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A Novel H2A-E⁺ Transgenic Model Susceptible to Human but not Mouse Thyroglobulin-Induced Autoimmune Thyroiditis: Identification of Mouse Pathogenic Epitopes

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Abstract

The A⁺E⁺ transgenic mouse is highly susceptible to human thyroglobulin (hTg)-induced thyroiditis, but strongly tolerant to a challenge by mouse thyroglobulin (mTg), in stark contrast to traditionally susceptible strains, wherein mTg induces stronger thyroiditis. To identify mouse thyroid epitopes recognized by destructive, hTg-primed T cells, we selected the three hTg epitopes known to be presented by H2E^b, as the basis for synthesizing potential mTg epitopes. One 15-mer peptide, mTg409, did prime T cells, elicit Ab, and induce thyroiditis. Moreover, cells primed with corresponding, pathogenic hTg410 cross-reacted with mTg409, and *vice versa*. mTg409 contained 4/4 anchor residues, similar to the corresponding hTg peptide. Based on this finding, a second mTg epitope, mTg179, was subsequently identified. These mTg autoepitopes, identified by using thyroiditogenic hTg epitopes, help to explain the severe thyroiditis seen in this novel A⁺E⁺ transgenic model.

Keywords

Autoimmunity; Class II transgene; Experimental autoimmune thyroiditis; H2E transgene; Thyroglobulin epitopes

1. Introduction

Susceptibility or resistance to experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis induced by immunization with thyroglobulin (Tg), is encoded by the H2A gene [1]; for example, A^k encodes susceptibility and A^b resistance. The contribution of

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the other class II locus, *H2E*, is less clear. A functional *H2E* class II molecule is not expressed in every strain, and it is found in both susceptible and resistant haplotypes. However, surface expression of *H2E* molecules does influence susceptibility to several autoimmune diseases. *De novo H2E* expression in transgenic models reduced pathology of autoimmune diabetes, myasthenia gravis and thyroiditis [2–4]. To further study the role of *H2E* in EAT induction, we have used a transgenic B10 mouse that expresses *H2E^b* in the absence of endogenous *H2A^b* molecules that normally mediate resistance [5]. This *E⁺B10.Ab⁰* (*A⁻E⁺*) strain is distinct from both traditionally resistant and susceptible strains. While traditionally resistant strain B10 (*A⁺E⁻*) produces mouse (m) Tg autoantibody without thyroid infiltration, the *A⁻E⁺* strain is profoundly nonresponsive to mTg, with not even a humoral response after mTg immunization with adjuvant [5]. This *A⁻E⁺* strain also deviates from traditionally susceptible strains; heterologous Tg, such as human (h) and porcine (p), induces severe thyroiditis, whereas mTg does not induce any infiltration. This starkly contrasts *H2A*-based susceptibility wherein mTg induces more severe thyroiditis than hTg, due to unique and shared epitopes [6]. Moreover, hTg-primed cells do not proliferate in response to mTg [5], even after prior depletion of regulatory T cells (Tregs) [7]. Thus, the target of pathogenic, hTg-primed cells remains undefined. Nevertheless, thyroid destruction is mediated by typical, mononuclear cell infiltration [5]. We hypothesized that hTg-primed T cells recognize yet unidentified epitopes in the mouse thyroids, leading to thyroid destruction. The necessity of thyroidal Ag presentation was verified by the inability of hTg-primed and -activated cells to transfer thyroiditis to MHC class II-deficient mice [8]. Preliminary studies searching for *H2E^b*-binding peptides of hTg identified three immunogenic sites on hTg [9]. These peptides were *H2E^b*-restricted, stimulated hTg-primed T cells *in vitro*, and two were thyroiditogenic.

That immunization of *A⁻E⁺* mice with hTg results in strong thyroiditis with no detectable response to a self antigen makes this strain unique among all mouse strains studied. To determine the molecular targets of hTg-primed cells responsible for perpetuating thyroid destruction, we focused on mTg epitopes that could be presented by *H2E^b*, using the *H2E^b*-restricted hTg peptides as a guide. We identified two *H2E^b*-restricted T cell epitopes, mTg179 and mTg409, which were immunogenic for *A⁻E⁺* mice, as well as thyroiditogenic.

