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Autoimmune thyroiditis: A model uniquely suited to probe regulatory T cell function

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Abstract

Murine experimental autoimmune thyroiditis (EAT) is a model for Hashimoto's thyroiditis that has served as a prototype of T cell-mediated autoimmunity for more than three decades. Key roles for MHC restriction and autoantigen influence on susceptibility to autoimmunity have been demonstrated in EAT. Moreover, it has served a unique role in investigations of self tolerance. In the early 1980s, self tolerance and resistance to EAT induction could be enhanced by increasing circulating levels of the autoantigen, thyroglobulin (Tg), by exogenous addition as well as endogenous release. This observation, directly linking circulating self antigen to self tolerance, led to subsequent investigations of the role of regulatory T cells (Tregs) in self tolerance. These studies revealed that protection against autoimmunity, in both naive and tolerized mice, was mediated by thymically-derived CD4⁺CD25⁺Foxp3⁺ Tregs. Moreover, these naturally-existing Tregs required proper costimulation, in context with autoantigen presentation, to maintain and enhance self tolerance. In particular was the selected use of MHC- and heterologous Tg-restricted models from both conventional and transgenic mice. These models helped to elucidate the complex interplay between autoantigen presentation and MHC class II-mediated T cell selection in the development of Treg and autoreactive T cell repertoires determining susceptibility to autoimmunity. Here we describe these investigations in further detail, providing a context for how EAT has helped shape our understanding of self tolerance and autoimmunity.

Keywords

Regulatory T cells; Autoimmune thyroiditis; Regulatory T cells and autoimmunity; MHC influence and autoimmunity; Enhancing self tolerance; Tolerance augmentation; Circulatory thyroglobulin

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1. Introduction

Experimental autoimmune thyroiditis (EAT) has functioned as an early prototype for studies of T cell-mediated, organ-specific autoimmune diseases since the early '70s. As a murine model for the hypothyroid syndrome, Hashimoto's thyroiditis (HT), EAT exhibits many of the characteristics of HT, including mononuclear cell infiltration destroying the thyroid follicles, autoantibody production and T cell proliferative response to thyroid autoantigens. EAT has several important features that make it an ideal model for investigation of autoimmunity and self tolerance. In addition to a clear correlation of susceptibility with major histocompatibility complex (MHC) class II alleles [1,2], it was used to demonstrate the presence of autoreactive T cells responsive to syngeneic mouse thyroglobulin (mTg), the principal autoantigen [3]. Investigation into the role of autoantigen in EAT revealed several important and unique findings; EAT can be induced with mTg in conjunction with bacterial LPS or IL-1 β as adjuvant with no need for CFA emulsion [4,5], and, moreover, thyroiditis can be induced by administration of repeated doses of mTg alone [3]. Indeed, EAT induction may simulate physiologic triggering mechanisms in thyroid disease.

Our studies with EAT also made use of the well established physiologic feedback loop in thyroid metabolism in which baseline circulatory Tg is released as a byproduct with thyroid hormone (T₄) [6], enabling indirect manipulation of circulating levels of autoantigen. The possibility of elevating circulatory mTg levels by either intravenous administration of exogenous mTg [7], or the release of endogenous mTg via administered thyroid-stimulating hormone (TSH) [7,8] is a unique and powerful feature of the EAT system. This antigen pretreatment of susceptible mice resulted in strong resistance to subsequent EAT induction even with adjuvant, and strongly suggested a role for circulatory Tg in maintenance of self tolerance [7,9].

Pioneering studies in the mid-70s provided early clues to the existence of T cells that suppress autoimmune disease development; depletion of T cells by thymectomy and radiation in mice and rats resulted in low incidence of multi-organ autoimmune diseases over time [10,11]. The coexistence of oophoritis, gastritis and thyroiditis made it difficult to investigate these regulatory (suppressor) T cells (Tregs) in terms of antigen-specificity, capacity to expand, duration of suppression, and interrelationship with MHC class II-restriction. Utilizing some of the unique attributes of EAT, we reported, beginning in 1981–82, the optimal protocols, the dose and time requirement for mTg to enhance existing self tolerance, the antigen-specificity, and the role of Thy1⁺ T cells as mediators of tolerance [7,9]. As monoclonal antibody (mAb) and transgenic technology advanced, we gradually narrowed the Treg lineage in EAT tolerance to the CD4⁺CD25⁺Foxp3⁺ subset, and studied their interrelationship with MHC class II control of susceptibility and resistance. These findings are summarized in this review.

