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Thymic CD4 T cell selection requires attenuation of March8-mediated MHCII turnover in cortical epithelial cells through CD83

Julia von Rohrscheidt,^{1*} Elisabetta Petrozziello,^{1*} Jelena Nedjic,¹ Christine Federle,¹ Lena Krzyzak,² Hidde L. Ploegh,³ Satoshi Ishido,⁴ Alexander Steinkasserer,² and Ludger Klein¹

¹Institute for Immunology, Biomedical Center Munich, Ludwig-Maximilians-University Munich, 82152 Planegg-Martinsried, Germany

²Department of Immune Modulation, University Hospital Erlangen, 91052 Erlangen, Germany

³Department of Biology, Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02142

⁴Department of Microbiology, Hyogo College of Medicine, Nishinomiya 663-8501, Japan

Deficiency of CD83 in thymic epithelial cells (TECs) dramatically impairs thymic CD4 T cell selection. CD83 can exert cell-intrinsic and -extrinsic functions through discrete protein domains, but it remains unclear how CD83's capacity to operate through these alternative functional modules relates to its crucial role in TECs. In this study, using viral reconstitution of gene function in TECs, we found that CD83's transmembrane domain is necessary and sufficient for thymic CD4 T cell selection. Moreover, a ubiquitination-resistant MHCII variant restored CD4 T cell selection in *Cd83*^{-/-} mice. Although during dendritic cell maturation CD83 is known to stabilize MHCII through opposing the ubiquitin ligase March1, regulation of March1 did not account for CD83's TEC-intrinsic role. Instead, we provide evidence that MHCII in cortical TECs (cTECs) is targeted by March8, an E3 ligase of as yet unknown physiological substrate specificity. Ablating March8 in *Cd83*^{-/-} mice restored CD4 T cell development. Our results identify CD83-mediated MHCII stabilization through antagonism of March8 as a novel functional adaptation of cTECs for T cell selection. Furthermore, these findings suggest an intriguing division of labor between March1 and March8 in controlling inducible versus constitutive MHCII expression in hematopoietic antigen-presenting cells versus TECs.

INTRODUCTION

CD83, an evolutionary conserved immunoglobulin superfamily member, was discovered as an activation marker on DCs. Besides, CD83 is also induced in activated T and B cells and is constitutively expressed by thymic epithelial cells (TECs). The physiological significance of CD83 expression and its mode of action in these diverse cellular contexts are only beginning to emerge (Prechtel and Steinkasserer, 2007; Breloer and Fleischer, 2008; Prazma and Tedder, 2008).

Remarkably, CD83 harbors the capacity to exert distinct cell-intrinsic and -extrinsic functions through discrete protein domains. In DCs, CD83's transmembrane (TM) domain stabilizes surface MHCII by opposing the association of MHCII with the ubiquitin ligase March1, thereby interfering with MHCII ubiquitination and internalization (Tze et al., 2011). An analogous mechanism explains how CD83 enhances CD86 expression on mature DCs (Baravalle et al., 2011). Of note, the full spectrum of targets that are controlled

by CD83 in this manner remains to be determined and may vary between cell types. Besides this cell-autonomous function, CD83 may transmit immune regulatory signals in trans during intercellular interactions or, when present in soluble form, even systemically. Loss- or gain-of-function approaches suggested a costimulatory function of CD83 (Kruse et al., 2000; Prechtel et al., 2007). Experiments using various species of recombinant soluble CD83 (sCD83) indicated that CD83's extracellular (EC) domain can modulate several biological processes. sCD83 interfered with DC maturation in vitro and inhibited DC-dependent allogeneic and peptide-specific T cell proliferation (Lechmann et al., 2001). When administered in vivo, the EC domain of CD83 prevented the induction of experimental autoimmune encephalomyelitis and other autoimmune diseases (Zinser et al., 2004; Starke et al., 2013; Eckhardt et al., 2014). Importantly, sCD83 of natural derivation is found in DC and B cell supernatants and in the serum, whereby its origin, for instance via generation of alternative transcripts or shedding from the membrane, remains to be clarified (Hock et al., 2001; Dudziak et al., 2005). The nature of the presumed CD83 receptor remains enigmatic.

*J. von Rohrscheidt and E. Petrozziello contributed equally to this paper.

Correspondence to Ludger Klein: ludger.klein@med.lmu.de

J. Nedjic's present address is Boehringer Ingelheim Pharma, Immune Modulation and Biotherapeutics Discovery, 88397 Biberach an der Riss, Germany.

Abbreviations used: cTEC, cortical TEC; EC, extracellular; EGFP, enhanced GFP; EpCAM, epithelial cell adhesion molecule; MFI, mean fluorescence intensity; mTEC, medullary TEC; RTOC, reaggregation thymus organ culture; SP, single positive; TEC, thymic epithelial cell; TM, transmembrane.

