T cells specific for post-translational modifications escape intrathymic tolerance induction

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Establishing effective central tolerance requires the promiscuous expression of tissue-restricted antigens by medullary thymic epithelial cells. However, whether central tolerance also extends to post-translationally modified proteins is not clear. Here we show a mouse model of autoimmunity in which disease development is dependent on post-translational modification (PTM) of the tissue-restricted self-antigen collagen type II. T cells specific for the non-modified antigen undergo efficient central tolerance. By contrast, PTM-reactive T cells escape thymic selection, though the PTM variant constitutes the dominant form in the periphery. This finding implies that the PTM protein is absent in the thymus, or present at concentrations insufficient to induce negative selection of developing thymocytes and explains the lower level of tolerance induction against the PTM antigen. As the majority of self-antigens are post-translationally modified, these data raise the possibility that T cells specific for other self-antigens naturally subjected to PTM may escape central tolerance induction by a similar mechanism.
Central T cell tolerance is established in the thymus where developing thymocytes that react strongly with self-antigens are either negatively selected and deleted or alternatively deviated into the T regulatory cell lineage. Central tolerance not only encompasses ubiquitous and circulating self-antigens, but also a large set of tissue-restricted self-antigens (TRAs) that are ectopically expressed in medullary thymic epithelial cells (mTEC). The expression of TRA by mTEC is to a large extent controlled by the autoimmune regulator (Aire) protein, and dysfunction of Aire is associated with defective central tolerance and autoimmune disorders. Nevertheless, autoreactive T cells exist in the periphery of healthy individuals, indicating that central tolerance is incomplete.

Most proteins are subject to different types of post-translational modifications (PTMs), e.g., phosphorylation or glycosylation, which often change the structure and function of the protein. Moreover, PTMs are also likely to change the way the protein is processed and recognized as a self-antigen by immune cells. T cell reactivity to PTM self-antigens has been considered to be an initiating and/or perpetuating factor in the progression of autoimmune diseases. PTM of self-antigens have been reported in patients with rheumatoid arthritis (RA) and type 1 diabetes. Such modifications have been shown to affect the binding of the antigen to the major histocompatibility complex (MHC) molecule, and consequently affect T cell activation. A similar mechanism has been described in detail for celiac disease, in which modification of gliadin peptides enables recognition by gut T cells. PTM can occur spontaneously, like oxidation or nitrosylation, or be enzyme-mediated like citrullination and glycosylation. In either case, the occurrence and degree of PTM is dependent on a number of host factors such as the compartmentalization of the enzyme or protein, regions flanking the amino acid to be modified, as well as physiological factors like pH and redox states. PTM of a self-antigen may occur naturally, in order to generate the desired biological activity of a protein, or in response to infection, inflammation, or physical damage. In the latter scenarios, creation or exposure of neo-epitopes to which the immune system has not been previously tolerized is possible. Nevertheless, leaky self-tolerance might also arise under non-pathological conditions. In this situation, the PTM self-antigen would only occur in peripheral tissues, while being absent from central lymphoid organs such as the thymus.

**Fig. 1** Summary of the autologous CIA model. **a** The MMC mouse expresses the immunodominant T cell epitope of heterologous (rat/human) CII as a self-antigen in the cartilage. A D266E amino acid substitution is the only difference between mouse and heterologous CII. A lysine at position 264 can become post-translationally modified by hydroxylation (not shown) and subsequent glycosylation with a monosaccharide. These modifications are recognized by distinct T cell clones (shown in **d**). MMC mice are immunized with heterologous CII (in this study rat CII) in order to induce an autoreactive T cell response. **b** The Nrfl mutation was inserted onto the BQ background in order to render arthritis susceptibility in MMC mice. IFN-γ ELISPOT of lymph node cells isolated 10 days after immunization with rat CII and following re-stimulation with the non-modified (native) or glycosylated (PTM) CII peptide are shown. Bars indicate the mean number of spots ± SEM. **c** Statistical significance (p = 0.0195) between native and PTM responses in BQ.Ncfl/MMC mice, using a Wilcoxon’s test. **d** Arthritis susceptibility of MMC mice in the BQ.Ncfl background is reduced but not completely abrogated, when compared to non-MMC littersmates. Number in brackets indicates cumulative number of animals that developed arthritis over total number of animals. **d** IL-2 production of T cell hybridoma clones specific to either the non-modified (HCQ.4) or PTM (HCQ.3) variants of the CII260-270 epitope after stimulation with the different peptides as well as whole CII protein obtained from different species (hu, human (two independent preparations/samples); bov, bovine (calf) CII from joint cartilage. The chondrosarcoma is a rat tumor line that was used as a positive control as it produces CII that is very heterogeneous in terms of PTMs. Mann-Whitney U test was used in ELISPOT assays. *p < 0.05; ***p < 0.001

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![Diagram](https://example.com/diagram.png)
With regard to central tolerance against TRA, it is unknown whether antigen-presenting cells (APC) in the thymus have all the necessary machinery and/or whether the intracellular environment allows for all potential PTM to be generated and displayed in order to impose tolerance. There are many examples of autoreactive T cell responses specifically directed against PTM variants of self-antigens\(^{10-12}\); however, a formal demonstration that PTM are exempt from central tolerance is required.

