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Limited Peripheral T Cell Anergy Predisposes to Retinal Autoimmunity¹

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Autoimmune uveoretinitis accounts for at least 10% of worldwide blindness, yet it is unclear why tolerance to retinal Ags is so fragile and, particularly, to what extent this might be due to defects in peripheral tolerance. To address this issue, we generated double-transgenic mice expressing hen egg lysozyme, under the retinal interphotoreceptor retinoid-binding promoter, and a hen egg lysozyme-specific CD4⁺ TCR transgene. In this manner, we have tracked autoreactive CD4⁺ T cells from their development in the thymus to their involvement in uveoretinitis and compared tolerogenic mechanisms induced in a variety of organs to the same self-Ag. Our findings show that central tolerance to retinal and pancreatic Ags is qualitatively similar and equally dependent on the transcriptional regulator protein AIRE. However, the lack of Ag presentation in the eye-draining lymph nodes results in a failure to induce high levels of T cell anergy. Under these circumstances, despite considerable central deletion, low levels of retinal-specific autoreactive CD4⁺ T cells can induce severe autoimmune disease. The relative lack of anergy induction by retinal Ags, in contrast to the same Ag in other organs, helps to explain the unique susceptibility of the eye to spontaneous and experimentally induced autoimmune disease. The Journal of Immunology, 2007, 178: 4276–4283.

The frequency of subclinical and clinically significant autoimmune disease in the retina suggests that actively acquired tolerance to retinal Ags can be easily broken (1–5). Furthermore, experimental autoimmune uveoretinitis (EAU),¹ induced by immunization with retinal Ags, and sympathetic ophthalmia, a human disease caused by autoimmunization following eye trauma, suggest that retinal tolerance is maintained to some extent by the physical sequestration of Ags behind the blood-retinal barrier (1–3, 6, 7). However, the relative importance of different tolerogenic mechanisms controlling retinal autoimmunity remains poorly defined. 

Central tolerance plays an important role in EAU, through the clonal deletion of autoreactive CD4⁺ T cells and probably by the induction of regulatory T (Treg) cells (8–11). Strain-specific resistance to the uveitogenic retinal Ag interphotoreceptor retinoid-binding promoter (IRBP) correlates with the level of IRBP expression in the thymus (12). In addition, the transplantation of thymi from IRBP-deficient mice into wild-type (WT) recipients exacerbates IRBP-induced EAU. This is due to the escape of autoreactive T cells from the transplanted organ and possibly due to a failure to induce IRBP-specific Tregs (8). Thymic expression of tissue-specific Ags in medullary epithelial cells, which mediate the subsequent deletion of autoreactive T cells, is largely controlled by the autoimmune transcriptional regulator AIRE (13, 14). The failure to delete autoreactive T cells in AIRE-knockout mice (AIRE-KO) leads to multiple organ-specific autoimmunity which includes retinal inflammation and anti-retinal Abs (13, 14). The fact that uveoretinitis is blocked in animals that are deficient in both AIRE and endogenous IRBP suggests that IRBP-specific T cells play an essential role in disease (8, 15).

In contrast to central tolerance, the role of peripheral tolerogenic mechanisms for controlling retinal autoimmunity is unclear. Anergy is induced when peripheral self-Ag presentation occurs in the absence of immunogenic costimulation (16–18). However, this mechanism may be deficient for retinal proteins that are sequestered from circulating lymphocytes behind the blood-retinal barrier. Resting β-galactosidase-specific CD4⁺ T cell clones transferred into transgenic mice expressing retinal β-galactosidase appear to be immunologically ignorant (19, 20) while the systemic expression of retinal Ags reduces susceptibility to EAU, presumably by converting a state of immunological ignorance to one of active tolerance (21, 22). However, the exact contribution, if any, of peripheral tolerance, Ag sequestration, and local forms of tolerance, such as anterior chamber-associated immune deviation (20, 23–26) vs central tolerance, remains to be determined.