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The most striking phenotype of CD83-deficient mice is a dramatic defect in thymic CD4 T cell selection (Fujimoto et al., 2002; Kuwano et al., 2007). An identical phenotype was reported for defective CD83 alleles generated through *N*-ethyl-*N*-nitrosourea mutagenesis (García-Martínez et al., 2004; Tze et al., 2011). Impaired CD4 T cell selection in *Cd83*^{-/-} mice reflects a requirement for CD83 in TECs (Fujimoto et al., 2002). Importantly, control of MHCII was considered an unlikely explanation for CD83's role in CD4 T cell selection because MHCII hemizygous mice, despite showing a similar reduction in MHCII on TECs as *Cd83*^{-/-} mice, generate normal CD4 T cell numbers (Kuwano et al., 2007). Hence, it was suggested that CD83, conceivably through interacting with a CD83 receptor on thymocytes or via an as yet undefined cell-intrinsic function in TECs, provides a thymocyte differentiation signal that is mechanistically separated from MHCII interactions (Lüthje et al., 2006; Kuwano et al., 2007; Breloer and Fleischer, 2008). In the present study, we have addressed whether CD83 might directly transmit essential signals from TECs to developing thymocytes via its EC domain or whether CD83's essential role in the thymus reflects a TEC-intrinsic effect through the control of MHC II or possibly also other modulators of CD4 T cell selection.

RESULTS AND DISCUSSION

Because we used a novel *Cd83*^{-/-} strain (Krzyzak et al., 2016), we first verified the reduction of CD4 single-positive (SP) thymocytes (Fig. 1 A). The thymic phenotype of *Cd83*^{-/-} mice was recapitulated in WT→*Cd83*^{-/-} BM chimeras, confirming the crucial requirement for CD83 in TECs (Fig. 1 B). We assessed two alternative explanations for the diminution in CD4 SP cell numbers: (1) CD4 T cells selected in the absence of CD83 may be subject to excessive negative selection, or (2) the paucity of CD4 SP cells may reflect a genuine defect in positive selection. In MHCII^{-/-} (*H2-ab1*^{-/-})→WT BM chimeras, lack of negative selection by hematopoietic APCs results in an enlarged CD4 SP compartment (Fig. 1 C). MHCII^{-/-}→*Cd83*^{-/-} chimeras displayed a slight increase in the CD4 compartment as compared with MHCII^{+/+}→*Cd83*^{-/-} chimeras. However, their CD4 compartment was not restored to the values observed in WT recipients (Fig. 1 C). Selection of CD4 T cells expressing two anti-foreign TCR transgenes specific for human C-reactive protein or OVA was dramatically reduced in *Cd83*^{-/-} BM recipients (Fig. 1, D and E). Together, these findings indicated that CD83 deficiency did not result in excessive negative selection but affected the size of the CD4 SP compartment through a defect in positive selection.

Two different modes of action were conceivable of how CD83 may support CD4 T cell selection: (1) in trans, through binding and signaling of its EC domain to an as yet unknown receptor on thymocytes or (2) in cis, through protecting substrates of ubiquitin ligases in TECs (Tze et al., 2011). To address this issue, we established an approach to express truncated or chimeric CD83 variants in *Cd83*^{-/-} TECs

in vivo (Travers et al., 2001; Aichinger et al., 2012). Reaggregation thymus organ cultures (RTOCs) from fetal TECs that had been transduced with bicistronic lentiviral vectors encoding CD83 variants and GFP were transplanted under the kidney capsule of recipient mice (Fig. 2 A). Such organoids are seeded by host-derived progenitors so that a steady-state flux through T cell differentiation was established within ~4 wk (Anderson and Jenkinson, 2007). We achieved transduction rates of 20–60%, with equal efficiency in cortical TECs (cTECs) and medullary TECs (mTECs; Fig. 2 B) and stable expression of virus-encoded GFP for at least 5 wk (Fig. 2 C).

The coexistence of transduced and nontransduced TECs in these RTOCs represented a caveat for the interpretation of gain-of-function experiments with CD83 or variants thereof. To ask whether CD83 expression by only a fraction of TECs was sufficient for normal CD4 T cell selection, we generated mixed RTOCs with titrated ratios of WT and *Cd83*^{-/-} TECs to emulate different transduction efficiencies (Fig. 2 D). Even at the lowest ratio of WT TECs tested (1:9), a marked increase in CD4 SP cells occurred.

The CD4 SP compartment in transplanted RTOCs transduced with full-length CD83 (construct #2) was indistinguishable in size from the CD4 SP population in WT RTOCs (Fig. 2, E and F). The respective roles of the EC, TM, and cytoplasmic domains of CD83 were then dissected through introduction of truncated or chimeric forms of CD83 (Fig. 2 F). Expression of a chimeric CD83 molecule harboring the TM and cytoplasmic domain, yet bearing the EC portion of human CD4 (hCD4; construct #3), rescued CD4 T cell selection. Likewise, a truncated CD83 molecule (construct #6) lacking the cytoplasmic domain restored the CD4 SP compartment to normal size. In contrast, a construct (#4) containing the EC domain of CD83 yet bearing a TM domain derived from hCD4, despite a higher transduction efficiency and very similar expression level, failed to restore CD4 T cell selection (Fig. 2, F and G). The crucial requirement for the TM domain was confirmed with a second construct (#5). These observations identified the TM domain as the minimal functional unit through which CD83 supports CD4 T cell selection.

We next addressed the expression pattern of CD83 in cTECs and mTECs. Surface CD83 was substantially more abundant on cTECs compared with mTECs (Fig. 3 A). This differential expression was confirmed at the level of RNA expression (Fig. 3 B). Consistent with a cTEC-specific function of CD83, surface MHCII was diminished on cTECs but not mTECs from *Cd83*^{-/-} mice (Fig. 3 C). Surface MHCI was not affected by lack of CD83 (Fig. 3 C).

