinities to study how small changes in affinity translate into cellular fate (39). The investigators found that thymocytes, which were exposed to different variants of the OVA peptide, differed in levels, rate, and localization of CD3ζ, ZAP70, LAT, Erk phoshorylation. Negative selecting peptides induced very rapid Ca^{2+} flux and faster CD3ζ and ZAP70 phosphorylation compared to positive selectors. Further, negative selectors induced faster LAT phosphorylation peaking early, while positive selecting peptides mediated steadier phosphorylation throughout the interaction. Additionally, these findings revealed a surprisingly narrow threshold window between positive and negative selection.

Besides negative selection, an additional tolerance inducing mechanism exists: thymocytes with a TCR of borderline or above threshold affinity for self-pMHC can be deviated into the regulatory T cell lineage (Tregs) (dominant tolerance). Tregs are phenotyped as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells and characterized by their immunosuppressive potential (40). It has been found that some auto-reactive thymocytes are deviated into Tregs and released into the periphery with the purpose to keep auto-reactive T cells in check that potentially escaped negative selection (41). Activated Tregs release cytokines that suppress immune reactions and therewith can dampen the activity of auto-reactive T cells that would otherwise cause autoimmunity.

Clonal deletion and dominant tolerance are not mutually exclusive as has been seen in several TCR-transgenic systems: For example, hemaglutinin (HA)-specific thymocytes were both clonally

deleted and deviated into Tregs when the antigen HA was expressed in the thymus (transgenic HA expression under the control of the Aire promoter). When the antigen was absent, the HA-specific cells developed normally into mature naïve T cells (42). Also in TCR-transgenic systems, which recognize a naturally expressed antigen (e.g. H-Y), both central tolerance mechanisms apply (43). The factors determining the fate of thymocytes with high affinity for self-peptide/MHC complexes towards clonal deletion or deviation into the Treg lineage are still poorly understood.

Finally, the remaining thymocytes are mature, either CD4 or CD8 single positive (CD4SP, CD8SP), non-responsive to self-peptides, and egress as naïve T cells from the thymus into the periphery. In conclusion, T cell development is a well-controlled and ingenious process with astonishing consistence.

3.1.5 The selection paradox

The paradox that positive and negative selection of thymocytes employ the same TCR-pMHC interaction but results in opposing cell fates (survival and death), has been challenging immunologist for decades. Several models have been suggested to resolve the selection paradox. One theory implicates a spatial and temporal segregation of positive and negative selection and together with a different integration of signals depending on the differentiation stage of the thymocyte. Changes in the gene expression profile might impinge on the sensitivity of the TCR signal. Indeed, it was shown that the activation threshold of mature T cells is higher compared to DP cells (44). It is believed that the signaling molecules proximal of the TCR play a fundamental role in translating the TCR stimuli into quantitative signals. For example, the TCR signaling threshold can be fine-tuned by de-/phosphorylation of CD3 (34).

As described earlier, the classical affinity model postulates that TCRs with low affinity for pMHC die by neglect, whereas high affinity TCRs are clonally deleted. Only TCRs of intermediate affinity pass thymic selection processes. The basic principle of this model is supported by a substantial body of evidence, but in its present form, it does not account for other central tolerance mechanisms than negative selection, nor does it incorporate a reason for the spatial and temporal compartmentalization of the thymic selection processes (45).

The avidity model suggests that the quantity of TCR-pMHC interactions per cell determines the thymocyte's fate. In case only a few TCRs of a thymocyte are being triggered by the pMHC repertoire of a cTEC (low avidity), this tickling would result in moderate downstream signaling and subsequent positive selection. While the engagement of many TCRs (high avidity) would create a

stronger accumulated downstream signal, resulting in negative selection of the thymocyte (46). The density of a given self-antigen presented per niche could add additional signal tuning options (Fig 3).

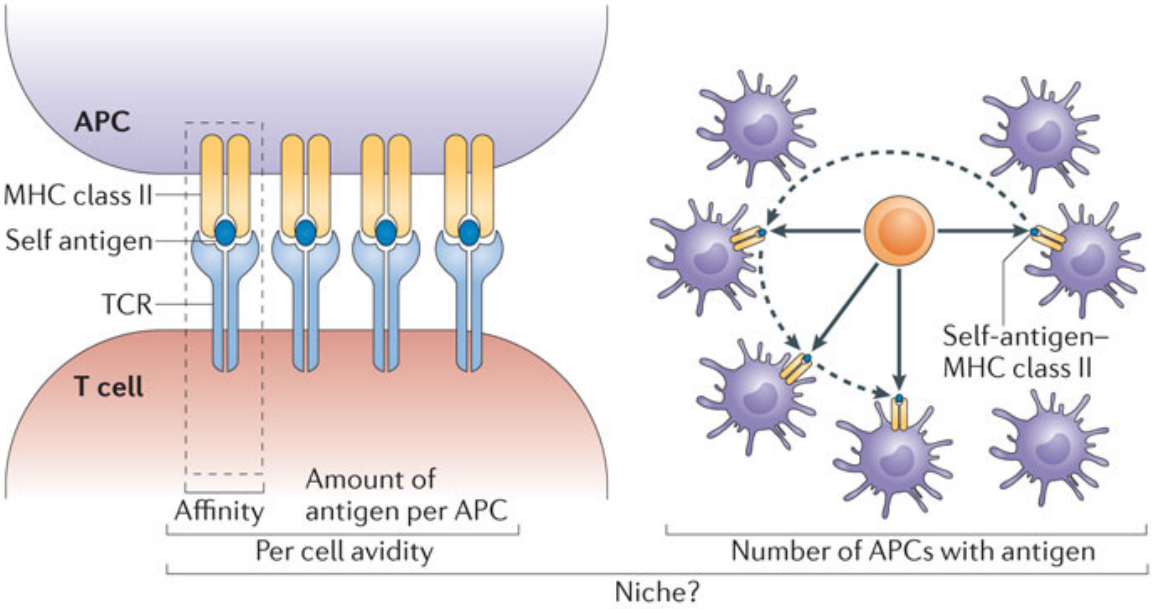

Figure 3: Potential criteria to modify the TCR stimulus by APCs.

The mechanisms that determine positive and negative selection of thymocytes are not fully understood. This illustration summarizes potential criteria that might lead in sum to distinct fine-tuned TCR triggering, which then determines the fate of the T cell. In the thymus, the fine-tuning of the transmitted TCR signal determines between survival vs. death in positive selection, and later in the medulla between tolerance induction vs. maturation of T cells. *Adopted from Hsieh et al., Nature Reviews Immunology, 2013 (47).*

The 'altered peptide' model predicts that cTECs have a distinct ligandome to present and to positively select thymocytes on. This would resolve the paradox by the means that the TCR could not receive an identical signal again, as the presented peptides are exclusively produced in cTECs. Sequencing of peptides eluted from MHCII of cTECs and splenic APCs have disproven this theory of peptide exclusiveness in cTECs, though (48). However, at that time, only 17 of the most abundant peptides could be sequenced, which most likely do not reflect the variety of the peptidome. Later, it was discovered that cTECs express different enzymes involved in the proteolytic pathways, indicating that cTECs might be able to display partially different peptides than other APCs (Fig 4).

In particular, cTECs predominately express cathepsin L (CtsL) whereas other thymic APCs produce cathepsin S. Cathepsins are lysosomal proteases implicated in the degradation of lysosomal proteins and the cleavage of the invariant chain (Ii), which blocks the peptide binding groove of MHCII and, therewith, protects from premature peptide-loading of MHCII. CtsL-deficiency resulted in a drastic reduction of CD4SP cells, which was caused by radical clonal deletion. These results indicate that thymocytes, which re-encounter the same peptides (created by the same cathepsins) in positive and negative selection, are clonally deleted (49).

Additionally, in CtsL-deficient mice it is indicated that the pMHC repertoire might be altered: A transgenic TCR specificity that developed normally in WT thymi, was not selected in CtsL-deficient mice (49). An additional cue supporting the 'altered peptide' model was brought by the finding that cTECs exclusively express the thymus-specific serine protease (TSSP, encoded by Prss16), which is involved in lysosomal degradation processes. Also TSSP-deficient mice showed substantial decreases in the positive selection of two MHCII-restricted transgenic TCR specificities that develop normally in WT mice, again indicating an altered pMHC repertoire (50).

Also for the MHCI loading pathway, a cTEC-specific antigen processing machinery was found: The proteasome subunit β5t (encoded by Psmb11) is exclusively expressed in cTECs and in Psmb11-deficient mice a drastic reduction of the CD8SP compartment is observed (51). In addition, the positive selection of several MHCI-restricted transgenic TCRs was impaired in Psmb11-deficient mice, suggesting an altered composition of MHCI ligands (52).

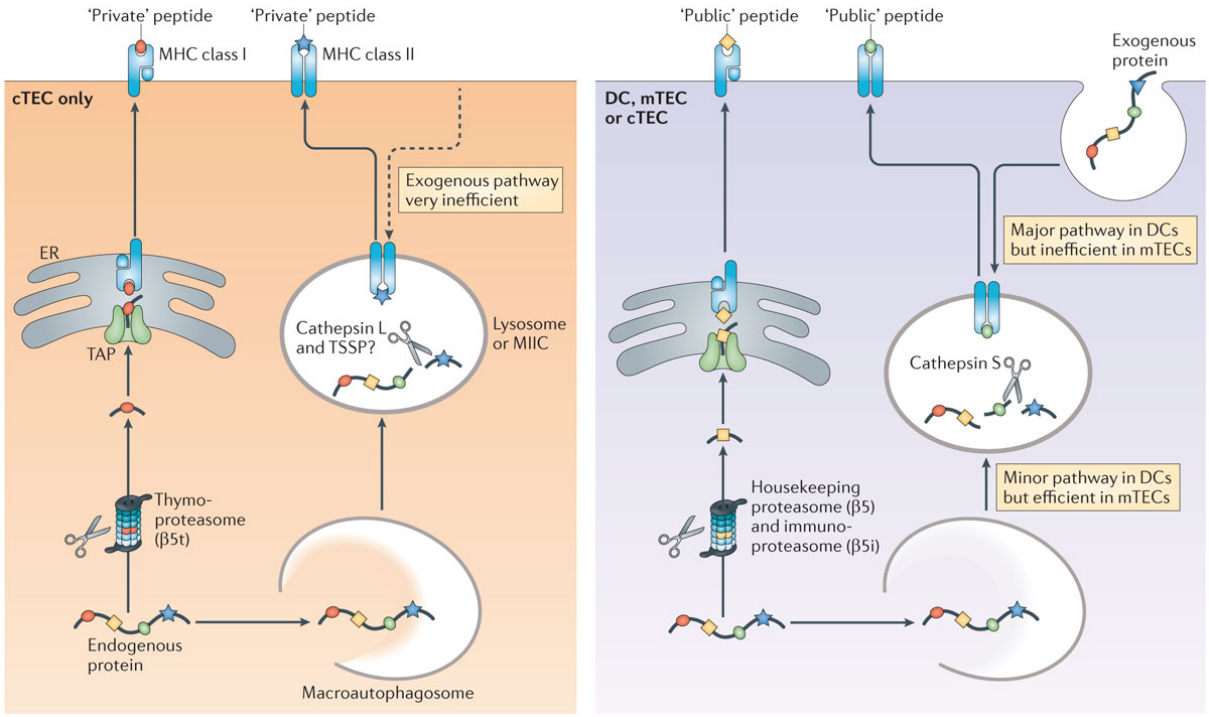

Figure 4: Unique proteolytic pathways generate 'private' MHC-bound peptides in cTECs.

cTECs express distinct proteolytic enzymes/subunits which allow for the generation of cTEC-specific peptides (Cathepsin L, TSSP, β5t). The pMHC of cTECs present a mixture of these cTEC-specific 'private' peptides and 'public' peptides that are also generated in other APCs. *Adopted from Klein et al., Nature Reviews Immunology, 2014 (53).*

Taken together, for the generation of a diverse and functional T cell repertoire, it seems necessary that cTECs express a different machinery which produces an altered peptide repertoire compared to other APCs. Therefore, the altered peptide model has been reinforced by the identification of cTEC-specific proteases yielding a somewhat different but not completely distinct pMHC repertoire from other APCs as initially proposed.

In summary, none of the present models trying to explain the selection paradox succeeded in incorporating all open questions. However, some of the suggested mechanisms are not mutually exclusive.

3.2 CD83

3.2.1 Membrane-bound CD83

In 1992, a new cell surface glycoprotein was identified and named HB15. It was found to be expressed by dendritic cell subsets and lymphocytes upon activation (54). Later, HB15 was integrated in the cluster of differentiation (CD) nomenclature and was termed CD83. It is a 45 kD, type-1 membrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. It comprises 175 amino acids with a single extracellular Ig domain, a transmembrane region, and a cytoplasmic tail. The murine CD83 has 63% amino acid sequence homology with human CD83, the greatest homology being between their transmembrane and cytoplasmic domains (55).

CD83 has been used as a maturation marker for DCs for decades, but its actual function has remained elusive. Besides DCs, also lymphocytes express CD83 highly and rapidly upon activation: Both $CD4^+$ and $CD8^+$ T cells up-regulate cell surface CD83 following stimulation via CD3 and CD28. In B cells, CD83 expression can be induced by IgM, CD40, or TLR-4 signaling (56). Based on the pattern of expression and its structural similarity with co-stimulatory molecules, CD83 is considered to play an important role in interactions between cells of the immune system (54). Several different virus strains (HIV, HSV, HCMV) evolved CD83-modulating mechanisms. Immune evasion mechanisms targeting specific molecules generally indicate that these molecules are import for efficient immune responses. Thus, this suggests that CD83 has functional importance for the activation of T cells (57-59). These mechanisms include CD83 degradation, repression, and shedding of the extracellular domain (58, 60).

Interestingly, allogeneic T cell stimulation by human DCs was reduced when CD83 expression was down-regulated via RNA interference (61, 62). However, in murine mixed lymphocyte reactions CD83-deficient and -sufficient DCs and B cells are equally potent to stimulate T cells (63, 64). Reciprocally, the overexpression of CD83 leaded to enhanced cell surface expression of MHCII and CD86 (co-stimulatory molecule) (65). Accordingly, Aerts-Toegaert et al. found enhanced T cell stimulation in a CD83 overexpression setting (62). Summarizing these results, it was found that overexpression of CD83 leads to enhanced MHCII and CD86 surface levels and to enhanced T cell stimulation, and *vice versa*. These studies suggest an important function of CD83 for the regulation of immune responses.

The importance of CD83 for the development of $CD4^+$ T cell was revealed by the study of CD83-deficient mice. $CD83^{-/-}$ mice have a strong impairment in the $CD4^+$ T cell development in the thymus which also translates into the periphery (63). This drastic reduction by 80% in $CD4^+$ T cells suggests that CD83 represents an additional regulatory component for the development of $CD4^+$ T

cells. A study using a mouse mutagenesis screening confirmed the observation that CD83 deficiency derogates the development of $CD4^+$ T cells (66).

The impaired development of $CD4^+$ T cells was shown to be not a T cell-intrinsic defect but rather an effect mediated by the thymic epithelium. Interestingly, the $CD8^+$ T cells developed normally in $CD83^{-/-}$ mice (63). Further characterization of the $CD83^{-/-}$ mouse model revealed reduced MHCII surface levels on several APCs (splenic B cells and DCs, peritoneal macrophages, and TECs) (64). It was also shown that the reduced MHCII levels were limited to the cell surface, since intracellular MHCII densities were normal in $CD83^{-/-}$ B cells. Additionally, Kuwano et al. could demonstrate an increased cell surface MHCII turnover in those cells, as shown by a more rapid internalization in the absence of CD83. On the other hand, antigen processing, pMHC complex formation and antigen presentation by splenic DCs and B cells were not affected by CD83-deficiency (64).

Recently, it was shown that CD83 inhibits MARCH1-mediated MHCII and CD86 ubiquitination and subsequent degradation in DCs (67). Furthermore, using truncated versions of CD83, it was shown that the transmembrane domain of CD83 mediates the inhibition of MARCH1. These findings revealed a first mechanism by which the lack of CD83 may cause reduced MHCII surface levels (Fig. 5). The exact mode of interaction between CD83, MARCH1, and MHCII remains to be elucidated.

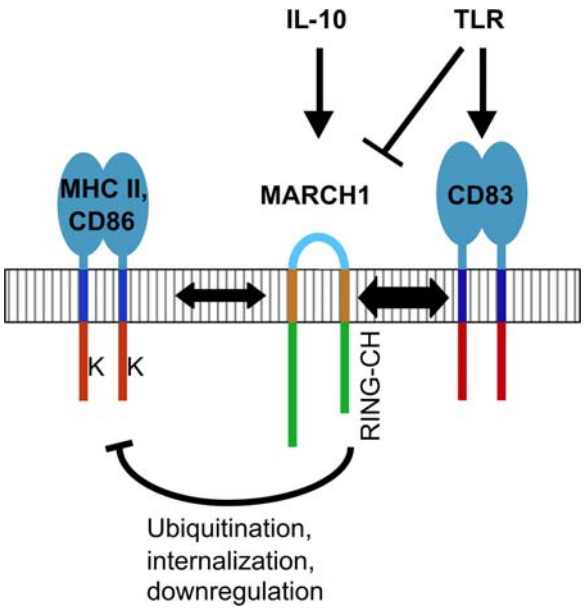

Figure 5: Proposed mechanism of CD83 regulating the display of MHC II and CD86 in DCs.

CD83 was found to inhibit MARCH1. The MARCH1 E3 ubiquitin ligase ubiquitinates the lysine225 residue of the MHCIIβ chain. The MARCH-mediated degradation of MHCII was found to be induced by IL-10, and blocked by TLR signalling. *Adopted from Tze et al., J Exp Med, 2011.*

3.2.2 Soluble CD83

Interestingly, a soluble form of CD83 (sCD83) has been found which consists of only the extracellular domain. Shedding and alternative splicing are the proposed mechanisms for the generation of sCD83 (59, 68). sCD83 has been reported to possess highly immunosuppressive potential: HCMV-infected mature DCs release sCD83, leading to an inhibition of the immunostimulatory capacity of DCs (59). Furthermore, recombinant human sCD83 completely inhibited DC-mediated T cell stimulation in a dose-dependent manner (69). The treatment of immature DCs with sCD83 led to a maturation block even in the presence of a potent maturation cocktail (IL-1β, TNF-α, and PGE_2). Recently, it was shown that the administration of sCD83 increases the frequency of Tregs after allogeneic transplantations. The immunomodulatory effect was mediated via the induction of TGFβ and the immunoregulatory enzyme Indoleamine 2,3-dioxygenase (IDO) (70).

The immunosuppressive potential of sCD83 has been further demonstated in a mouse model for autoimmunity, i.e. autoimmune encephalomyelitis (EAE). It was reported that the injection of sCD83 prevented the paralysis that is associated with the progression of EAE. It was even shown that the administration of sCD83 was acting curative as well as preventive in different therapeutic settings (71).

Regarding the signalling of CD83, a lot of unsuccessful effort was made to identify the ligand of CD83. The high glycosylation level of CD83 might be a reason for these difficulties (72).

In summary, CD83 is expressed upon activation of lymphocytes and DCs, but its function and its potential ligand remain elusive. Several viruses adapted immune evasion mechanisms that target CD83 expression, including the release of the highly immunosuppressive sCD83. Recently, sCD83 was found to induce tolerance by increasing the frequency of Tregs after allogeneic transplantations. These findings suggest an immunomodulatory effect of sCD83. Furthermore, CD83 expression by TECs was shown to be crucial for $CD4^+$ T cell development as revealed by CD83-deficient mice. In conclusion, CD83 is a highly interesting molecule and a potential target for the manipulation of the immune response.

3.3 MARCH ubiquitin E3 ligase family

Ubiquitination is a highly conserved pathway fine-tuning the proteome of a cell. Ubiquitination is the covalent conjugation of proteins with ubiquitin, which is an important regulatory mechanism and best known for its role in protein degradation. Multiple ubiquitinated proteins are targeted to the proteasome for degradation. Several manners of ubiquitination are known: the parameters like the length of the ubiquitin chain (poly- vs. mono-ubiquitination) and the number of mono-ubiquitinated lysine residues (or others) determine the protein's fate. For membrane-bound proteins, the type of ubiquitination may determine between internalization only or subsequent degradation (73).

