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Lymphotixin Pathway-Directed, Autoimmune Regulator-Independent Central Tolerance to Arthritogenic Collagen

Robert K. Chin, Mingzhao Zhu, Peter A. Christiansen, Wenhua Liu, Carl Ware, Leena Peltonen, Xuejun Zhang, Linjie Guo, Shuhua Han, Biao Zheng, and Yang-Xin Fu

Ectopic expression of peripherally restricted Ags by medullary thymic epithelial cells (mTECs) is associated with negative selection. Autoimmune regulator (AIRE) is considered to be the master regulator of these Ags. We show in this study that the ectopic expression of type II collagen (CII) in mTECs and the corresponding central tolerance to CII are AIRE independent but lymphotixin dependent. The failure to properly express CII in mTECs of Aire−/− and Ltbr−/− mice leads to overt autoimmunity to CII and exquisite susceptibility to arthritis. These findings define the existence of additional pathways of ectopic peripheral Ag expression, parallel to and independent of AIRE, which may cover an extended spectrum of peripheral Ags in the thymus. The Journal of Immunology, 2006, 177: 290–297.

A TCR repertoire that is exquisitely fitted to self-MHC is crucial for the sensitivity with which T cells must respond to foreign Ags. This requirement is balanced against the necessity of eliminating or otherwise controlling the imminent threat of overly self-active TCR specificities. Autoimmune regulator (AIRE), considered a master regulator (1), drives the presentation of a robust and comprehensive immunological self in the thymus through the ectopic expression of peripherally restricted Ags by key medullary thymic epithelial cells (mTECs). Mutation of AIRE is responsible for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive, monogenic disorder characterized by organ-specific autoantibodies and multiorgan autoimmune destruction (2–4). The expression of AIRE in mTECs is itself directed by signaling through the lymphotixin-β receptor (LTβR) (5). Its ligand LT is expressed predominantly in the lymphoid compartment and is up-regulated on activated thymocytes (6). Autoimmunity arising from perturbed tolerance in the thymus has been documented for Lta−/− and Ltbr−/− mice and indeed mice deficient in several downstream signaling molecules (5, 7–9).

Clinical findings in APECED patients with mutations in AIRE, and analysis of Aire−/− mice, have revealed autoimmunity and autoimmune destruction focused predominantly on features the endocrine system (1–4). The targeting of predominantly endocrine organs in APECED and Aire−/− mice raises the possibility that, even in the absence of AIRE, hosts can remain tolerized to the wide spectrum of outstanding peripherally restricted Ags. To explore the possibility that, in addition to AIRE, lymphotixin signaling drives an additional host of parallel pathways for the ectopic expression of peripheral Ags, we investigated self-Ags known to be targeted in autoimmune disease, but not targeted in APECED. Type II collagen (CII) is a prominent target of autoimmune destruction in rheumatoid arthritis (RA) and is the arthritogenic Ag in the mouse model of RA, collagen-induced arthritis (CIA) (10, 11). It is unclear whether there is a defect at the level of central or peripheral tolerance in the pathogenesis of disease. There is a preponderance of evidence implicating CD4+ T cells as the central mediators of disease induction in arthritis (12). Not only does susceptibility segregate with class II MHC (MHC-II) polymorphism, administration of mAbs in mouse models against TCRαβ, CD4, and I-A all prevent disease (13–15). Recent gene array analysis of mouse MECs revealed many gene transcripts that may not be controlled by Aire (16, 17). Interestingly, CIA also is selectively expressed in human MECs, raising the possibility that impaired central tolerance may contribute to human RA (18). Given the essential contributions of autoreactive T cells to the pathogenesis of both RA and CIA, we sought to define the role of central tolerance in forestalling anti-CII autoimmunity and examine the input of both AIRE and LT to this process.

Materials and Methods

Mice

Lta−/− and Ltbr−/− mice, originally established on the 129 strain, were backcrossed to C57BL/6 mice for at least 13 and 11 generations, respectively, and maintained under specific pathogen-free conditions as described. C57BL/6 and Rag1−/− mice were purchased from The Jackson Laboratory. Ltbr−/− mice were obtained from Taconic Farms.
mTEC isolation

TECs were isolated and purified using the previously described technique (1). Briefly, five thymi from young (5- to 7-wk-old) mice were digested with collagenase (0.1 mg/mL), dispase (2 U/mL), and DNase (10 U/mL) in RPMI 1640 (5% FCS) for 30 min to three rounds at 37°C. Cells were resuspended in PBS (5 mM EDTA, 1% FCS), incubated on ice for 10 min, and then separated on a discontinuous Percoll density gradient. The TEC-enriched fraction was harvested and stained with G8.8, 6C3 (Ab to cortical epithelial cell marker with the same reactivity as CDR1; BD Pharmingen), and CD45.2. mTECs were sorted on a MoFlo (DakoCytomation) according to the phenotype of CD45.2^+ G8.8^+ 6C3^+ with purity of >90%.