DCs and B cells from mice lacking CD83 or carrying a mutation in CD83's TM domain display reduced surface MHCII owing to accelerated MHCII turnover (Kuwano et al., 2007; Tze et al., 2011). We hypothesized that the reduced MHCII on CD83-deficient cTECs may likewise stem from increased MHCII internalization. To test this idea, purified cTECs from WT or *Cd83*^{-/-} mice were cul-

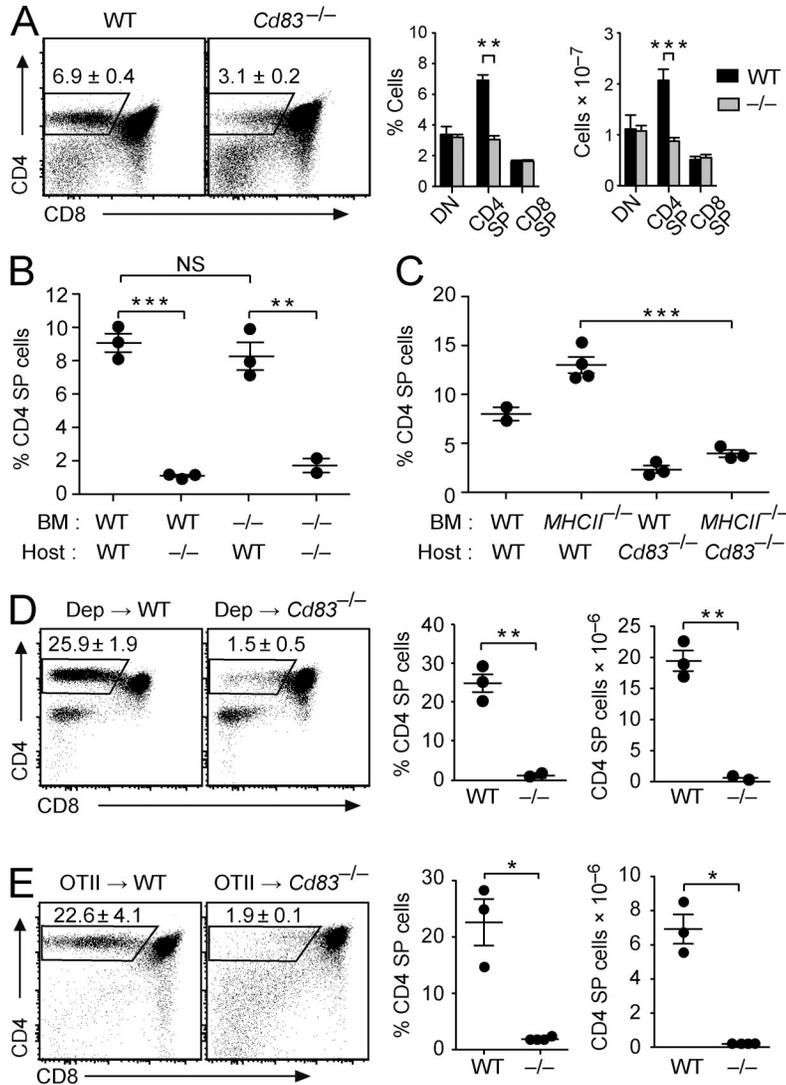


Figure 1. Impaired positive selection of CD4 T cells in *Cd83*^{-/-} mice. (A) Thymocyte subsets in WT and *Cd83*^{-/-} mice. The mean frequency ± SEM of CD4 SP cells is indicated in the dot plots. The bar diagrams show the percentage and absolute number of thymocyte subsets ($n \geq 22$ for each genotype). DN, double negative. (B) Irradiated WT or *Cd83*^{-/-} recipients were reconstituted with WT or *Cd83*^{-/-} BM. Frequencies of CD4 SP cells ± SEM were assessed 6–8 wk after reconstitution. (C) Irradiated WT or *Cd83*^{-/-} recipients were reconstituted with WT or *MHCII*^{-/-} BM. Frequencies of CD4 SP cells ± SEM were assessed 6–8 wk after reconstitution. (D) Irradiated WT or *Cd83*^{-/-} recipients were reconstituted with TCR-Dep-transgenic BM. Frequencies and absolute numbers of CD4 SP cells ± SEM were assessed 5 wk after reconstitution. (E) Irradiated WT or *Cd83*^{-/-} recipients were lethally irradiated and reconstituted with TCR-OTII-transgenic BM. Frequencies and absolute numbers of CD4 SP cells ± SEM were assessed 5 wk after reconstitution. (B–E) Data are representative of at least two independent experiments with two to four mice per donor genotype. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test).

tured in vitro with or without brefeldin A (BFA; Fig. 3 D). BFA is an ER to Golgi transport inhibitor that prevents the delivery of newly synthesized proteins to the cell surface, thus abrogating de novo MHCII routing to the surface, thus abrogating de novo MHCII routing to the surface. With WT cTECs, BFA did not have any discernable effect on the surface MHCII, consistent with the previously reported remarkably long $t_{1/2}$ of MHCII complexes on TECs (Müller et al., 1993). In contrast, surface MHCII on *Cd83*^{-/-} cTECs was significantly reduced after culture in the presence of BFA (Fig. 3 D). BFA treatment resulted in a similar degree of MHCII reduction on WT and *Cd83*^{-/-} mTECs (Fig. 3 E). Thus, CD83 controls MHCII surface levels on cTECs but not mTECs through opposing MHC II turnover. However, the mechanistic basis of CD83's influence on MHCII remained open, and most importantly, it was unclear whether impaired CD4 T cell selection in the absence of CD83 was causally (and solely) related to its effect on MHCII.