2. Inducing Resistance to EAT in Genetically Susceptible Strains

2.1. Role of circulatory autoantigen

Despite the evidence for autoreactive T cells [3], genetically susceptible mice do not spontaneously develop thyroiditis, suggesting that a mechanism exists to inhibit these cells from inducing autoimmunity. As mentioned above (Sect. 1), earlier studies by neonatal thymectomy and irradiation had indeed provided evidence for naturally-existing Tregs (nTregs) preventing the development of organ-specific autoimmunity, including oophoritis, gastritis, and thyroiditis [10–13]. We hypothesized that, under normal physiologic conditions, Tregs were kept at a basal state of activation by low levels of circulating autoantigen; the homeostatic level was sufficient to prevent the development of autoimmunity but clonal balance between Tregs and autoreactive T cells could be overcome by immunogenic stimuli, such as the administration of autoantigen and adjuvant [7]. We further hypothesized that Treg-

mediated resistance to EAT induction could be strengthened by increasing the levels of circulating Tg in the absence of proinflammatory stimuli, such as polyadenylic:polyuridylic acid complex (poly A:U). Indeed, pretreating mice with intravenous administration of mTg, but not irrelevant autoantigen, resulted in strengthened peripheral tolerance and prevented thyroiditis induction [7]. Resistance to challenge with mTg and adjuvant was similarly induced when mice were given daily injections of TSH or TSH-releasing hormone (TRH) for 13 days to stimulate endogenous mTg release [7]. As seen in Table 1, exogenous high doses of mTg resulted in both T and B cell tolerance with reduced incidence of thyroiditis incidence and negligible mTg Ab titers. Daily injections of TSH or TRH were effective in preventing T cell-mediated infiltration with intermediate Ab levels compared to controls. The response to ovalbumin (OVA) was not affected in mice given mTg, TSH or TRH, demonstrating the specificity of induced tolerance. The antigen specificity of Treg-mediated inhibition of autoimmunity has subsequently been demonstrated in other models of autoimmunity [14].

Several other protocols designed to elevate circulating mTg levels all led to strong resistance to EAT induction: two 100- μ g doses of deaggregated mTg (dmTg) given i.v. 7 days apart, ten 10- μ g doses of mTg given daily [8]; 20 μ g LPS given i.v. 24 hours prior to two 20- μ g doses of mTg [15], or TSH infusion with a peritoneal osmotic pump for 3 days [8]. All of these protocols resulted in elevation of circulating levels of mTg >3- to 5-fold above baseline for a critical period of 2 – 3 days, which correlated directly with the development and establishment of enhanced resistance in susceptible mice [7,8,15].

Antigen-specific tolerance induced by either exogenous or endogenous mTg released by TSH required only 2 – 3 days of elevated mTg levels, but was long-lasting, persisting for at least 73 days [16]. Thus, transient elevation of circulating mTg resulted in alterations of the balance between autoreactive and regulatory lymphocytes. These alterations could have been produced by elimination of functional thyroiditogenic T cells, either by deletion or anergy, or by enhancing the function of Tregs suppressing autoimmunity. The development of anti-CD4 mAbs enabled unequivocal demonstration of the role for an active mechanism of suppression mediated by Tregs; susceptibility to thyroiditis induction could be restored in tolerized mice only after depletion of CD4⁺ T cells [17]. Conversely, CD4⁺ T cells from tolerized mice transferred resistance to subsequent EAT challenge [16]. These studies correlated the elevation of circulating mTg level directly with CD4⁺ Tregs.

2.2. Further definition of Tregs mediating tolerance

While CD4⁺ T cells were critical in mediating induced resistance to EAT induction [17], they represented a large and functionally heterogeneous population of T cells responsible for both thyroiditis development and peripheral tolerance. An important advance in the ability to examine Tregs was the seminal description of a small (~10%) but constant subset of peripheral CD4⁺ T cells constitutively expressing CD25 (IL-2R α) as a marker for CD4⁺ Tregs critical in maintaining peripheral tolerance. CD4⁺CD25⁺ T cells from naïve mice inhibited multi-organ autoimmunity, including thyroiditis, that developed following transfer of CD4⁺CD25⁻ T cells into athymic BALB/c mice [18]. Moreover, they arose naturally in the thymus, but not until day 5 postnatal, indicating that they were absent from the day 3 neonatally thymectomized mice that exhibited multi-organ autoimmunity [19]. Tregs were subsequently defined at the molecular level by the description of Foxp3 (forkhead box P3) as the necessary and sufficient master transcription factor determining Treg development [20]. Since we had reasoned that Tregs mediating induced tolerance were derived from activation or expansion of nTregs responsible for mediating self tolerance under normal conditions [17], we hypothesized that these Tregs would be phenotypically similar to CD4⁺CD25⁺Foxp3⁺ nTregs. Indeed, we showed that Tregs induced by pretreatment with either exogenous (Fig. 1A) or endogenous (Fig. 1B) mTg expressed CD25, as CD25 mAb given in vivo abrogated established tolerance

and the mice regained susceptibility to challenge with mTg and LPS [21]. These CD4⁺CD25⁺ Tregs also expressed Foxp3 and thus were phenotypically similar to the nTregs essential in maintaining naturally-existing tolerance in mice with an intact thymus [22].