2. Materials and Methods

2.1. Generation of *E⁺B10.Ab⁰* (*A⁻E⁺*) mice

All mice were bred at the Immunogenetics Mouse Core Facility at the Mayo Clinic. Generation of the transgenic *A⁻E⁺* mouse on the B10 background was previously described [5]. Briefly, an MHC class II-knockout strain, *H2Ab⁰*, obtained by a targeted mutation of the *H2A^b* β -chain gene in an *H2^b* mouse, which does not normally express *H2E* due to a nonfunctional *H2Ea* gene [10], was made congenic by backcross to C57BL/10 (B10) [5]. *A⁺E⁺* (*E⁺B10*) transgenic mice were generated by mating and repeated backcross of *Ea^k* transgenic mice [11] to B10 mice. *A⁻E⁺* (*E⁺B10.Ab⁰*) mice were then produced by intercrosses between *B10.Ab⁰* mice and *E⁺B10* mice [5]. Mice of either sex were kept on acidified, chlorinated water, and used at 10–16 weeks of age.

2.2. Tgs and peptides

mTg and hTg were obtained from frozen thyroids fractionated on a Sephadex G-200 column as described [12]. Tg was checked for the presence of LPS by the *Limulus* amoebocyte assay (Associates of Cape Cod, Woods Hole, MA) (a 40 μ g dose contained <1 ng of LPS) and diluted in nonpyrogenic saline.

The mTg and hTg sequences were previously searched for *H2E^b*-binding peptides, and three hTg peptides (15- to 16-mer) were confirmed immunogenic [9]. For this study, the

corresponding mTg-derived peptides were also synthesized (Fig. 2A). Peptides were synthesized by the Mayo Peptide Synthesis Facility using orthogonal solid-phase methods on Rink resin (Novabiochem, San Diego, CA) and N α -9-fluorenyl-methoxycarbonyl protected L-amino acids [13]. Each peptide was then purified to >90% homogeneity by reverse-phase HPLC, and the molecular mass of each peptide was confirmed by electrospray-ionization mass spectroscopy (MSQ single quad instrument, ThermoFisher-Scientific, Rockford, IL).

2.3. Immunization

Immunization with hTg was performed by i.v. injection of 40 μ g hTg followed 3 h later by 20 μ g of *Salmonella enteritidis* LPS on Days 0 and 7. For proliferative response to peptides, mice were immunized s.c. on Day 0 with peptide emulsified in complete Freund's adjuvant (CFA) at four sites (hind footpads and inner thighs), and sacrificed between Days 10 and 12. Each site received 50 μ l of emulsion containing 50 μ g peptide and 150 μ g *Mycobacterium tuberculosis* (strain H37Ra; Difco Laboratories, Detroit, MI). To induce EAT, mice were immunized s.c. with peptide-CFA on Days 0 and 7 into the thighs and hind footpads on alternate sides, and sacrificed between Days 28 and 35. To enhance induction of EAT, a separate group of mice were injected i.p. with 1 mg CD25 mAb (PC61, rat IgG1 λ ; ATCC, Manassas, VA) on Days -14 and -10 [7]. CD25 mAb was produced by culture in a FiberCell module (Fiber Cell Systems, Frederick, MD), and its concentration in the supernatant was determined by anti-rat λ (BD Pharmingen, San Jose, CA) ELISA. CD25⁺ T cell depletion was verified on Day -4 by FACS analysis of peripheral blood leukocytes with a different CD25 mAb, PE-7D4 (rat IgM; Southern Biotech, Birmingham, AL), as previously described [7].

2.4. Bone marrow (BM)-derived dendritic cells (DC)

BM-derived DC were propagated as described [14]. Briefly, BM cells from normal mice were cultured for 7 days with 20 ng/ml recombinant murine GM-CSF (Peprotech, Rocky Hill, NJ) in RPMI (RPMI 1640, supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol) and 5% fetal bovine serum (FBS). Cells were washed and replenished with fresh medium every 2 days. On Day 6, cells were pulsed with 200 μ g/ml Tg overnight in RPMI/5% FBS supplemented with 100 ng/ml *Escherichia coli* LPS (Sigma, St. Louis, MO). Pulsed DC were harvested on Day 7 and used as antigen-presenting cells (APC). An aliquot was examined by FACS, using PE-labeled CD11c (hamster IgG, clone HL3, BD Pharmingen), and CD86 (rat IgG2a, clone GL-1, ATCC) followed by FITC-labeled anti-rat IgG2a (clone RG7/1.7).

2.5. Thyroid follicle isolation

Thyroid follicles were isolated by slight modification of a previous protocol [15]. Briefly, thyroids were removed from four normal mice and minced with scissors. Minced thyroids were incubated with 2 ml of 1.5 mg/ml type I collagenase (Sigma) at 37°C for 15 min. The thyroids were then vigorously disrupted by pipette and incubated again for 10 minutes. This last procedure was repeated two more times. The enzymatic reaction was terminated by the addition of 20% FBS. The follicles were washed in RPMI, resuspended in 400 μ l RPMI and used as stimulators in proliferation cultures. Since the thyroid follicles cannot be counted accurately, the same exact protocol was used in repeat experiments.