**Results**

Aire mediates tolerance to the native antigen and not PTMs. In order to investigate how physiological PTM of autoantigens may affect central tolerance and the susceptibility to a tissue-restricted autoimmune disease, we made use of the autologous collagen-induced arthritis (CIA) model for RA. In this model, mice expressing a point-mutated collagen type II (CII) molecule mimicking the human/bovine/rat T cell epitope (MMC mouse, for mutated mouse collagen\(^{13}\); Fig. 1a) can be immunized with either of these CII molecules. Due to accessibility, we have used rat CII in our immunization protocols. Whereas in the traditional CIA model the T cell response is raised solely against the immunized foreign CII protein, with no cross-reactivity to mouse self-CII; in the autologous CIA model, the T cell responses are directed against the heterologous CII expressed in the joint cartilage of the MMC mouse. Hence, efficient tolerization of T cells specific for the immunodominant CII epitope present on human, rat, bovine, or chicken CII (amino acids 260–270; CII\(_{260-270}\)) can only take place in the MMC mouse\(^{14}\). Importantly, expression of the heterologous CII molecule (in MMC mouse) has been shown to be naturally regulated and to occur exclusively at sites of known physiological CII expression, e.g., joint cartilage and the eye\(^{13}\).

The tolerance status in MMC mice can be determined by analyzing the ex vivo phenotype and activation of CII\(_{260-270}\) specific T cells, and by monitoring the susceptibility of MMC mice to develop autoimmune arthritis. Most importantly, the CII\(_{260-270}\) epitope is naturally subjected to PTM. The lysine at position 264 constitutes a critical TCR contact point and is naturally subjected to hydroxylation and glycosylation, where each of the two variants is recognized by distinct T cell clones\(^{15,16}\). To induce autoimmunity against self-CII, MMC mice and control wild-type littermates (WT) were immunized with rat CII and monitored for development of arthritis. All mice had a mutation in the Ncf1 gene, which enhances arthritis susceptibility and allows for the development of arthritis in MMC mice\(^{17}\). The expression of heterologous CII in the joints of MMC mice resulted in fewer numbers of activated CII-reactive T cells, after antigen immunization (Fig. 1b). Consequently, MMC mice were less susceptible to arthritis (Fig. 1c). These observations suggest that T cells in the MMC mouse are tolerized to heterologous CII. Nevertheless, such tolerance remained incomplete. Whereas an almost completely abrogated response was observed against the native form of CII\(_{260-270}\), T cell reactivity to the PTM CII\(_{260-270}\) peptide remained significant (\(p=0.0195\), Wilcoxon’s test)\(^{18}\). The absence of response to the non-modified CII\(_{260-270}\) epitope suggests the possibility that the native variant is abundantly available for tolerance induction in vivo. To test this, we extracted CII from healthy joint cartilage of both human and bovine sources, and evaluated their level of PTMs. In both cases, extracted CII was only able to activate T cell clones specific for the PTM variant (Fig. 1d), supporting an earlier observation that CII from healthy cartilage is uniformly glycosylated in both rodents and humans\(^{19}\).

Based on these observations, we note an apparent discrepancy in the relative abundance of CII expression in the periphery and the overall level of tolerance. Hence, the data suggest that tolerance towards the two variants of the antigen may be differentially regulated. In order to investigate a potential role of the thymus in this biased tolerance regulation, we generated Aire-sufficient and Aire-deficient MMC mice (hereafter denoted as MMC.Aire\(^{\text{Suf}}\) and MMC.Aire\(^{\text{KO}}\), respectively). Arthritis protection in the autologous CIA model was found to be Aire-dependent, as MMC.Aire\(^{\text{KO}}\) mice developed more arthritis, and a stronger humoral response, when compared to MMC.Aire\(^{\text{Suf}}\) littermates (Fig. 2a and Supplementary Fig. 1a and b).

Analyses of T cell recall responses early after immunization, or during the chronic phase of disease, showed that the higher arthritis susceptibility of MMC.Aire\(^{\text{KO}}\) mice was coupled to a specific increase in reactivity against the non-modified antigen (Fig. 2b, c). Whereas MMC.Aire\(^{\text{Suf}}\) mice mounted a substantial response only to the PTM peptide, MMC.Aire\(^{\text{KO}}\) mice displayed an immunodominant response to the non-modified peptide, with an unaltered PTM peptide response. Similar data were also obtained for IL-17A-producing cells (Supplementary Fig. 2b).