In immature DCs, MHCII is ubiquitinated at lysine 225 of the I- α chain, earmarking it for lysosomal degradation (Shin et al., 2006). During DC maturation, up-regulation of CD83 antagonizes this process and hence enhances MHCII surface expression (Tze et al., 2011). To test whether decreased MHCII on *Cd83*^{-/-} cTECs may similarly be caused by ubiquitination-dependent destabilization of MHC II, we used mice in which the I- α chain is modified with a lysine to arginine substitution at position 225 (MHCII^{K225R}; McGehee et al., 2011). MHCII surface expression on MHC II^{K225R} cTECs was indistinguishable from that on WT cTECs (Fig. 4 A), and the size of the CD4 SP compartment in MHCII^{K225R} mice was very similar to WT controls (Fig. 4 B). Superimposition of CD83 deficiency with expression of ubiquitination-resistant MHCII not only restored normal MHCII expression on cTECs, but also rescued CD4 T cell development (Fig. 4, A and B). These findings supported the idea that CD83 opposes the ubiquitination-dependent

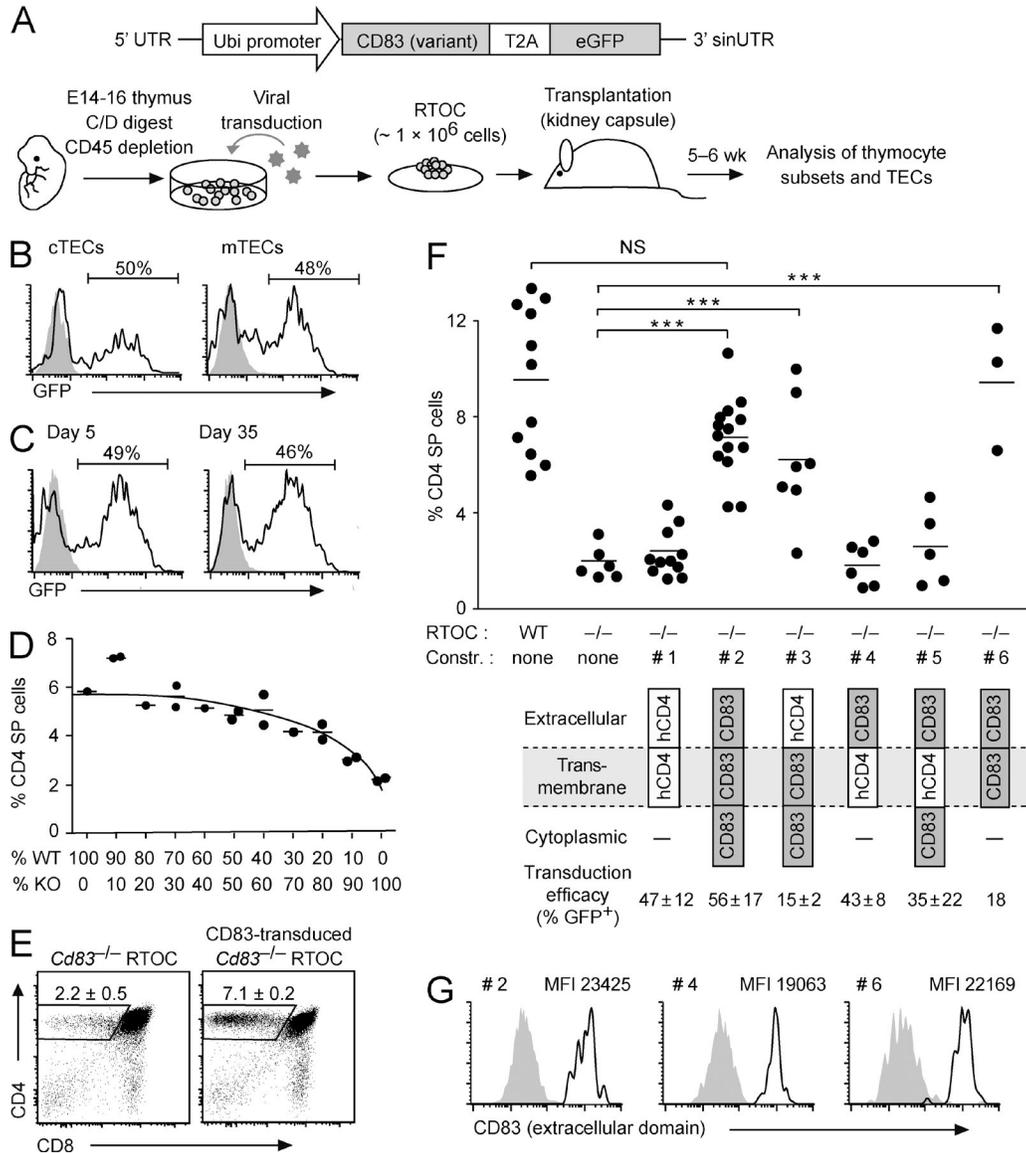


Figure 2. CD83's TM domain is necessary and sufficient for CD4 T cell selection. (A) Experimental strategy to reconstitute expression of CD83 or chimeric variants thereof in TECs *in vivo*. WT or *Cd83*^{-/-} embryonic TECs, obtained by collagenase/dispase (C/D) digestion and CD45 depletion of E14–16 thymuses, were transduced with vectors encoding equimolar amounts of the construct of interest and EGFP via a T2A linker. RTOCs were prepared by 48-h cultivation on a nylon membrane and subsequently transplanted under the kidney capsule. Thymocyte subsets and TECs were analyzed 5–6 wk after transplantation. sinUTR, self-inactivating UTR; Ubi, ubiquitin; UTR, untranslated region. (B) Expression of the virally encoded GFP reporter in gated cTECs (CD45⁻EpCAM⁺Ly51⁺CD80⁻) and mTECs (CD45⁻EpCAM⁺Ly51⁻CD80⁺) from RTOCs prepared with a full-length CD83-encoding vector (#2 in F) on day 7 after transplantation. Gray histograms are noninfected control cTECs or mTECs. (C) GFP expression in TECs from RTOCs on days 5 and 35 after transplantation. Gray histograms are TECs from nontransduced RTOCs. (D) CD4 SP thymocyte frequencies in mixed RTOCs with titrated ratios of WT and *Cd83*^{-/-} TECs. Individual