Real-time PCR

Real-time PCR was conducted on cDNA prepared from DNase-treated RNA extracted from whole thymus or sorted mTECs after four-color analysis and gating. RNA from mTECs was further amplified with the Riboprobe Affinity RNA amplification kit (Arcturus) before cDNA preparation. For Aire, the following primers were used: forward CCAGTTGACCCAGGTATAC, (reverse) CAGACGGCTCAACACAGTAGA, (probe) FAM-TGACCCTGCAACACCG-TAMRA. For insulin, the following primers were used: (forward) TCTCAGACCTTGGGCTTGA, (reverse) ATGCTGGTGCAGCACTGATC, (probe) FAM-TCACCTCCGTCGTGGCACACG-TAMRA. For keratin-14 (K14), the following primers were used: (forward) ATTGAGAGCATCCGCAGCC, (reverse) GTTTCAGGTCTTGTAGTCC, (probe) FAM-CCCGGCAACCCAGGCG-ACTGATC, (reverse) ATGCTGGTGCAGCACTGATC, (probe) FAM-TCACCTCCGTCGTGGCACACG-TAMRA. For GAPDH, the following primers were used: (forward) CTTCAGACCTTGGCGTTGGA, (reverse) ATGCTGGTGCAGCACTGATC, (probe) FAM-GGAGACCTTGGGCTTGA, (reverse) ATGCTGGTGCAGCACTGATC, (probe) FAM-TCACCTCCGTCGTGGCACACG-TAMRA. For collagen, the following primers were used: (forward) CAGACGGCTCAACACAGTAGA, (reverse) CAGACGGCTCAACACAGTAGA, (probe) FAM-TGACCCTGCAACACCG-TAMRA. Duplex real-time PCR, with GAPDH as an internal control, was conducted in a final volume of 25 μL with 900 nM of the forward and reverse primers and 200 nM of the probe using 2× Taqman Master Mix (Applied Biosystems) containing AmpliTaq Gold polymerase. Reactions were run on the Cepheid SmartCycler. Analysis of AIRE, insulin, keratin 14, and GAPDH gene expression were performed with a concurrently prepared standard curve and divided by 10^5 before plotting. For statistical analysis, t values were determined by Student’s two-tailed t test.

Splenocyte and T cell transfer

Splenocytes were obtained by mechanically spleening the spleens of 5- to 7-mo-old age-matched Ltb^-/- or WT control mice. RBCs were depleted with ammonium chloride (ACK) RBC lysis buffer. Forty million cells were injected retro-orbitally into sublethally irradiated (350 rad) Rag1^-/- mice. Mice were bled 4 wk after transfer and analyzed for autoantibodies. For T cell transfer, B cells were purified from C57BL/6 WT mice by MACS column (Miltenyi Biotec), and CD4^- T cells were similarly purified by MACS column from age-matched C57BL/6 WT and Ltb^-/- mice. Two million purified CD4^- T cells from either C57BL/6 WT or Ltb^-/- mice were combined with 10 million purified WT B cells, and injected retro-orbitally into Rag1^-/- mice. Mice were bled 8 wk after transfer and analyzed for autoantibodies.

Thymus transplantation

Thymi were isolated from newborn Ltb^-/- or WT mice and cultured in 1.35 mM 2-deoxyglucose (Sigma-Aldrich) for 6–8 days to deplete bone marrow-derived cells. Thymi were then transplanted under the kidney capsule of 6-wk-old adult C57BL/6 mice that were thymectomized at 4 wk of age. Recipients were lethally irradiated and reconstituted with WT bone marrow immediately before thymus transplantation. Whole blood was collected 6 wk after transplantation. T cell reconstitution was assessed by FACs analysis of whole blood for CD4, CD8, and B220. Serum was used for autoantibody ELISA as described above.