2.3. Relationship between nTregs and Tregs mediating induced tolerance

The brief, rapid 2–3 day period required for the development of EAT tolerance suggested that the responsible CD4⁺CD25⁺ Tregs were derived from a functional expansion of pre-existing cells, rather than de novo generation of Tregs. We detected nTregs in traditionally resistant B10 mice, where depletion of CD25⁺ T cells enabled induction of mild thyroiditis [23]. In susceptible CBA mice, we made use of our adjuvant-free model of EAT induction that results in mild mononuclear cell infiltration in ~50% of the mice [3]. Following depletion of CD25⁺ T cells, mice demonstrated increased susceptibility to adjuvant-free thyroiditis induction with higher incidence and thyroiditis severity [24]. Moreover, mice depleted of CD25⁺ T cells failed to respond to tolerogenic doses of mTg to establish resistance to subsequent EAT induction. Thus, in genetically susceptible CBA mice, nTregs maintaining the basal state of self tolerance can be directly linked to Tregs mediating the heightened state of tolerance induced by elevated levels of circulating mTg.

2.4. Context of antigenic stimulation is critical to tolerance induction

The induction of tolerance to EAT by administration of soluble mTg, the same autoantigen used to induce thyroiditis, clearly demonstrates the importance of the context of antigen encounter. The earliest studies of induced tolerance to EAT showed that, while mTg pretreatment in the absence of adjuvant leads to strengthened peripheral tolerance, the same antigen in the presence of an inflammatory stimulus, such as poly A:U, results in thyroiditis [7]. Subsequent investigations demonstrated that administration of proinflammatory cytokine, IL-1 [5] or IL-12 [25], within hours of tolerogenic doses of mTg inhibits tolerance induction and instead results in priming of the inflammatory autoimmune response. This activation of thyroiditogenic T cells induced by inflammatory stimuli is not affected by administration of neutralizing anti-IFN- γ mAb, but is inhibited by anti-CD40L and anti-CD28 mAbs [25], indicating the important role of costimulation in the context of tolerogen presentation. Similarly, administration of mAbs to CD137 (4-1BB) or GITR, members of the TNFR superfamily, concomitant with tolerogenic doses of mTg, interfered with tolerance induction, and resulted in priming of inflammatory thyroiditogenic T cells [21,26]. The inhibition of tolerance induction by GITR mAb is particularly interesting, as other models have described proliferation of Tregs induced in vitro by GITR mAb and IL-2 [27,28]. We reconcile our result with the view that Tregs and autoreactive T cells are in continuous balance in the periphery, and administration of GITR mAb likely activates both T cell subsets, with more vigorous expansion of the autoreactive population.

It is clear that a variety of proinflammatory stimuli can divert the tolerogenic signaling directed to nTregs to immunogenic signaling directed to autoreactive T cells. It is as yet unknown if polarization occurs at the level of antigen presentation by dendritic cells. Our studies demonstrated that the clonal balance between Tregs and autoreactive T cells in maintaining self tolerance is easily perturbed, but circulatory autoantigen greatly influences the T cell subset dominance. Thus, it has been of extreme interest to determine the mechanisms by which Tg activates or expands Tregs. Subsequent to the discovery of Foxp3 as the critical transcriptional regulator of Treg development, it was described that TGF- β was capable in vitro of directing naïve cells to express Foxp3 and develop a Treg phenotype [29–31], providing a possible mechanism to explain EAT tolerance induction. However, administration of neutralizing TGF- β mAb at the time of tolerance induction had no effect on the ability to induce tolerance to subsequent thyroiditis induction (unpublished data). Similarly, neutralizing IL-4 or IL-10 mAbs given during tolerance induction had no effect [32]. While we had described a role for

proinflammatory cytokines in inhibiting tolerance induction, we have yet to observe any known cytokine involvement in promoting the rapid Treg differentiation observed in EAT tolerance.