data points (one or two per time point) are indicated. (E) Thymocyte subsets in *Cd83*^{-/-} RTOCs or *Cd83*^{-/-} RTOCs from full-length CD83-transduced material. (F) Frequency of CD4 SP cells in RTOCs from *Cd83*^{-/-} TECs transduced with viral constructs (Constr.) #1–6 (schematically depicted below the respective data points). The mean transduction efficacy (percentage of GFP⁺ cells among gated CD45⁻EpCAM⁺ TECs) ± SEM is shown. $n \geq 3$. (G) TECs from RTOCs transduced with the indicated lentiviral constructs as indicated in F were stained for the EC domain of CD83. Unshaded histograms are gated on GFP⁺ TECs (CD45⁻EpCAM⁺ cells). Gray histograms are gated on GFP⁻ TECs. The MFI of gated GFP⁺ TECs is indicated. Data in B, C, and E are representative of $n \geq 6$ each in at least two independent experiments. In D and F, each data point corresponds to an individual RTOC. ***, $P < 0.001$ (Student's *t* test).

down-modulation of MHCII in cTECs and established that the detrimental effect of CD83 deficiency on CD4 T cell selection is solely attributable to CD83's effect on MHCII.

MHCII ubiquitination in DCs and other hematopoietic APCs is mediated by the E3 ligase March1 (Matsuki et al., 2007; De Gassart et al., 2008). We therefore asked whether

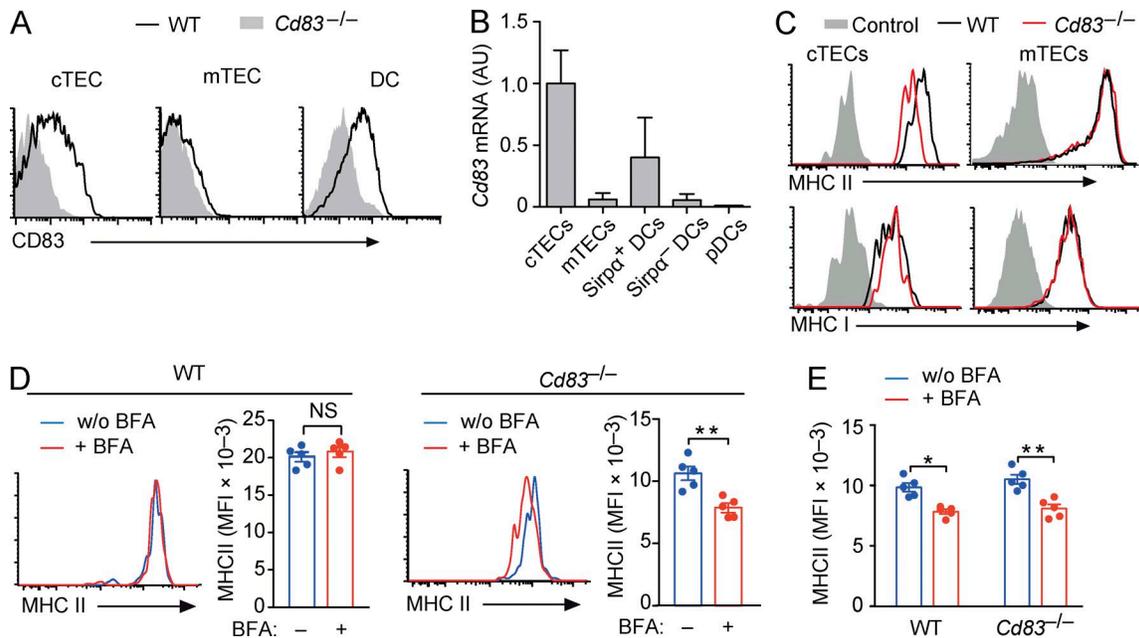


Figure 3. CD83 controls MHCII turnover in cTECs but not mTECs. (A) Surface staining of CD83 on cTECs, mTECs, and thymic DCs from WT or *Cd83*^{-/-} mice. (B) Relative abundance of *Cd83* mRNA in cTECs, mTECs, and three subsets of thymic DCs. Values indicate the mean \pm SD from three biological replicates. AU, arbitrary units; pDCs, plasmacytoid DCs. (C) Surface staining of MHCII or MHC I on cTECs and mTECs from WT or *Cd83*^{-/-} mice. Gray histograms are staining controls from *MHCI*^{-/-} or *MHCII*^{-/-} mice. Data are representative of $n \geq 5$ each. (D) MFI \pm SEM of MHCII on cTECs from WT or *Cd83*^{-/-} mice after in vitro culture for 16 h in the presence or absence of BFA. (E) MHCII expression (MFI \pm SEM) on mTECs from WT or *Cd83*^{-/-} mice after in vitro culture for 16 h in the presence or absence of BFA. Data in D and E are from $n \geq 4$ each in at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$ (paired Student's *t* test).