In vitro T cell stimulation and collagen-induced arthritis

For the induction of CIA, 12-wk-old C57BL/6 WT or Lta^-/- mice were injected i.d. at the base of the tail, with 100 μg (in 100 μl) of chicken CII (Sigma-Aldrich) dissolved in 0.01 M acetic acid and emulsified in an equal volume of CFA prepared by grinding 100 mg of heat-killed Mycobacterium tuberculosis (H37Ra; Difco) in 20 ml of IFA (Sigma-Aldrich). Mice were boosted with the same dose of CII in IFA by i.p. injection at day 21, then observed daily for the onset of arthritis. The arthritis index was derived by grading the severity of each paw from 0 to 3 as described (19), based on the degree of swelling and periarticular erythema. The scores of all four paws were added up to yield the arthritis index (19). For in vitro T cell stimulation, CD4^- T cells were purified from the spleen by MACS column (Miltenyi Biotec) from C57BL/6 WT and Lta^-/- mice 10 days after secondary CIA immunization or primary KLH immunization, respectively. Splenocytes from young, normal congenic mice were irradiated with 2000 rad and used as APCs. A total of 4 × 10^6 purified T cells and 1 × 10^6 APCs per well were used for proliferation assays. Denatured Arthrogenen CII T cell grade type II chicken collagen (Chondrex) or keyhole limpet hemocyanin (Sigma-Aldrich) was added as stimulating Ag at concentrations of 0, 10, and 50 μg/mL. Cells were cultured in flat-bottom 96-well plates with DMEM supplemented with 1.5% homologous normal serum, penicillin-streptomycin (1×), and 2-ME (2-ME, 5 × 10^-5 M) for 5 days. [3H]Thymidine was added 16 h before cell harvest. Cell culture supernatants were collected at 72 h, and IFN-γ concentration was analyzed by mouse Th1/Th2 cytokine CBA kit (BD Biosciences).

Results

Ectopic thymic expression of CII is independent of AIRE

To determine whether CII, like insulin, is expressed in the thymus, real-time PCR was used to measure CII (α1) mRNA expression in 4-wk-old WT B6 thymus. CII was robustly expressed in WT thymus (Fig. 1a). To determine whether AIRE also is required for the ectopic expression of CII, thymi from age-matched Aire^-/- mice

Autoantibody ELISA

For detection of anti-insulin autoantibodies, ELISA plates (Dynex Technologies) were coated with 2 μU/mL insulin (Lilly Research Laboratories) at 4°C overnight. For the detection of anti-collagen Abs, ELISA plates were coated with ELISA-grade bovine CII (Chondrex) according to the manufacturer’s instructions. Plates were washed with water and blocked with PBS/0.1% BSA for 1 h at 37°C. Serum samples were serially diluted in PBS/0.1% BSA from 1/300 to 1/8100. Bound Abs were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotechnology Associates). The OD was measured at 405 nm by a spectrophotometer ( Molecular Devices). Following dilution factor correction, adjacent OD readings on linear portion of titration curve were averaged, Arbitrary units were calculated from standard curve and divided by 10^5 before plotting. For statistical analysis, p values were determined by Student’s two-tailed t test.

Histology

Joint tissues collected for histologic examination were obtained 2 wk following secondary immunization, in 10% buffered Formalin, and embedded in paraffin. Sections (4–5 μm) were obtained from the paraffin blocks and stained by H&E methods. All sections were then examined qualitatively by a pathologist in a blinded fashion.

Immunofluorescence staining

Age-matched thymi from WT, Lta^-/-, and Aire^-/- mice were embedded in OCT and snap-frozen. Sections (8 μm) were obtained from the frozen blocks, fixed in acetone, rehydrated in PBS/0.1% saponin, and blocked with 5% goat serum in PBS at room temperature for 1 h. mAb to Ep-CAM (clone G8.8; BD Biosciences), biotin-conjugated anti-CII mAb (Chondrex). This Ab also crossreacts with type XI collagen, because both types of collagen comprise the same chain, α1(II). To simplify the presentation in this study, CII represents α1(II), polyclonal rabbit anti-AIRE (a gift from Dr. P. Peterson, University of Tartu, Tartu, Estonia), and FITC-conjugated mAb to CD11c (clone N418; Biologend) were incubated with tissue sections at 4°C overnight. Appropriate fluorescence-labeled secondary Abs were used for anti-Ep-CAM and anti-AIRE detection. Tyramid Signal Amplification-biotin amplification (PerkinElmer) was used for anti-CII-biosignal amplification in accordance with the product protocol. For immunofluorescence staining of CII, strepavidin PE-cytochrome 5 was used as the final reagent in TSA-biotin amplification.
were analyzed for relative CII mRNA levels and found to be statistically similar to WT (Fig. 1a). This is in striking contrast with the ectopic expression of insulin, which was undetectable in the thymus of Aire<sup>−/−</sup> mice (Fig. 1a). These findings raise the possibility that the ectopic expression of CII, and possibly a host of other autoantigens, is AIRE independent.