To address a number of these issues, we generated a transgenic mouse expressing the neo-self-Ag, hen egg lysozyme (HEL), under the retinal-specific IRBP promoter and crossed these animals to the 3A9 HEL-specific CD4⁺ TCR-transgenic line. In this way, we have tracked retinal-specific autoreactive T cells during the development of spontaneous uveoretinitis, and by breeding to AIRE-KO mice we have been able to separate contributions due to

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3 Abbreviations used in this paper: EAU, experimental autoimmune uveoretinitis; Treg, regulatory T cell; IRBP, interphotoreceptor retinoid-binding protein; AIRE, autoimmune regulator; KO, knockout; WT, wild type; HEL, hen egg lysozyme; rHEL, retinal HEL; mHEL, membrane-bound HEL; insHEL, pancreatic expressed HEL; sHEL, soluble HEL; DC, dendritic cell; APC, Ab-forming cell.

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central and peripheral tolerance. We demonstrate that central tolerance to retinal HEL (rHEL) is comparable to that seen in other models which express this neo-self Ag in a tissue-restricted manner (pancreas, thymus, and skin) (27, 28). However, in rHEL double-transgenic mice, severe disease is seen at an early age, which despite a similar level of clonal deletion, is in striking contrast to that seen when HEL is expressed under the rat insulin promoter (insHEL) (27). Increased disease susceptibility correlates with reduced functional inactivation in the periphery principally due to a failure to present self-Ag in the eye-draining lymph nodes, which is in contrast to the insHEL model (27, 29). The lack of peripheral anergy helps to explain the fragility of retinal tolerance and the unique susceptibility of the retina to spontaneous and experimentally induced autoimmune disease.

Materials and Methods

Generation of rHEL mice

The rHEL transgene was prepared by replacing the promoter of the previously described membrane-bound HEL (mHEL) expressing KLK construct (30) with the retinal-specific IRBP promoter (31). A ClaI restriction site was introduced between HincIII and XhoI, 5’ to the retinal promoter by inserting an oligonucleotide linker. The 1.9-kb IRBP promoter fragment was then isolated by digesting with ClaI and BglII. The original KLK construct was linearized with Clal, and the MHC class I promoter removed by partial digest with BamHI to yield a 10.4-kb fragment, containing the lysozyme gene linked to MHC class 1 transmembrane region and vector sequence. The final construct (rHEL) was obtained by ligating the 1.9-kb IRBP fragment with the 10.4-kb fragment containing the gene-encoding membrane-bound lysozyme. The rHEL transgene was excised from the vector by digestion with SaII and ClaI and prepared for microinjection into C57BL/6 × CBA/CasJF1 oocytes as previously described (32). Transgenic founders were identified by PCR and animals were kept in specific-pathogen-free conditions. Mice were backcrossed to B10.BR mice, or to the 3A9 TCR transgenic on the B10.BR background, for three to nine generations and to A-KO (33, 34). The 3A9 TCR transgenic carries rearranged TCRα (Vα3) and TCRβ (Vβ8.2) genes, which recognize with high affinity the immunodominant HEL peptide (46-61) in the context of I-κA (27, 33, 35). All the animal experiments were approved by the Oxford University Ethical Review Committee and were performed under Home Office license.

Induction of EAU by immunization

Mice were immunized i.p. with 100 μg of HEL (Sigma-Aldrich) emulsified with 100 μl of CFA (H37Ra; Difco Laboratories) in a total volume of 200 μl. Mice were sacrificed after 3 wk and tissues were taken for histological examination.

Real-time RT-PCR

Real-time RT-PCR was performed as previously described (36) using the Quantitect SYBR Green RT-PCR kit (Qiagen). The amount of HEL in each experimental sample was quantified using a standard curve of known HEL transcripts. The samples were run on a 15% SDS-PAGE gel under nonreducing conditions. HEL was detected using HyHEL-9-biotin and streptavidin-HRP second stage (Amersham Biosciences). Detection was by ECL (Amersham Biosciences). A total of 10 ng of HEL was run as a control and blots were analyzed with Scion Image software. Serum HEL was measured as previously described (41).

In vivo assays

CD4+ cells were purified from splenocytes using anti-CD4 microbeads and miniMACS columns (Miltenyi Biotec) according to the manufacturer’s instruction. The number of purified CD4+ cells was typically 90% or above.