The important role of costimulation observed in our tolerance induction model, as well as in others [22,33] suggested that a specific costimulatory pathway was likely required for proper tolerogenic stimulation of the immune system. We blocked multiple costimulatory pathways, including CD28 and CD40L, by in vivo administration of blocking mAbs concomitant with tolerogenic doses of mTg; only blockade of CTLA-4 specifically inhibited tolerance induction [24]. The role we describe for CTLA-4 as a critical costimulatory molecule for maintaining and expanding the pool of peripheral Tregs in our model of induced tolerance fits well with observations in other systems, where CTLA-4 deficiency results in generalized autoimmunity, and CTLA-4 is required for protection against autoimmunity mediated by transferred Tregs [33]. The exact mechanism by which CTLA-4 promotes tolerance induction is not clear, though evidence in other models has demonstrated both cell-intrinsic and cell-extrinsic roles in Treg activation and function [34–36]. The nature of CTLA-4 signaling in induction of tolerance to EAT requires further investigation.

2.5. Treg function in EAT tolerance

The mechanisms by which Tregs mediate suppression of autoreactive T cells are unclear. In various models of autoimmunity, production of IL-4, IL-10, and TGF- β , as well as cell-contact dependent mechanisms and competition for IL-2 have assumed some importance [37,38]. We have not detected any effect of in vivo blockade of IL-4, IL-10, TGF- β , or CTLA-4 on the mediation of established tolerance [32] (and unpublished data). These negative results could signify a lack of involvement of these molecules in mediating tolerance, or be examples of functional redundancy in Treg-mediated suppression.

While the most important demonstration of the role of Tregs suppressing thyroiditis results from in vivo studies, we used an in vitro co-culture model with the goal of overcoming the difficulties inherent with the complexity of in vivo studies. The identification of CD25 as a marker for Tregs was critical not only for in vivo experimentation, but also in enabling isolation of a sufficiently concentrated pool of Tregs from mice with established tolerance to EAT. Enriched CD4⁺CD25⁺ T cells suppressed the in vitro mTg-specific proliferative response of thyroiditogenic T cells in a dose-dependent co-culture assay [21,24]. However, this suppression could not be blocked by neutralizing IFN- γ , IL-10, TGF- β , or CTLA-4 mAbs and significant production of IL-4, IL-10, or TGF- β by Tregs following in vitro culture with mTg was undetectable [26] (and unpublished data). In contrast, the proliferative response of mTg-primed splenocytes to mTg in co-culture could be rescued by adding CD137 and GITR mAbs [21, 26]. The presence of these mAbs did not alter the nonproliferative phenotype of CD4⁺CD25⁺ T cells in vitro, indicating that they instead interfered with Treg suppression rather than conversion of Tregs away from a regulatory function. Combined with the observation that in vivo administration of CD137 and GITR mAbs at the time of challenge of mice with established tolerance enabled thyroiditis induction, these data suggest that the mAbs acted to render mTg-responsive cells resistant to Treg suppression, although these mAbs could act to temporarily disable the suppressive action of Tregs.

A critical caveat relating to the limited utility of the co-culture model of Treg function is that co-culture does not reflect the heightened tolerant state in vivo after the increase of circulatory Tg level, nor the same degree of antigen specificity. Tregs from mice with established tolerance to mTg were capable of suppressing the proliferative responses of both mTg-primed T cells and T cells primed to an irrelevant antigen, OVA (unpublished data). This is a critical limitation, as the antigen specificity of EAT and tolerance is a unique and important feature of this model. Furthermore, modeling of Treg-mediated suppression of autoreactive T cells by in vitro assay has additional limitations, including non-physiologic ratios of Tregs to autoreactive T cells and

ignoring the temporal and spatial dynamics of their interactions in vivo. All of these are important considerations going forward with investigation into the mechanism of Treg-mediated suppression in EAT.

3. Treg Function Does Not Supersede the Requirement for Correct MHC Class II Presentation in EAT Susceptibility

3.1. Roles of H2A and H2E class II genes

While EAT susceptibility was traditionally defined by MHC class II *H2A* haplotype [1,2], we have demonstrated that changes in Treg function markedly influence susceptibility and/or resistance to thyroiditis. Enhancing Treg function by tolerance induction with mTg converts an EAT-susceptible *H2^k* strain to resistant. On the other hand, eliminating CD4⁺CD25⁺ Tregs converts traditionally resistant strains, *H2^b* [23] and *H2^d* [39], to susceptible, albeit not to the extent of a susceptible haplotype. Although heterologous hTg can induce EAT in susceptible strains, Treg depletion in resistant *A^b* mice does not enable thyroiditis induction with hTg even in the presence of adjuvant, despite 73% homology with mTg [40], presumably due to an inability of the *A^b* molecule to properly process/present thyroiditogenic hTg epitopes.