CD83 controls MHCII in cTECs through opposing *March1*. However, unlike several hematopoietic APC subsets, neither cTECs nor mTECs expressed *March1* mRNA above the detection limit (Fig. 5 A), and MHCII levels on *March1*^{-/-} cTECs were unaffected (Fig. 5 B). Moreover, combined deficiency in CD83 and *March1* did not restore MHCII surface expression on cTECs, and the CD4 SP compartment in *Cd83*^{-/-}*March1*^{-/-} mice was similarly reduced as in *Cd83*^{-/-} mice (Fig. 5 C). Thus, the critical requirement of CD83 in cTECs for MHCII expression and CD4 T cell selection was not a reflection of *March1* antagonism.

The *March* protein family member most closely related to *March1* is *March8* (Ishido et al., 2009). Forced expression of *March8* in hematopoietic APCs reduces surface MHCII, and transgenic overexpression of *March8* in TECs results in a defect in CD4 T cell selection reminiscent of that in CD83-deficient mice (Ohmura-Hoshino et al., 2006). However, the physiological targets of *March8* remain unknown (Ishido et al., 2009). For instance, unlike *March1*, *March8* is not dynamically regulated during DC maturation, and hence, it is not considered a critical player in MHCII regulation in hematopoietic APCs (De Gassart et al., 2008). Within different types of thymic APCs, *March8* mRNA was most strongly expressed in cTECs (Fig. 6 A). Indeed, superimposing CD83 and *March8* deficiency restored MHCII levels on cTECs (Fig. 6 B), and the size of the CD4 SP com-

partment in *Cd83*^{-/-}*March8*^{-/-} mice was indistinguishable from that in WT mice (Fig. 6 C).

In sum, we show that CD83's crucial role for CD4 T cell selection reflects its capacity to attenuate MHCII turnover in cTECs by counteracting *March8*-mediated MHCII ubiquitination. MHCII hemizygous mice, despite a similar reduction of MHCII on TECs as in *Cd83*^{-/-} mice, generate normal CD4 T cell numbers. Thus, a relatively slow MHCII turnover rather than absolute MHCII surface levels as such may be crucial for efficient CD4 T cell selection. This adds to our understanding of how cTECs have adapted their cell biology for T cell selection and how their MHC ligandome is shaped (Klein et al., 2014). The kinetics of cellular interactions during positive selection are only beginning to emerge, but it is tempting to speculate that a long MHCII half-life on cTECs provides a platform for stable and efficient selection events (Müller et al., 1993). Intriguingly, the efficient selection of CD4⁺ but not CD8⁺ T cells seems to be contingent upon prolonged or even repetitive interactions with selecting ligands on cTECs (Singer et al., 2008).

Viral strategies are commonly used to manipulate gene expression in hematopoietic cell types. The transplantation of RTOCs generated with transduced embryonic TECs represents an analogous strategy to study gene function in thymic epithelium in vivo (Travers et al., 2001; Aichinger et al., 2012). By expressing chimeric CD83 variants in *Cd83*^{-/-}