To determine whether the divergent expression profiles of CII and insulin in the Aire<sup>−/−</sup> thymus manifests themselves into different susceptibility to autoimmune reactivity to these Ags with age, we analyzed, by ELISA, spontaneously arising anti-insulin and anti-CII Ab titers of 5- to 7-mo-old age-matched WT and Aire<sup>−/−</sup> mice. Although there was a significant increase in the titers of anti-insulin Ab in Aire<sup>−/−</sup> mice relative to WT (Fig. 1b), there was no perceptible change in anti-CII titers (Fig. 1c). These findings suggest that ectopic expression of peripheral Ags in the thymus may play a crucial role in forestalling the development of autoimmunity to those specific Ags. In contrast with the failure of ectopic insulin expression in Aire<sup>−/−</sup> thymus and subsequent raising of anti-insulin Abs, ectopic expression of CII and tolerance to CII were unaffected by the absence of AIRE.

**Thymic expression of CII is controlled by LT**

As an Ag whose expression in the periphery is tightly restricted, the ectopic expression of CII in the thymus is likely to be similarly regulated, even if independent of AIRE. Previous studies have demonstrated the rapid kinetics and acute sensitivity with which Aire and ectopic insulin expression respond to signaling through the LT pathway (5). Grossly, we did not observe a major reduction of the medulla area by H&E staining nor by various anti-mTEC Abs. To determine whether the LT pathway controls the ectopic expression of CII, as it does insulin, we used real-time PCR to measure CII mRNA expression in age-matched WT, Lta<sup>−/−</sup>, and Libr<sup>−/−</sup> thymi. Both Lta<sup>−/−</sup> and Libr<sup>−/−</sup> thymi showed a 75–80% reduction in their expression of CII, the level of reduction similar to WT mice (Fig. 1a). Thus, not only does CII expression respond to LTβR stimulation, but also the kinetics of its regulation by LTβR signaling is rapid, demonstrating the exquisite sensitivity by which CII is controlled by the LT pathway.

To demonstrate that the induction of CII by stimulation with agonistic LTβR Ab is specific to appropriate downstream targets, we used K14 as a negative control. K14 is a marker constitutively expressed by medullary epithelial cells and not known to be inducible by LTβR. Under all conditions of stimulation, we found ample expression but negligible induction of K14 (Fig. 2, c and d). Together, these data suggest that thymic expression of CII is controlled by LT.

**AIRE and CII expression may be segregated into distinct cell populations**

Having determined by real-time PCR that Aire<sup>−/−</sup> leave ectopic expression of CII in the thymus undisturbed, whereas Lta<sup>−/−</sup> and Libr<sup>−/−</sup> thymi present with serious disruption of CII expression, we undertook to confirm these findings at the protein level by immunofluorescence localization. Double staining in WT thymi of ectopically expressed CII (red) and the MEC marker G8.8 (green) localized the ectopically expressed CII within the epithelial cells of the thymic medulla (Fig. 3a). To assess whether ectopically expressed CII is disturbed in Aire<sup>−/−</sup> and Lta<sup>−/−</sup> thymi, age-matched thymi from these mice were similarly stained. Although Aire<sup>−/−</sup> thymi presented with no perturbation in ectopic CII expression (Fig. 3b), Lta<sup>−/−</sup> thymi appeared to have substantially lost CII expression (Fig. 3c).

These findings confirm our real-time PCR data, that although ectopic expression of CII is independent of AIRE, it is critically dependent on LT signaling.

Having demonstrated that CII is expressed in the thymus independently of AIRE, we wished to determine further whether the pathway responsible for CII expression localizes to the same cell population as the AIRE pathway. Double staining of WT thymi for AIRE (green) and CII (red), revealed no significant colocalization (Fig. 3, d–f). Additionally, staining of dendritic cell markers has excluded these lineages as the source of ectopic CII (Fig. 3, g and h). Together, these data suggest that AIRE-dependent and parallel AIRE-independent pathways reside in distinct cohorts of mTECs, together forming a patchwork representation of the peripheral self.
Breakdown of central tolerance leads to spontaneous anti-collagen autoimmunity