CII is promiscuously expressed in mouse and human mTECs. Taken together, these observations strongly suggest that tolerance to self-CII is dependent on its molecular structure (native or PTM form), and that it is controlled at the thymic level. The Aire
dependence of thymic CII expression has been controversial.\textsuperscript{20,21} The discrepancies may, in part, be explained by different mouse strains and normalization of gene expression data.\textsuperscript{22} Using insulin (Ins2) and glutamic acid decarboxylase 67 (Gad67) as Aire-dependently and Aire-independently expressed prototypic genes, respectively, we found CII (Col2a1) to be expressed in the thymus in a partially Aire-dependent manner (Fig. 3a, b; and Supplementary Fig. 3 for WT mice). CII mRNA transcripts were only detected in the thymus but not in secondary lymphoid organs (e.g., spleen), and included the CII 260–270 epitope, as determined by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3b) and Sanger sequencing. Using monoclonal antibodies specific for triple helical CII,\textsuperscript{23,24} we were able to confirm expression at the protein level in murine and human thymus samples (Fig. 3c, d, respectively). Interestingly, CII+ cells coincided with medullary regions and mTEC (Fig. 3d and Supplementary Fig. 4). However, contrary to CII in the joints, the K264 on CII was not glycosylated when CII was expressed in the thymus (Fig. 3e, f). Quantification of CII+ cells in murine and human thymus (Supplementary Table 1) suggests, once again, that CII constitutes a self-antigen involved in central selection of thymocytes.

Central tolerance is induced by presentation of native epitope. The specificity of the T cell receptor is promiscuous.\textsuperscript{25} To exclude the possibility that thymic selection towards the non-modified CII 260–270 peptide (Figs. 1b and 2b, c) did not result from thymic presentation of an unrelated (but Aire-dependent) autoantigen to which these T cells would cross-react, we grafted 3–4 weeks old naturally athymic nude mice (H-2Aq) with MMC or WT thymi. After establishment of a peripheral T cell pool (>95% of recipient
Fig. 4 mTEC-mediated central tolerance to self-CII is limited to the native epitope. a Four-week-old nude mice were grafted with neonatal thymus from either WT or MMC donors (n = 5/group). Eleven weeks later, mice were immunized with rat CII and draining lymph node cells were re-stimulated in vitro with different CII peptides. b MMC-positive (n = 8) and MMC-negative (n = 6) nude mice were grafted with neonatal thymus from wild-type donors. Eleven weeks later, recipients were immunized with rat CII and draining lymph node cells were re-stimulated in vitro with different CII peptides. Values shown are the mean ± SEM number of spots recorded for IFN-γ-producing and IL-17A-producing cells. p values were calculated using Mann–Whitney U test. *p < 0.05; **p < 0.01; NS not significant.

Migratory APCs can transfer PTM CII to the thymus. To investigate to what extent the thymus contributes to central tolerance to the PTM epitope variant, we developed a TCR transgenic mouse line expressing an αβ TCR (HCQ3)15, which is specific for the PTM variant of the heterologous CII260–270 peptide in the context of A^k. Importantly, transgenic HCQ3 T cells do not cross-react with the non-modified CII peptide (Supplementary Fig. 6a). When transferred to naive WT or MMC recipients, naive HCQ3 T cells proliferated vigorously in joint-draining lymph nodes of MMC, but not WT mice, as assessed by carboxyfluorescein succinimidyl ester (CFSE) dilution (Supplementary Fig. 6b). Furthermore, HCQ3.MMC double-positive mice displayed a reduced recall response to the PTM peptide (Supplementary Fig. 6c). Together, these data show that the PTM peptide is indeed expressed for immune recognition in the periphery of naive MMC mice. Moreover, it shows that HCQ3 T cells are only tolerated when in the context of MMC co-expression.

Next, we determined the frequency of thymocytes specific for the PTM peptide in HCQ3.MMC compared with single-positive HCQ3 littersmates. There was a minor but significant decrease of CD4 single-positive (CD4SP) thymocytes in HCQ3.MMC mice (Fig. 5a). Due to lack of reliable peptide–MHC class II tetramers for CII260–270, we assessed the immediate up-regulation of CD40 ligand (CD40L) on stimulated T cells. CD40L has previously been shown to serve as a specific and unbiased marker for antigen-specific T cells regardless of their cytokine profile26. Upon ex vivo stimulation with the PTM peptide, we observed that the frequency of HCQ3.MMC thymocytes able to up-regulate CD40L was significantly reduced, in comparison to HCQ3 thymocytes (Fig. 5b).