CD4+ cells were cultured from splenocytes using anti-CD4 microbeads and miniMACS columns (Miltenyi Biotec) according to the manufacturer’s instruction. The number of purified CD4+ cells was typically 90% or above. A total of 1.5 × 10^5 purified cells were cultured as described (41) in the presence of irradiated WT syngeneic splenocytes with increasing concentrations of HEL (Sigma-Aldrich) for 3 days. To measure proliferation, [3H]thymidine (Anachem) was added for the last 12 h of stimulation, cells were harvested onto a filter membrane (Packard Biosciences), and counts were read on β counter. Alternatively, culture supernatants were collected and assayed with the Mouse Th1/Th2 Cytokine Cytometric Bead Assay (BD Biosciences). For the Treg suppressor assays, cells were purified from the pooled splenocytes and lymph nodes, of at least two animals, using the CD4+ CD25+ Regulatory T Cell Isolation kit (Miltenyi Biotec) according to the manufacturer’s instruction. Isolated CD4+ cells were cultured on their own, as 6.0 × 10^4 CD4+ CD25+ or 1.0 × 10^5 CD4+ CD25+ per well, or in a ratio of 2:3 of CD4+ CD25+ to CD4+ CD25- in the presence or absence of 10^-8 M HEL for 3 days. CD4+ CD25+ cells were typically 98% of isolated CD4+ (62-74% of small lymphocytes).

Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 4.0. Statistical analysis was performed using the unpaired, two-tailed t test, with a 95% confidence interval: *p < 0.05; **p < 0.01.

Results

Generation of IRBP-HEL (rHEL)-transgenic mice

To study retinal tolerance, we generated a series of transgenic mice expressing mHEL in retinal photoreceptor cells. This neo-self-Ag, which we named rHEL, comprised an extracellular lysozyme domain and transmembrane region derived from the MHC class I molecule H-2Kb under the control of the IRBP promoter (Fig. 1). This strategy would enable us to make comparisons with identical Ag expressed in other tissues, notably the insHEL pancreas model. This promoter was chosen as IRBP is highly uveitogenic in a number of mouse strains and can induce EAU effectively (2, 12). The rHEL Ag is similar to potentially uveitogenic membrane-bound Ags, such as rhodopsin (3), but is not secreted like IRBP. Transgenic mice expressing rHEL were produced from CBA/Ca × C57BL/6F1 mice and backcrossed three to nine generations to B10.BR. All lines appeared healthy and bred well with no differences seen between backcross number or sex. One transgenic line was selected for detailed analysis. Confocal and conventional microscopy of single-transgenic rHEL mice expressed HEL in the
EAU, together with the anti-HEL-transgenic CD4 + cells expressing endogenously rearranged receptors, are required to cause disease. Double-transgenic mice revealed that additional T or B cells expressing anti-HEL transgenes are not sufficient alone to cause disease. The anti-HEL-transgenic CD4 + cells crossed the rHEL-transgenic mice with the HEL-specific 3A9 TCR. Immunization with HEL/CFA induced EAU in the majority of animals (Fig. 2). Confocal microscopy of established lesions in TCR-rHEL mice confirmed the recruitment of DCs and macrophages into the inflamed retina (data not shown).