In addition to the *H2A* region control, the *H2E* region, expressed in many strains, also exerts a great influence. The role of the *H2E* region was best revealed in the absence of the *H2A* region. These studies made use of the class II-deficient B10.Ab⁰ mice, where the normally expressed *H2A^b* molecule is genetically altered, and the *H2E* region is naturally non-expressed [41,42]. When an *H2E* transgene was introduced into this *H2A⁻* mouse, the resultant E⁺B10.Ab⁰ (*A⁻E⁺*) mouse developed EAT only in response to immunization with heterologous Tgs, human, porcine and bovine [42]. In contrast to *H2A⁺* mice, mTg was unable to induce thyroiditis in this *A⁻E⁺* mouse. To determine the relative influence of Tregs and MHC expression in this strain, *A⁻E⁺* mice were depleted of CD4⁺CD25⁺ T cells in vivo prior to thyroiditis induction with hTg or mTg. Increased susceptibility was observed only to hTg-induced EAT, while resistance to mTg-induced EAT was unaltered [23]. These data demonstrate that, while Tregs function in susceptibility to EAT, correct presentation of autoantigen by MHC class II molecules is an absolute, invariant requirement for the development of autoimmune thyroiditis. The susceptibility and resistance to EAT of various strains and the extent of Treg influence on hTg or mTg immunization are summarized in Table 2.

3.2. How does heterologous Tg-restricted response induce EAT in mice?

To understand how a heterologous Tg-restricted response induces thyroiditis in a mouse which, by definition, only expresses mTg, we reasoned that these hTg-primed cells were recognizing mTg epitopes presented in the thyroid. One mechanism of thyroid damage is by CD8⁺ cytotoxic T lymphocytes (CTL) [43]. To determine if hTg-primed mice generated CTL to specific Tg peptides, we selected two hTg-derived peptides, hTg410 and hTg2344 (Table 3), which had thyroiditogenic properties [44]. CTL from hTg-immunized *A⁻E⁺* mice were generated by in vitro expansion with the peptides and mediated killing of target cells loaded with intact hTg or hTg-derived peptide (Fig. 2A). These responses were antigen-specific, as CTL generated by hTg410 activation did not recognize peptide hTg2344, and vice versa. Furthermore, when lymph node cells from *A⁻E⁺* mice immunized with hTg410 or hTg2344 were cultured with the immunizing peptide, the resultant CTL specifically killed hTg peptide-labeled target cells (Fig. 2B). As shown in a preliminary report, these peptide-specific CTL also killed hTg-loaded targets, demonstrating that the target epitopes are naturally processed from intact hTg [45].

That hTg immunization of *A⁻E⁺* mice generated CTL capable of recognizing specific, naturally-processed epitopes of hTg suggested that such effector cells may be recognizing

mTg-derived peptides to induce thyroid destruction in vivo. Using as a guide three hTg-derived peptides (including hTg410 and hTg2344 described above), previously shown to be recognized by hTg-primed cells [44], we searched for and synthesized corresponding peptides on mTg. Table 3 lists the pairing of some hTg- and mTg-derived peptides. Indeed, one peptide, mTg409, was shown to be H2E^b-restricted and immunogenic for A⁺E⁺ mice, inducing thyroiditis [46]. Of particular note, mTg409 contained 4/4 predicted MHC-binding anchor residues, equivalent to the corresponding, pathogenic hTg peptide, hTg410. We identified a second mTg epitope, mTg179, homologous to peptide hTg179; both shared 4/4 MHC anchor residues and were pathogenic for A⁺E⁺ mice (Table 3). These findings yet again underscore the importance of proper MHC class II presentation for thyroiditis induction.

4. Possible Reciprocal Role of Tregs in the Suppressive effect of MHC Class II Co-expression on Thyroiditis Susceptibility

In our studies to differentiate H2A-mediated from H2E-mediated thyroiditis, we also noted that A⁺E⁺ mice displayed significantly less severe thyroiditis upon H2E^b-restricted hTg immunization, compared to A⁺E⁺ mice (Table 2) [23,42], suggesting that the H2A^b class II molecule exerted a suppressive role in this system. Similarly, we have shown that the presence of an HLA-DQ8 transgene plays a moderating role in HLA-DR3-mediated response to hTg in DR3/DQ8 double transgenic mice [47]. The existence of Tregs influencing EAT induction in the context of this H2A^b class II molecule [23] suggested that they may be responsible for the suppressive effect of H2A expression on H2E-mediated thyroiditis. Also, in an earlier study, transgenic H2E^s molecules were found to reduce the severity of EAT encoded by *H2A^s* in B10.S mice [48], as reported in other models of autoimmune disease [49,50].