Having found profound reduction in CII expression in the thymi of Lta−/− and Ltbr−/− mice, in a manner reminiscent to that of ectopic insulin expression, we investigated further whether, similar to insulin, this reduction in ectopically expressed CII was severe enough to represent a breakdown of tolerance. Autoantibodies to CII, a T cell-dependent response, have been shown to initiate joint inflammation by binding to articular cartilage and activating complement and are sufficient on passive transfer to induce severe acute arthritic damage (21, 22). We assayed for the production of spontaneously arising anti-CII Abs as an indicator of defective T cell-dependent tolerance in Lta−/− and Ltbr−/− mice by ELISA. Consistent with a breakdown in tolerance to this self-Ag, we found significantly higher titers of CII Ab and anti-insulin Ab in both Lta−/− and Ltbr−/− mice (Fig. 4, a and b). Despite overt anti-CII autoimmunity, analysis of both Lta−/− and Ltbr−/− mice age >1 year revealed no evidence of spontaneously arising arthritis, presumably because the absence of peripheral LT signaling blocks disease pathogenesis, as demonstrated recently (23).

To sidestep the constraint imposed by the absence of LT signaling in the periphery, and to confirm that the autoimmunity in Lta−/− and Ltbr−/− mice represents a defect within the lymphoid compartment, 40 million splenocytes from 7-mo-old WT and Ltbr−/− mice were transferred into lightly irradiated (350 rad) Rag1−/− mice. Similar to anti-insulin Ab, serum titers of anti-CII Ab also were significantly elevated in Rag1−/− recipients of Ltbr−/− donor splenocytes (Fig. 4, c and d). Ltbr−/− donor splenocytes in Rag1−/− hosts were thus able to recapitulate the findings of Lta−/− and Ltbr−/− mice, demonstrating both the lymphoid-autonomous nature of the autoimmunity shown in these mice and the magnitude of autoimmune destruction that can arise from a defect in LT-regulated central tolerance. To attribute the autoimmunity exclusively to the T cell compartment, we transferred 2 million purified Ltbr−/− or WT T cells, in conjunction with 10 million purified WT B cells, into Rag1−/− mice. These autoantibody titers were similarly elevated in Rag1−/− recipients of purified Ltbr−/− donor T cells after 8 wk (Fig. 4, e and f). These data suggest that there is a potential increase of autoreactive T cells in the spleen leading to increased anti-CII Ab production.

Although the above splenocyte and T cell transfer experiments have focused the autoimmune defect to the T cell compartment, they still do not conclusively define whether this breakdown in T cell tolerance occurs centrally or in the periphery. To unequivocally demonstrate that the absence of LT signaling in the thymus results in a breakdown of T cell central tolerance in Lta−/− and Ltbr−/− mice, we performed thymus transplantation experiments. Thymi from Ltbr−/− and WT newborn pups were cultured for 6–8 days in the presence of 1.35 mM 2-deoxyguanosine to deplete bone marrow-derived cells. These thymi were then transplanted under the kidney capsule of 6-wk-old thymectomized B6 mice. Recipients were lethally irradiated before surgery and reconstituted with WT bone marrow the same day. Screening of recipients at 12 wk postop found comparable reconstitution of the T cell compartments (data not shown). Screening for spontaneously arising autoantibodies, we found significant higher levels of anti-insulin and anti-CII Abs in recipients of Ltbr−/− thymi (Fig. 5, a and b). An increase in tissue infiltration of T cells is often seen in Ltbr−/− and Aire−/−
mice and as an important feature of autoimmunity. To determine whether recipients of Ltb−/− thymi recapitulate this increase in tissue infiltration, various tissues were collected and submitted for histological analysis. These studies indeed revealed increased tissue infiltration in reconstituted mice, especially in the liver (Fig. 5c). These data clearly demonstrate not only that T cell central tolerance is critical for suppression of anti-CII autoimmunity, but also that the disruption of the ec-topic expression of insulin and CII in the thymi of Lta−/− and Ltb−/− mice results in autoimmune responses to those Ags.

Lta−/− mice are susceptible to CIA despite their resistant C57BL/6 background

Susceptibility to CIA is profoundly influenced by MHC-II genetics, and in genetically resistant strains, such as C57BL/6, even repeated immunizations fail to produce disease (10). Our Lta−/− and Ltb−/− mice, despite their resistant C57BL/6 background and lack of draining LNs, spontaneously elaborate anti-CII Abs and increased T cell response to CII in a manner reminiscent of strains susceptible to CIA, such as DBA/1(10). To determine whether the disruption of central tolerance to CII in

FIGURE 4. Autoimmune inflammation in LT-deficient mice is lymphocyte autonomous. a and b, Production of anti-insulin (a) and anti-CII Ab (b) from age-matched (5–7 mo) WT, Lta−/−, and Ltb−/− mice was measured by serum ELISA. c and d, Age-matched (5–7 mo) WT and Ltb−/− splenocytes were adoptively transferred into sublethally irradiated Rag1−/− recipients. Recipient sera were analyzed by ELISA for the presence of anti-insulin (c) and anti-collagen Abs (d). e and f, Age-matched (5–7 mo) WT or Ltb−/− CD4+ T cells were combined with WT B cells and transferred to Rag1−/− mice. Recipient sera were analyzed by ELISA for the presence of anti-insulin (e) and anti-collagen Abs (f) Representative figure of two independent experiments.