Thymic selection by Aire-expressing mTEC depends on the interaction between specific TNF family members, such as RANK–RANKL and CD40–CD40L27-30. Mice treated with anti-RANKL monoclonal antibody (mAb) are transiently depleted of Aire-expressing mTEC, which consequently affects negative selection31. We then treated HCQ3.MMC mice with anti-RANKL mAb and assessed T cell selection. Indeed, Aire-expressing mTEC were depleted after mAb treatment (Fig. 5c). Nevertheless, considering the similar levels of HCQ3 cells able to respond to the PTM peptide, ablation of Aire+ mTECs did not affect negative selection of HCQ3 thymocytes (Fig. 5d). Taking into consideration the data shown in Figs. 2 and 4, this observation supports the notion that tolerance to the non-modified antigen is established in the thymus and is dependent...
Peripheral antigen migration mediates thymic tolerance to the PTM epitope. Frequency of (a) CD4 single-positive and (b) CD4+CD40L+ thymocytes from naive HCQ3 and HCQ3.MMC mice, after antigen stimulation in vitro. Cells left unstimulated (No Ag) or concanavalin A (ConA) stimulated were used as negative and positive controls, respectively. Treatment with anti-RANKL antibody induces selective depletion of Aire-expressing mTECs in the thymus. Indicated number of mice was treated with 100 μg of anti-RANKL or an isotype control antibody every second day for 2 weeks, and thymi were collected and analyzed the following week. Dot plots show representative examples of outcome following antibody treatment. Treatment with anti-RANKL antibody does not alter central tolerance of HCQ3 transgenic T cells in HCQ3.MMC mice as determined by frequencies of CD4SP thymocytes (left) or up-regulation of CD40L, as described in (c). CII260-270 peptide. Cells cultured in the absence of antigen (No Ag) and in the presence of PMA/ionomycin (PMA/Ion) were used as negative and positive controls, respectively. (d) Three- to four-week-old HCQ3-nude and HCQ3.MMC-nude mice were grafted with neonate thymus from wild-type mice. Thymus and pooled spleen and lymph nodes from individual mice were recovered 14–21 weeks after transplantation, when recipient-derived T cells constituted more than 95% of the peripheral T cell pool (as determined by CD45.1 expression) and investigated for up-regulation of CD40L, as described in (d). (e) CD11c+ cells were enriched from spleens and lymph nodes of naive MMC or WT donors and transferred to the indicated number of naive HCQ3 mice. Two weeks later, recipient mice were sacrificed and thymocytes were prepared and investigated ex vivo for frequency of CD4SP cells (left) and up-regulation of CD40L, as described for in (d). Data from two pooled experiments are shown. *p values were calculated by unpaired t test. *p < 0.05; **p < 0.01. Gating strategies used for analysis of flow cytometry data are shown in Supplementary Fig. 8.
Whether T cells specific for other PTM self-antigens escape central tolerance of T cells speciﬁc for other PTM self-antigens escape central tolerance. It has been difﬁcult to answer this question due to the low levels of the self-antigen in the thymus. However, recent studies have shown that some PTM self-antigens are able to induce central tolerance. A critical event in the development of central tolerance is the selection of developing thymocytes. However, despite its availability in the thymus, and sufﬁcient presentation of iodinated thyroglobulin in the thymus is unlikely to occur due to its very low blood levels. Consequently, T cells speciﬁc for thyroglobulin-derived peptides harboring iodinated tyrosine are likely to escape central tolerance, while iodinated thyroglobulin constitutes the physiological form in the tissue. In case of multiple sclerosis (MS), the autoreactive responses are thought to be in part directed against myelin basic protein (MBP). The N-terminal part of MBP (MBPα,1–11), where the alanine at position 1 is acetylated (MBPα,1–11), constitutes the primary target for encephalitogenic T cells in an animal model of MS. In contrast, T cells do not respond to the non-acetylated MBPα,1–11 peptide. Thymic expression of MBP protein is limited, but the MBPα,1–11 sequence is readily available through the more widely expressed golli-MBP protein. However, as golli-MBP expression is regulated by distinct upstream promoters, the MBPα,1–11 sequence is not situated N-terminally in golli-MBP and therefore it is not presented in its acetylated form as to induce tolerance. Finally, with regard to the autoantigen studied here, the regulation of glycosylation is very complex and differs between different proteins, cell types, and species. It is not clear how glycosylation of CII occurs, and how it is regulated enzymatically in vivo. CII is normally produced by chondrocytes, which have the capacity to hydroxylate and glycosylate lysine residues. However, analyses of recombinant CII protein, or CII260–270 peptides produced from different cell types, have suggested that ﬁbroblast-like cells, but not B cells, DCs or macrophages can produce galactosylated CII. This clearly shows that the capacity for PTM of lysine residues is not a ubiquitous phenomenon. It is well documented that immune homeostasis. However, from an immunological point of view, PTM constitute a challenge to the immune system, since they greatly increase the number of self-antigens against which tolerance must be established. Because some PTM only occur in speciﬁc cell lineages in a given tissue, we raised the hypothesis that some of these PTM may not be generated and/or presented in the thymus at a sufﬁcient level as to establish T cell tolerance. Using CII as a model autoantigen that displays a critical involvement of PTM in T cell reactivity, we report here that tolerance towards the non-modiﬁed and PTM variants of the same antigen are independently and differentially regulated. We show that, in contrast to the non-modiﬁed antigen, the PTM variant obviously failed to be generated by mTEC in the thymus. However, the perceptible impact of the PTM epitope on intrathymic T cell selection could be traced back to its exclusive expression in peripheral tissues. Hence, peripheral expression of the PTM antigen is potentially able to establish tolerance via anergy or ignorance induction, as well as by negative selection and/or Treg induction of developing thymocytes. However, despite its availability in both peripheral and central lymphoid organs, PTM-speciﬁc autoreactive T cells persisted within the peripheral repertoire and allowed for development of autoimmunity (Fig. 2).