4.1. Probe of Treg influence with native Tg molecule

We investigated the possibility that H2A-restricted Tregs might be suppressing H2E-restricted thyroiditis by in vivo elimination of CD4⁺CD25⁺ T cells in E⁺B10 (A⁺E⁺) transgenic mice, which express both the native H2A molecule nonpermissive for hTg-induced EAT, and the transgenic H2E molecule permissive for hTg-induced EAT [23]. Treg-depleted mice developed more severe thyroiditis following induction with either mTg or hTg, suggesting Treg involvement. However, we could not discriminate between H2A- or H2E-derived Tregs, as all CD25⁺ T cells were depleted in vivo. Extending these studies of MHC-restricted Tregs to DR3- or DQ8-transgenic mice, CD4⁺CD25⁺ T cells were depleted prior to EAT induction with either mTg or hTg. Exacerbation was dependent upon the class II genes and the Tg species used for immunization. As seen in Fig. 3, thyroiditis severity was enhanced in DR3-transgenic mice only after induction with hTg, indicating that Tregs exert an inhibitory role. On the other hand, since mTg-induced EAT was already strong in DR3⁺ mice on the NOD background [51], Treg depletion had no additional effect. In DQ8⁺ mice, which are less susceptible than DR3⁺ mice after hTg immunization [52], thyroiditis severity was also increased after depleting Tregs. Even in DQ8⁺ mice, which normally are not susceptible to mTg-induced EAT [52], removal of Tregs led to mild thyroiditis. These results demonstrate that, for HLA-restricted Tregs, differences in function also exist between class II-restricted Tregs. The findings in both *H2* and *HLA* transgenic mice demonstrate that the expression of a new class II molecule can alter susceptibility to autoimmunity by selecting for Tregs with different function.

4.2. Probe of Treg influence with Tg peptides

We further verified the presence of H2A-restricted and H2E-restricted Tregs by using Tg peptides restricted to either class II molecule (Table 3). We delineated five mTg peptides that bind to H2A^b and are immunogenic for A⁺E⁺ and A⁺E⁻ mice [53]. One of these H2A-restricted peptides, mTg1677, is pathogenic for A⁺E⁺ mice and Treg-depleted A⁺E⁻ mice [53], mirroring intact mTg [23]. Likewise, we found responses to H2E-restricted peptides to be higher in both

$A^{-}E^{+}$ and $A^{+}E^{+}$ mice after in vivo depletion of Tregs [46]. These findings demonstrate that Tregs restricted to either MHC class II molecule exist, and suggest that the decrease in hTg-induced thyroiditis severity of $A^{+}E^{+}$, compared to $A^{-}E^{+}$, mice may be a result of an increased number or function of hTg-specific Tregs restricted to A^b . Alternatively, there could be an alteration of the T cell repertoire, including potential deletion of autoreactive cells, and qualitative changes in T cell responses via a combination of T cell epitopes presented in the context of A^b and E^b . Intriguingly, others have observed that epitopes from the H2E molecule inhibit diabetes in the NOD mouse [54,55], and similar presentation of an HLA-DR-derived peptide by HLA-DQ can suppress autoimmunity in HLA-transgenic mice [56]. An analogous event may be occurring in $A^{+}E^{+}$ mice for hTg-induced EAT, although we have not examined whether the E^b molecule can present A^b -derived peptides, nor whether, in a similar B10.S system, A^s molecules can present E^s -derived peptides [48].

5. Role of Thymic Expression of Tg in Susceptibility or Resistance to thyroiditis

As described above, H2A expression has a suppressive effect on the development of H2E-mediated thyroiditis. On the other hand, H2E expression leads to an increase in susceptibility to mTg-induced thyroiditis [23]. Although this increase in susceptibility could be due to a decrease in Treg function in $A^{+}E^{+}$ mice, compared to $A^{+}E^{-}$ mice, we recently proposed a different mechanism for this effect. We used our H2A^b-binding peptides (Table 3), along with a peptide derived from the H2E sequence, Ea52–68, known to bind to H2A^b with high affinity [57]. Indeed, we found that this H2E-derived peptide blocked the binding of mTg and a subset of mTg-derived peptides, including mTg1677, a thyroiditogenic peptide [53]. We proposed that this, or similar, peptide competition in the thymus leads to a decrease in clonal deletion of mTg-specific autoreactive T cells, thereby increasing the frequency in the periphery of these cells with pathogenic potential, as illustrated in Fig. 4. Moreover, mTg-specific Tregs would not be selected. It has been shown that Tg is expressed in the thymus [58,59], and is subject to control by the transcription factor, autoimmune regulator (AIRE) [60,61], as other tissue-specific antigens. Decreased AIRE expression leads to a subtle decrease in thymic expression of Tg and an increase in peripheral autoreactive T cells [60]. Furthermore, studies in humans and mice have suggested that AIRE, via its ability to upregulate tissue-specific antigens, is intimately involved in the selection of Tregs [62,63]. Thus, our recent findings serve to emphasize the importance of thymic expression of Tg in the context of proper MHC class II in the selection of both naive autoreactive T cells and Tregs, thereby influencing susceptibility or resistance to thyroiditis.