Lta−/− and Ltb−/− mice is sufficiently severe to overcome the inhibitions of their resistant C57BL/6 background in vivo, we challenged WT and Lta−/− mice with heterologous CII emulsified in CFA, boosted at day 21 with i.p. injection of IFA-emulsified CII. At day 11 after boost, both groups display 100% incidence of arthritis symptoms. Although WT B6 mice remained only mildly symptomatic in response to repeat immunizations, all Lta−/− mice developed fulminant disease phenotype after the secondary immunization (Fig. 6a). In Lta−/− mice, histological analysis of the interphalangeal joints 2 wk after secondary immunization showed typical synovial hyperplasia and infiltration of the subsynovial tissue with inflammatory cells, including lymphocytes, whereas WT joints were histologically normal(Fig. 6b). These findings demonstrate that the breakdown in central tolerance to CII in Lta−/− and Ltb−/− mice may be sufficiently severe to render them now susceptible to CIA, despite their resistant C57BL/6 background.

To confirm that CII-responsive autoreactive T cells in Lta−/− mice play an integral role in the inexorable progression of CIA in Lta−/− mice, we analyzed CIA-specific T cell responses. CD4+ T cells were purified from WT and Lta−/− mice 10 days after secondary immunization, before the appearance of clinical symptoms in vivo. These purified CD4+ cells were challenged in vitro with standardized irradiated WT splenic APCs titrated with the appropriate CII Ag. Thymidine incorporation assays demonstrated that, whereas WT CD4+ T cells remained firmly unresponsive to CII challenge, CD4+ T cells from Lta−/− mice responded with robust proliferation. (Fig. 6c) Moreover, activation of CD4+ T cells from Lta−/− mice led to their efficient maturation into effector cells, as IFN-γ, the prototypic effector cytokine in CIA pathogenesis, was also significantly increased in Lta−/− cell cultures relative to WT (Fig. 6d). In contrast with the self-Ag CII, immunization with the exogenous Ag KLH resulted in robust in vitro CD4+ T cell responses from WT mice, but only weak response in Lta−/− mice (Fig. 6e), suggesting that the excessive inflammatory response to CII seen in

FIGURE 5. Thymic LtbR expression is critical for central tolerance. Neonatal WT and Ltb−/− thymi were cultured in 1.35 mM 2-deoxy-2-thioguanosine (Sigma-Aldrich) for 6–8 days to deplete bone marrow-derived cells. Thymi were then transplanted under the kidney capsule of 6-wk-old thymectomized C57BL/6 mice. Recipients were lethally irradiated and reconstituted with WT bone marrow the same day. Recipient sera were analyzed by ELISA for the presence of anti-insulin (a) and anti-collagen Abs (b) 3 mo following transplant. Titers expressed in arbitrary units. Student’s t test is used for statistical analysis. Increase of infiltration of lymphocytes into liver was clearly visualized in nude mice reconstituted with Ltb−/− thymi (c).

Lta−/− and Ltb−/− mice
combined clinical score was recorded (mean 8 mice). The incidences of both groups were 100% after day 11. Individual WT and

Average cpm values

calculated cell culture.

provided a crucial insight into how central tolerance covers

sion of a subset of peripherally restricted Ags in the thymus

Discussion

Lta

C57BL/6 background.