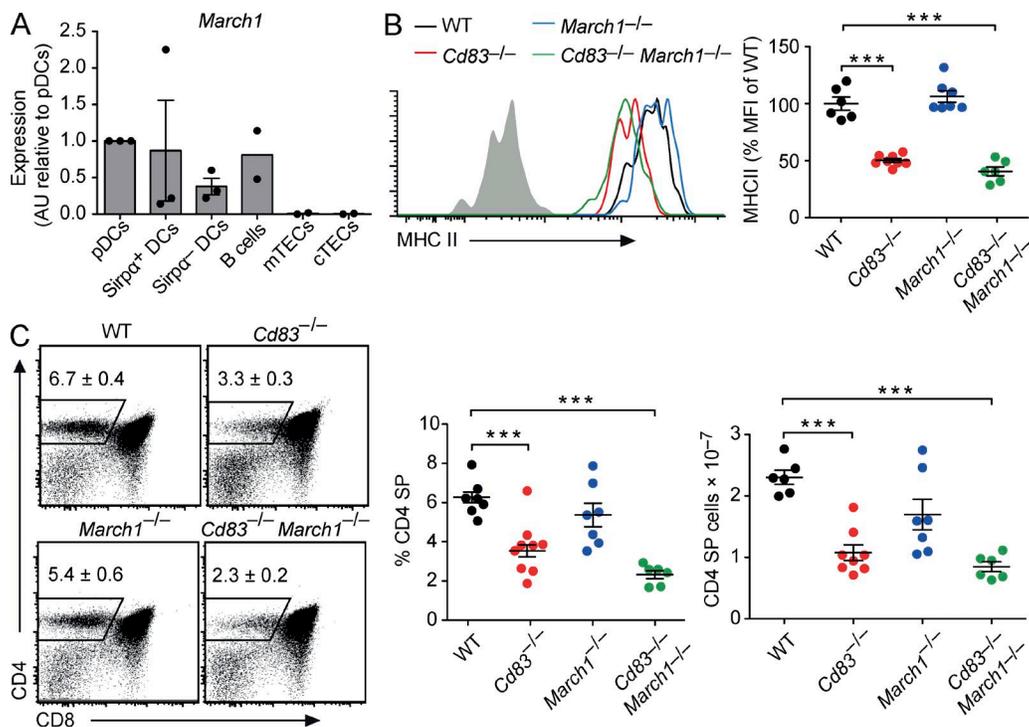


Figure 5. *March1* does not regulate MHCII in cTECs. (A) Relative expression of *March1* mRNA in thymic APCs. Bars indicate the mean \pm SEM of two to three biological replicates that are shown as individual data points. Data are representative of two independent experiments. AU, arbitrary units; pDCs, plasmacytoid DCs. (B) Surface MHCII on cTECs from WT, *Cd83*^{-/-}, *March1*^{-/-}, and *Cd83*^{-/-} *March1*^{-/-} mice. The graph on the right depicts the MFI of MHCII in individual mice relative to WT. Horizontal bars are mean values \pm SEM from at least six mice per group. (C) CD4 and CD8 expression on thymocytes from WT, *Cd83*^{-/-}, *March1*^{-/-}, and *Cd83*^{-/-} *March1*^{-/-} mice. Values indicate the mean frequency of CD4 SP cells \pm SEM among total thymocytes. Cumulative data are summarized on the right. Each data point represents an individual mouse. Data are from $n \geq 6$ for each experimental group. ***, $P < 0.001$ (Student's *t* test).

(M5/114.15.2), H-2K^b (AF6-88.5.5.3), epithelial cell adhesion molecule (EpCAM; G8.8), CD45 (Ly-5), CD11c (N418, HL3), Sirp α /CD172a (P84), Ly51 (6C3), CD83 (Michel-19), CD80 (16-10.A1), CD19 (6D5), and CD317/PDCA-1 (927), conjugated to different fluorochromes or biotin. Dead cells were excluded by gating on DAPI-negative cells. Surface stainings were performed according to standard procedures with $1\text{--}2 \times 10^6$ cells in a volume of 50 μl . FACS measurements were performed on a cell analyzer (FACS Canto II; BD) and analyzed using FlowJo software (Tree Star). Cell sorting was performed on a FACS Aria III cell sorter (BD).

Chimeric or truncated CD83 constructs and lentiviral vectors

Truncated or chimeric CD83 constructs have been described before and were provided by C. Goodnow (Garvan Institute of Medical Research, Darlinghurst, Australia) and K. Hori-kawa (John Curtin School of Medical Research, Australian National University, Acton, Australia). Construct numbers in Fig. 2 correspond to the following construct names in Tze et al. (2011): #1, hCD4; #2, CD83 WT; #3, hCD4 chimera 1; #4, CD83 chimera 1; #5, CD83 chimera 2; and #6, CD83 Δ C. Constructs were subcloned into the lentiviral vector FUGW followed by a T2A peptide and enhanced GFP

(EGFP; pFUGW-T2A-GFP) provided by T. Brocker (Ludwig-Maximilians-University, Munich, Germany). For lentivirus production, confluent HEK293FT cells in a 10-cm culture dish were transiently transfected with 8 μg of the respective lentiviral vector, 6 μg PAX2 packaging plasmid, and 6 μg of VSVG envelope plasmid using a standard calcium phosphate protocol. The supernatant was collected and replaced with fresh medium at 48 and 72 h after transfection, pooled, and centrifuged at 14,000 rpm for 4 h to pellet the virus particles. The pellets were resuspended in 3–4 ml of fresh DMEM and stored at -80°C .

RTOC

Single cell suspensions of E14–E16 fetal thymic lobes were prepared by collagenase/dispase I (Roche) digestion. CD45⁺ cells were depleted using magnetic-activated cell-sorting beads (Miltenyi Biotec) according to standard procedures. Aliquots of 10^6 cells were infected with lentivirus (mean fluorescence intensity [MFI] of 1) in 1 ml DMEM (8% FCS) supplemented with 10 $\mu\text{g}/\text{ml}$ polybrene. After 3 h, cells were washed three times, spun down, and resuspended in $\sim 1 \mu\text{l}$. The cell slurry was deposited onto 0.45- μm nylon membranes (EMD Millipore) floating in 6-well plates containing 6 ml DMEM (8% FCS). RTOCs were incubated for 48 h before transplantation.