6. Perspectives

As an autoimmune disease model to probe the contribution of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs, EAT offers the following unique attributes. 1) It can be induced by Tg immunization with or without adjuvant and its MHC-based susceptibility is well defined according to *H2A* haplotypes, enabling the determination of the extent of Treg control. Indeed, Treg influence does not supersede *H2A*-restriction. 2) The contribution of the *H2E* region can be assessed in the absence of *H2A*, revealing *H2E*-restriction of autoreactive T cells recognizing naturally processed heterologous Tg epitopes. 3) When both *H2A* and *H2E* molecules are expressed, an Ea-chain peptide can be shown to compete with mTg epitope presentation, enabling mTg-specific T cells to escape clonal deletion and ostensibly de-select mTg-specific Tregs.

But the most important attribute of EAT studied over the past two decades is its contribution to our understanding of self tolerance mechanisms. By raising circulatory Tg levels above baseline with either exogenous mTg or endogenously released mTg via TSH infusion, naturally-existing Tregs are activated/expanded to withstand challenge with mTg plus

adjuvant. Autoreactive T cells subject to polyclonal stimulators in the ready presence of circulatory Tg could explain the prevalence of autoimmune thyroid disease in the general population. As described in a recent review by Kong *et al.* [64], thyroid autoimmunity is indeed a prominent autoimmune disorder arising from immunotherapy with either systemic immunomodulators, such as IFN- α , or specific targeting of cells of the immune network, such as CTLA-4 mAb (ipilimumab) or CD52 mAb (alemtuzumab). We have recently investigated the consequences of perturbing Treg function in several breast cancer immunotherapy and EAT combination models [65]. Indeed, EAT can serve as an appropriate indicator of autoimmune sequelae. On the other hand, the ability to specifically activate/expand Tg-specific Tregs offers promise in potentially overcoming opportunistic autoimmune disorders. We are pleased to contribute this paper to the special issue of the Journal of Autoimmunity. We recognize Dr. Noel Rose's contributions in many fields, including his recent work with the American Autoimmune Related Disease Association (AARDA) [66]. We note his continued contributions to the field of autoimmunology [67–74]. This volume is part of the Journal's series in the recognition of outstanding figures in immunology and autoimmunity [75–77].

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Fig. 1.

In vivo depletion of $CD4^+CD25^+Foxp3^+$ T cells abrogates tolerance induced by exogenous or endogenous mTg. Circulatory mTg level was raised either by 100 μ g deaggregated mTg i.v. on days -24, -17 (A), or by TSH-containing osmotic pump i.p. on days -26 or -22 (B). All mice were then injected i.v. with two 1-mg doses of CD25 mAb and challenged with 40 μ g mTg + 20 μ g LPS on days 0 and 7. Thyroiditis was assessed in individual mice on day 28. Adapted from Morris *et al.* (A) [21] and Morris *et al.* (B) [24].

Fig. 2.

Immunization with hTg induces Tg-specific cytotoxic T cells. (A) $E^+B10.Ab^0$ (A^+E^+) mice were immunized with 100 μ g hTg and 20 μ g LPS on days 0 and 7 and their splenocytes were cultured with 10 μ g/ml hTg410 or hTg2344 for 5 days. (B) A^+E^+ mice were immunized with 150 μ g peptide hTg410 or hTg2344 in CFA and their lymph node cells were cultured for 5 days with the priming peptide. The cultured cells were then used as effectors in a ^{51}Cr -release assay; EL-4 lymphoma cells (10^4 /well) loaded with hTg or peptide served as target cells; effector:target ratio = 100:1.

Fig. 3.