Central CD4 + T cell tolerance is dependent on AIRE
To measure the level of tolerance achieved through clonal deletion in the TCR-rHEL mice, we enumerated autoreactive CD4 + T cells in the thymus, spleen, and peripheral lymph nodes. Flow cytometry analysis showed a 4-fold reduction in the number of clonotype-specific (1G12) single-positive CD4 + T cells in the thymus of TCR-rHEL compared with controls (3.6 x 10^6 vs 1.7 x 10^7; p < 0.0001), and a 7-fold reduction in the spleen (1.9 x 10^6 vs 1.3 x 10^7; p < 0.0001). Clonotype-specific cells were also reduced in the mesenteric lymph node (1.4 x 10^6 vs 8.3 x 10^5; p = 0.0015) and in pooled submandibular and superficial cervical lymph nodes (3.2 x 10^5 vs 1.5 x 10^6; p = 0.0138) (Fig. 3A). Efficient clonal deletion of autoreactive T cells reduces the total fraction of clonotype-specific CD4 + T cells to <50% of thymic CD4 + T cells and the fraction falls to <25% in the spleen (Fig. 3A). Flow cytometry analysis of lymphocytes from eye-draining submandibular lymph nodes showed no down-modulation of the Vβ8.2 chain in double-transgenic mice and there was no expansion of CD4 + T cells with low affinity for the clonotype-specific 1G12 Ab (Fig. 3B). No differences in disease severity or cell numbers were found between mice of early backcross generations and later pedigrees.
To quantify the importance of central tolerance and investigate the contribution of AIRE-dependent protein expression, we crossed our double-transgenic TCR-rHEL with AIRE-KO mice. AIRE deficiency reversed negative selection in the thymus of TCR-rHEL mice and restored peripheral T cell numbers to those seen in the absence of Ag (Fig. 3A), confirming the pivotal role of AIRE in central tolerance to retinal Ags. Because the severity of disease in adult TCR-rHEL mice was already maximal, it was not surprising to find no additional affect of AIRE deficiency on this phenotype (Fig. 3C). However, loss of central tolerance did accelerate disease onset. Disease is absent at 20 days (6 of 6 TCR-rHEL mice) but is routinely seen by 21–22 days (9 of 12 TCR-rHEL mice). In contrast, 18-day TCR-rHEL AIRE-KO mice have developed disease (5 of 7 TCR-rHEL AIRE-KO mice) (Fig. 3D). These findings demonstrate the limiting role of thymic deletion in retinal tolerance and suggest that the risk of disease relates to the number of peripheral autoreactive CD4\(^+\) T cells.

**FIGURE 3.** AIRE-dependent central tolerance to retinal Ags. A, Absolute number of CD4\(^+\) single-positive cells (and 1G12\(^+\)) in thymus, spleen, mesenteric lymph node (MLN), and pooled submandibular and superficial cervical lymph nodes (SLN) from TCR-rHEL double transgenics and 3A9 TCR-transgenic mice (WT or AIRE-KO). Results are shown as means with error bars indicating SD. Differences in cell numbers were considered significant if t test p values (two-sample assuming equal variances) were below 0.05. Values of p were denoted as follows: *, 0.01–0.05; **, 0.001–0.01; ***, <0.001. B, Histograms of CD4\(^+\) T cells from submandibular lymph nodes stained with the 3A9 clonotype-specific Ab 1G12 (upper panel) and Vβ8.2/8.3 (lower panel), comparing TCR-rHEL double transgenics or a nontransgenic (filled histograms) with a TCR single transgenic (line histogram). C, EAU scores of TCR-rHEL AIRE HET/KO and 3A9 TCR-transgenic KO. D, EAU scores of young TCR-rHEL (●) and TCR-rHEL AIRE-KO (○); age in day number.

FIGURE 4. Tregs are not induced by thymic self-Ag in TCR-rHEL mice. A, Flow cytometry of SLN lymphocytes from TCR-rHEL double transgenics and 3A9 TCR transgenic, gated on CD4\(^+\) cells and stained with anti-CD25 and anti-CD45RB Abs. The numbers indicate percentages of gated CD4\(^+\) cells. B, Absolute numbers of clonotype-specific CD4\(^+\)CD25\(^+\) and Foxp3\(^+\)CD4\(^+\)CD25\(^+\) cells in the thymus and spleen of TCR-rHEL double transgenics and 3A9 TCR transgenics. Results are shown as means with error bars indicating SD. No statistically significant differences were found. C, Sorted CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells (nominated CD25\(^+\) and CD25\(^-\)) from TCR-rHEL and 3A9 TCR transgenics were cultured on their own or in a 2:3 ratio of CD4\(^+\)CD25\(^+\) to CD4\(^+\)CD25\(^-\) for 72 h with allogeneic APCs in the presence or absence of 10\(^{-6}\) M HEL for 3 days. The mean level of proliferation in medium was subtracted from that in the presence of HEL and is displayed as mean with error bars indicating SD.
Recent studies have suggested a role for Tregs in the control of EAU (8, 10, 11) and examination of CD4+ T cells from TCR-rHEL double-transgenic mice revealed a small number of CD45RBlowCD25+ cells, which were enriched as a fraction of total CD4+ T cells (Fig. 4A) but not increased in absolute numbers of clonotype-specific CD4+CD25+ or Foxp3+CD4+CD25+ compared with TCR controls (Fig. 4B). To assess Treg function in more detail, we sorted CD4+CD25+ and CD4+CD25− cells from TCR and TCR-rHEL mice and cultured them singly or in a 3:2 ratio in the presence of HEL (10−6 M). This analysis showed no difference in the level of proliferation of equivalent numbers of CD4+CD25− from TCR or TCR-rHEL an indication that anergy is largely absent at this level of Ag (Fig. 4C). The purified CD4+CD25+ from the TCR-single and TCR-rHEL double-transgenic mice suppressed the HEL-induced proliferation of CD4+CD25− by 10- and 3-fold, respectively (Fig. 4C). However, the basal level of proliferation of CD4+CD25− cells from double transgenic was higher than in TCR single transgenics (Fig. 4C). Although Foxp3+ cell numbers are equivalent in TCR and TCR-rHEL mice (Fig. 4B) a small number of Ag-experienced CD4+CD25− cells from TCR-rHEL mice might be sufficient to mediate this effect.