FIGURE 6. LTα-deficient mice are susceptible to CIA despite resistant

C57BL/6 background. a. Incidence of arthritis and clinical scores of

C57BL/6 WT (open symbols, n = 8 mice) and Lta−/− (filled symbols, n = 8 mice). The incidences of both groups were 100% after day 11. Individual arthritic paws were evaluated for the severity of inflammation, and a combined clinical score was recorded (mean ± SEM, p < 0.01). Results are representative of four similar independent experiments. b. Severe infiltration found in the joints of challenged Lta−/− mice. Peripheral joints of B6 WT and Lta−/− mice following secondary immunization were fixed in 10% buffered formalin and paraffin mounted. Sections (4–5 μm) were stained by H&E and analyzed. Representative sections are shown. c, CD4+ T cells from C57BL/6 WT and Lta−/− mice were purified 10 days after the secondary CIA immunization and cocultured with irradiated normal congeneric splenocytes in the presence of denatured chicken CII for 5 days. Each well contained 4 × 10^5 T cells and 1 × 10^6 irradiated splenic APC. [3H]Thymidine was added 16 h before cell harvest. Average cpm values ± SD of triplicate wells were plotted after being divided by 10^3. Representative of two experiments. d, Cell culture supernatants from triplicate were collected and pooled together at 72 h, and IFN-γ concentration was analyzed by CBA Kit. Data represent the IFN-γ concentration in 50 μg of CIA-stimulated cell culture. e, CD4+ T cells from C57BL/6 WT and in Lta−/− mice were purified 10 days after the primary KLH immunization and cocultured with irradiated normal congeneric splenocytes in the presence of denatured KLH for 5 days. Each well contained 4 × 10^5 T cells and 1 × 10^6 irradiated splenic APC. [3H]Thymidine was added 16 h before cell harvest. Average cpm values ± SD of triplicate wells. Representative of four independent experiments.

Lta−/− mice is not due to a global T cell dysregulation. Rather, these findings argue that the susceptibility of Lta−/− mice to CIA is the result of a failure of T cell tolerance; that rather than the abortive response seen in WT T cells, Lta−/− T cells improperly tolerated to CII actively participate in the autoimmune destruction of joint structures.

Discussion

Appreciation of AIRE’s role in promoting the ectopic expression of a subset of peripherally restricted Ags in the thymus provided a crucial insight into how central tolerance covers peripherally restricted Ags (1). Our recent demonstration that AIRE and AIRE-dependent peripherally restricted Ags are absent in Lta−/− and Libr−/− thymi defined LT’s role in directing this machinery (5). Another study has shown that, although mTECs in Libr−/− thymi appear reduced by anti-Ulex europaeus agglutinin staining, they are unchanged by other markers for mTECs (5, 7–9). Recent gene array analysis of mouse MECs found numerous tissue-restricted genes up-regulated in mature mTECs independent of AIRE. Most, but not all, of these genes are induced in functionally mature CD80hi mTECs (16, 17). Given the focused targeting of endocrine organs in APECED and the limited reduction in expression of tissue-specific Ags in the Aire−/− thymus, we sought to determine whether AIRE-independent pathways exist for the remainder of peripheral Ags in autoimmune diseases that are unrelated to AIRE. In this study, we find that while ectopic expression of CII is AIRE independent, it is nevertheless under LT control. The significant reduction of ectopic CII expression in both Lta−/− and Libr−/− thymi and isolated mTECs, and the rapid response of thymi to in vivo agonist LTβR Ab stimulation, suggest that LT acts on mTECs directly. It remains to be determined whether the absence of LT signaling could impair the proper function or organization of a subset of mTECs. Very little is known about the signals that permit AIRE access to particular peripheral Ags, and even less is known about AIRE-independent pathways. Given the prominent polyendocrinopathy seen in APECED patients and Aire−/− mice, it is interesting to speculate that as AIRE controls endocrine system Ags, other pathways may similarly direct peripheral Ags that are grouped, and restricted along developmental lines. Indeed, our finding that ectopic CII and AIRE expression do not colocalize, coupled with data from others that mTECs from AIRE deficient mice express only a limited number of Ags (16, 17), it is possible that AIRE and AIRE-independent pathways are mutually exclusive in the individual mTECs.

CII is a major constituent protein of cartilage in diarthrodial joints, the predominant site of inflammation in RA, and is targeted specifically in the autoimmune response of RA patients (24, 25). The notion that central tolerance to arithitogenic Ags preempts the initiation of autoimmune destruction in the CIA model is somewhat overlooked, mostly from conceptual misgivings of how these Ags can come to be presented in the thymus. The uncovering of AIRE’s role in the ectopic thymic expression of peripheral Ags, and our findings relating to AIRE-independent pathways for ectopic expression of CII and likely others, remove this inhibition. Suggestive work by Lamhamedi-Cherradi et al. (26) in TRAIL−/− mice found an association between defects in thymocyte apoptosis and the susceptibility of C57BL/6 mice to CIA, although their system did not permit isolation of the TRAIL−/− defect exclusively to the thymus. We determined not only that CII was expressed in murine mTEC in a LT-dependent fashion, but also that transplantation of Libr−/− thymi into resistant hosts was sufficient to break tolerance, demonstrating the crucial protective contribution of central tolerance for autoimmune arthritis. Our findings are mirrored by recent studies demonstrating human mTEC expression of a highly diverse selection of tissue-specific genes, including CII (18). Our study raises the possibility that the breakdown of central tolerance for CIA may be critical for the development of human RA. It’s noteworthy that both CIA and CXI comprise the same chain, α1(XI) (II). Although CIA is composed of three identical α1(XI) chains, CXI has additional two chains of α1(XI) and


c2(XI). Whether these additional chains for CXI also are involved in LT-mediated tolerance and arthritis remains to be determined.