Exacerbation of hTg- or mTg-induced thyroiditis by depletion of CD4⁺CD25⁺ T cells is more pronounced in less susceptible DQ8⁺ mice. DR3⁺ Ab⁰/NOD (A) or DQ8⁺ Ab⁰/NOD mice (B) were injected with 1-mg doses of CD25 mAb on days -14 and -10 prior to EAT induction with 100 µg hTg or 40 µg mTg + 20 µg LPS on days 0 and 7. Thyroiditis was assessed in individual mice on day 28.

Fig. 4.

Competition by Eα52-68 peptide interferes with clonal deletion of mTg-specific autoreactive T (Tauto) cells and selection of Tregs in H2A^b mice. (A) In the absence of H2E class II molecules, Tg-derived peptides are efficiently presented by H2A class II molecules for clonal deletion and selection of Tregs. (B) In the presence of H2E class II molecules, Eα-derived peptides, such as Eα52-68, compete with Tg-derived peptides for presentation by H2A class II molecules. Reduced presentation of Tg-derived peptides allows Tg-specific Tauto cells to escape clonal deletion, and decreases the selection of Tg-specific Tregs.

Table 1

Induction of tolerance in susceptible CBA mice by pretreatment with mTg, TSH or TRH.

Protocol	Pretreatment (days) ^a	Thyroiditis incidence (%) ^b	Log ₂ titer (mean ± SE) ^b	
			mTg Ab	ovalbumin Ab
1	mTg (-10, -3)	30	1.6 ± 0.3	ND
2	TSH (-21 --8)	0	7.7 ± 1.4	9.3 ± 0.7
2	TRH (-21 --8)	17	7.8 ± 0.4	9.8 ± 0.9
2	None	67	13.8 ± 0.4	7.5 ± 0.4

^a mTg, 200 µg i.v.; daily TSH, 0.25 U or TRH, 1.0 µg i.p. on the days shown.

^b Immunized i.v. with 20 µg each of mTg and LPS on days 0 and 7, and i.p. with 100 µg ovalbumin on days 0 and 18; sacrificed on day 28 (protocol #1) or day 24 (protocol #2).

ND, not determined.

Adapted from Kong *et al.* [7].

Influence of Tregs and MHC class II expression on response to EAT induction with mTg or hTg.

Table 2

Strain	Class II phenotype		Prior depletion of Tregs ^a		EAT induction with mTg ^b	EAT induction with hTg ^b
	H2A/HLA-DQ	H2E/HLA-DR				
B10	H2A ^b	–	–	–	–	–
E ⁺ B10	H2A ^b	H2E ^b	+	+	+	+
E ⁺ B10.Ab ^{0c}	–	H2E ^b	–	++	++	++
DR3 ⁺ Ab ^{0d}	–	HLA-DR3	+	–	++	++
DQ8 ⁺ Ab ^{0d}	HLA-DQ8	–	–	+++	+++	+++
			+	–	±	±
			+	±	+	+

^aMice were injected i.v. with control IgG or CD25 mAb (PC61) to deplete CD25⁺ Tregs prior to immunization.

^bMice were immunized with mTg or hTg and thyroiditis was assessed on day 28.

^cDeficient in A^b molecule due to knockout of Aβ chain.

^dNon-obese diabetic (NOD) background strain deficient in A^b molecule due to knockout of Aβ chain.

Table 3

MHC Class II-binding synthetic peptides used to probe interaction of Tregs and class II expression

MHC Class II restriction	Name	Derivation	Amino acid sequence ^a	Thyroiditogenic ^b
H2E ^b	hTg179	hTg	NTTDMMI F DLVHSYNRFPD	Yes
	mTg179	mTg	NTTDMMI F DLIHNYNRFPD	Yes
	hTg410	hTg	QSQQ F SVSENL L KEA	Yes
	mTg409	mTg	HYQRLSESL L REA	Yes
	hTg1241	hTg	GPLIC S LESGRWESQ	No
	hTg2344	hTg	L TWVQ T HIRGFGGDP	Yes
H2A ^b	mTg1677	mTg	QNVLSGLY S PVVF S AS	Yes
	mTg1744	mTg	WRDTS A TQ A NAT C AG	-
	mTg2051	mTg	S FCPS A ALQ S L T EEK	-
	mTg2086	mTg	KHFDV A H I ST A ATSN	-
	mTg2342	mTg	LTWVQ S H I GAFGGDPQ	-
	Ea52-68	H2Eα	ASF E AQ G AL A NI A VDKA	-

^a Bolded amino acid indicates predicted MHC class II anchor residue.^b Indicates whether peptide is able to expand Tg-primed cells to transfer thyroiditis to naive mice and/or is itself directly thyroiditogenic. Peptide Ea52-68 is not a Tg-derived peptide, and so was not tested for thyroiditogenicity. -: not determined.