To explore the role of Tregs in vivo, we isolated CD4+CD25+ cells from rHEL expressing double transgenics and coadaptively transferred naive CFSE-labeled 3A9 T cells with and without the isolated CD4+CD25− T cells, in a 1:2 ratio, into ML5 recipients expressing soluble (sHEL) at 10−20 ng/ml (~10−8 M). Despite enriching for CD4+CD25−, we could detect no difference in proliferation due to suppression (data not shown).

Limited T cell anergy

To assess the apparent lack of T cell anergy in more detail (Fig. 4C), we purified splenic CD4+ cells from TCR and TCR-rHEL mice by positive selection, using magnetic bead columns, and stimulated them in vitro with varying amounts of HEL in the presence of irradiated syngeneic APCs (Fig. 5A). Our findings showed that the proliferative response of double-transgenic CD4+ T cells to high levels of Ag was similar to controls (Figs. 4C and 5A), allowing for a 3-fold reduction in the number of clonotype-specific cells in the CD4+ population. The proliferation of autoreactive CD4+ T cells to low levels of Ag (10−8 M) was <10-fold reduced and was significantly greater than background (Fig. 5A). Purified CD4+ T cells from TCR-rHEL mice also produced Th1 cytokines IL-2, TNF-α, and IFN-γ at levels comparable to TCR single-transgenic mice when stimulated with HEL (10−6 M) in vitro (Fig. 5B), and up-regulated CD69 after an overnight stimulation with HEL (data not shown). To measure the functional impairment in vivo, we used a sensitive and well-validated assay of T cell help (27), which tests the ability of CD4+ T cells to recognize Ag and drive differentiation of B cells into Ab-forming plasma cells (AFCs). Previous work has demonstrated that anergic T cells cannot effectively induce AFC generation (27, 48, 49). To measure this, we isolated CD4+ cells from both TCR and TCR-rHEL double-transgenic mice and mixed 2 × 105 cells with splenocytes from anti-HEL Ig-transgenic mice (MD4, H-2b) and transferred them into partially irradiated soluble HEL expressing recipients (ML5, H-2b) for a period of 5 days. CD4+ T cells from double-transgenic mice were no different from naive CD4+ T cells in their ability to provide help and induce AFC formation (Fig. 5C). Adjusting for the mean number of clonotype-specific cells had no effect on this result (data not shown). This is in stark contrast to the results from other HEL-expressing mice, including TCR-insHEL mice, which show significantly impaired T cell help due to anergy (27, 28).