The ubiquitination of substrates is performed by a catalytic cascade of three protein classes, E1, E2, and E3. They interact subsequentially to transfer ubiquitin moieties to substrates: ubiquitin activation via an E1 enzyme, transfer of activated ubiquitin to an E2 enzyme and targeting of ubiquitin to the lysine residue of the substrate protein. This last step is mediated by an E3 ubiquitin ligase, which facilitates the transfer of ubiquitin from E2 to the substrate by bringing them in close vicinity (73).

Several hundred mammalian E3 ligases have been identified. A large subgroup of these contains a RING (really interesting new gene) domain. RING type E3s have been further subdivided depending on the order of their cysteine (C) and histidine (H) residues into RING-HC (C3HC4), RING-H2 (C3H2C3), and RING-CH (C4HC3). RING-CH ligases with multiple transmembrane domains are called MARCH (membrane-associated RING-CH). The MARCHs are unique amongst ubiquitin ligases in that they are anchored to the membrane, suggesting a specialized role in regulation of membrane protein trafficking. They have been shown to ubiquitinate and downregulate several immunological-relevant transmembrane proteins, such as MHCI and MHCII, CD86, and ICAM1 (74).

Also viruses express MARCH-like ligases named MIRs (modulator of immune recognition), which are believed to have been pirated from the host genome. Viruses have evolved for millions of years under the selective pressure of their host's immune system. Therefore viruses elaborated immune evasion mechanisms that interfere with various aspects of antigen processing and presentation (75). The adoption of MARCH E3 ligases by several virus strains implicates their central role for the adaptive immune system.

The MARCH family consists of eleven genes with low overall sequence similarity and diverse predicted structures. However, some of the MARCH members share especially high homology to one other member as well as subcellular location, expression profile, or substrate specificity. Of all MARCH members, MARCH4 and MARCH9 have been found to ubiquitinate MHCI exclusively

(76). MARCH1 and MARCH 8 (also referred to as c-MIR) share high levels of sequence and functional similarity: both target MHCII for degradation, as well as CD86 and CD95 (Fas) (3, 77-80). Since it has been shown that CD83 inhibits MARCH1-mediated MHCII degradation in DCs, the MARCH1/8 pair will be introduced in more detail in the following.

Regarding the substrate specificity, some MARCH members affect a wide range of targets, while others are rather limited. However, almost all MARCH proteins recognize their substrates via transmembrane domain interactions (81). For example, within the transmembrane domain of the MHCII β-chain a linear amino acid cluster of ^{217}LFIYF^{221} has been found to control the efficiency of recognition by MARCH ligases. Furthermore it was shown, that MARCH ligases ubiquitinate the same lysine residue (K225) of the MHCII β-chain. This highly conserved residue was mutated to an arginine in the MHCII(K>R) mouse model, thus revealed that the MHCII endocytosis/turnover rate in DCs relies mainly on ubiquitination by MARCH E3 ligases (1, 2).

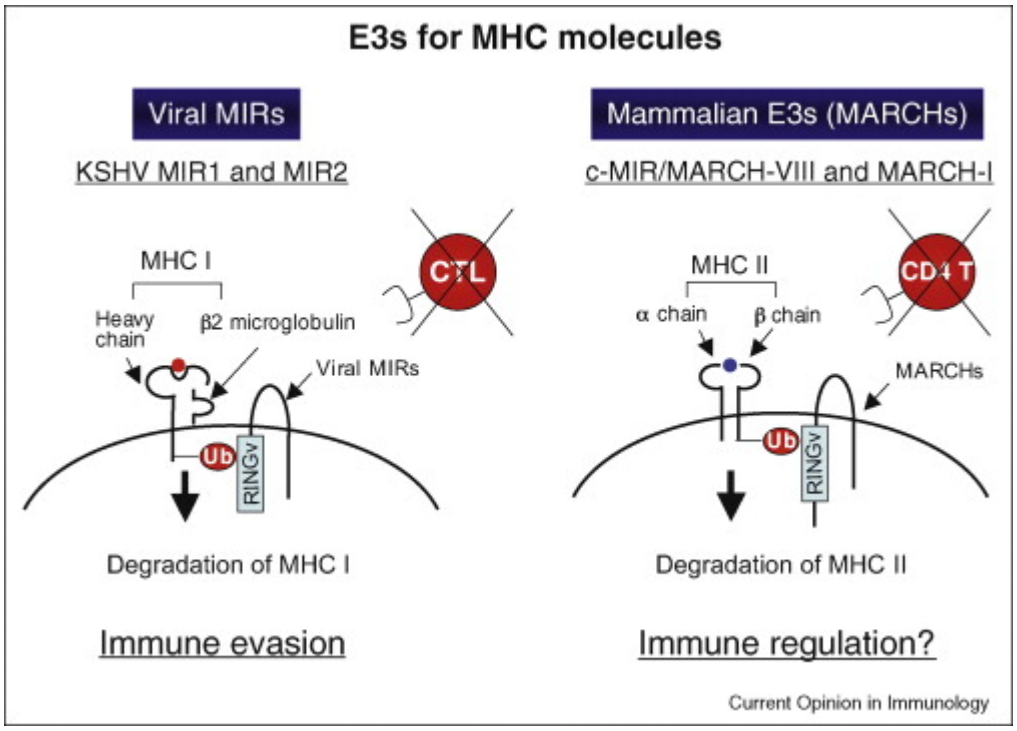

Figure 6: Ubiquitin E3 ligases targeting MHC molecules.

Viral MIRs and cellular MARCH ubiquitin ligases were found to target MHCI and MHCII, respectively, for degradation, leading to reduced antigen presentation. *Adopted from Ishido et al., Current Opinion in Immunology, 2009 (82).*

3.3.1 MARCH8 ubiquitin E3 ligase

MARCH8 overexpression in B cell lines caused a decrease in MHCII and CD86 levels (4). The effects of MARCH8 on the immune system were further demonstrated in a transgenic mouse which limited the MARCH8 overexpression to APCs (4). The expression of the transgene was controlled by the invariant chain promoter which is only active in APCs. In accordance with previous studies, these mice showed diminished MHCII levels and a diminished antigen presenting capacity. In addition, these mice also showed a reduced $CD4^+$ T cell compartment in the thymus and in the periphery. Therewith, these MARCH8-overexpressing mice resemble the phenotype of $CD83^{-/-}$ mice.

3.3.2 MARCH1 ubiquitin E3 ligase

MARCH1-deficient B cells displayed a prolonged half-life of surface MHCII and a concomitant disappearance of ubiquitinated forms of MHCII, resulting in remarkably increased surface MHCII levels. This increase is also apparent in cDCs and pDCs. MARCH1 expression was reported to be restricted to secondary lymphoid organs (76). Within those, B cells and DCs are the predominant cell types expressing MARCH1 (83). Regarding its physiological role, it was found that upon DC activation, MARCH1 is down-regulated which then initiates the up-regulation of MHCII surface levels. In particular, LPS and TLR signaling have been shown to mediate MARCH1 downregulation (23).

On the other hand, IL-10 was reported to induce MARCH1 and reciprocally repress CD83 expression in DCs, leading to reduced MHCII and CD86 levels and a more tolerogenic DC phenotype (84). Interestingly, $MARCH1^{-/-}$ mice have reduced thymus-derived Treg numbers, suggesting an important role of MARCH1-mediated MHCII ubiquitination for Treg selection in the thymus (83). Recently, CD83 was found to inhibit MARCH1-mediated MHCII degradation in DCs (67). This finding complements the phenotype of the $CD83^{-/-}$ mice, showing reduced MHCII levels in APCs.

Taken together, MARCH1 and MARCH8 demonstrate an important role for the immune system by regulating the endocytosis of MHCII from the surface of DCs. Since they share sequence homology and substrate specificity, it had been a possible scenario that MARCH1 und MARCH8 have completely redundant functions *in vivo*. However, only MARCH1 was found to respond to external stimuli such as LPS and IL-10. Furthermore, a differential expression of MARCH ligases in various organs was reported, suggesting distinct roles for MARCH1 and MARCH8 (76).

4 AIM OF THE STUDY

CD83 has been used as maturation marker for DCs for decades, as it is highly expressed on the surface of these cells upon their activation. Its functional importance for immune regulation was unraveled by CD83-deficient mouse models, which display a strong reduction in $CD4^+$ T cell numbers and further, reduced MHCII surface levels on APCs (63, 64, 66). Regarding the reduced MHCII levels in DCs, it has been shown that CD83 inhibits MARCH1-mediated MHCII and CD86 ubiquitination and degradation. However, the underlying molecular mechanism leading to the perturbed T cell development in these mice remained elusive. The aim of this study was to elucidate the role of CD83 for T cell development in the thymus.

We hypothesized two possible scenarios: Firstly, CD83 could deliver signals to developing thymocytes via cell-cell-interaction. The fact that sCD83, the 'shedded' extracellular domain of CD83, acts highly immunosuppressive, suggests that CD83 has a regulatory function for immune responses. Therefore, a potential CD83-CD83 ligand interaction during T cell development appears conceivable. Secondly, CD83 was found to inhibit MARCH1 via its transmembrane domain and, therewith, contributes to MHCII regulation. Thus, it seems plausible that the reduced MHCII levels of $CD83^{-/-}$ APCs are causative for the impaired $CD4^+$ T cell development.

To investigate the molecular mechanism, which leads to impaired $CD4^+$ T cell development in $CD83^{-/-}$ mice, we tested truncated versions of CD83 *in vivo* for their potential to rescue the $CD4^+$ T cell compartment. To this end, we established a lentiviral transduction system for TECs, which allowed us to test gene functions, i.e. truncated versions of CD83, *in vivo.* Furthermore, we addressed the expression pattern of CD83 and MARCH ligases, i.e. MARCH1, MARCH8 and MARCH9, in the main thymic APC subsets.

Since CD83 agonists and sCD83 are potential therapeutics for autoimmune diseases, the identification of CD83's mode of action is highly anticipated. In addition, clarifying the role of CD83 in T cell development could contribute to resolve longstanding questions such as the selection paradox.

5 RESULTS

5.1 Impaired development of $CD4^+$ thymocytes in $CD83^{-/-}$ mice

5.1.1 Phenotypic characterization of thymocytes developing in $CD83^{-/-}$ thymi

The phenotype of the CD83-deficient mouse has already been described in previous publications (63, 64, 66). Most striking is the reduced number of $CD4^+$ T cells in the thymus and the periphery. We could confirm this previous data: Flow cytometry of $CD83^{-/-}$ thymi showed a strong $CD4^+$ T cell reduction. In one-week old $CD83^{-/-}$ thymi, the CD4SP thymocytes were decreased by 63% compared to WT thymi and made up only 1.4% ± 0.5% of thymocytes instead of 3.6% ± 1.2% in WT. The $CD8^+$ T cell compartment was not altered (Fig 7A). A similar reduction of $CD4^+$ T cells was also detected in the periphery. The percentage of $CD4^+$ T cells of splenocytes in $CD83^{-/-}$ mice was reduced by 56% ± 17% compared to WT (Fig 7B).
Also in older mice, the CD4SP cells in the thymus were reduced by 66%: 2.7% ± 0.3% of the $CD83^{-/-}$ thymocytes were CD4SP, while 7.9% ± 0.9% of the WT thymocytes were CD4SP (Fig 7A, representative plots are shown).

It was reported that $CD83^{-/-}$ APCs (B cells, DCs, macrophages and TECs) express reduced levels of MHCII. As this reduction could be causative for the reduced $CD4^+$ T cell development, we examined for a potential correlation between MHCII expression levels and $CD4^+$ T cell development. To this end, we investigated the T cell development of $MHCII^{+/-}$ mice as it has been shown that $MHCII^{+/-}$ APCs display a 50% reduction in their pMHCII levels (64). However, in $MHCII^{+/-}$ mice no significant differences for CD4SP cell development were observed compared to WT mice (Fig 7B).

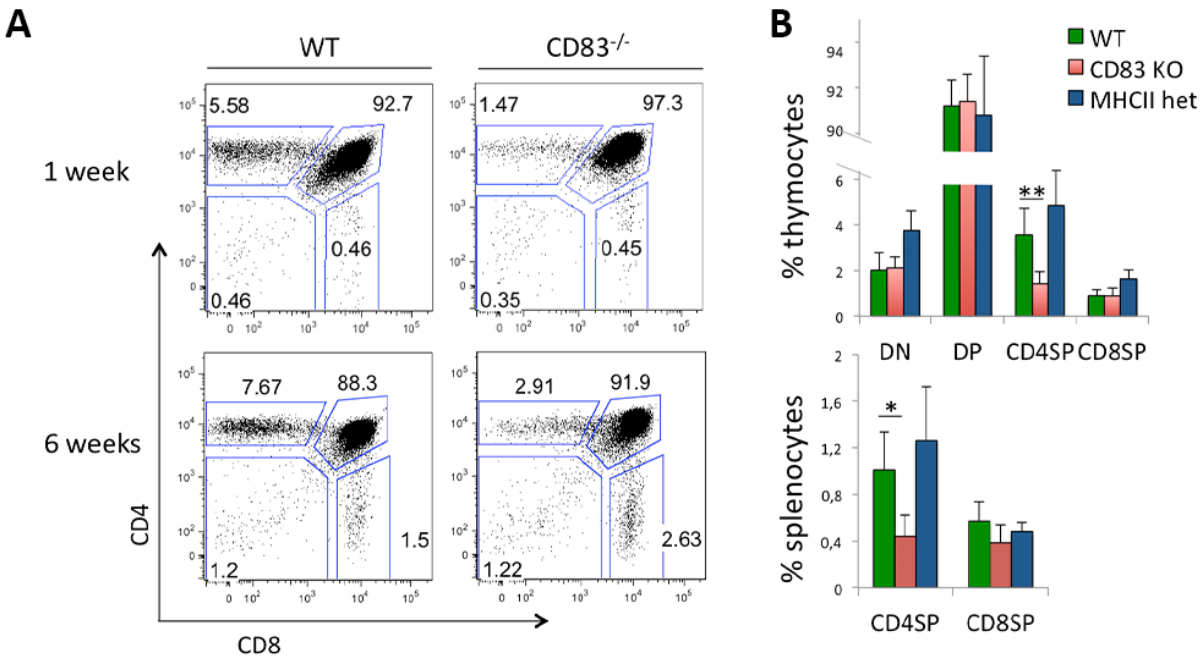

Figure 7: Characterization of T cells developing in CD83-deficient mice.

A) Thymi of 1- and 6-week old WT, CD83$^{-/-}$, and MHCII$^{+/-}$ mice (n=3) were analyzed by FACS. Representative CD4/CD8 plots of live gated lymphocytes for WT and CD83$^{-/-}$ are shown.
B) The bar diagrams show the quantified thymocyte subsets (DN= CD4^{-} CD8^{-}, DP= CD4^{+} CD8^{+}, CD4SP= CD4^{+} CD8^{-}, CD8SP= CD4^{-} CD8^{+}) in thymus and spleen of 1-week old mice, including the respective MHCII$^{+/-}$ data. A student's t-test has been performed: * indicates p≤0.05, ** indicates p≤0.01.

Next, we investigated if the Treg compartment, a subset of CD4SP thymocytes, is affected by the CD83-deficiency. The percentage of Treg cells within the CD4SP compartment of CD83$^{-/-}$ thymi (2.0% ± 0.1% of CD4SP cells) is comparable to WT thymi (2.1% ± 0.4% of CD4SP cells) (Fig 8). These results suggest that the high affinity TCR-bearing Treg cells do neither have an advantage nor disadvantage during the development in CD83$^{-/-}$ thymi.

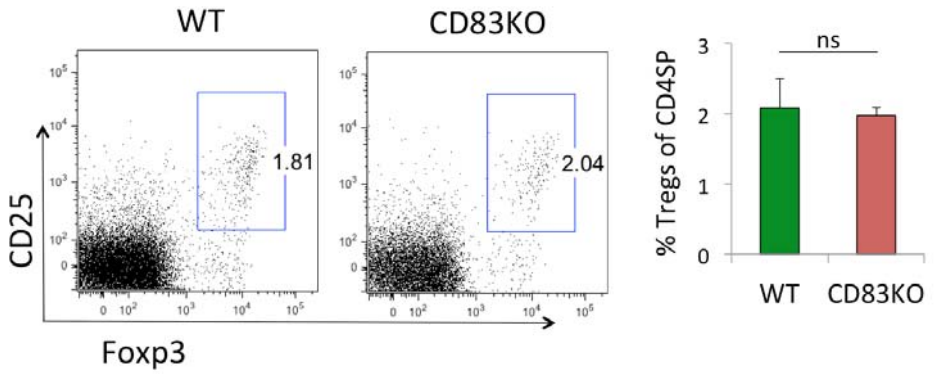

Figure 8: The percentage of thymic Treg cells within the CD4SP compartment is not affected by CD83-deficiency.

Representative plots depict the percentage of Tregs (Foxp3^{+} CD25^{+}) of CD4SP cells of WT and CD83$^{-/-}$ thymi. The bar diagram depicts the quantification of 6 mice each. A student's t-test yielded no statistical significance, p≥0.05.

In order to gain more insights what might be the cause for the diminished CD4^{+} T cell compartment in CD83-deficient mice, we further characterized the phenotype of their thymocytes. Firstly, we examined the maturation status. The CD4SP cells of CD83$^{-/-}$ thymi were found to display a more immature phenotype than their WT counterpart, which is evident from a higher proportion of CD24^{hi} CD69^{hi} cells (82% ± 2% vs. 69% ± 2% in WT) (Fig 9).

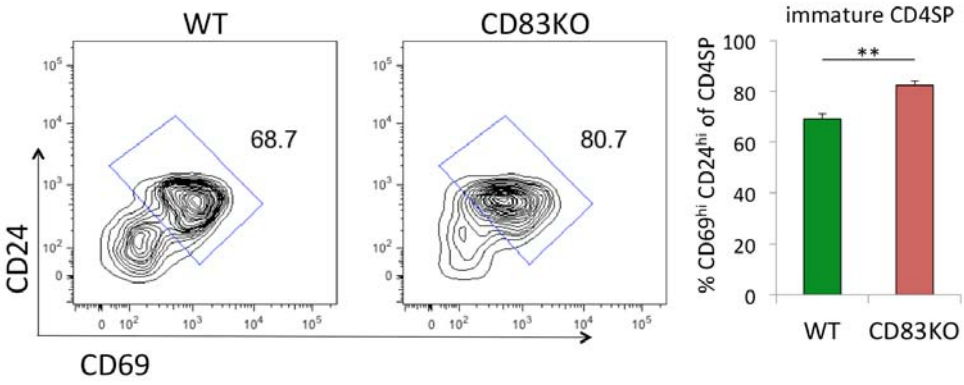

Figure 9: CD4SP cells developing in the CD83$^{-/-}$ thymus display a more immature phenotype.

Representative plots display the maturation status of CD4SP cells of WT and CD83$^{-/-}$ thymi, which were analyzed for their CD69/CD24 profile. CD69^{hi} CD24^{hi} represent immature, CD24^{lo} CD69^{lo} cells represent mature CD4SP cells. The cells shown were gated as live CD4SP cells. The results were quantified in the bar diagram on the right (WT n=4, CD83KO n=6). A student's t-test has been performed: ** indicates p≤0.005.

To investigate the signal quality thymocytes receive during positive selection in CD83-deficient thymi, we measured the CD5 levels of WT and CD83$^{-/-}$ thymocytes by FACS. It has been reported that a T cell's CD5 expression level directly correlates with the signal strength of the TCR-pMHC interaction during positive selection (85). We found reduced CD5 levels in the CD4SP thymocytes of CD83$^{-/-}$ mice. In CD8SP cells, however, no difference in CD5 levels was seen (Fig 10). This suggests that CD4SP thymocytes developing in CD83-deficient mice may receive weaker TCR-pMHCII interaction signals during positive selection, while the signal strength of the TCR-pMHCI interaction is the same regardless of CD83 expression.