Theoretically, most self-antigens could be carried into the thymus by APC, and establish tolerance to newly generated/ modiﬁed proteins. However, as shown here and earlier, as well as evidenced by the strong phenotype of autoimmune polyendocrine syndrome I patients, peripheral tolerance mechanisms and/or transport of peripheral self-antigens to the thymus cannot compensate for the lack of autochthonous thymic-induced central tolerance. A critical event in the development of MHC class II-associated autoimmune diseases is likely to be associated with T cell recognition of poorly tolerized PTM neoepitopes. One example is thyroglobulin, which constitutes a target in autoimmune thyroiditis, and in which case PTM through iodination have been proposed as a mechanism leading T cells to escape tolerance. Although thyroglobulin is available in the thymus for central tolerance induction, iodination does not occur in the thymus, and sufﬁcient presentation of iodinated thyroglobulin in the thymus is unlikely to occur due to its very low blood levels. Consequently, T cells speciﬁc for thyroglobulin-derived peptides harboring iodinated tyrosine are likely to escape central tolerance, while iodinated thyroglobulin constitutes the physiological form in the tissue. In case of multiple sclerosis (MS), the autoreactive responses are thought to be in part directed against myelin basic protein (MBP). The N-terminal part of MBP (MBPα,1–11), where the alanine at position 1 is acetylated (MBPα,1–11), constitutes the primary target for encephalitogenic T cells in an animal model of MS. In contrast, T cells do not respond to the non-acetylated MBPα,1–11 peptide. Thymic expression of MBP protein is limited, but the MBPα,1–11 sequence is readily available through the more widely expressed golli-MBP protein. However, as golli-MBP expression is regulated by distinct upstream promoters, the MBPα,1–11 sequence is not situated N-terminally in golli-MBP and therefore it is not presented in its acetylated form as to induce tolerance. Finally, with regard to the autoantigen studied here, the regulation of glycosylation is very complex and differs between different proteins, cell types, and species. It is not clear how glycosylation of CII occurs, and how it is regulated enzymatically in vivo. CII is normally produced by chondrocytes, which have the capacity to hydroxylate and glycosylate lysine residues. However, analyses of recombinant CII protein, or CII260–270 peptides produced from different cell types, have suggested that fibroblast-like cells, but not B cells, DCs or macrophages can produce galactosylated CII. This clearly shows that the capacity for PTM of lysine residues is not a ubiquitous phenomenon. It is well documented that immune

Discussion

The relevance of PTM antigens has been extensively discussed as a potential cause or perpetuation of autoimmunity (reviewed in ref. 24). In fact, T cell reactivity to PTM antigens are frequently found in patients suffering from RA or type I diabetes. However, unambiguous experimental validation of this scenario has been missing. Here, we demonstrate for the ﬁrst time that central tolerance to non-modiﬁed and PTM variants of the same self-antigen is differently regulated. It is important to stress that the current study only addresses immunological tolerance to CII. Whether T cells speciﬁc for other PTM self-antigens escape central tolerance in a similar manner as described here needs to be addressed in subsequent studies.

Previous studies using TCR transgenic mice have made clear that thymic central selection plays an important role in achieving T cell tolerance. Promiscuous presentation of TRA on mTEC through Aire, Fz2, or yet unknown mechanisms, induces self-tolerance via negative selection and/or Treg induction in the nascent T cell repertoire. It has also been demonstrated that peripheral expressed antigens can be transported to the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction. Despite the different roads leading to central tolerance, it has also been demonstrated that peripheral mechanisms can achieve non-responsiveness to self-antigens that are not available for presentation in the thymus by migratory APC, and thus contribute to central T cell tolerance induction.
responses to CII, including PTM with citrullination, oxidation and glycosylation, are frequently observed in RA patients. Furthermore, T cell autoimmunity to CII is biased towards the glycosylated CII_{260–270} PTM epitope in RA patients, implying that T cell tolerance to non-modified CII is strictly imposed. In this regard, the increased arthritis susceptibility observed in Aire-deficient MMC mice may at first glance seem contradictory, as the non-modified form of the CII epitope is not a target structure in peripheral tissue. However, the rat CII used for immunization is derived from a Swann rat chondrosarcoma that, in contrast to healthy joint-derived cartilage, also expresses the non-modified epitope (Fig. 1d). Following immunization, the more frequent T cells specific for the non-modified CII epitope in Aire-deficient mice may become activated and deliver help to B cell-producing CII-reactive antibodies that cross-react to mouse CII and thus induce clinical arthritis.

Our finding that T cell tolerance to distinct variants of the same self-antigen operates at different levels exemplifies a mechanism whereby autoreactive T cells can escape central tolerance induction. Although the generalization to other autoantigens needs to be further investigated, the scenario described for CII can in principal be applicable to any autoantigen which may be post-translationally modified, but where the capacity to perform these modifications is restricted to certain cell lineages, tissues, or environments. Importantly, PTM may also alter proteolysis of the self-antigens and consequent peptide sequence presentation and signal strength required for T cell selection and/or induction of thymic Tregs.