Although we have found that Libr−/− mice are more susceptible to CIA, others have described LTβR-Ig fusion protein as an effective inhibitor of CIA development, arresting even established disease (23). There are several possible explanations. First, the LT pathway could play different, even opposing roles in central tolerance vs peripheral inflammation. The thymic defect in the absence of LT overcomes the absence of LT in the peripheral environment. For example, CII-specific T cell proliferation and IFN-γ secretion was significantly higher in primed Lta−/− mice than that in WT mice in our study. Earlier studies (23) with LTβR-Ig blockade had an isolated anti-inflammatory response peripherally, without affecting central tolerance, and thus did not result in higher CII-specific T cell proliferation and IFN-γ secretion. Second, different murine strains were used. DBA is a CII-susceptible strain, meaning that it may already harbor defects in central or peripheral CII-tolerance. Thus, LTβR-Ig blockade in DBA models probably could not further exacerbate the central defect but would be active only in inhibiting peripheral inflammation. In contrast, B6 is a resistant strain, susceptible to CIA only with a defect in central tolerance as the result of LT deficiency. In the B6 background, LT deficiency affects both central tolerance and in peripheral inflammation, with the central defect predominant. Third, the effect of genetic LT deficiency could be more comprehensive than those seen with LTβR-Ig blockade. LT deficiency during development permits appearance of compensatory mechanisms, such as increased TNF expression in place of LT. Short-term LTβR-Ig blockade may not lead to such compensatory effects. Fourth, because of the promiscuous nature of TNF ligand/receptor pairings, LTβR-Ig also blocks the costimulatory function of LIGHT to HVEM, leading to decreased cell activation in treated mice. Lta−/− does not affect LIGHT or HVEM signaling.

The role of AIRE in establishing self-Ags specific central tolerance is not limited to transcriptional control of thymic expression of peripheral Ags. A recent report by Kuroda et al. (27) showed that, although thymic fodrin expression is normal in Aire−/− mice, they nevertheless go on to develop autoimmunity against fodrin. These data suggest that another mechanism of AIRE, in addition to thymic transcriptional control, is responsible for tolerance to fodrin. In contrast with the anti-fodrin immunity in Aire−/− mice, we found neither perturbation of thymic expression of CII nor autoimmunity to CII. This additional mechanism is thus unlikely to be involved in CII tolerance.

Although clonal deletion has occupied the center of attention as the mechanism of central tolerance mediated by ectopically expressed peripheral Ags, this may be not the full story (28). It has been demonstrated that resistant MHC-II, such as EBα2, EBβ2, and I-Aβ2, not only resist CIA in their native hosts, but also will actively protect genetically susceptible hosts when transgenically expressed (29–31). This protection is difficult to be explained by clonal deletion alone, because the endogenous autoreactive repertoire would not be deleted by the resistant MHC-II. Regulatory cells could provide a more elegant explanation. Ectopic expression of CII by mTECs, presented on resistant MHC-II, may instead facilitate efficient production of CII-specific regulatory T cells. Coexpression of susceptible and resistant MHCs on all APCs permits these regulatory T cells to restrain even endogenous autoreactive clones raised on susceptible MHCs, a finding revealed only in systems using endogenous self-Ag and a polyclonal T cell repertoire. It remains to be determined whether and how peripheral tolerance may play a role in the limiting autoimmune destruction in both LT and AIRE deficiency.

The revelation of an AIRE-independent pathway for controlling different peripheral Ags in the thymus opens new avenues for exploration of the complex mechanisms of negative selection beyond AIRE. The ability of LT to induce ectopic expression of peripherally restricted Ags, and the loss of AIRE-dependent and independent Ags in Lta−/− and Libr−/−, permit dissection of the LT-AIRE pathway, uncover the contributions of other mechanism of tolerance in the context of impaired negative selection, and the identification of possible AIRE family members. Understanding of the regulation of AIRE and parallel pathways responsible for central tolerance may offer novel diagnostic possibilities and therapies in the treatment of autoimmune disease.

Disclosures

The authors have no financial conflict of interest.

References


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