In line with the findings on CD5 levels, TCRβ levels were likewise specifically reduced in CD4SP cells of CD83$^{-/-}$ thymi, which is indicative of a weaker TCR induction upon positive selection (Fig 10) (86).

In summary, the phenotype of the CD4SP cells suggests a less intense signaling interaction during positive selection in CD83$^{-/-}$ mice.

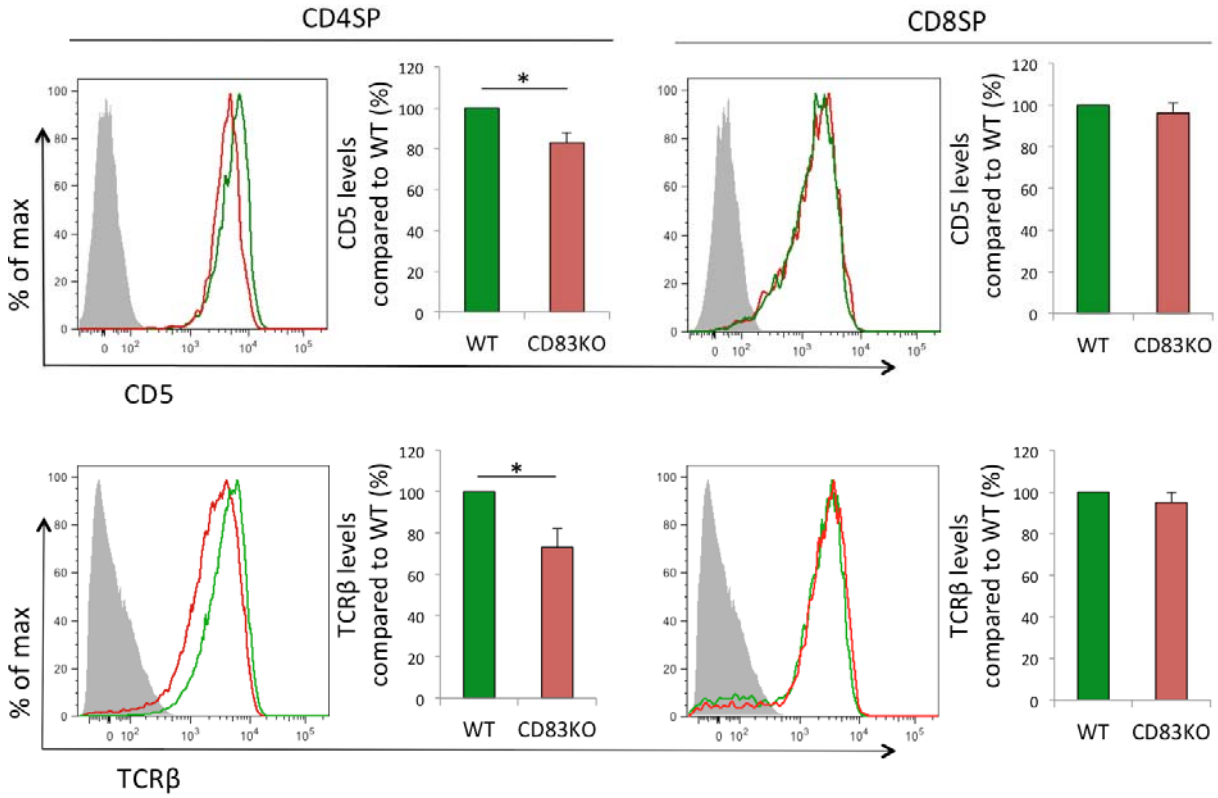

Figure 10: CD4SP cells display reduced CD5 and TCRβ levels in CD83$^{-/-}$ thymi.

The expression level of CD5 and TCRβ of CD4SP and CD8SP cells of WT and CD83$^{-/-}$ thymi were analyzed by FACS. The gMFI values were quantified (n=3). Unstained controls are shown in grey. A student's t-test has been performed: * indicates p≤0.05.

5.1.2 CD83 expression in TECs is crucial for $CD4^+$ T cell development

In order to investigate if the impaired $CD4^+$ T cell development is caused by a T cell-intrinsic defect or rather mediated extrinsically, we generated bone marrow chimeras. In bone marrow chimeras it is possible to distinguish between effects mediated by the hematopoietic compartment and the radio-resistant stroma. Reconstituting lethally irradiated WT recipients with WT or $CD83^{-/-}$ bone marrow into resulted in a normal CD4SP compartment (9.1% ± 1.0% and 8.3% ± 1.4%, respectively), while the transfer of WT or $CD83^{-/-}$ bone marrow into $CD83^{-/-}$ mice resulted in a similarly impaired $CD4^+$ T cell development as seen in $CD83^{-/-}$ mice (1.1% ± 0.1% and 1.7% ± 0.6%, respectively) (Fig 11). This indicates that the genotype of the recipient determines the phenotype, which means that the presence of CD83 in the radio-resistant TEC compartment is crucial for $CD4^+$ T cell development.

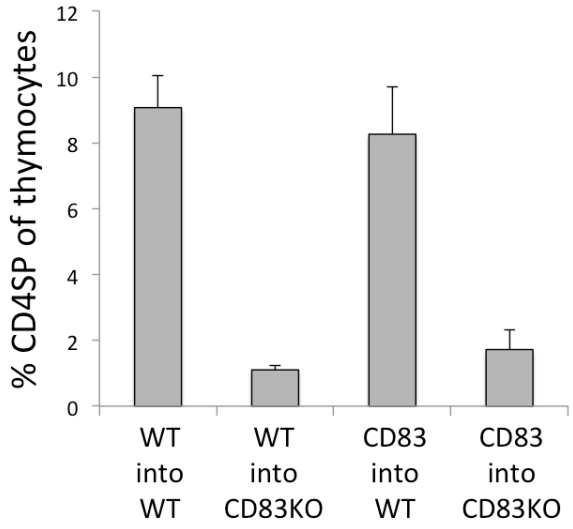

Figure 11: CD83-deficient TECs are responsible for impaired $CD4^+$ T cell development.

The bar diagram depicts the average CD4SP percentages of thymocytes in the four sets of bone marrow chimeras analyzed 6 weeks after bone marrow reconstitution by FACS. WT or $CD83^{-/-}$ recipients were reconstituted with either WT or $CD83^{-/-}$ bone marrow (each set n=4).

5.1.3 Reduced CD4$^+$ T cell numbers in CD83$^{-/-}$ mice are not mediated through excessive deletion by DCs

The reduced CD4$^+$ T cells could be due to either impaired positive selection or excessive negative selection of thymocytes. To investigate the contribution of DC-mediated negative selection to the reduced CD4$^+$ T cell compartment, we generated bone marrow chimeras in which the hematopoietic cells were incapable of participating in negative selection. To this end, we reconstituted irradiated WT or CD83$^{-/-}$ recipients with WT or MHCII$^{-/-}$ bone marrow (Fig 12). The difference between "WT into WT" chimeras to "MHCII$^{-/-}$ into WT" chimeras displayed the contribution of hematopoietic APCs to the negative selection of CD4SP thymocytes (38% of CD4SP). Interestingly, a similar proportion of deletion by hematopoietic APCs (42% of CD4SP) was found in CD83$^{-/-}$ recipients, although the overall CD4$^+$ thymocyte numbers were still strongly reduced. This shows that thymocytes, which have been positively selected in the CD83$^{-/-}$ cortex, proceed to the medulla where they undergo negative selection to the same ratio as WT thymocytes. In conclusion, the reduced CD4$^+$ T cell numbers in CD83$^{-/-}$ mice are not due to excessive deletion by DCs. However, mTECs were still able to mediate negative selection in this experimental setting.

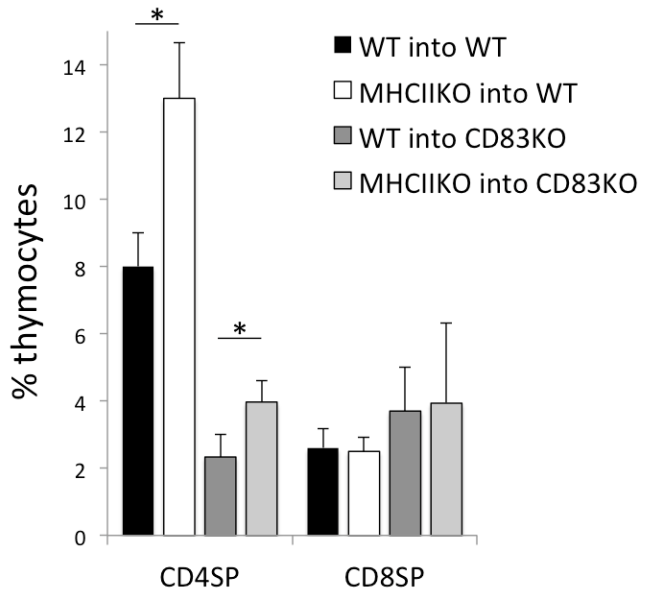

Figure 12: Thymic DCs contribute to negative selection but are not responsible for the CD83$^{-/-}$ phenotype.

The bar diagram depicts the average CD4SP and CD8SP cell percentages of thymocytes in bone marrow chimeras analyzed 6 weeks after generation by FACS. WT and CD83$^{-/-}$ recipients were reconstituted with either WT or MHCII$^{-/-}$ bone marrow (each set n=3). A student's t-test has been performed: * indicates $p \leq 0.05$.

5.1.4 Impaired positive selection of TCR-transgenic thymocytes in CD83$^{-/-}$ mice

To address if specifically the positive selection of thymocytes is affected in CD83$^{-/-}$ mice, we investigated the development of TCR-transgenic T cells, which are usually positively selected at a high frequency in the H-2^{b} background. Therefore, we generated chimeras with TCR-transgenic bone marrow. In these chimeras, all developing T cells express the same MHCII-restricted TCR of known specificity, OTII or DEP. Furthermore, due to their specificity towards foreign antigen (Ovalbumin and human CRP, respectively), these TCRs are not subject to clonal deletion (mechanism to delete auto-reactive thymocytes).

Remarkably, in the "DEP into CD83$^{-/-}$" chimeras, only 1.5% ± 0.8% of thymocytes were CD4SP cells, while 26% ± 3.2% of thymocytes were CD4SP in the "DEP into WT" chimeras (Fig 13A).

A similar result was observed in "OTII into CD83$^{-/-}$" mice. Here, 2.4% ± 0.3% of thymocytes developed into CD4SP cells, whereas, in "OTII in WT" mice 25% ± 10.8% were CD4SP cells (Fig 13B).

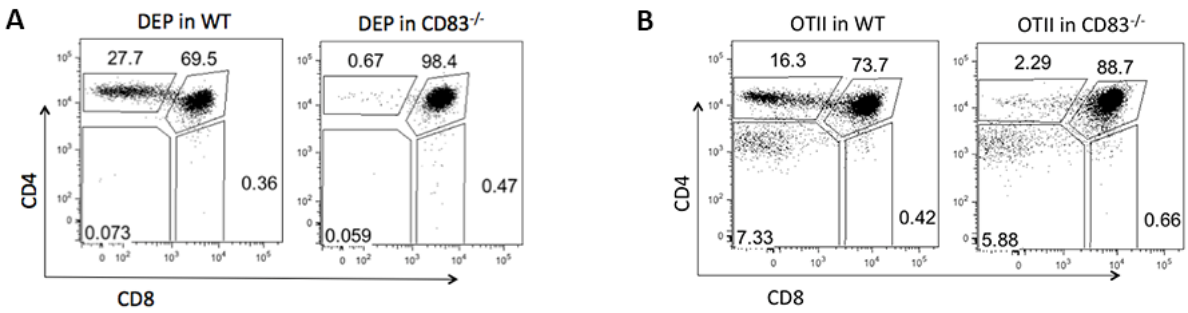

Figure 13: The selection of two MHCII-restricted transgenic TCRs is impaired in CD83$^{-/-}$ thymi.

A) The plots depict representative CD4/CD8 profiles of thymi of 2 different sets of bone marrow chimeras analyzed by FACS, 5 weeks after bone marrow reconstitution. Either WT or CD83$^{-/-}$ recipients were reconstituted with DEP TCR-transgenic bone marrow (each set n=3).
B) The plots depict representative CD4/CD8 profiles of thymi of 2 different sets of bone marrow chimeras analyzed by FACS, 5 weeks after reconstitution. WT or CD83$^{-/-}$ recipients were reconstituted with OTII TCR-transgenic bone marrow (each set n=3).

Taken together, the fact that even the development of thymocytes with transgenic TCRs with foreign specificity was affected, indicates that impaired positive selection is causative for the reduced CD4^{+} T cell development in CD83$^{-/-}$ mice.

Our results are in line with the results of the CD83$^{-/-}$ chimeras reconstituted with AND-TCR-transgenic bone marrow, which also showed a strong reduction in the CD4SP compartment (63).

Interestingly, the DEP-TCR^{+} CD4SP thymocytes developing in CD83$^{-/-}$ recipients also displayed reduced CD5 and TCRβ levels (Fig 14A+B). Also for OTII-TCR-transgenic CD4SP thymocytes we found reduced CD5 levels when selected in CD83$^{-/-}$ thymi (Fig 14C). By investigating a single TCR specificity, the reduction in the CD5 and TCRβ levels was more pronounced than in the polyclonal repertoire. These data support the notion that CD4SP thymocytes receive a weaker TCR stimulus by CD83$^{-/-}$ TECs.

To investigate if reduced MHCII levels might cause the impaired CD4^{+} T cell development, we also generated DEP- and OTII-TCR transgenic chimeras with MHCII$^{+/-}$ recipients. Both DEP^{+} and OTII^{+} CD4SP cells were developing in MHCII$^{+/-}$ mice equally well as in WTs (not shown) and furthermore displayed no reduction of their CD5 and TCRβ levels (Fig 14A+B+C).

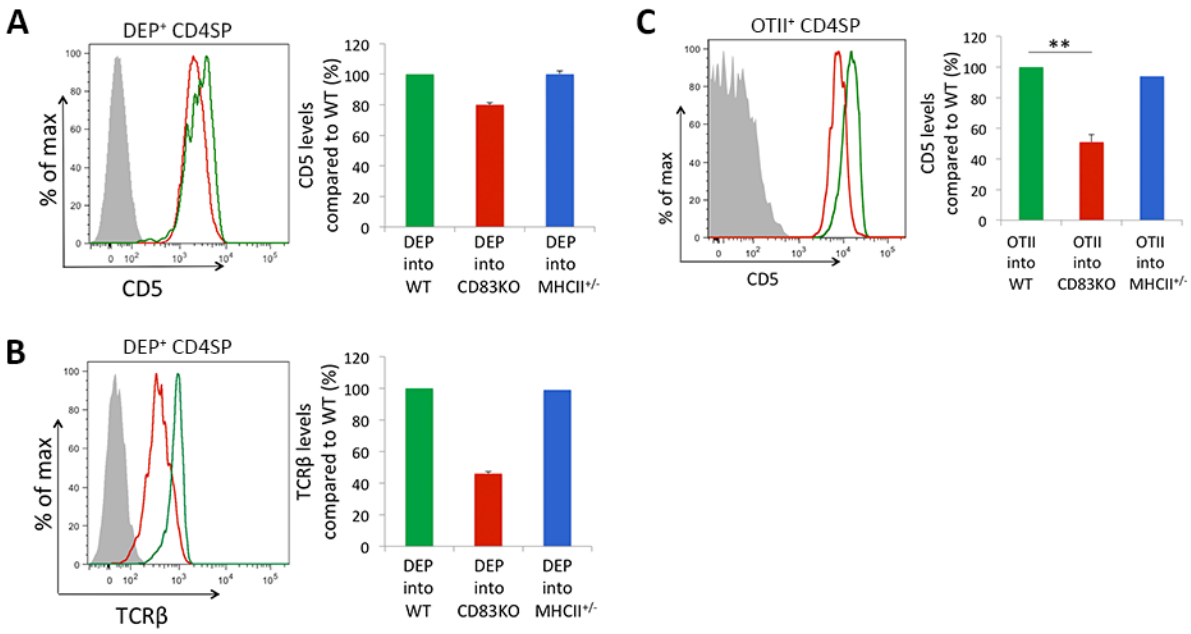

Figure 14: TCR-transgenic CD4SP cells display reduced CD5 and TCRβ levels in CD83$^{-/-}$ thymi.

A + B) The expression level of CD5 (A) and TCRβ (B) of DEP^{+} CD4SP cells of the DEP-transgenic bone marrow chimeras (n=2) were analyzed by FACS. The data of "DEP into MHCII$^{+/-}$ " chimera are also shown in the bar diagram. The gMFI values were quantified and depicted relative to WT levels.
C) The expression level of CD5 of OTII^{+} CD4SP cells of the OTII-transgenic bone marrow chimeras (n=3) were analyzed by FACS. The results of one "OTII into MHCII$^{+/-}$ " chimera is also shown in the bar diagram. The gMFI values were quantified and depicted relative to WT levels. A student's t-test has been performed: ** indicates p≤0.005.

In summary, we showed that CD4SP cells which are developing in CD83$^{-/-}$ thymi are reduced and display a phenotype that indicates a weak TCR signaling during positive selection. By generating

bone marrow chimeras, we could show that this defect is caused by CD83-deficient TECs. Furthermore, we could demonstrate that the reduced $CD4^+$ T cell numbers are caused by impaired positive selection and not by excessive negative selection.

These results strongly suggest that cTECs, i.e. the mediators of positive selection, are responsible for the observed $CD83^{-/-}$ phenotype.

5.2 CD83-deficient cTECs show a strong reduction in MHCII surface levels

Besides the reduced number of $CD4^+$ T cells in the thymus and the periphery, the $CD83^{-/-}$ mice were reported to display reduced levels of MHCII expression on the surface of various APCs (DCs, B cells, TECs) (63, 64, 66). As outlined above, the CD83-deficiency affects the cTEC-mediated positive selection of $CD4^+$ thymocytes. To elucidate a potential correlation between the MHCII levels of cTECs and the $CD4^+$ T cell development, we investigated the MHCII levels of the thymic APC subsets.

We analyzed cTECs, mTECs and thymic DCs of one-week old mice for their surface levels of MHCII, MHCI, and CD80 (a TEC maturation marker correlating with MHCII expression) (Fig 15). We analyzed such young mice, because at that age most cTECs can be isolated from the thymus.

Comparing $CD83^{-/-}$ with WT cells, the surface levels of MHCI were not significantly different in cTECs, mTECs and DCs, confirming previous data (64). We observed also comparable levels of CD80 in WT and CD83-deficient subsets. However, in cTECs the MHCII level was reduced to 46% ± 8% of WT levels. CD83-deficient mTECs displayed a milder MHCII reduction as their expression is reduced to 78% ± 9% of WT levels. Thymic $CD83^{-/-}$ DCs showed a reduction to 75% ± 7% of WT levels.

Kuwano et al. reported that the MHCII level of $MHCII^{+/-}$ APCs would be reduced by 50% compared to WT levels (according to mean density values) (64). Since we have shown that $CD4^+$ T cell development is not impaired in $MHCII^{+/-}$ mice, the MHCII levels in these mice seem to be sufficient for $CD4^+$ T cell development. In case there is a correlation between the MHCII level and the ability to positively select thymocytes, it was interesting to compare the MHCII levels between $CD83^{-/-}$ and $MHCII^{+/-}$ thymic APC subsets. Therefore, we included the MHCII levels of $MHCII^{+/-}$ APCs to our analysis. Surprisingly, we observed that the reduction in the MHCII level (measured by gMFI) in $MHCII^{+/-}$ APCs is weaker than previously reported: In $MHCII^{+/-}$ mice, the MHCII surface expression of cTECs was reduced to 68% ± 11% of WT levels, mTEC levels to 61% ± 10% of WT levels, and DC levels to 65% ± 3% of WT levels (Fig 15).

Of importance, our data show a stronger MHCII reduction in CD83-deficient cTECs (46% ± 8% of WT) than in $MHCII^{+/-}$ cTECs (68% ± 11% of WT). While the MHCII levels of $MHCII^{+/-}$ cTECs are still

sufficient for the positive selection of $CD4^+$ thymocytes, they may be reduced below a potentially required threshold level in CD83-deficient cTECs, thus accounting for the observed effects.