Methods

Mice. All mouse strains used were on the C57BL/10.Q (B10.Q, or simply BQ) genetic background. This strain expresses the MHC class II allele Aq, which presents the CII_{260–270} peptide. Other genes have been introduced by backcrossing mice to the respective littermates, if used for experimental purposes (see below). Ncf1 mutant mouse strain differs from B10.Q simply by the Ncf1m1j mutation, which results in a reduced function of the NOX2 complex.

Ncf1-mutated mice are thus highly susceptible to arthritis development. Aire-deficient mice were obtained from The Jackson Laboratory on a C57BL/6 background, and were subsequently backcrossed onto B10.Q for six generations. The MMC transgenic mouse carries a principal be applicable to any autoantigen which may be post-translational modified, whereas the capacity to perform these modifications is restricted to certain cell lineages, tissues, or environments. Importantly, PTM may also alter proteolysis of the self-antigens and consequent peptide sequence presentation and signal strength required for T cell selection and/or induction of thymic Tregs.

Antigens. The rat CII was obtained from peptide-digested SWARM, and subsequently processed as previously described. CII peptides, containing the 259–273 sequence of rat CII with a non-modified lysine at position 264 with a [β]-galactospyrano/ residue on l-hydroxylysine at position 264 (PTM), were synthesized as previously described.

Collagen-induced arthritis. Mice were injected at the base of the tail with 100 μl emulsion consisting of 100 μg rat CII emulsified 1:1 in CFA (Difco, Detroit, MI, USA). Development of clinical arthritis was followed three times weekly through visual scoring of the paws, starting 2 weeks after immunization. The arthritis was scored using a scale ranging from 1 to 15 for each paw, with a maximum score of 60 per mouse. Each arthritis toe and knuckle was scored as 1, with a maximum of 10 per paw. A score of 5 was given to an arthritic ankle.

Thymus transplantation. Noninvasive thymus transplantation was performed as described by Basso et al. Thymic lobes removed from newborn pups were immediately grafted into the axillary cavities of 3–4-week-old nude mice. The incision was closed with sutures, and the mouse was placed in a warm environment until the incision healed from anasthesia. All mice were grafted with one entire thymus in one of the axillary cavities. Peripheral blood from transplanted mice was collected at regular intervals and samples were analyzed by flow cytometry. The establishment of a peripheral T cell pool was followed by monitoring the expression of CD45.1 (recipient-derived) and CD45.2 (donor-derived) on CD4 T cells obtained from peripheral blood (Supplementary Fig. 5). Mice were immunized with CII in CFA 11 weeks after transplantation, when more than 95% of the peripheral T cell pool was of recipient origin.

Immunassays. ELISPOT assays were performed as previously described. Briefly, mice were immunized with CII in CFA, and 10 or 70 days later cells were prepared from the spleen and draining lymph nodes and re-stimulated with CII for 4 days. For detection of interferon-γ (IFN-γ) spots, plates were pre-coated according to the manufacturer's instructions with R46A2 clone (10 μg ml⁻¹; from our in-house collection) and detection was achieved with biotinylated AN18.17.24 (Mabtech, Nacka Strand, Sweden). For IL-17A detection, TC11-18H10 (4 μg ml⁻¹; BD Biosciences, San Diego, CA, USA) and biotinylated TC11-8H4.1 (2 μg ml⁻¹; BD Biosciences) were used as capture and detection antibodies, respectively. Binding of biotinylated antibody was revealed by using streptavidin-conjugated alkaline phosphatase and the substrate Sigma Fast BCIP/nitroblue tetrazolium (Sigma).

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Qualitative and quantitative RT-PCR. Complementary DNA (cDNA) was obtained by extraction (PureLink RNA Mini Kit, Ambion, Life Technologies) and conversion (High Capacity cDNA Reverse Transcription Kit, Life Technologies) of total RNA derived from thymi of Aire⁺⁻ mice and Aire⁺/+ mice. Gene-specific primers and probe were designed (Eurogentec). cDNA was synthesized from genomic DNA of HCQ3 cells, and inserted into the cassette vectors for expressing TCR α- and TCR β-chain. This mouse was subsequently used as a founder for the HCQ3 transgenic strain. Athymic nude mice (Nu/J, H-2q) were obtained from The Jackson Laboratory on a C57BL/6 genetic background and T cells were enriched from peripheral blood (Supplementary Fig. 5). Mice were immunized with CII in CFA 11 weeks after transplantation, when more than 95% of the peripheral T cell pool was of recipient origin.

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[β]-galactospyranosyl residue on l-hydroxylysine at position 264 (PTM), were synthesized as previously described. Collagen-induced arthritis. Mice were injected at the base of the tail with 100 μl emulsion consisting of 100 μg rat CII emulsified 1:1 in CFA (Difco, Detroit, MI, USA). Development of clinical arthritis was followed three times weekly through visual scoring of the paws, starting 2 weeks after immunization. The arthritis was scored using a scale ranging from 1 to 15 for each paw, with a maximum score of 60 per mouse. Each arthritis toe and knuckle was scored as 1, with a maximum of 10 per paw. A score of 5 was given to an arthritic ankle.