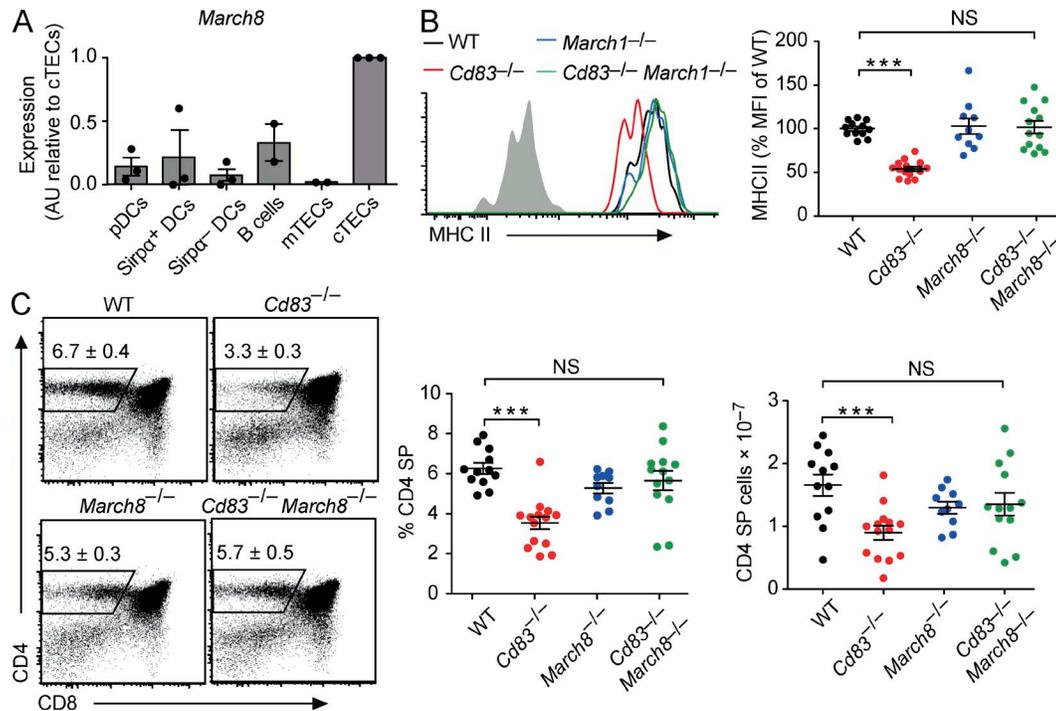


Figure 6. March8 deficiency rescues MHCII expression on cTECs and CD4 T cell selection in CD83-deficient mice. (A) *March8* mRNA expression in thymic APCs (mean \pm SEM of two to three biological replicates). Data are representative of two independent experiments. AU, arbitrary units; pDCs, plasmacytoid DCs. (B) Surface MHCII on cTECs from WT, *Cd83*^{-/-}, *March8*^{-/-}, and *Cd83*^{-/-} *March8*^{-/-} mice. The diagram on the right depicts the MFI of MHCII in individual mice relative to WT. Horizontal bars are mean values \pm SEM from at least nine mice in each group. (C) CD4 and CD8 expression on thymocytes from mice of the indicated genotype. Values indicate the mean frequency of CD4 SP cells \pm SEM among total thymocytes. Cumulative data are summarized in the graph on the right. Each data point represents an individual mouse. Data are from $n \geq 9$ for each experimental group. ***, $P < 0.001$ (Student's *t* test).

Preparation of thymic APCs

Thymuses of 3–5-wk-old animals were cut into pieces, and thymocytes were mechanically released by pipetting up and down. The supernatant containing thymocytes was discarded. The thymus fragments were digested with 0.5 U/ml Liberase Thermolysin medium (Roche) at 37°C in two consecutive rounds of 15 min. Cells were washed and resuspended in 1 ml of high-density Percoll ($\rho = 1.115$; GE Healthcare) and overlaid with 1 ml of low-density Percoll ($\rho = 1.055$) followed by a layer of 1 ml RPMI. The gradient was centrifuged at 1,350 *g* for 30 min at 4°C (without brake). The upper interphase containing the low-density cell fraction was harvested, washed, and stained for FACS sorting. TECs, DC subsets, and B cells were sorted according to surface expression of CD45, Ly51, EpCAM, CD80, CD19, CD11c, PDCA-1 (CD317), and Sirp α (CD172a) as follows: cTECs, CD45⁻EpCAM⁺Ly51⁺CD80⁻; mTECs, CD45⁻EpCAM⁺Ly51⁻CD80⁺; B cells, CD45⁺CD11c⁻CD19⁺; plasmacytoid DCs, CD45⁺CD11c^{int}CD317⁺; Sirp α ⁺ classical DCs, CD45⁺CD11c^{high}CD172a⁺; and Sirp α ⁻ classical DCs, CD45⁺CD11c^{high}CD172a⁻.

MHCII decay on isolated TECs

The low-density fraction of enzymatically prepared thymic cells (see the previous section) was plated in a 96-well plate (2×10^5 cells/well in 200 μ l) in DMEM (8% FCS) with or

without 5 μ g/ml BFA (Sigma-Aldrich). After 16-h incubation at 37°C, cells were collected and stained for FACS analyses. In parallel, some cells were kept for 16 h at 4°C as controls.

BM chimeras

BM was depleted of T cells using biotinylated CD8 α and CD4 monoclonal antibodies and streptavidin magnetic-activated cell-sorting beads (Miltenyi Biotec). Recipient mice were irradiated with 2×550 rad and reconstituted with 10^7 BM cells.

Quantitative PCR

RNA was isolated from thymic APCs using the Arcturus PicoPure RNA isolation kit (Applied Biosystems). Purified RNA was subjected to DNase digestion (QIAGEN) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories) containing both oligo deoxythymidine and random hexamer primers. Quantitative PCR reactions were performed on a real-time thermal cycler (CFX96 C1000; Bio-Rad Laboratories) using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories). Fluorescence was recorded at the annealing step, and relative expression levels were calculated with the comparative cycle threshold method, using β -actin as the housekeeping gene. The primers were *March1*-5', AAGAGAGCCCACTCATCACACC;

March1-3', ATCTGGAGCTTTTCCCACTTCC; March8-5', AGTAGTCCTCCATCCACGAC; March8-3', GATGACGAGAGCCCTCTGAT; CD83-5', GCCTCCAGCTCCTGTTTCTA; CD83-3', AGTGTTTTGGATCGTCAGGG; β -actin-5', GCCTTCCTTCTTGGGTAT; and β -actin-3', GGCATAGAGGTCTTTACGG.

Statistical analysis

Unless indicated otherwise, statistical significance was assessed using the two-tailed unpaired Student's *t* test with Welch's correction for unequal variances.

Online supplemental material

Fig. S1 provides details on the targeting of the *March8* gene locus. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20160316/DC1>.

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