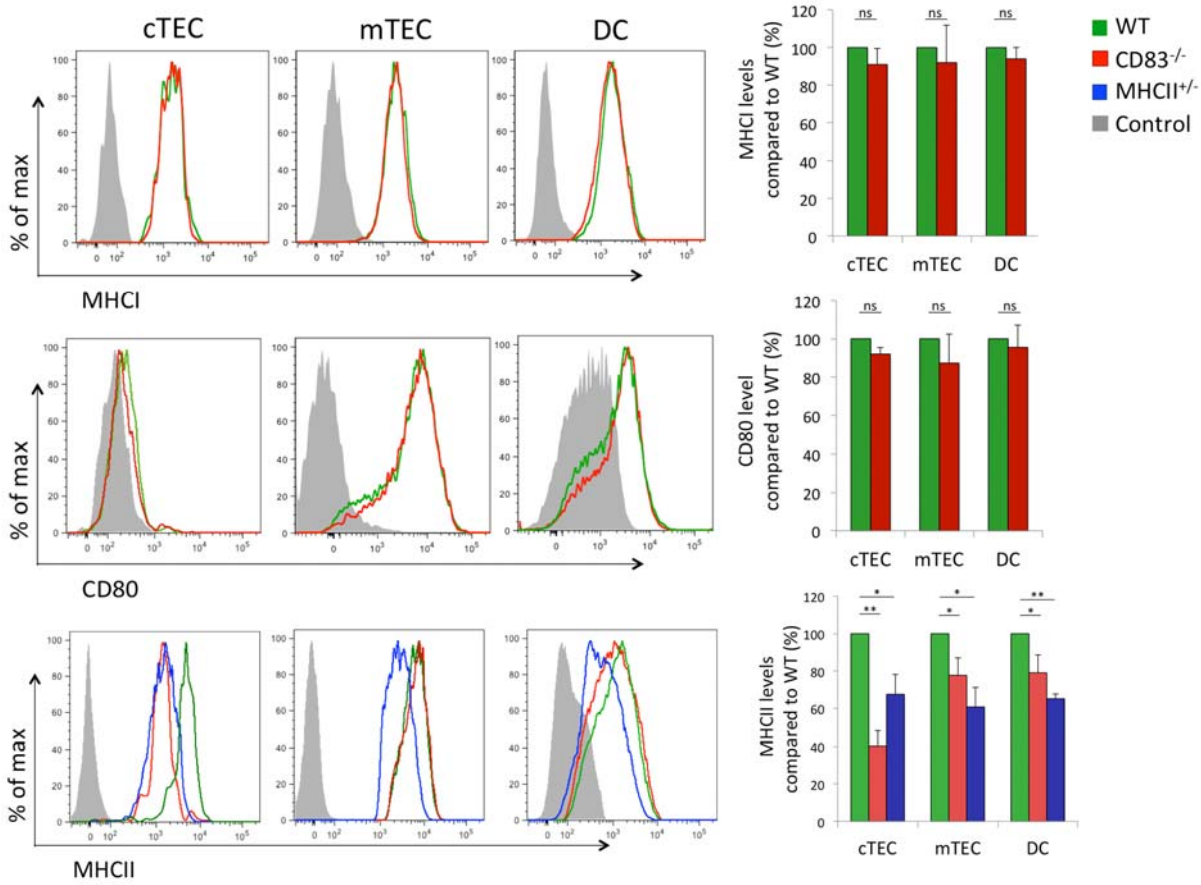

Figure 15: Reduced MHCII surface levels in thymic APC subsets of $CD83^{-/-}$ mice.

Representative histograms of MHCI and CD80 surface levels of cTECs, mTECs and cDCs of 1-week old WT and $CD83^{-/-}$ mice analyzed by FACS are shown (n=3). The geometrical mean fluorescent intensities (gMFI) have been quantified in the bar diagrams. As controls for the MHCI and CD80 staining we used isotype controls. For the MHCII staining, we used the respective $MHCII^{-/-}$ cells. The gMFI of WT was set to 100%, and the relative levels of expression in $CD83^{-/-}$ cells are depicted. For the surface levels of MHCII, age-matched $MHCII^{+/-}$ mice were included in the analysis. A student's t-test has been performed: ** indicates $p \leq 0.005$, * indicates $p \leq 0.05$, ns indicates $p > 0.05$.

Taken together, we showed reduced MHCII surface levels in CD83-deficient APCs, while MHCI and CD80 were not affected. Specifically, we found $CD83^{-/-}$ cTECs to display the greatest reduction of MHCII surface levels, which were even more reduced than in $MHCII^{+/-}$ cTECs. These results support the hypothesis that the reduced MHCII levels of cTECs are causative for the impaired positive selection of $CD4^+$ T cell.

5.3 CD83 is predominately expressed by cTECs in the thymus

The expression of CD83 by activated APCs and T cells, and also on thymic epithelium is known (54, 63, 87). Our findings that positive selection of thymocytes is affected in $CD83^{-/-}$ mice, and that cTECs express only about half as much MHCII as WT cTECs, suggest that cTECs depend on CD83 expression to maintain their MHCII levels and to mediate positive selection. To substantiate this dependency, we investigated the expression of CD83 in the major thymic APC subsets.

In flow cytometric analysis, we observed a pronounced CD83 surface staining of cTECs and DCs, while only a minor CD83 expression was detected on mTECs (Fig 16A).

In addition, we investigated the CD83 mRNA expression levels of various thymic APC subsets, and also of immature and LPS-matured bone marrow–derived DCs (BMDCs) as positive control (Fig 16B). BMDCs were known to upregulate CD83 upon activation. Therefore we included immature and mature BMDCs to measure this induction. Consistent with previous reports, we detected an induction of CD83 expression in mature BMDCs compared to immature BMDCs. Strikingly and in line with the flow cytometric results, cTECs showed the highest expression level of CD83 mRNA, which is about 3-fold higher than in mature BMDCs and $Sirpa^{+}$ cDCs. mTECs and other thymic DC subsets expressed low levels.

Taken together, we identified cTECs and $Sirpa^{+}$ cDCs as the APC subsets expressing CD83 most prominently in the thymus by mRNA quantification and surface staining.

In summary, the CD83 and MHCII expression data further substantiate that cTECs, i.e. the mediators of positive selection, are responsible for the observed phenotype in $CD83^{-/-}$ mice.

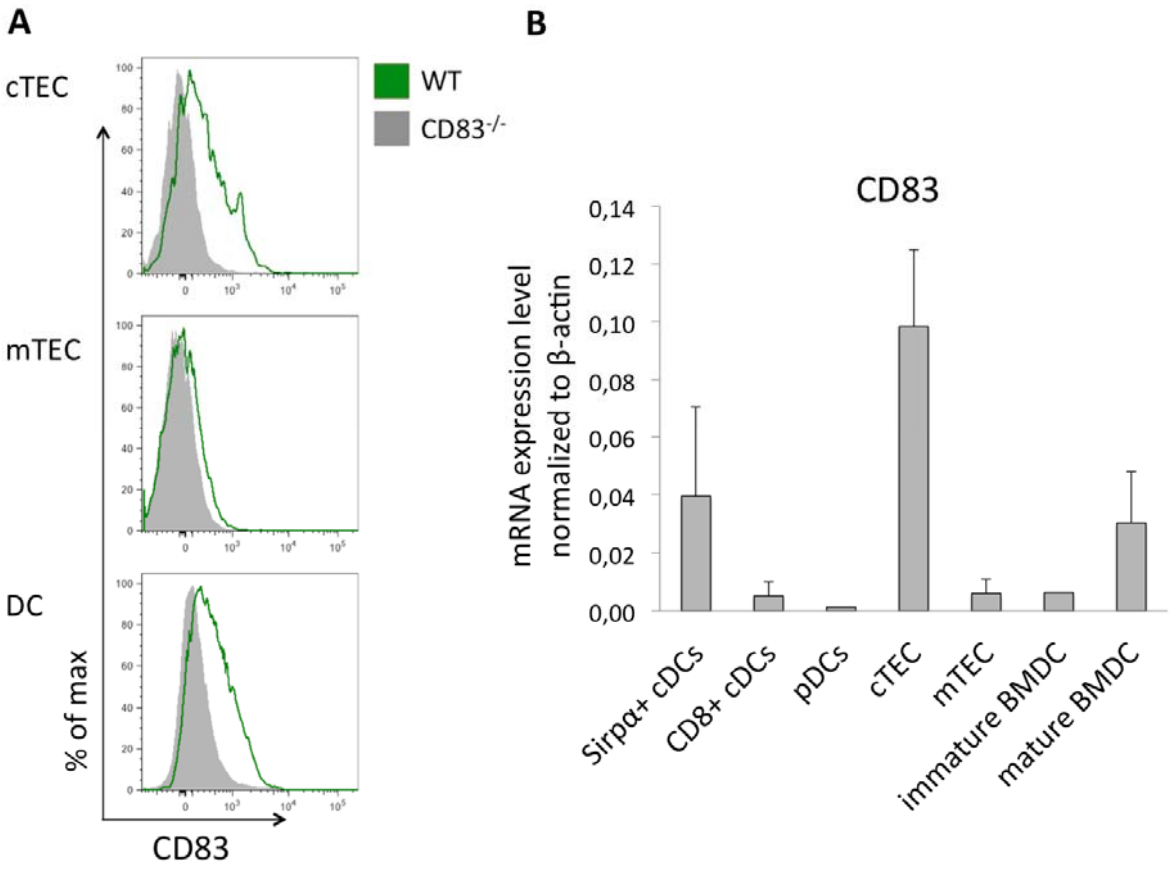

Figure 16: CD83 expression in thymic APC subsets.

A) Representative histograms show the surface expression of CD83 in cTECs, mTECs, and thymic CD11c^{hi} DCs, analyzed by FACS. Thymi of 1-week old mice were stained with lineage markers and αCD83-mAb (clone Michel-19). cTECs were gated as DAPI^{-}, CD45^{-}, EpCAM^{+}, Ly51^{+}; mTECs as DAPI^{-}, CD45^{-}, EpCAM^{+}, Ly51^{-}; and DCs as DAPI^{-}, CD45^{+}, CD11c^{hi}. The respective cells of CD83$^{-/-}$ mice were used as negative staining control.
B) The bar diagram displays the average relative mRNA expression levels of CD83 in cTECs, mTECs, and thymic DC subsets analyzed by real-time PCR. The thymic subsets were FACS sorted from 1-week old B6 WT mice. Subsets were defined as follows: Sirpa^{+} cDCs (DAPI^{-}, CD11c^{hi}, Sirpa^{+}, CD24^{-}), CD8^{+} cDCs (DAPI^{-}, CD11c^{hi}, Sirpa^{-}, CD24^{+}), pDCs (DAPI^{-}, CD11c^{lo}, B220^{+}), immature BMDC (with GMCSF cultured bone-marrow cells), mature BMDC (with LPS stimulus matured BMDC). β-actin was used for normalization. The results of 3 independent experiments for Sirpa^{+}, CD8^{+} DCs, cTECs, mTECs, and mature BMDC are depicted, while for pDCs and immature BMDC, the results of one experiment are shown.

5.4 Reconstitution of CD83 function by lentiviral transduction of CD83$^{-/-}$ RTOCs - Rescue experiments

After having demonstrated that the positive selection of CD4^{+} thymocytes is impaired when cTECs lack CD83, we were interested which function CD83 had in cTECs. The requirement of CD83 during positive selection could reflect a) that CD83 provides a crucial signal to thymocytes via CD83 ligand interaction or b) that CD83 functions to regulate the pMHCII level in cTECs. By elucidating which domain of CD83 (extracellular, intracellular, or transmembrane domain) is required for CD4^{+} T cell development, we could scrutinize between these two scenarios. In this respect, it has previously been shown in DCs that the transmembrane domain of CD83 affects the MHCII levels by inhibiting MARCH1-mediated MHCII degradation (67). To study if this MHCII stabilizing mechanism of CD83 is also involved in CD4^{+} T cell development, we introduced truncated versions of CD83 into TECs and examined their effects on the CD4^{+} thymocyte development *in vivo*.

To this end, we established a technique to reconstitute CD83 function in CD83$^{-/-}$ TECs. By lentiviral transduction of embryonic CD83$^{-/-}$ TECs, followed by the generation of a 'Reaggregate Thymic Organ Culture' (RTOC) and subsequent transplantation of these RTOCs under the kidney capsule (explained in more detail in the following), we were able to study the effects of transduced genes expressed in TECs *in vivo*.

The lentiviral infection approach was used to stably introduce the genes of interest, i.e. the truncated CD83 versions, into the genome of the non-dividing TECs. The infectivity of lentivirus is most efficient in single cell suspensions. For that reason, we applied the RTOC technique, as it has been shown that embryonic TECs are able to reaggregate and form thymic tissue after having been digested to a single cell suspension (19). By means of the RTOC technique and the preceding depletion of hematopoietic cells, we were able to target exclusively stromal cells with the lentiviral transduction approach. After the infection of TECs and their reaggregation into RTOCs, these were transplanted under the kidney capsule of recipient mice. There, they grew for 5-6 weeks and were seeded by the recipient's hematopoietic cells. Thus, we created chimeric organs where about 50% of the cells were positive for the transgene. The experimental setup is illustrated in Figure 17.

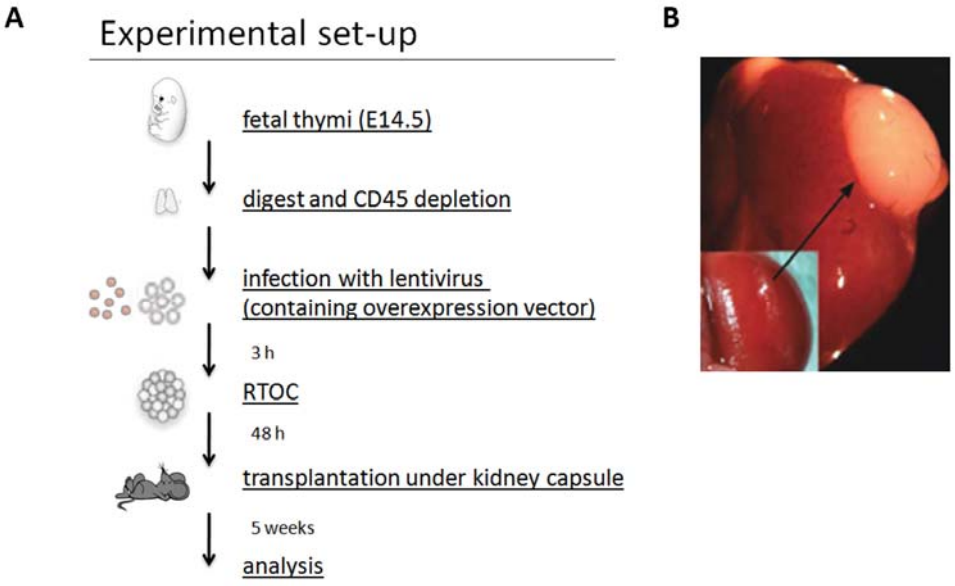

Figure 17: Lentiviral transduction of RTOCs.

A) Scheme of our experimental set-up for the lentiviral transduction of RTOCs. (See methods section for more details.)
B) Photograph showing the size of the RTOC under the kidney capsule at transplantation and 5 weeks later. *Adopted from Aichinger et al., Eur J Cell Biol, 2002.*

5.4.1 RTOCs foster T cell development comparable to endogenous thymi

First, to assure a normal cell development and functionality within the reaggregated organ, we investigated the T cell compartment in transplanted WT RTOCs. Regarding the proportion of CD4SP, CD8SP, DP, and DN cells, we found comparable results in endogenous thymi and RTOCs, indicating a normal regulation of thymocyte development in RTOCs (Fig 18).

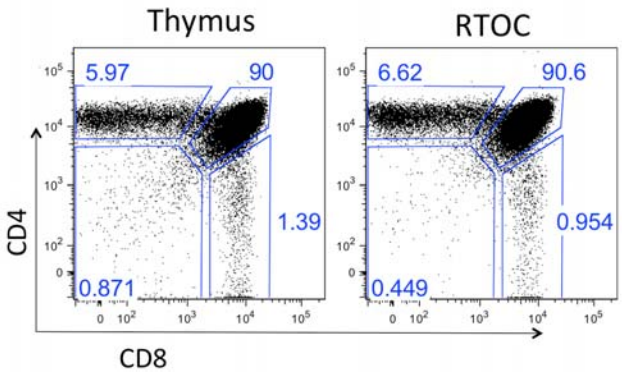

Figure 18: Reaggregate thymic organ culture (RTOC) fosters comparable T cell development as endogenous thymus.

Representative plots display the CD4/CD8 profile of thymocytes of a RTOC and an age-matched endogenous thymus. Both tissues were grinded and FACS analyzed. The cells shown were gated as live small lymphocytes.

To study the functionality of tolerance mechanisms (negative selection and Treg induction of auto-reactive $CD4^+$ thymocytes) in RTOCs, we transplanted Aire-HA RTOCs (expressing the neo antigen hemagglutinin (HA) under the control of the Aire promoter) into TCR-HA transgenic mice (high proportion of T cells expressing a transgenic TCR that recognizes HA). We observed deletion of a large proportion of HA-specific T cells from the repertoire (from 33% to 6% of CD4SP cells) when the cognate antigen is expressed by TECs, which demonstrates that negative selection occurs in RTOCs. Also the Treg induction from 0.2% in WT RTOCs to 31% in the Aire-HA RTOCs indicated functional tolerance mechanisms of RTOCs (Fig 19). The percentages measured in our RTOCs were similar to published data, where fetal thymic lobes of the Aire-HA mouse model were transplanted into TCR-HA recipients (42).

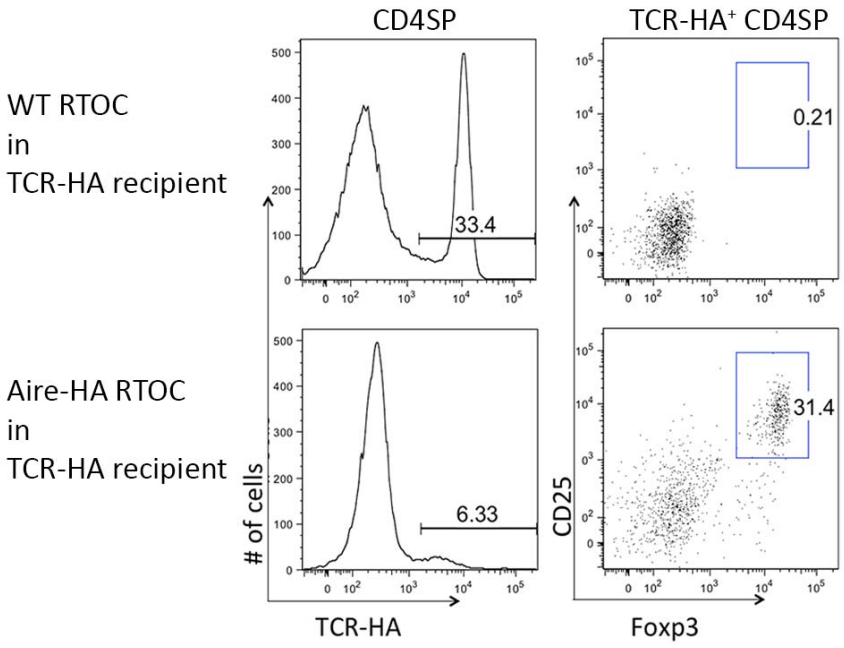

Figure 19: Functional tolerance mechanisms in RTOCs.

The histograms display the frequency of recipient-derived TCR-HA-specific CD4SP cells, which developed in the transplanted WT or Aire-HA RTOCs. RTOCs were grinded and analyzed by FACS, the cells shown were gated as live small lymphocytes. The dot plots depict the frequency of Tregs ($CD25^+$ $Foxp3^+$) of TCR-HA-specific CD4SP cells, which developed in either WT RTOCs or Aire-HA RTOCs.

In summary, we showed that RTOCs are equally capable to foster T cell development and to induce tolerance as thymi, indicating no differences in functionality between RTOC and thymus. Therefore, the RTOC technique is applicable to investigate the effect of CD83 expression on thymocyte development *in vivo*.