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coupling steps were monitored using the Kaiser test. After incorporation of the second Gly residue and Fmoc deprotection, the orthogonally protected amino acids Fmoc-Lys(tBu)-OH and Fmoc-Lys(tBu)-OH were coupled sequentially to the peptide resin. The three identical α-chains were assembled simultaneously using amino acid (5 equiv), HCTU (4.5 equiv), and DIPEA (10 equiv) with respect to each amine, and coupled for 2 x 20 min. For THPGal, the automated synthesis was paused at position 264. (S)-N-acetyl-2-fluorenylmethoxycarbonyl-L-n-benzylxycarbonyld-3-oxa-L-azepanopyrrold-8-yl-lysine (1.5 equiv) was activated with HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (1.4 equiv) and DIPEA (4 equiv) and coupled manually for 3 h. Following Kaiser test to verify complete coupling, the THP-Gal peptide was precipitated and washed three times with cold diethyl ether (Et2O), followed by lyophilization. The crude peptide was dissolved in 0.1 M acetic acid (4 ml per 20 mg batch) and purified with reverse-phase high-performance liquid chromatography (HPLC) (Variosphere C18/100) with triethylamine/formic acid/H2O/triisopropylsilane/ethanedithiol (94:2:2:1:1 (millidegrees) at 222 nm while the temperature was continuously increased over a range of 5–85 °C at a rate of 1 °C per minute. The in silico reverse-phase HPLC. Neutralization was achieved by the addition of acetic acid (9–10 ml) and the solution was then concentrated under reduced pressure, after which the residue was purified using reverse-phase HPLC followed by lyophilization. The yields of the final THPs were 7.7% and 13.0%, respectively, for THPGal and THPK.

The identity of THPGal and THPK was verified by MALDI-TOF-MS (Voyager-DE Pro, Applied Biosystems) using a sinapinic acid matrix and with detection in the positive mode. The m/z value for THPGal [M+H]+ 16,626.61, found 16,624.86 (13 ppm) for THPK [M+H]+ 16,091.96, found 16,081.41 (Circumstantial evidence was supposed to be used, both to verify the triple helical structure of the two THPs and to analyze their thermal stability, i.e., their melting temperature (Tm). Both THPGal and THPK showed triple helical conformations at 20 °C, characterized by a maximum wavelength around 222 nm and a minimum wavelength around 195 nm. The thermal transition curves were obtained by recording the ellipticity (mildegrees) at 222 nm while the temperature was continuously increased over a range of 5–85 °C at a rate of 1 °C per minute. The inflection point in the transition region in the obtained sigmoidal melting curves was defined as the melting temperature (Tm). Tm (THPGal) 52.5 °C, Tm (THPK) 51.7 °C. The chemical structure of the triple helical CII peptides is presented in Supplementary Figure S2a.

Generation of a mAb specific for PTM CII. A B10.Q mouse was immunized twice (7 weeks apart) with 50 µl of an emulsion containing 20 µg of the triple helical CII259-273 peptide harboring a galactosylated hydroxylysine at position 264 (THPGal) in incomplete Freund's adjuvant (IFA). Inguinal lymph node cells were prepared 5 days after the secondary immunization and fused with myeloma cells (P3X63-Ag8.653) as previously described28. Supernatants from growing cultures were tested by ELISA for anti-β2m (enzyme-linked immunosorbent assay) plates coated with rat CII (10 µg ml−1), THPGal (2.5 µg ml−1), and the control triple helical CII peptide harboring a non-modified lysine at position 264 (THPK, 2.5 µg ml−1). Cells in wells positive for binding to both CII and THPGal but negative for THPK were expanded and further sub-cloned. After four additional rounds of screening and sub-cloning, one clone specific for CII and the THPGal peptide of the IgG2b isotype was obtained, and denoted T8.

Histology and cytospin. Thymi from 1-week-old to 2-week-old mice were harvested without blood contamination and cut into small pieces in RPMI. Thymi were then digested using collagenase IV/dispase/DNase in RPMI (200/20/20 µg ml−1) in rounds of 15 min at 37 °C, until full digestion was achieved. An equal volume was aspirated and the resultant supernatant after each digestion round. The resulting cell suspension was then enriched for CD45-negative cells by magnetic beads (Miltenyi Biotec). The obtained cells were diluted accordingly and spun onto microscope slides (Superfrost, VWR). Staining of cells was performed in PBS containing 1% bovine serum albumin and 0.05% Triton X-1. For CD11c+ cells, 1:100 FcRn+ (15-0081-82, BioLegend). The obtained cells were washed and incubated with streptavidin-PEcaja (Alexa Fluor 647 conjugated, BioLegend) antibodies overnight at 4 °C. Slides were washed and incubated with secondary antibodies (Alexa 680 (Invitrogen, USA), followed by an LSR-II (BD Biosciences) and mounted in DAPI (4,6-diamidino-2-phenylindole) containing media. Pictures were acquired using a Zeiss LSM700 (Carl Zeiss AG) confocal microscope.