FIGURE 5. Limited peripheral tolerance is associated with a lack of Ag presentation in eye draining lymph nodes. A, In vitro proliferation of purified CD4+ cells from TCR-rHEL double transgenics and 3A9 TCR transgenic, stimulated with HEL for 3 days. Dots represent the mean of triplicate samples and results are typical of four separate experiments. B, In vitro production of Th1 cytokines from purified CD4+ cells from TCR-rHEL double transgenics and 3A9 TCR transgenic, stimulated with HEL (10−6 M) for 3 days. Results are the average of duplicate plates, less cytokine production in the absence of HEL, and are displayed as mean with error bars indicating SD. Data are typical of two experiments. C, The number of AFCs, per spleen, 5 days after adoptive transfer of an enriched CD4+ T cell population from TCR-rHEL, which had been mixed with 1.0 × 107 anti-HEL Ig-transgenic MD4 H-2b cells, into ML5 (H-2b). Results are adjusted for the number of CD4+ cells and are shown as means with SD. D, Flow cytometry of CFSE-labeled naive CD4+ cells isolated from submandibular and superficial cervical lymph nodes 72 h after adoptive transfer into: nontransgenic, rHEL and ML5 (sHEL) recipients. Results are typical of four experiments.

The induction of T cell anergy in the periphery requires self-Ag presentation in tissue-draining lymph nodes (16, 50). To determine whether anergy induction in rHEL-TCR mice was impaired due to a lack of Ag presentation, we transferred CFSE-labeled splenocytes from 3A9 CD4+ TCR donors into rHEL single-transgenic recipients. Similar experiments show vigorous proliferation of CD4+ T cells in lymph nodes draining the insHEL-expressing pancreas (Ref. 29 and C. C. Goodnow, personal communication) and in the skin draining nodes of mice expressing HEL in melanocytes (28). However, after 3 days we found no significant proliferation of lymphocytes isolated from pooled submandibular and superficial cervical lymph nodes of rHEL recipients, despite a strong proliferative response to sHEL at 10−20 ng/ml (Fig. 5D). These findings suggest that the failure to induce significant levels of anergy in TCR-rHEL mice is due to the low level of peripheral Ag presentation.

Discussion

We have developed a transgenic model that has allowed us to track autoreactive CD4+ T cells from their development in the thymus to their involvement in retinal autoimmunity. By crossing to
AIRE-KO mice, we have measured the level of self-tolerance due to the thymic expression of this retinal Ag. Additionally, we have examined the relative contributions of clonal deletion and peripheral tolerance in retinal autoimmunity and have demonstrated a relative lack of peripheral tolerance induction due to inefficient Ag presentation and anergy induction. The ability to compare tolerance to the same self-Ag across a variety of organs has allowed us to delineate the role of anergy in organ-specific autoimmune susceptibility (27, 36).

Our findings agree with earlier reports that thymic deletion of autoreactive T cells is a dominant mechanism of tolerance to retinal Ags including IRBP (8, 12) and that this is dependent on AIRE (13–15). Loss of AIRE-dependent expression of rHEL accelerates the stage of onset of disease, presumably by increasing the release of autoreactive T cells into the periphery. A high level of central deletion is also seen in transgenic mice expressing HEL in the retina under the rhodopsin promoter on a mixed FVB/N and B10BR background (9). However, in contrast to our model, rhodopsin-HEL single-transgenic mice are resistant to HEL-induced EAU (9). It is not known to what extent this is due to the interplay of background modifier genes or transgene and promoter-specific differences in Ag expression. In addition individual variation in disease susceptibility may due to different levels of Ag expression in the thymus (51) and strain-specific resistance to IRBP has been shown to correlate with the level of thymically expressed IRBP (8, 12).

AIRE-dependent expression of tissue-specific Ags is also responsible for central tolerance to HEL expressed in the endocrine pancreas and thyroid glands (34, 36). In this model, clonal deletion reduces the number of clonotype-specific CD4+ T cells to 3.6 × 10^6 in TCR-insHEL double-transgenic mice (A. Liston, personal communication), which is directly comparable to the number of cells in TCR-rHEL mice. However, the fulminant disease seen in TCR-rHEL mice, evident by 42 days of age, contrasts with the milder forms of insulinitis in TCR-insHEL mice, where the cumulative incidence of diabetes is <25% even in aged mice (36). These differences are not due to a difference in the level of antigenic target as total insHEL has been estimated at 21–60 ng/pancreas (27) and our results estimate total rHEL to be between 55 and 77 ng/eyes (Fig. 1E). This implies that there must be significant differences in the efficiency of peripheral tolerance to retinal and pancreatic self-Ags.