5.4.2 Lentiviral transduction of RTOCs allows stable transgene expression *in vivo*

To reconstitute the CD83 gene function, we introduced CD83-encoding vectors into $CD83^{-/-}$ TECs. To achieve this, we used lentiviral infections because lentiviral particles even target non-dividing cells. Furthermore, they integrate the lentiviral vector into the genome of these cells to allow for a stable transgene expression (88).

The used expression vector pFUGW encodes the ubiquitin promoter followed by the gene of interest (GOI), which is linked via T2a to the reporter eGFP. The GFP reporter allows to identify infected/transgenic cells. To ensure equimolar expression of the reporter and the gene of interest, we used a T2a-linker, which is proteolytically cleaved after translation (89). The vector construct is illustrated in Figure 20.

Figure 20: Schematic representation of lentiviral expression vector.

The Ubiquitin promoter drives the expression of the gene of interest (GOI), which is linked to the GFP reporter via a T2a linker. (The vector is described in more detail in the methods section.)

To verify a stable integration and long-term expression of our construct, we investigated the GFP expression in RTOCs over time. To do so, we infected cells with a GFP-containing vector, prepared two RTOCs from these cells, and transplanted them after 2 days in culture. One RTOC was analyzed after 3 days; the second was analyzed 5 weeks after transplantion. The GFP signal was detected 5 days and 5 weeks post infection in a similar percentage of TECs (49% and 46%, respectively), demonstrating a stable integration into the genome and long-term expression (Fig 21).

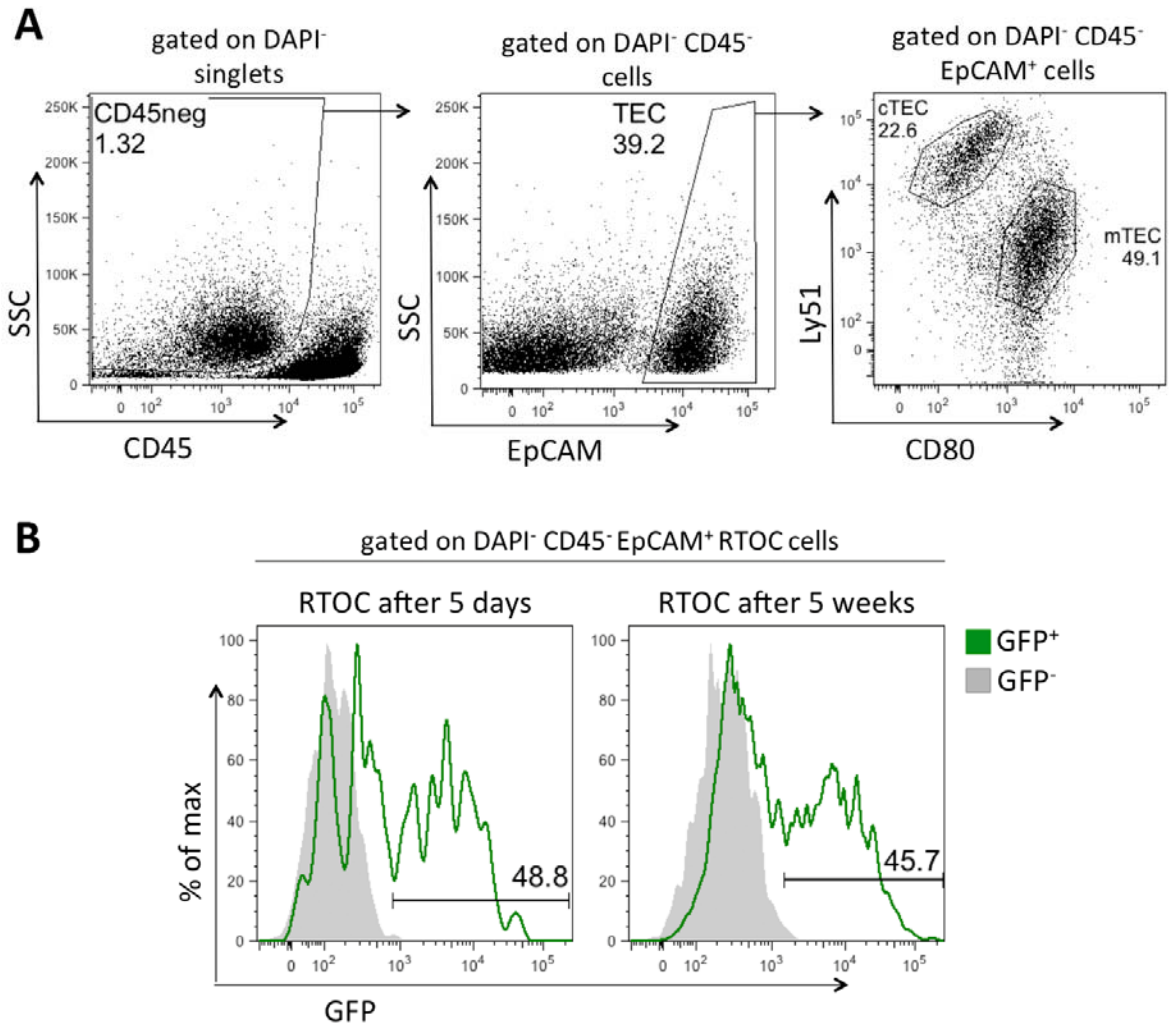

Figure 21: Lentiviral transduction enables stable GFP expression in RTOC TECs.

A) The gating strategies for cTECs and mTECs are depicted. TECs are gated as DAPI$^-$ singlets (FSC-A/FSC-H) CD45$^-$ EpCAM$^+$ cells. cTECs are gated as Ly51$^+$ CD80^{lo} TECs. mTECs are gated as Ly51$^-$ CD80^{hi} TECs.

B) The histograms depict the GFP expression (i.e. infection rate) of RTOC TECs 5 days and 5 weeks after infection. The RTOCs were digested and TECs were enriched via a density gradient. The cells shown were gated as live CD45$^-$ EpCAM$^+$ cells. TECs of uninfected RTOCs were used as negative staining control, shown in grey.

Since we could not isolate cTECs from 5-week old RTOCs, we investigated if cTECs and mTECs are equally infected by the lentiviral particles in 1-week old RTOCs. To study this, we infected RTOCs and analyzed the percentage of GFP^+ TECs 5 days after transplantation. Both cTECs and mTECs were targeted with comparable efficiency: 43% and 47%, respectively (Fig 22). The respective cells of uninfected RTOCs were used as GFP^- control.

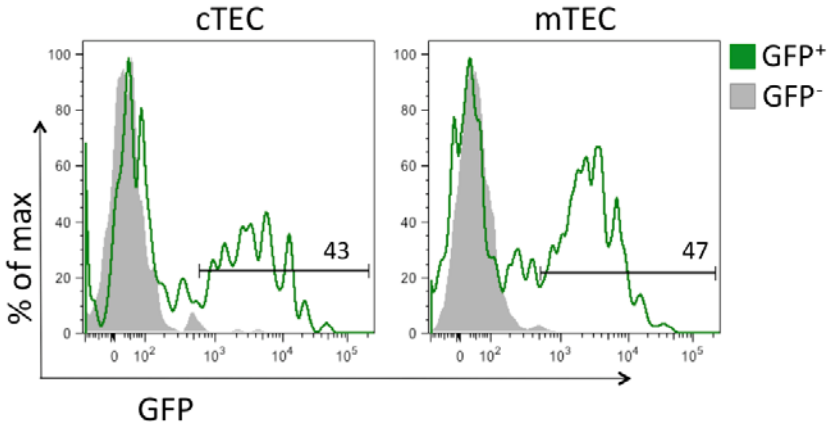

Figure 22: cTECs and mTECs are equally transduced by lentiviral infection.

The histograms display the GFP expression (infection rate) of cTECs and mTECs of a RTOC 7 days after infection/5 days after transplantation. The RTOC was digested and TECs were enriched via density gradient. cTECs were gated as live $CD45^-$ $EpCAM^+$ $Ly51^+$ $CD80^-$ cells, mTECs were gated as $CD45^-$ $EpCAM^+$ $Ly51^-$ $CD80^+$ cells. The respective cells of uninfected RTOCs were used as GFP-negative control, shown in grey.

Next, we assessed if the infection or the GFP expression negatively affects the cells. To this end, we examined the MHCII and CD80 expression profiles (markers indicating the maturation of TECs) of infected vs. non-infected RTOC TECs. We could observe that the lentiviral infection had no influence on the MHCII or CD80 expression pattern of RTOC TECs *per se*, suggesting a similar maturation in infected vs. uninfected TECs (Fig 23).

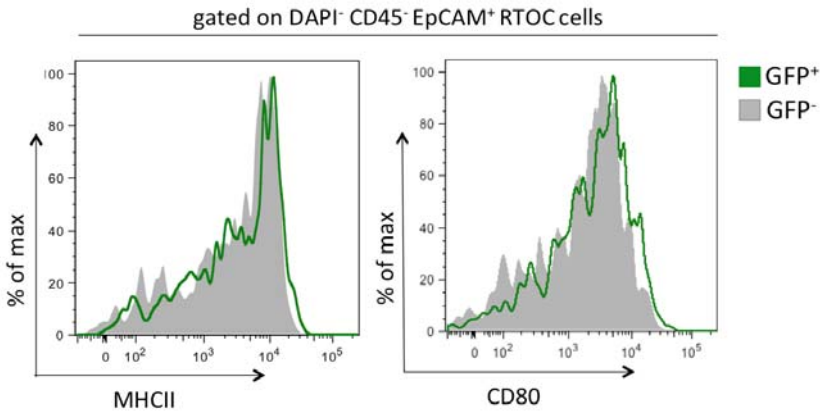

Figure 23: Infected and non-infected TECs display similar maturation pattern.

The representative plot depicts the MHCII and CD80 expression profile of both GFP^+ (green line) and GFP^- TECs (grey filled) of the same infected RTOC. Prior to analysis, RTOCs were digested and TECs were enriched via a density gradient. The cells shown were gated as live $CD45^-$ $EpCAM^+$ cells.

The quantification of infectious units (IU) of the different virus stocks is crucial for yielding similar infection rates in the RTOCs. To prove that our quantifications were correct, we compared two dilutions each of three different virus stocks (A, B, and C) for their infectivity of RTOCs. After infection of the TECs with the different virus stocks, the generated RTOCs were kept in culture. After 5 days, the RTOCs were analyzed, and we observed comparable percentages of GFP^+ cells for the same dilutions of the different virus stocks (Fig 24). These data validate our quantification of the IU/ml.

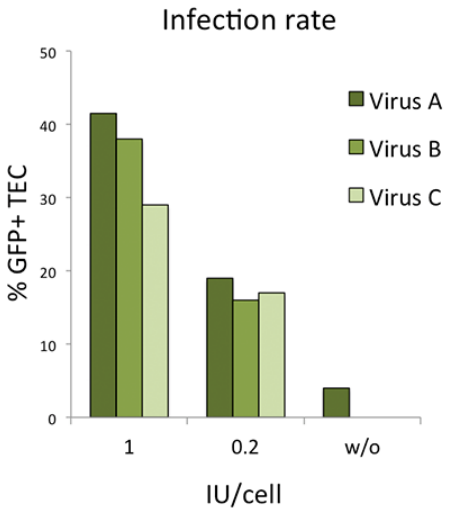

Figure 24: Quantification of infectious units allows similar infection rates.

Bar diagram shows the percentage of GFP^+ TEC of RTOCs infected with titrated amounts of infectious units (IU) of three independently prepared and quantified virus stocks. The RTOCs were analyzed after 5 days in culture. The percentages were calculated from live $CD45^-$ $EpCAM^+$ TECs.

5.4.3 A fraction of 10% CD83-sufficient cells is sufficient for a pronounced increase in CD4SP cells in CD83$^{-/-}$ RTOCs

For the reconstitution of CD83 gene function, we determined the minimal infection rate (i.e. the minimum percentage of CD83-sufficient TECs), which allows to detect a rescue of gene function (i.e. an increase of the CD4SP cell percentage) in CD83$^{-/-}$ RTOCs. To do so, we set up mixed RTOCs of CD83-deficient and WT cells at indicated ratios and transplanted those into CD83$^{-/-}$ recipients. After 5 weeks, the percentage of CD4SP cells of thymocytes was analyzed. We found that CD83-deficient RTOCs containing 10% WT TECs showed an increase in CD4SP cells from 2% to 4% compared to CD83$^{-/-}$ RTOCs. This increase was becoming more pronounced, the higher the percentage of WT cells was (Fig 25). Based on the observation that 10% of CD83-sufficient cells are capable to significantly increase the CD4SP percentage, we concluded, that a minimal infection rate of 10% should be sufficient to detect a rescue of CD4SP cells in CD83$^{-/-}$ RTOCs.

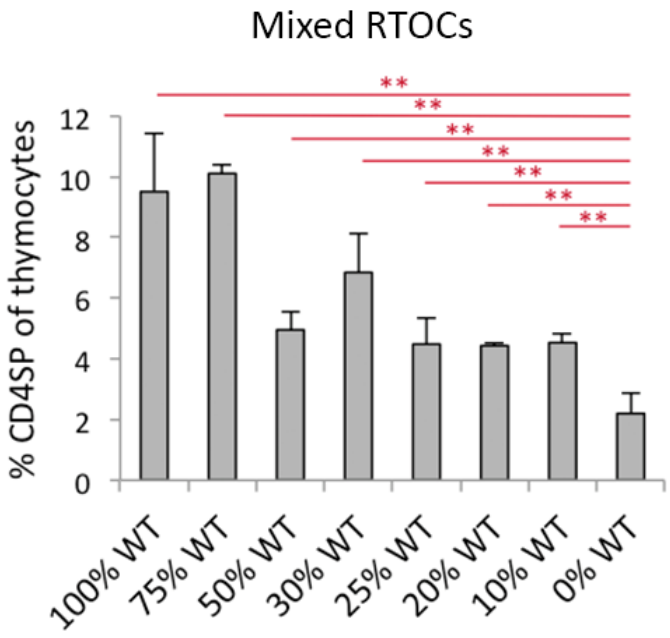

Figure 25: Supplementing CD83$^{-/-}$ RTOCs with WT cells rescues the CD4^{+} T cell compartment.

The bar diagram displays the average percentage of CD4SP cells of thymocytes of mixed RTOCs. CD83$^{-/-}$ RTOCs were generated, mixed with indicated ratios of WT cells, and analyzed 5 weeks later by FACS (each data point n=4).the The results of 2 experiments were combined. A student's t-test has been performed: ** indicates p≤0.005.

5.4.4 Dose-dependent infection rate in RTOCs

After having determined that the minimum infection rate was 10% in our experimental setting, we assessed the required ratio of infectious units (IU) to cells to achieve higher infection rates

than 10%. By titrating the amount of IU to TECs, we observed a dose-dependent infection rate (Fig 26; also see above Fig. 24 comparing different IU). Comparing the infection rates of 4 different virus stocks, we found that a ratio of 0.2 IU/cell yielded an infection rate of about 23% ± 10%. A ratio of 1 IU/cell yielded an infection rate of 42% ± 14% (Fig 24, 26). Thus, an infection with a ratio of 0.2 - 1 IU/cell should be sufficient for our rescue experiments.

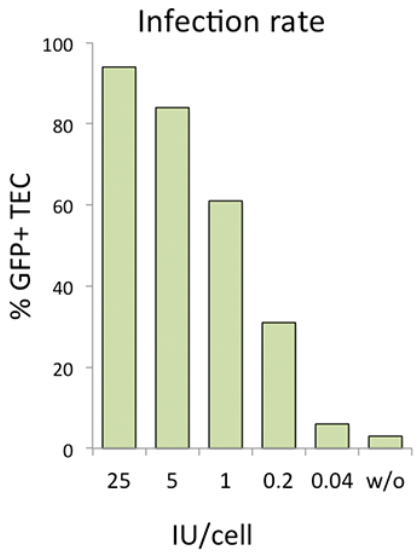

Figure 26: RTOC infection rate is dependent on the ratio of IU/cell.

Bar diagram shows the percentage of GFP^+ TECs of RTOCs that have been infected with titrated amounts of infectious units (IU) per cell. 5 days after infection, the RTOCs were analyzed by FACS and the cells were gated for live $CD45^-$ $EpCAM^+$ TECs.

5.4.5 Rescue of CD4$^+$ T cell development by lentiviral transduction of CD83 in CD83$^{-/-}$ RTOCs

To validate that the CD4$^+$ T cell reduction in CD83$^{-/-}$ mice was reproducible in CD83$^{-/-}$ RTOCs, we analyzed the thymocytes generated in WT and CD83$^{-/-}$ RTOCs. We observed the same phenotype as seen in the thymi of CD83$^{-/-}$ mice: 2.2% ± 0.7% CD4SP cells developed in CD83$^{-/-}$ RTOCs compared to 9.5% ± 2.3% in WT RTOCs (Fig 27).

As a proof of principle, that the reconstitution of CD83 gene function can be achieved by our approach, we reconstituted CD83$^{-/-}$ RTOCs with full-length CD83 and analyzed the CD4SP cell development 5 weeks later. Indeed, the lentiviral transduction of the full-length CD83 expression vector in CD83$^{-/-}$ RTOCs, with an infection rate of 56% ± 17%, rescued the impaired CD4$^+$ T cell development in 14 out of 14 RTOCs analyzed. The percentage of CD4SP cells of full-length CD83-transduced CD83$^{-/-}$ RTOCs was not significantly different from WT RTOCs (Fig 27).

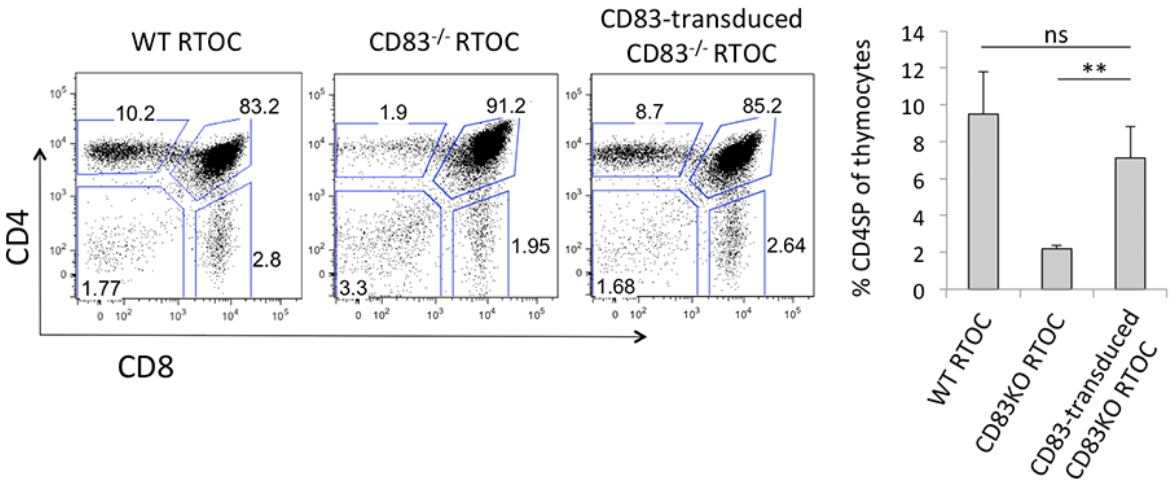

Figure 27: Lentiviral transduction of CD83 rescues CD4$^+$ T cell development in CD83$^{-/-}$ RTOCs.

Representative plots display the CD4/CD8 profile of thymocytes of WT RTOCs (n=11) and CD83$^{-/-}$ RTOCs (n=7) and CD83$^{-/-}$ RTOCs which have been lentivirally transduced with a full-length CD83-expression vector (n=14). The average percentages of CD4SP cells have been quantified in the bar diagram on the right. A student's t-test has been performed: ** indicates p≤0.01, ns indicates p>0.05.

5.4.6 CD83's transmembrane domain crucial for $CD4^+$ T cell development

After having shown that the transduced full-length CD83 is able to rescue the impaired $CD4^+$ T cell development in $CD83^{-/-}$ RTOCs, we addressed the question which domain of the CD83 molecule is capable of rescuing the impaired $CD4^+$ T cell development. This could give further insight on CD83's mode of action.