Human thymus was obtained from three children undergoing corrective cardiac surgery. Parents gave informed consent, and the study was approved by the Regional Ethical Board at the University of Gothenburg, Sweden (no. 217-12, 2012-04-26). Human thymus tissue was imbedded in OCT and cut to 7 µm sections with a cryostat. The sections were fixed with cold acetone and blocked with Protein block (X0909, Dako) with 5% donkey serum (D9663, Sigma). The tissue was stained in 4°C for 1 h with a cocktail of four anti-CII mAbs (clones C16C, C12C, UL-1, and M2139, in-house produced and biotinylated) and anti-AIRE (sc-17985, Santa Cruz Biotechnology). The tissues were washed and incubated in 4°C for 30 min with Streptavidin Alexa Fluor 635 (S32364, Life Technologies), anti-goat donkey Alexa Fluor 555 (A21432, Life Technologies), and Hoechst (H21486, Life Technologies). Sections were mounted with Vectashield (H-1000, Vector Laboratories) and images acquired using a LSM510 (Carl Zeiss AG) confocal microscope. Cytospins of human samples were prepared from the sorted thymi samples were mounted with Protein block (X0909, Dako) and stained with a PBTM CII-specific mAb (clone T8) for 1 h, followed by streptavidin Alexa Fluor 555 (S32361, Life Technologies) for 20 min. The slides were subsequently incubated in PBS containing biotinylated SY3200 (Sony). Cytospins of human thymus samples were prepared from the sorted mTECs defined as CD45+EpCam+CD2R2+HLA-DR- for immunohistochemistry. The samples were stained and mounted as described above.

Anti-RANKL treatment. Depletion of Aire+ mTECs was done as previously described31. Briefly, mice were injected intraperitoneally every other day for 2 weeks with 100 µg of anti-RANKL mAb (clone IIK2/5) or isotype control (clone 2A3; BioXcell, West Lebanon, NH, USA). After 2 weeks, mice were sacrificed and thymi harvested. Organs were enzymatically digested as described above for mouse thymus. TECs were enriched by negative anti-CD45 labeling (Miltenyi Biotec, Bensheim, MA, USA) and further stained for flow cytometry analysis.

Flow cytometry. The following anti-mouse antibodies and staining reagents were used: anti-Vß8 FITC (clone F23.1), anti-CD40L PE (clone M1), anti-CD80 PE (clone 16-10A1), anti-CD45 Brilliant Violet 650 (clone 63-5.7, and biotinylated anti-Ly51 from BD; anti-CD4 Pacific Blue (clone RM4-5), anti-Ep-CAM APC (clone G8.8), and anti-IA/IE Pacific Blue (clone MS/114.13.2) from BioLegend; anti-Aire FITC (clone SH12) from eBioscience; Qd655.2 anti-EpCam conjugate and Cy7.9-PE anti-CD20 from eBioscience; Fixable Aqua 680® C594 (eBioscience) and Fixable Inius™ FarRed 780® C560 from Life Technology as well as anti-FcγRIII/II (clone 2.4G2) in-house produced. For detection of CD40L, single-cell suspensions were processed as described earlier25. In brief, 1.2 x 10⁶ cells ml−1 were cultured for 6 h in the presence of the peptides (10 µg ml−1), anti-CD28 (1 µg ml−1), and brefeldin A (2 µg ml−1). Fixable Aqua 680® C594 (eBioscience) and Fixable Inius™ FarRed 780® C560 from Life Technology as well as anti-FcγRIII/II (clone 2.4G2) in-house produced. For detection of CD40L, single-cell suspensions were processed as described earlier25. In brief, 1.2 x 10⁶ cells ml−1 were cultured for 6 h in the presence of the peptides (10 µg ml−1), anti-CD28 (1 µg ml−1), and brefeldin A (2 µg ml−1). Fixable Aqua 680® C594 (eBioscience) and Fixable Inius™ FarRed 780® C560 from Life Technology as well as anti-FcγRIII/II (clone 2.4G2) in-house produced. For detection of CD40L, single-cell suspensions were processed as described earlier25. In brief, 1.2 x 10⁶ cells ml−1 were cultured for 6 h in the presence of the peptides (10 µg ml−1), anti-CD28 (1 µg ml−1), and brefeldin A (2 µg ml−1). Fixable Aqua 680® C594 (eBioscience) and Fixable Inius™ FarRed 780® C560 from Life Technology as well as anti-FcγRIII/II (clone 2.4G2) in-house produced.
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Author contributions
B.R. designed experiments, conducted animal studies, cellular assays, data analyses and interpretations, and wrote the manuscript. P.M. performed animal studies, cellular assays, data analyses, and interpretations. H.Y. generated the TCR transgenic mouse and assisted in data interpretations. V.U. performed cellular assays, data analyses, and interpretations. C.L. performed immunofluorescence on sections and sorted cells from human thymus. C.N. performed confocal microscopy of murine samples and helped in data analyses. J.V. synthesized and characterized the THPs. J.K. synthesized and characterized the THPs. B.K. contributed with manuscript preparation and data interpretation. O.E. contributed with human samples, data analyses, and interpretations. R.H. initiated and designed the project, was co-supervisor, and helped writing the manuscript. J.B. designed the project, conducted animal experiments and cell assays, compiled and analyzed data, co-supervised the project, and wrote the manuscript.

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