Thymic control of T cell responses in the periphery involves not just the direct elimination of autoreactive T cells, but also the release of Ag-specific Tregs a process which is independent of AIRE (52–54). However, despite an increase in CD4+CD25+ Foxp3+ positive cells as a fraction of the T cell pool, we were unable to detect an absolute increase in Tregs as a result of the Ag in TCR-rHEL mice. In addition, we were only able to detect a modest suppressive activity in vitro even when these cells were cultured 3:2 to CD4+CD25− and we were unable to detect Treg function in an adoptive transfer assay or in our Th assay, where the ratio of CD4+CD25+ to other CD4+ T cells is not manipulated. Broadly similar findings have also been reported in TCR-insHEL mice (34, 36) and therefore it is unlikely that these cells account for the difference in the severity of pancreatic and retinal disease. The relative absence of a significant role for this population in both models may be due to the high affinity of the 3A9 TCR and may also reflect strain differences as endogenous Treg number has been shown to be dependent on background (55, 56). Our findings do not exclude an important role for Tregs in retinal tolerance and previous work has demonstrated that the severity of IRBP-induced EAU in WT mice is increased following depletion of CD4+CD25+ cells (8). However, it is difficult to exclude nonspecific effects of anti-CD25 treatment. Future studies with Foxp3-deficient mice will be useful to address this issue (57, 58).

Instead, our data suggest that the greater susceptibility to autoimmunity in TCR-rHEL mice compared with TCR-insHEL is caused by a failure to induce similar levels of T cell anergy in the periphery, particularly against high levels of Ag (16, 18, 27, 59). Although, there is a <10-fold reduction in proliferative response at low levels of Ag, the anergic response against rHEL is strikingly absent compared with insHEL at higher levels of Ag (27). Typically, naive autoreactive T cells encountering Ag in tissue draining lymph nodes undergo an initial burst of proliferation followed by deletion or anergy (16, 18, 59). An inability to present Ag will inevitably fail to induce significant levels of anergy (16, 18) and indeed naive 3A9 CD4+ T cells transferred into rHEL mice did not proliferate in the eye-draining lymph nodes. This result is again in striking contrast to the vigorous proliferation of transferred naive 3A9 CD4+ T cells in the pancreatic lymph nodes of insHEL mice (Ref. 29 and C. C. Goodnow, personal communication). Normally, the absence of immunogenic Ag presentation would be sufficient to prevent activation and proliferation of rare autoreactive cells that escape central tolerance (16, 18, 59). However, the failure to induce sufficient T cell anergy leaves little protection against immunogenic forms of Ag presentation that will inevitably occur by chance and possibly at high level, after eye trauma or by cross-reactivity during infections (61). Under these circumstances, our results suggest that tolerance depends primarily upon thymic deletion and the preservation of the blood-retinal barrier, which prevents the free trafficking of lymphocytes (62).

Anergy can be induced, in most organs, when tissue-specific Ags are presented by immature DCs (59, 63–66). For CD8+ T cells in the pancreas, this function depends on CD11c+CD80+ DCs (67–69), while CD4+ T cells in gut and pancreas depend on dermal/interstitial CD11b+ DCs, possibly in conjunction with CD11c+CD80+ cells (70–72). CD8+ T cell anergy requires signals through T cell inhibitory receptors CTLA-4 and PD-1 (73) and it is likely that similar mechanisms will apply to CD4+ T cells. In contrast, immunogenic signaling through a DC requires additional innate inflammatory signals and up-regulation of B7 receptors (74). As DCs are not recruited to a noninflamed retina, sampling of retinal self-Ag will not occur, which will prevent the induction of peripheral anergy (47, 75).

In conclusion, while the present study reveals the important role of AIRE-dependent central tolerance to retinal Ags, it also demonstrates that clonal deletion is insufficient to prevent autoimmunity and that poor Ag presentation in the eye-draining lymph nodes fails to induce significant levels of peripheral anergy. The relative lack of peripheral tolerance is exacerbated by the absence of DC immune surveillance in the retina and the failure to induce effective tolerance in the periphery leaves the retina susceptible to autoimmune attack.

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Disclosures
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