By testing various truncated versions of CD83 (kindly provided by Christopher Goodnow), we found that only those constructs, which contained the transmembrane (TM) domain of CD83, rescued the impaired $CD4^+$ T cell development in $CD83^{-/-}$ RTOCs (Fig 28). RTOCs expressing the mutants, which contain the CD83-TM domain, developed comparable CD4SP percentages (Mutant1: 9.4% ± 2.0%, Mutant4: 6.2% ± 1.9%) to WT RTOCs (9.5% ± 2.3%), while mutants expressing the extracellular and/or intracellular domain (Mutant2: 2.6% ± 1.2%, Mutant3: 1.8% ± 0.8%) were not significantly different from control-infected $CD83^{-/-}$ RTOCs (2.4% ± 0.8%). These findings are in line with the data of Tze et al. who reported that the reduced MHCII level in $CD83^{-/-}$ DCs can only be rescued by TM domain-containing mutants (67).

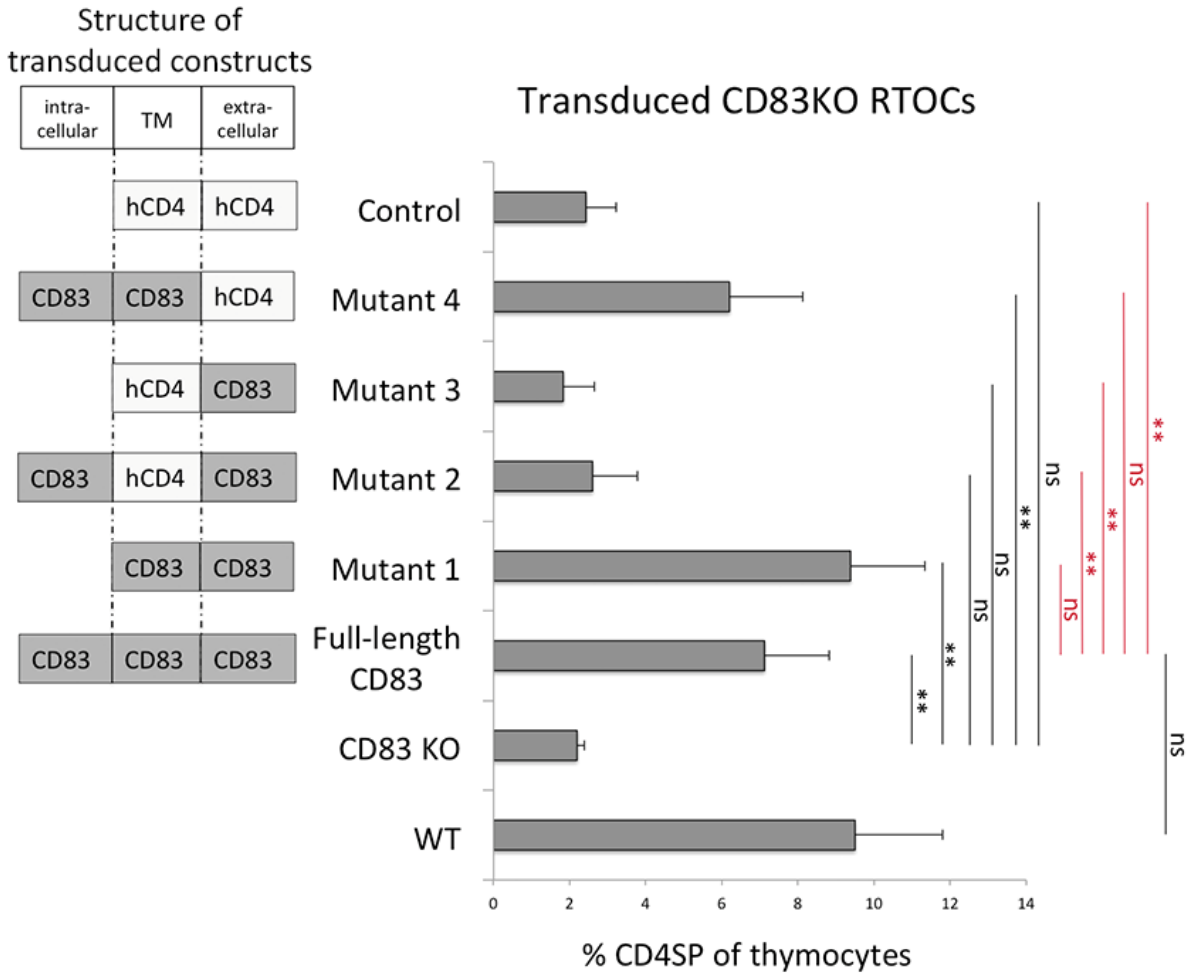

Figure 28: The transmembrane domain of CD83 is crucial for CD4SP cell development.

The structures of lentivirally-transduced constructs are schematically illustrated on the left. On the right, the bar diagram depicts the average percentage of CD4SP cells of thymocytes of WT and CD83$^{-/-}$ RTOCs and of CD83$^{-/-}$ RTOCs transduced with the indicated mutants. RTOCs were transduced with: Full-length CD83 n=14, 56% ± 17% infection rate (IR); Mutant1 n=3, 13% IR of pooled cells; Mutant2 n=4, 35% ± 22% IR, Mutant3 n=6, 43% ± 8% IR; Mutant4 n=7, 15% ± 2% IR of pooled cells; Control n=11, 47% ± 12% IR. A student's t-test has been performed: ** indicates p≤0.005, ns indicates p>0.05.

In summary, we developed a system that can be used to probe gene function in TECs *in vivo*. Furthermore, we found that the transmembrane domain of CD83 is sufficient to rescue the CD4^{+} T cell development in CD83$^{-/-}$ RTOCs. This suggests that the extracellular and the intracellular domain of CD83 are dispensable for the positive selection of thymocytes. Furthermore, we conclude that the mechanism by which CD83 is involved in positive selection of thymocytes is not mediated via cell-cell contact, inside-out, or outside-in signaling but rather by transmembrane interactions.

5.5 Differential expression of MARCH family members in thmyic APC subsets

It has recently been suggested that CD83 blocks the association of MARCH1, an ubiquitin E3 ligase, with MHCII and, therewith, prevents MARCH1-mediated ubiquitination and degradation of MHCII (67). These studies were performed *in vitro* by transfection of DCs. To address the question if such regulatory network involving MARCH1, CD83 and MHCII is also present in cTECs, the expression level of MARCH1 mRNA in various thymic cell types was investigated. Interestingly, MARCH1 mRNA was only detected in DC subsets, while TECs showed no MARCH1 mRNA expression (Fig 29A).

We continued to investigate the expression of other MARCH family members to determine if the role of MARCH1 in DCs is possibly fulfilled by one of its close homologues in cTECs. In particular, the relative mRNA expression level of its closest relatives MARCH8 and MARCH9 (which have also been reported to ubiquitinate human MHCII complexes (HLA-DP, -DR, and -DQ (81)) were analyzed. Indeed, cTECs expressed high levels of MARCH8 mRNA. Also mTECs and Sirpa$^+$ cDCs were found to express MARCH8 mRNA (albeit at lower levels than cTECs), whereas in CD8$^+$ cDCs it was completely absent (Fig 29B).

Only low levels of MARCH9 mRNA were detected in all subsets analyzed (Fig 29C).

Taken together, we identified a differential expression of MARCH ubiquitin ligases, especially of MARCH1 and MARCH8, in thymic APC subsets. cTECs were found to express high levels of MARCH8 mRNA, while MARCH1 and MARCH9 mRNA was expressed at low levels only.

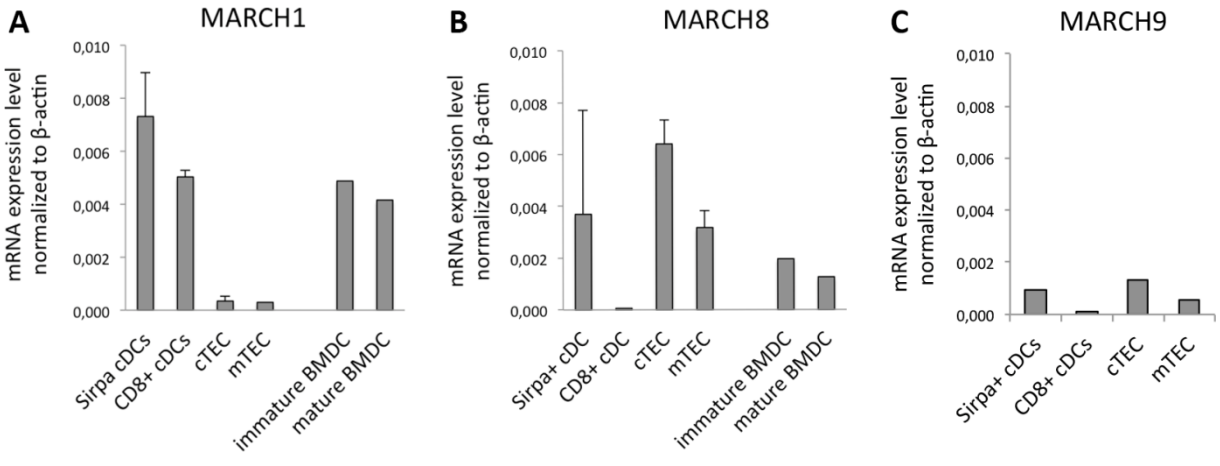

Figure 29: Differential expression of MARCH E3 ligases in thymic APC subsets.

The bar diagrams display the average relative mRNA expression levels of MARCH1 **(A),** MARCH8 **(B)** and MARCH9 **(C)** in cTECs, mTECs, thymic cDC subsets as analyzed by real-time PCR. The thymic subsets were FACS sorted from 1-week old B6 WT mice. cDC subsets were defined as following: Sirpa$^+$ cDCs (DAPI$^-$, CD11c^{hi}, Sirpα$^+$, CD24$^-$), CD8$^+$ cDCs (DAPI$^-$, CD11c^{hi}, Sirpα$^-$, CD24$^+$). Immature BMDC (with GMCSF cultured bone-marrow cells), mature BMDC (with

LPS cultured immature BMDC) were included to the analysis of one experiment as positive control. β-actin was used for normalization. The MARCH1 diagram depicts the results of 2 independent experiments. The MARCH8 diagram shows the results of 3 independent experiments. The MARCH9 diagram shows the results of 1 experiment. Each experiment was performed with triplicates of each sample, and negative controls (samples synthesized without RT, not shown).

5.6 The ubiquitination-resistant MHCII(K>R) mutant rescues the CD83-deficiency phenotype

We have demonstrated that the role of CD83 in $CD4^+$ T cell development is dependent on its transmembrane domain. These results in combination with those of Tze et al., who have shown that CD83's transmembrane domain inhibits MARCH1-mediated ubiquitination of MHCII in DCs, suggest that the transmembrane domain of CD83 may similarly be responsible in cTECs to regulate MHCII levels by a ubiquitin-dependent process. In order to investigte this regulatory mechanism for CD83 in cTECs, we used the MHCII(K>R) mouse model which is resistant to MHCII ubiquitination (see introduction for more detail). We crossed the MHCII(K>R) knock-in (KI) alleles to the $CD83^{-/-}$ background. In CD83-deficient MHCII(K>R)KI mice, we observed a full rescue of the $CD83^{-/-}$ phenotype: The percentage of the CD4SP cells (4.7% ± 0.7%) was comparable to CD83-sufficient MHCII(K>R)KI littermates (5.7% ± 1.0% CD4SP) and WT littermates (4.3% ± 0.9% CD4SP) (Fig 30). These results indicate that CD83 regulates MHCII levels via ubiquitination.

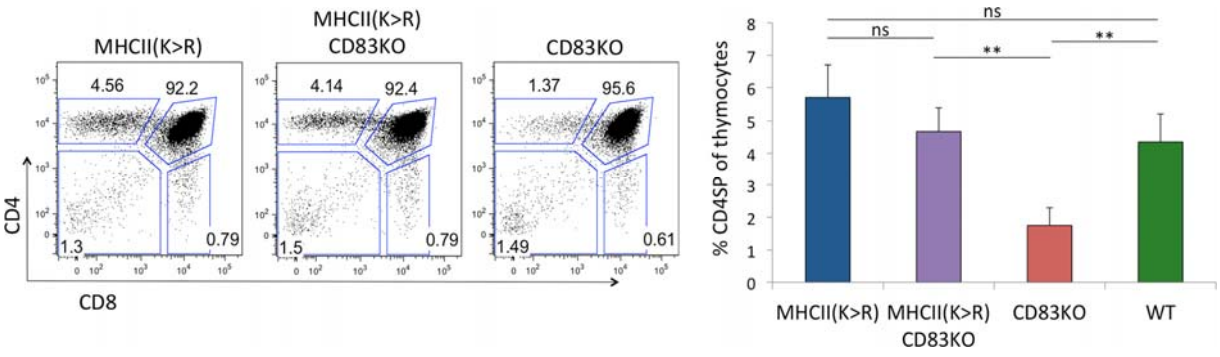

Figure 30: Ubiquitination-resistant MHCII(K>R) mutation rescues CD83-deficiency phenotype – $CD4^+$ T cell development.

The FACS plots depict representative CD4/CD8 profiles of thymi of 2-week old MHCII(K>R)KI, MHCII(K>R)KI CD83KO, and CD83KO mice. On the right, the bar diagram depicts the average CD4SP cell percentage of thymocytes per genotype (n=5). A student's t-test has been performed: ** indicates p≤0.005, ns indicates p>0.05.

Besides the thymocyte development, we also analyzed the MHCII levels of cTECs in these mice to investigate if the ubiquitination-resistant MHCII mutant fully restores MHCII levels to WT levels in the CD83-deficient mice. Indeed, we found similar MHCII levels in cTECs of CD83-sufficient and CD83-deficient MHCII(K>R)KI littermates: CD83-deficient (MHCII(K>R)KI cTECs expressed 95% ± 12% of the MHCII gMFI of CD83-sufficient MHCII(K>R)KI littermates (Fig 31). These results indicate that CD83 acts prior/upstream of MHCII ubiquitination since the CD83-deficency phenotype is rescued by the resistance to ubiquitination.

These results demonstrate that the lack of CD83 in CD83$^{-/-}$ mice (CD4SP compartment and MHCII level reduction) can be compensated when MHCII is resistant to ubiquitination, suggesting that CD83 regulates MHCII levels via inhibiting ubiquitination in cTECs.

Furthermore, we found comparable MHCII levels in MHCII(K>R)KI and WT cTECs. Since increased MHCII levels in DCs and B cells were reported for the MHCII(K>R)KI mouse model, our data suggest a different MHCII regulation in cTECs compared to DCs and B cells (90).

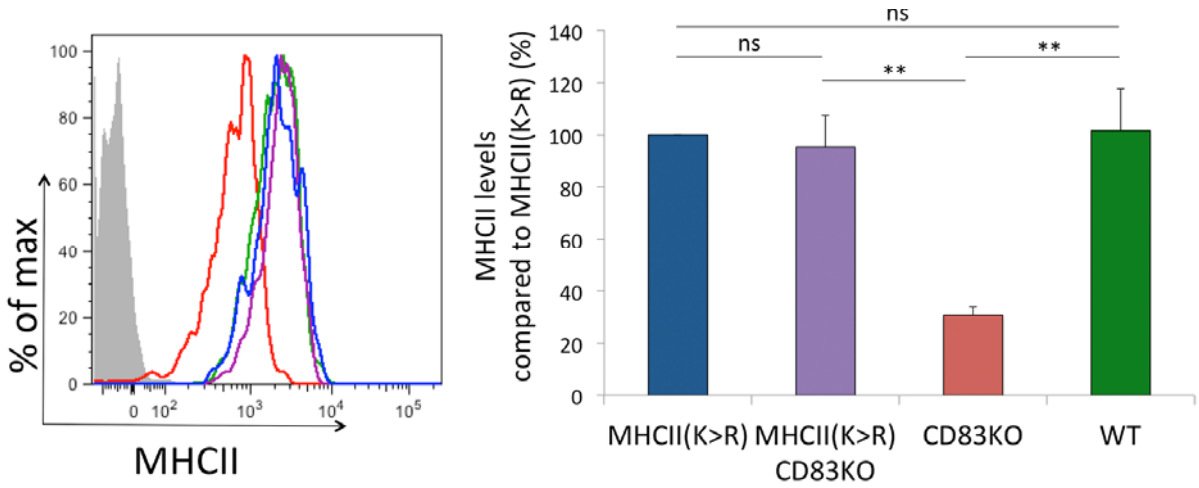

Figure 31: Ubiquitination-resistant MHCII(K>R) mutation rescues CD83-deficiency phenotype – MHCII levels.

Representative histograms of MHCII surface levels of cTECs of 2-week old MHCII(K>R)KI, MHCII(K>R)KI CD83KO, CD83KO, and WT mice, analyzed by FACS, are shown. The gMFI values were quantified in the bar diagram on the right. The gMFI of MHCII(K>R)KI was set to 100%, and the relative levels of MHCII(K>R)KI CD83KO, CD83KO, and WT cTECs are depicted (n=5).

5.7 Investigations on the retention time of MHCII molecules at the surface of CD83$^{-/-}$ TECs

We demonstrated that CD83 functions to stabilize pMHCII at the surface of cTECs. Therefore, we hypothesized two possible scenarios, which might lead to the reduced CD4^{+} T cell development in CD83$^{-/-}$ mice: a) the pMHCII level in CD83$^{-/-}$ cTECs is below a required threshold of avidity to mediate positive selection of thymocytes or b) CD83-deficient cTECs cycle their pMHCII molecules at increased rates, causing a shorter retention time at the surface, which is insufficient for the positive selection of thymocytes.

We addressed the later theory by investigating the pMHCII turnover in CD83$^{-/-}$ vs. WT cTECs *in vitro*. To this end, we isolated TECs from 1-week old mice, pulsed them with E$\alpha_{52\text{-}68}$ peptide, and analyzed the E$\alpha_{52\text{-}68}$ presentation after 0, 2, 4, and 8 hours of incubation at 37°C. For the analysis, we took advantage of the YAe antibody which recognizes specifically the E$\alpha_{52\text{-}68}$ peptide bound on MHCII, which allowed us to measure the E$\alpha_{52\text{-}68}$ presentation by FACS. Preliminary results, however, showed no differences in the retention time of E$\alpha_{52\text{-}68}$-MHCII for CD83$^{-/-}$ mTECs and cTECs, as compared to the respective WT cells (Fig 32). Of note, due to massive cell death within 8 hours of incubation, further studies will be necessary to validate these preliminary results. Further investigations could elucidate the role of CD83 in the MHCII turnover and MHCII regulation in general.

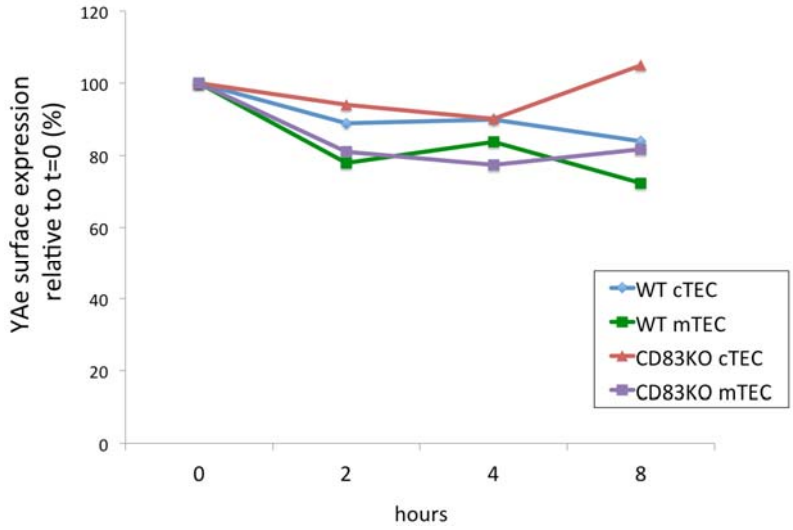

Figure 32: The retention time of MHCII at the surface seems not altered in CD83$^{-/-}$ TECs *in vitro*.

1-week old thymi were isolated, digested, and TECs were enriched via density gradient and CD45^{+} cell depletion. The remaining cells were loaded with E$\alpha_{52\text{-}68}$ peptide (100µg/ml) for 2 hours at 37°C, washed 3 times, and distributed into 4 wells (pre-coated with 0.5% gelatine) for further incubation at 37°C. After the indicated time periods the cells were transferred to 4°C. After 8 hours, the cells were stained for DAPI, EpCAM, CD80 and Ly51 to differentiate between cTECs and mTECs. YAe (E$\alpha_{52\text{-}68}$-MHCII complex) and Y3P (panMHCII) were stained to calculate the ratio of E$\alpha_{52\text{-}68}$-MHCII/panMHCII. The ratio of E$\alpha_{52\text{-}68}$-MHCII/panMHCII at t=0 was set to 100%, representing the maximal presentation of E$\alpha_{52\text{-}68}$-MHCII (after pulse and wash). The later time

points are depicted relative to the ratio at t=0. The results of one out of three independent experiments are shown.

In summary, this study elucidated a MHCII-stabilizing role of CD83 in T cell development. We demonstrated the transmembrane domain of CD83 to be crucial for cTECs to mediate positive selection of thymocytes. In particular, CD83's transmembrane domain inhibits the ubiquitination of the MHCII β-chain at position K^{225}. According to our mRNA expression profiles, the ubiquitination of MHCII is most likely mediated by MARCH8 in cTECs.

6 DISCUSSION

6.1 cTECs are the predominant cell type expressing CD83 in the thymus

In this study, we demonstrated that in the thymus CD83 is predominantly expressed by cTECs (Fig 16). So far, most thymic expression studies of CD83 focused on thymic DCs in the medulla (54, 66), only Fujimoto et al. hinted into the direction of CD83 expression in other parts of the thymus, as they detected CD83 expression in the thymic cortex by *in situ* hybridizations (63). It had also been known that TECs express CD83, while our study demonstrates that CD83 expression is confined to the cTEC population. In mTECs, we detected only marginal CD83 surface staining and CD83 mRNA levels.

6.2 CD83 has a crucial role in the stablilization of pMHCII, while its signalling *in trans* has no major role in T cell development

Regarding the mode of action of CD83, we demonstrated that the transmembrane domain of CD83 is sufficient to mediate positive selection of $CD4^+$ thymocytes (Fig 28). In other words, both the intra- and extracellular domains of CD83 are dispensable for its role during T cell development. Therefore, we can exclude, that cTEC-thymocyte interactions via CD83-CD83 ligand binding are required for positive selection.

In line with the finding that the transmembrane domain of CD83 inhibits MARCH1-mediated MHCII degradation in DCs, we could show that CD83 has a MHCII-stabilizing functions also in cTECs (Fig 15). The observation, that $CD83^{-/-}$ APCs display reduced MHCII expression levels indicates that CD83 is generally involved in the regulation of MHCII surface levels. Surprisingly, although mTECs express only marginal CD83 levels, they also display reduced MHCII levels in CD83-deficient mice. A possible explanation for this could be that in a $CD83^{-/-}$ thymus, mTECs receive less crosstalk signals by the diminished CD4SP compartment, which causes their improper maturation to $MHCII^{hi}$ cells (91). An alternative explanation is that CD83 is expressed in mTECs at low levels, but is still of functional significance.

The hypothesis, that the reduced MHCII level could be causative for the impaired CD4SP development in $CD83^{-/-}$ mice, was addressed by Kuwano et al. (64). They argued that $MHCII^{+/-}$ TECs, which are able to foster normal $CD4^+$ T cell development, showed a stronger reduction in their MHCII surface levels than $CD83^{-/-}$ TECs and thus concluded that the reduced MHCII levels of

the $CD83^{-/-}$ TECs cannot be responsible for the impaired CD4SP cells in $CD83^{-/-}$ mice. Here, we examined cTECs and mTECs separately and found that $CD83^{-/-}$ cTECs actually display a stronger MHCII reduction than $MHCII^{+/-}$ cTECs (Fig 15). Since cTECs generally only make up 10% of all TECs, this behaviour was likely overseen by Kuwano et al. Hence, the reduced MHCII level in CD83-deficient cTECs being causative for the impaired $CD4^+$ T cell development remains a feasible hypothesis.

To further elucidate the correlation of CD83 expression with MHCII regulation in cTECs, we examined the expression of MARCH1 in these cells. As outlined, in DCs, CD83 regulates MHCII levels via the inhibition of the ubiquitin ligase MARCH1. Surprisingly, we found that cTECs do not express MARCH1. Further investigations revealed that cTECs express MARCH8, a close homolog of MARCH1 with similar target specificity (Fig 29).

We thus hypothesized that CD83 may regulate MHCII levels via MARCH8-mediated ubiquitination of MHCII (similarly to MARCH1-mediated ubiquitylation of MHCII in DCs). To test if MHCII becomes regulated by MARCH-mediated ubiquitination in cTECs, we took advantage of the MHCII(K>R) mouse model, whose pMHCII molecules are resistant to MARCH-mediated ubiquitination (90). Due to an amino acid exchange from lysine to arginine at residue 225 of the β-chain, the MHCII complex cannot be targeted by any MARCH E3 ligase, since all of them ubiquitinate this one highly conserved lysine at position 225 (K^{225}) (4). Combining this ubiquitination-resistant MHCII allele with the $CD83^{-/-}$ background, allowed normal $CD4^+$ T cell development: In these mice, the T cells developed normally irrespective of the presence or absence of CD83, which indicates that CD83 acts upstream of/prior to the ubiquitination of K^{225} (Fig 30).

Furthermore, the MHCII levels of cTECs in MHCII(K>R) mice were likewise not affected by the presence or absence of CD83 (Fig 31). This data indicates that the MHCII reduction in $CD83^{-/-}$ cTECs is also mediated via an enhanced ubiquitination process. In line with the *in vitro* data of Tze et al., that CD83 inhibits MARCH1-mediated ubiquitination of MHCII in DCs, our results suggest that ubiquitination of MHCII in cTECs is likely mediated by the MARCH8 ubiquitin ligase, which becomes inhibited by CD83. This hypothesis is further supported by the finding that mice with overexpressed MARCH8 display a phenotype similar to $CD83^{-/-}$ mice (reduced $CD4^+$ T cells, reduced MHCII levels) (4).

However, the interaction between CD83 and MARCH8 in cTECs has not been verified yet. It formally remains possible that CD83 affects MHCII via other regulatory pathways. Possible scenarios include that CD83 also counteracts other MARCH ligases or other unknown regulators of MHCII expression which would target the same lysine^{225}. In this respect, it was shown that MARCH9 ubiquitinates

HLA-DQ in transfected human HEK cells (81). However, murine thymic subsets express negligible levels of MARCH9 mRNA compared to MARCH1/8 (Fig 29). Our hypothesis, that CD83 regulates MHCII via inhibiting MARCH8-mediated ubiquitination in cTECs, could be strengthened by demonstrating that the phenotype of $CD83^{-/-}$ mice would be rescued by its combination with MARCH8-deficiency. Since MARCH8- deficient mice were not available, this remains to be shown in future studies.

Furthermore, it remains to be shown, how CD83 and MARCH8 are regulated in cTECs. What is the physiological role of the interaction of these two molecules in cTECs? Why do cTECs express them at all? Would the MHCII level not be stable in the absence of both? Are there further targets to be regulated by MARCH8, which are making its presence crucial in cTECs, and therefore also the presence of a MHCII stabilizer, i.e. CD83? There seems to be more to it than only the stabilization or degradation of MHCII. For now, we can conclude, that the extracellular domain of CD83 and therefore any cell-cell interaction by cTECs is dispensable for T cell development, whereas the transmembrane domain of CD83 is required for proper $CD4^{+}$ T cell development.

In summary of the above, we have provided a plausible explanation for the reduced MHCII surface levels in $CD83^{-/-}$ cTECs, i.e. that, due to the absence of CD83, MARCH8 is no longer inhibited by this molecule and thus targets more MHCII for degradation.

It is tempting to speculate that the reduced number of CD4SP cells in these mice is due to a reduced overall level of surface MHCII molecules. However, it is also possible that the remaining MHCII level would principally be sufficient to support normal CD4SP cell development and that the observed CD4SP cell reduction in $CD83^{-/-}$ mice is rather due to a reduced residence time of the MHCII molecules on the surface of cTECs, which is insufficient for positive selection. In other words, $CD83^{-/-}$ cTECs may cycle their pMHCII complexes at such a high rate, that their surface retention time is too short to positively select thymocytes. Stochastically, some molecules may reside at the surface for periods long enough for a few CD4SP cells to develop, as seen in the CD83-deficient thymus. This "turnover model" is supported by the finding that $CD83^{-/-}$ B cells display a faster internalization rate of MHCII than WT cells (64).

To investigate a potentially increased turnover, we examined the retention time of the $E\alpha_{52\text{-}68}$ peptide-MHCII complex at the surface of cTECs *in vitro*. First experiments showed no difference in the turnover rate of these specific complexes (as recognized by the YAe antibody), between $CD83^{-/-}$ and WT cTECs and mTECs after an 8-hour chase period (Fig 32). Our results suggest that $CD83^{-/-}$

TECs have a more stable MHCII presentation and kinetics than splenic CD83$^{-/-}$ B cells, as Kuwano et al. showed an accelerated endocytosis for the later compared to WT (64). However, thymic DCs and cTECs are known to have prolonged MHCII half-lives compared to splenic DCs *in vivo* (20h and 17h vs. 3h, respectively) (24). Therefore, it might be required to chase the retention time of E$\alpha_{52\text{-}68}$ peptide-MHCII complexes for longer time periods to detect differences between WT and CD83$^{-/-}$ cTECs. Since a large fraction of cTECs, died within 8 hours of culture, it might be necessary to examine the retention time *in vivo*. To do so, the peptide could be injected intravenously into CD83$^{-/-}$ and WT mice, TECs analyzed for peptide presentation after 2 - 24 hours.
In summary, further examination of the MHCII retention time at the surface of CD83-deficient cTECs will be necessary to substantiate the "turnover model".

Interestingly, it was reported for the MHCII(K>R) DCs to express higher MHCII levels than WT DCs, because of their resistance to ubiquitination-induced endocytosis leading to an accumulation of MHCII at the surface (1, 2, 90). We did not observe these increased MHCII levels in cTECs (Fig 31). This difference suggests that cTECs express most of their MHCII at the surface and do not recycle them in endosomes in a way as immature DCs and B cells do. Therefore, the inhibition of MHCII ubiquitination does not enhance the surface MHCII level in cTECs, but may still impact on their MHCII half-life at the surface. Taken together, further studies are necessary to elucidate the influence of MHCII kinetics on the thymic selection processes.

Besides the “turnover model”, we suggest as an alternative scenario, that a certain MHCII expression level might be required to select thymocytes. To address this, we would need a mouse model whose cTECs express even less surface MHCII than CD83$^{-/-}$ cTECs. In case these mice’s T cell development would not be affected, this would indicate that the MHCII levels are not the limiting factor for CD4^{+} T cell development in CD83$^{-/-}$ mice. Unfortunately, mice with such a phenotype have not yet been reported.

Our studies may also lead to a better understanding of the selection paradox:
The different tasks within the thymus, i.e. positive and negative selection, may require different kinetics of pMHC. The selection paradox describes the phenomenon that the very same interaction between APC and T cell, namely the TCR-pMHC interaction, can trigger two opposing outcomes (survival and death). Many models have been proposed to explain this selection paradox. 1) Temporal and spatial segregation between positive and negative selection. 2) Different affinities or

3) Different avidities of the TCR-pMHC interactions. 4) A distinct peptidome of cTECs (private peptides) (see introduction for more details).

The active regulation of MHC molecules is a recently discovered mechanism, which was not taken into consideration before. The finding that cTECs differ from medullary APCs with respect to the MHCII kinetics/levels (due to the stabilizing function of CD83 and the differential expression of MARCH E3 ligases) and the diminished co-stimulatory molecule expression, implies that the immunologic synapse in the cortex might be different from the ones formed with medullary TECs, which may transmit a distinct digital signal to thymocytes.

6.3 CD83$^{-/-}$ cTECs are impaired to mediate positive selection of CD4^{+} thymocytes

The development of CD8^{+} T cells is not affected in CD83$^{-/-}$ mice, indicating that expression levels, degradation, or turnover of MHCI is not influenced by CD83. In line with this, we found no differences between the MHCI levels of thymic APCs of CD83$^{-/-}$ and WT mice, nor did we observe any abnormalities in the CD8^{+} T cell development of CD83$^{-/-}$ mice (Fig 7, 10, 12). The fact that the CD8^{+} T cells are normally selected by CD83-deficient cTECs, indicates that cTECs are *per se* functional, and that only the MHCII pathway is affected by the CD83-deficiency.

To mention, Fujimoto et al. reported an increase in the CD8SP compartment in AND TCR-transgenic CD83$^{-/-}$ mice (63). Since, we could not detect such a redirection into the CD8^{+} lineage in OTII or DEP TCR-transgenic systems on a CD83$^{-/-}$ background, nor in the polyclonal CD83-deficient repertoire, this observation probably results from a special feature of the AND-TCR model.

By investigating the phenotype of CD4SP cells developing in the CD83$^{-/-}$ mice, we confirmed the data of previous studies suggesting an impaired positive selection of CD4^{+} thymocytes. We and others found these cells to express lower levels of CD5 and TCRβ (Fig 10) (64). Both markers represent the signal strength that thymocytes receive during positive selection. In addition, a higher proportion of cells has an immature phenotype (CD24^{hi} CD69^{hi}) compared to WT (Fig 9). Taken together, by phenotyping we observed that CD4SP cells of CD83$^{-/-}$ mice receive weaker positive selecting signals than in WT mice.

Furthermore, we demonstrated that the development of OTII and DEP TCR-transgenic thymocytes (both TCRs with specificities towards foreign antigen) were also strongly impaired (Fig 13). For transgenic TCRs of foreign specificities we can exclude excessive negative selection (due to auto-reactivity) being causative for the strong CD4SP cell reduction. In conclusion, the developmental

impairment of $CD4^+$ T cells in $CD83^{-/-}$ mice is rather mediated by defects during positive selection than massive negative selection.

In line with the T cell phenotype, we and others found that the phenotype of $CD83^{-/-}$ mice is mediated by the radio-resistant stromal compartment, indicating that CD83-deficiency affects TECs to select thymocytes (Fig 11) (63). In line with this, we did not detect an enhanced contribution of DCs to negative selection in $CD83^{-/-}$ thymi (Fig 12). To address the contribution of DCs to negative selection, we reconstituted $CD83^{-/-}$ and WT recipients with MHCII-sufficient or -deficient bone marrow. We found similar percentages of CD4SP cells to be subjected to negative selection by DCs in WT and $CD83^{-/-}$ recipients, suggesting normal negative selection rates in $CD83^{-/-}$ thymi. To formally exclude any effects of CD83-deficiency on negative selection, we would have had to render both DCs and mTECs unable to clonally delete thymocytes. Since a mTEC-specific $MHCII^{-/-}$ mouse model was not available, the transgenic C2TA-KD mouse model would provide for a suitable experimental setup (92). mTECs of these mice express only 10% of WT levels of MHCII and are therefore strongly impaired in negative selection. Reconstituting C2TA-KD-transgenic $CD83^{-/-}$ mice with MHCII-deficient bone marrow should eliminate most negative selection of mTECs and DCs. This could give final certainty that negative selection is not affected in $CD83^{-/-}$ mice.

Interestingly, in $CD83^{-/-}$ thymi we observed a similar proportion of DC-mediated negative selection of CD4SP cells as in WT thymi (38% and 42%) (Fig 12). Although the CD4SP compartment is strongly reduced in $CD83^{-/-}$ thymi, the percentage of auto-reactive/high affinity TCR-expressing cells (to be eliminated by DCs) was comparable to WT thymi. Presumably, this indicates that the TCR repertoire that is positively selected in $CD83^{-/-}$ thymi, is normal, meaning not biased in any direction towards a TCR repertoire with rather high or low affinity for self-antigen.

6.4 Probing gene function in TECs

Since TECs cannot be stably cultured, our current understanding of their functions, interactions, and their mediation of thymocyte selection is mainly derived from gain- or loss-of-function studies *in vivo*. However, these techniques are associated with inherent problems, such as that the cell numbers that can be obtained per mouse are very small, and that gene targeting approaches are very costly and time consuming. All these hurdles led to slowing down the identification of further genes and interactions that are relevant for TEC development and function. To overcome these limitations, we established an approach, which allows a quick and specific assessment of gene function in TECs *in vivo*. By lentiviral transduction of embryonic thymic stromal cells, we were able

to introduce genes of interest into the genome of TECs. The reaggregation of these stromal cells into reaggregated thymic organ cultures (RTOCs) and subsequent transplantation of the RTOCs under the kidney capsule of recipient mice, enables long-term studies *in vivo*. Applying this system, we succeeded in reconstituting $CD83^{-/-}$ RTOCs with a CD83 expression vector, which resulted in a complete rescue of the $CD4^{+}$ T cell development (Fig 27).

We also tried to rescue the $CD83^{-/-}$ phenotype by a shRNA-knockdown approach. However, so far we have not been successful. We knocked down MARCH8 in $CD83^{-/-}$ RTOCs in order to rescue the CD4SP development (data not shown). According to the proposed model above, the knockdown of MARCH8 should rescue the $CD83^{-/-}$ phenotype just like the expression of CD83. However, although we yielded an infection rate of 50%, we did not observe a rescuing effect on T cells. Due to limited material we could not formally proof a successful knockdown by reduced mRNA level for MARCH8, therefore these results were not informative. We tested the construct for functionality in a classical luciferase assay, and observed a dose-dependent knockdown using this construct (data not shown). Furthermore, the identical shRNA construct sequence was used by Chen et al., who yielded efficient knockdown of MARCH8 mRNA in transfected 293 cells in vitro (78). These facts suggest a *per se* functional shRNA sequence. A possible explanation could be that the knockdown of MARCH8 is not efficient enough and that the remaining MARCH8 amounts are "sufficient" for degrading MHCII beyond the threshold level required for proper T cell development. Unfortunately, due to very limited cTEC numbers, which can be obtained from RTOCs, it has not been feasible to assess the change in MHCII, CD83 or MARCH8 levels upon CD83 expression or MARCH8 knockdown.

To proof that, in principle, our system is applicable for knockdown studies in TECs, we transduced a C2TA-knockdown construct. C2TA is the master regulator of MHCII expression and we thus expected reduced MHCII levels in TECs after the transduction of the C2TA-knockdown construct (similar to the transgenic C2TA-KD mouse model (92)). Surprisingly, the RTOCs transduced with the construct (ubiquitin promotor-GFP-C2TAsh) did not grow as usual, indicating a toxic/interfering effect on TEC growth (data not shown). Unlike our C2TA-knockdown construct, which, due to size limitations of the lentiviral construct, is driven by a ubiquitin promoter, the traditional C2TA-knockdown construct of the C2TA-KD mouse model relies on the expression of an Aire promoter-driven construct which allows TECs to develop and differentiate to mature mTECs (the Aire expression only starts at late stages of mTEC differentiation). Apart from the ubiquitin promoter, we also tested the CMV promoter in our system. However, both showed this destructive effect on RTOCs. Previous experiments in our laboratory indicate that the lentiviral transduction of constructs carrying these promoters *per se* does not harm the development of

RTOCs. This suggests that the C2TA-knockdown was indeed successful, but reduced the MHCII levels below a level required for early TEC development.

Taken together, we were able to show that our 'lentiviral transduction of RTOC' system allows the manipulation of TECs for gain-of-function studies. Further studies will be required to verify its usefulness for knockdown studies.

With the lentiviral transduction approach, we could fully reconstitute the $CD4^+$ T cell development in $CD83^{-/-}$ RTOCs. The infection rates, i.e. the percentage of GFP^+ TECs within the RTOCs, of the mutant screening were ranging from 56% to 13%. Even the lowest infection rate (13% of Mutant1) yielded a full reconstitution to WT levels. Compared to the mixed RTOCs ($CD83^{-/-}$ and WT cells mixed), these were rather surprising results. In $CD83^{-/-}$ RTOCs supplemented with 10% or 20% CD83-sufficient WT cells, we observed a rescue to 50% of WT $CD4^+$ T cell levels (Fig 25). In contrast to this, a TEC infection rate of 13% with the CD83-mutant1 overexpression vector yielded full reconstitution of $CD4^+$ T cell development in $CD83^{-/-}$ RTOCs. Possibly, the infected cells express so much CD83, i.e. more than endogenous levels, and accordingly have more than endogenous MHCII levels which can compensate for the non-infected, MHCII-reduced TECs. Yet, another explanation for the reduced rescue in the mixed RTOCs could be that the CD83-deficient cells have overgrown WT cells for unknown reasons. Unfortunately, there were no markers available which we could have used to control the ratio of WT to $CD83^{-/-}$ cells after 5 weeks. However, the key question we were addressing by the mixed RTOC experiments, namely the minimal infection rate needed to be able to detect an increase in $CD4^+$ T cells, was still answered (Fig 25, 10%) and was shown to be sufficient to show a full rescue (Fig 28).

On the side, in the mixed RTOC experiment, we observed by supplementing $CD83^{-/-}$ RTOCs with increasing amounts of WT cells an increase in CD4SP cell development. As a result, we would have expected either a linear- or a sigmoidal-shaped curve regarding the correlation between the ratio of WT cells and the CD4SP cell development. However, we neither observed a clear linear correlation, nor a clear sigmoidal correlation. Therefore, further investigations, will be necessary to differentiate between these two possible curve shapes and the possible interpretations for the positive selection process: According to the 'single niche model', a linear increase would indicate that the more $CD83^+$ cTECs are present, the more CD4SP cells are being selected, therefore, the number of CD83-sufficient cTECs (selecting cTECs) would be the limiting factor. The 'single niche model' was proposed by Merkenschlager et al. after having observed a linear correlation between transgenic T cell selection and the number of selecting TECs (of MHC haplotype able to select the

TCR-transgenic cells) titrated into RTOCs of non-selecting MHC haplotype (93). They proposed that thymocytes do not migrate between microenvironments and that they, therefore, would audit only few cTECs in their microenvironment (single niche) for positive selection. This would render the availability of selecting ligands they see in their niche the limiting factor for thymocyte maturation. This model implies that positive selection is mediated by a single interaction with a selecting cTEC.

On the other hand, a sigmoidal-shaped curve would suggest that a certain number of CD83-sufficient cells (threshold) is required to mediate positive selection of the majority of thymocytes. This would indicate that the availability of multiple interactions with CD83-sufficient cells is limiting. Supplementing RTOCs with increasing amounts of selecting TECs would, therefore, not increase the T cell output unless the required number of selecting cTECs is reached (threshold/turning point of curve). Accordingly, adding selecting TECs above the required threshold number would not yield a higher T cell output (saturation). Furthermore, a low threshold would signify that few interactions are required or that the thymocytes are actively screening their environment. Wheras a high threshold, meaning a requirement of many selecting cTECs, would suggest a rather low motility of thymocytes. These speculations require experimental evidence. Therefore, further investigations, such as repeating the mixed RTOC experiment with finer titration steps, could contribute to the question if the positive selection of a thymocyte is mediated by a single interaction with a selecting cTEC (linear correlation), or if positive selection is rather the result of multiple cTEC interactions (sigmoidal correlation).

7 CONCLUSION

The results of this study revealed that the transmembrane domain of CD83 is crucial for the $CD4^+$ T cell development, while cell-cell interactions via CD83 engagement are dispensable in that concern. Furthermore, we found differential expression patterns of CD83 and MARCH E3 ligases in the different thymic APC subsets, which have likely evolved to differentially regulate their MHCII surface expression. By elucidating a distinct MHCII regulation in cTECs, this study provides a new facette to the longstanding enigma about potential qualitative differences in cTECs' and mTECs' pMHC-TCR interaction.

8 MATERIAL and METHODS

8.1 Mice

Mice were bred in the animal facility of the Institute for Immunology of the LMU Munich in individually ventilated cages under SPF conditions. $CD83^{-/-}$ mice were a kind gift of Prof. Alexander Steinkasserer. MHCII(K225R)-GFP mice were kindly provided by Prof. Hidde Ploegh (90). C57BL/6N mice were purchased from Taconic. All other strains were from in-house breeding colonies. Local law regulation authorities approved all animal experiments.

8.2 Antibodies and reagents for flow cytometry

Surface stainings were performed according to standard procedures at a density of 1-2 x 10^6 per 50µl and volumes were scaled up accordingly. Flow cytometry measurements were performed on FACS Canto II (Becton Dickinson) using FACS DIVA software (Becton Dickinson) and Flowjo software (Treestar Inc., USA) was used for analysis.

Table 1: List of antibodies used.

Antigen	Conjugate	Clone	Supplier
CD4	APC	GK1.5	BD
CD8	PE-Cy7	53-6.7	BD
DAPI			Invitrogen
EpCAM	PE-Cy7	G8.8	BD
CD45	PerCP	Ly-5	BD
CD11c	PerCP	N418	BD
Va2	PE	B20.1	eBioScience
Vb5	FITC/bio	MR9-4	eBioScience
CD5	APC	53-7.3	BD
CD24	Pacific Blue	M1/69	BD
CD69	Pe-Cy7	H1.2F3	BD
TCRb	APC	H57-597	eBioScience
Streptavidin	PE-Cy7/APC		eBioScience
I-A/I-E	APC	M5/114.15.2	BioLegend
Va11	APC	RR8-1	eBioScience
CD83	bio	Michel-19	BioLegend
Keratin5	PerCP	AF138	Covance
CD80	bio	16-10A1	BD
Ly51	PE	BP-1	BD
H2-K	FITC	AF6-88.5	BD
YA3	bio	eBioYAe	eBioScience
Y3P	FITC		purified SN

8.3 Genotyping

For genotyping, mouse tails were digested in 50µl digestion buffer for 5 hours at 55°C, followed by Proteinase K heat inactivation at 95°C for 5 minutes.

Gitocher digestion buffer (10x)

670mM Tris pH 8.8
166mM Ammonium sulfate
65mM $MgCl_2$
0.1% Gelatine

Digestion buffer

3µl Proteinase K (10mg/ml stock)
2.5µl Triton (10% Stock)
5µl Gitocher Buffer (10x)
0.5µl β-Mercapto-ethanol
39µl H_2O

Subsequently 1µl of the digested tail DNA was used for genotyping.

PCR Red-buffer (5x)

250mM KCl
50mM Tris pH 8.3
43% Glycerol
7.5mM $MgCl_2$
2mM Cresol Red

PCR reaction

1µl tail digest
2.5mM primers (final)
200µM dNTP (final)
PCR Buffer (1x final)
1µl Taq Polymerase
Ad 30µl H_2O

All genotyping reactions were carried out using the TD54x30 program:

3min at 94°C

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

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

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

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

10min at 72°C

Table 2: Primer sequences for the genotyping of mice.

Genotype		Sequence (5' - 3')	Amplicon
I-Ab WT	fwd	AGGAGTACGTGCGCTACGACAG	332bp
	rev	GCAGAGGTGAGACAGGAGGGAGA	
I-Ab KO	fwd	CACTGAAGCGGGAAGGGAC	888bp
	rev	GCAGAGGTGAGACAGGAGGGAGA	
OTII	fwd	GCTGCTGCACAGACCTACT	160bp
	rev	CAGCTCACCTAACACGAGGA	
CD83 WT	fwd	CACGCTCCCTTATGTTAGG	300bp
	rev	TTCCATGAAGAAGCAAGGTGG	
CD83 KO	fwd	TTCTCCTTCCAGATCTCTGG	400bp
	rev	TTCCATGAAGAAGCAAGGTGG	
MHCII(K>R) WT	fwd	AGGGTCTTAGTGAATACAGTTGGT	600bp
	rev	TCCTCGCCATTGCCTGTGC	
MHCII(K>R) KI	fwd	AGGGTCTTAGTGAATACAGTTGGT	200bp
	rev	GTCCAGCTCGACCAGGATGG	
March1 KO	fwd	GTACTGGTGAGTTCATAATG	623bp
	rev	GTACGTGTGTGTACTGGAAG	
DEP	fwd	CGAGAGGAAGCATGTCTAAC	650bp
	rev	ACCGCGGTCATCCAACACAG	

8.4 Cloning

Plasmids containing truncated versions of CD83 were kindly provided by Prof. Chistopher Goodnow (67). 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 (NEB. Munich, Germany) were used to digest vector and PCR products at 10U/≤1µg DNA at 37°C for approximate 1h. Cut vectors or inserts were purified on a 1.5% TAE agarose gel and purified using a gel extraction kit (Qiagen, Hilden Germany). Before ligation vector backbones were dephosphorylated with 10U alkaline phosphatase (Roche, Mannheim, Germany) with the appropriate buffer at 37°C for 20min. Ligation was performed using T4 DNA Ligase (Roche, Mannheim, Germany) using equal stoichiometric amounts of insert and vector. All ligation reactions were heat-shock (20min on ice followed by 2min at 42°C) transfected in (DH5α), incubated shaking at 37°C for 1h, and plated out on LB-agar plates containing an appropriate antibiotic. Ampicillin (300µg/ml) and kanamycin (50µg/ml) were used as selection markers.

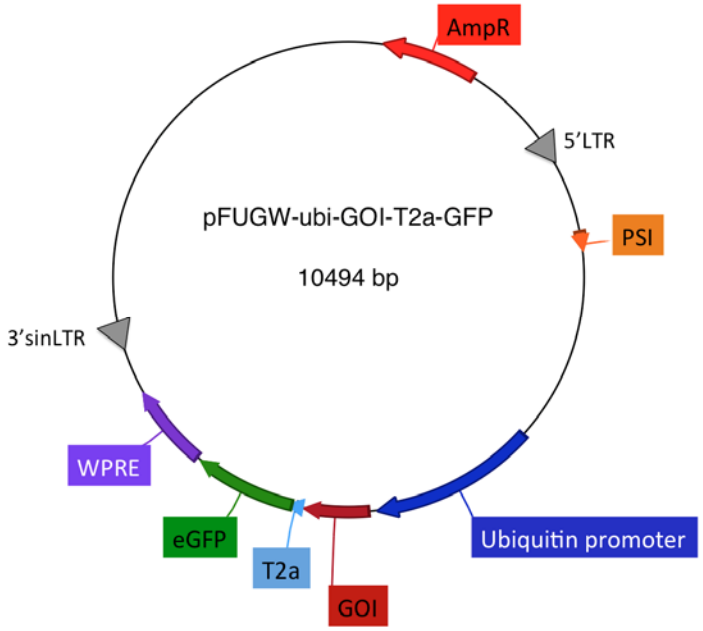

Figure 29: Scheme of lentiviral constructs used for virus production.

Important features are the ubiquitin promoter, the gene of interest (GOI) bridged to the eGFP reporter via a T2a linker, posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE), a Psi packaging sequence and an Ampicillin resistance cassette. LTR sequences flank the construct, which is integrated into the genome of infected cells. The viral construct was rendered replication-deficient (self-inactivated, sin) by deleting the essential U3 part from the 3' LTR sequence.

8.5 Lentivirus production

8.5.1 Transient transfection of HEK293FT cells for lentivirus production

$2x10^6$ cells were plated in 10cm cell culture dishes and left to grow up to 75% confluence. For transfection, a mixture of 8µg GOI DNA (construct is illustrated in Fig 33) with 6µg pax2 DNA and 6µg VSVg DNA in 0.5M $CaCl_2$ in HEBS per dish was added to the cells and incubated at 37°C for 6-8h. After that, cells were supplied with excessive fresh medium, which is then replaced after 24h with 5ml/plate fresh 5% FCS DMEM medium. The SN was collected and replaced with fresh medium at 48h and 72h after transfection, pooled and centrifugated at 38.500g for 4h to pellet the virus particles. The pellets were resuspended in 3-4ml fresh DMEM medium and stored at -80°C.

8.5.2 Transduction of NIH3T3 cells for lentivirus titration

NIH3T3 cells were plated at the concentration of $2x10^4$ cells/well in 24-well plates and incubated over night. 20µl of each virus preparation stock produced were used for the titration. 10µl of the stock were added to the first well, the following 5 wells received titrated concentrations of virus (with a dilution factor of 3). Each well was supplemented with polybrene (10µg/ml). After 3 days,

the expression of GFP as infection marker was measured. The amount of virus particles (IU) per ml is then calculated by the proportion of infected cells relative to the amount of virus added.

8.6 Isolation of thymic antigen presenting cells

Thymi were harvested and cleared from connective tissue and fat, cut into very small pieces using scissors and resuspended in digestion medium (RPMI containing 0.2mg/ml Collagenase (Roche), 0.2 mg/ml DispaseI (Roche), 2% FCS, 25mM HEPES (pH7.2) and 25µg/ml DNaseI (Roche)). 1ml digestion medium was used per thymus. Digestion was performed in a FACS tube at 37°C. The cell suspension was also pipetted up and down softly every 5min to apply additionally some mechanical force for better and faster digestion. After 30-40min the cell suspension was transferred to 4°C, filtered and washed in FACS buffer (2% FCS in PBS, 2mM EDTA) and resuspended in Percoll™ (ρ 1.115; GE Healthcare). A second layer of Percoll (ρ 1.055) and a third layer of FACS buffer was carefully added on top. After the layering, the gradient was centrifuged at 4°C and 1350g with slow acceleration and no break for 30min. The upper interface, containing the desired low density cell fraction, was harvested and washed in FACS buffer. Cells were now ready for staining.

Table 3: Percoll density gradient solutions.

ρ 1.115	ρ 1.055
9x Percoll stock (ρ 1.134) 1x PBS (10x) 25mM HEPES pH=7.2 (final)	1x Percoll (ρ1.115) 1.09x PBS (1x)

8.7 Reaggregate Thymic Organ Cultures (RTOC)

8.7.1 RTOC generation

Single cell suspensions of E14.5 – E16.5 fetal thymic lobes were prepared by enzymatic digestion (see above). $CD45^+$ cells were depleted using CD45 MACS beads (Miltenyi Biotech) according to standard procedures. Cells were infected with lentivirus in fresh 8% FCS DMEM medium at a ratio of 1:1, the cell suspension was supplemented with polybrene (10ug/ml). After 3 hours, the cells were washed 3 times and then every 1x 10^6 cells were distributed to a new 1ml screw-cap Eppi, spun down and the SN was completely removed. The cell slurry (<1.4µl) was taken up with a pipette and deposited onto a 0.45µm nylon membrane (Millipore) swimming in a 6-well culture plate filled with 6ml fresh 8% FCS DMEM medium. The RTOCs were incubated for 48h prior to transplantation under the kidney capsule of mice.

8.7.2 RTOC transplantation

Recipient mice were narcotized with 200µl/10g body weight of a combination of Ketamin (0.8mg/ml, Ketavet, Pfizer) and Xylazin (5mg/ml, Rompun, Bayer, Germany) in PBS. Their eyes were protected from light and from drying out with Bepanthen creme (Bayer, Germany). The site of incision was shaved and disinfected. A small incision was made through skin and peritoneum. The kidney was lifted out and fixed at an exposed position. The fragile kidney capsule is carefully scratched with a scalpel. Then, the capsule is lifted up with fine forceps and the RTOC was slipped underneath the capsule. Then, the kidney is pushed back to its normal position, the sticky peritoneum pulled back together, and the skin is stapled with 2-3 staples. Mice were sacrificed 5 weeks later, and RTOCs were FACS analyzed.

8.8 Bone marrow chimeras

Bone marrow was obtained from donor mice by isolating tibia and femur and flushing out the bone marrow mass using syringe and needle. The cells were resuspended in cold PBS, filtered, and depleted from T cells using CD8 and CD4 MACS beads (Myltenyi) according to standard procedures. Recipient mice were lethally irradiated with 2x 550rad and reconstituted by injecting 6-8x10^6 bone marrow cells in 200µl PBS into the tail vein. Chimeras were analyzed 5-6 weeks after reconstitution.

8.9 Quantitative 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 according to the manufacturers recommendations. Primers were used at 500nM each.

Cycling conditions:
94°C for 180sec
94°C for 10sec
56°C for 20sec
72°C for 20sec
} 40x (plate read)

Followed by a melting curve analysis. The melting curves for each reaction were analyzed between 56°C and 95°C with an increment of 0.5°C/30s.

Biorad's CFX Manager software was used for analysis. 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. Calculation of gene expression levels was carried out by using following formula: Expression level = $2^{-\Delta Ct}$.

Table 4: qPCR primer sequences.

Gene		Sequence (5' - 3')
βactin	fwd	GCCTTCCTTCTTGGGTAT
	rev	GGCATAGAGGTCTTTACGG
CD83	fwd	GCCTCCAGCTCCTGTTTCTA
	rev	AGTGTTTTGGATCGTCAGGG
MARCH1	fwd	AAGAGAGCCCACTCATCACACC
	rev	ATCTGGAGCTTTTCCCACTTCC
MARCH8	fwd	AGTAGTCCTCCATCCACGAC
	rev	GATGACGAGAGCCCTCTGAT
MARCH9	fwd	TAATCCGCTGGATCAGTG
	rev	AGCAATCTGGACCTTCTC

8.10 MHCII turnover

Thymi of 2 week old WT and $CD83^{-/-}$ mice were isolated, digested, and TEC were enriched via density gradient and $CD45^{+}$ cell depletion (described above). The cells were pulsed with the $E\alpha_{52-68}$ peptide (100µg/ml) for 2 hours at 37°C in a gelatin-coated well. After intensive washing, the cells were distributed into 5 wells and cultured for 2, 4, and 8 hours or immediately put on ice (t=0). After the indicated time points the cells were stored on ice. The cells were then stained with antibodies for FACS analysis. YAe stained $E\alpha_{52-68}$-MHCII complexes, Y3P stained all pMHCII complexes. For the ratio of $E\alpha_{52-68}$-MHCII complexes presented, we divided the gMFI of YAe by the gMFI of Y3P.

8.11 Statistical analysis

Geometrical mean fluorescence intensities (gMFI) were used to compare logarithmic expression data acquired by FACS. Statistical significance were assessed by the two-tailed unpaired Student`s t-test with unequal variance.

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10 ACKNOWLEDGEMENTS

The first and greatest thanks goes to my supervisor Prof. Ludger Klein for sharing his tremendous experience and knowledge and for teaching me valuable lessons in good scientific practice and analytical thinking. I learned a lot during my PhD time; those experiences surely will guide me in my future career.

I am thankful for the opportunity to be part of such a thriving, excellent, and pleasant research group. Therefore, I'd like to thank all lab members during that time for the nice and cooperative atmosphere: Ursula Jakobeit, Maria Hinterberger, Martin Aichinger, Gerald Wirnsberger, Christine Federle, Sonja Höflinger, Steffi Kirsch, Ksenija Jovanovic, Julia Winnewisser, Tomoyoshi Yamano, Lei Wang, Jelena Nedjic, Josep Soler, and Chunyan Wu. I also appreciate and honor the great work of our mouse facility staff, which was fundamental for my work.

Furthermore, I'd like to thank all the scientists who generously provided me with plasmids or mice: Prof. Alexander Steinkasserer, Prof. Christopher Goodnow, Prof. Hidde Ploegh, and Prof. Jannie Borst and Prof. Satoshi Ishido.

Especially, I'd like to thank Christine, who supported and guided and teached me on a daily basis. It was a priceless comfort of having her around, her expertise, and most importantly her helpfulness every minute. Christine, I am endlessly grateful!

Sonja, the heart of our group, helped me a lot during the hardest hours of dissecting mice; I appreciate it.

I thank Jelena for re-joining our lab, for the vivid discussions, and highly appreciate her suggestions regarding my project. I am glad to got to know her and her extraordinary passion for science.

I'd like to thank Julia, not only for being a work life-enriching colleague but also for being my best friend!

My dearest Lorenz, thank you for being by my side! I am fortunate to have you in my life!

Last but definitely not least, I thank my beloved parents for supporting and encouraging me the last 30 years!