2.6. Adoptive transfer of cells activated with and proliferative response to Tg or peptides

Between Days 28 and 35 after immunization with mTg409-CFA, spleen cells (SC) were cultured for 3 days (37°C) with mTg409 (1–10 μ g/ml) in RPMI and 1% normal mouse serum (NMS). The activated cells ($6\text{--}7 \times 10^7$ /recipient) were then transferred i.v. into irradiated (500 rads) recipients.

SC (6×10^5 cells/well) or lymph node cells (LNC) [6×10^5 cells plus 4×10^5 irradiated (2000 rad) SC/well] were cultured for 5 days in flat-bottomed 96-well plates in quadruplicate in RPMI/1% NMS; the medium contained either 20 $\mu\text{g/ml}$ PPD, 40 $\mu\text{g/ml}$ Tg or 1–10 $\mu\text{g/ml}$ peptide [12]. To assess MHC restriction, 5 $\mu\text{g/ml}$ purified anti-H2E^b mAb (clone Y-17 [16], mouse IgG2b) was added to the cultures. For proliferations with DC, Tg-primed SC were first panned on anti-macrophage (YBM 6.1.10, rat IgG2a) and anti-B cell (YMB 5.10.4, rat IgG2c) coated plates. The enriched T cells ($1\text{--}4 \times 10^5$ /well) were then cultured with Tg-pulsed DC (3×10^3 /well) in round-bottomed plates. [³H]thymidine (1.0 $\mu\text{Ci/well}$) was added 18–20 h prior to harvest onto glass fiber filter paper (Tomtech Mach3Man Cell Harvester, LKB Wallac, Gaithersburg, MD) and incorporation assessed by a Microbeta Plus 1450 liquid scintillation counter (LKB Wallac). Stimulation index was calculated as mean cpm $\pm$ SE of antigen-containing wells divided by mean cpm of control wells.

2.7. Analysis of secreted cytokines

LNC were cultured as described above and supernatants were harvested after two days. The IFN- γ , IL-4, IL-10 and IL-17 concentrations were measured by ELISA, using capture and biotinylated anti-cytokine monoclonal antibodies, per manufacturer's protocol (BD Pharmingen).

2.8. Determination of Abs to Tgs and peptides

Mice were bled from the tail artery on the day of sacrifice, and sera were stored at -20°C . Anti-Tg or anti-peptide titers were measured by ELISA, using plate-bound Tg or peptide (1 $\mu\text{g/well}$ in Immulon II microtiter plates) and alkaline phosphatase-labeled goat anti-mouse IgG (Sigma), as described [12]. The OD (405nm) values were corrected for nonspecific binding by subtracting the OD of normal mouse serum.

2.9. Evaluation of thyroiditis

Thyroid pathology was assessed by histological examination of H&E stained thyroids. Thyroid specimens were sectioned vertically through both thyroid lobes (50–60 sections from 10–15 step levels). Mononuclear cell infiltration was scored on an index of 0–4.0: 0, normal thyroid; 0.5 small interstitial foci of infiltration involving $>0\text{--}10\%$ of the thyroid; 1.0, follicular destruction with $>10\text{--}20\%$ involvement; 2.0, $>20\text{--}40\%$ involvement; 3.0, $>40\text{--}80\%$ involvement; and 4.0, $>80\%$ involvement [12].

2.10. Statistical analysis

Differences between groups of *in vitro* proliferation assays were analyzed using the unpaired Student's *t* test.

3. Results

3.1. hTg-primed E⁺B10.Ab⁰ (A⁺E⁺) cells do not respond to mTg presented by various APC

We have shown previously that A⁺E⁺ mice are highly tolerant to mTg; immunization with this self antigen induced neither a detectable humoral nor a cellular response to mTg [5]. Moreover, the strong anti-hTg T cell response observed after hTg immunization, which presumably is responsible for thyroid destruction, did not cross-react with mTg. However, since mTg and hTg are 72% homologous [17], and both induce thyroiditis in conventional, susceptible strains [6], the hTg-primed thyroiditogenic T cell response in the A⁺E⁺ mouse was most likely directed toward mTg, or its processed peptides. We first tried to detect such cross-reactivity by measuring the cytokine secretion of hTg-primed cells to mTg. Along with IFN- γ , IL-4 and IL-10, we determined the secretion of IL-17 by hTg-primed cells. IL-17 is secreted by a distinct inflammatory subset of CD4⁺ T cells, designated Th17, which has been shown to play an

important role in other CD4⁺ T cell-mediated autoimmune diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis [18]. While hTg-primed cells secreted significant amounts of IL-17 and IFN- γ in response to the immunogen hTg (48x and 490x over background for IL-17 and IFN- γ , respectively), IL-17 and IFN- γ secretions in response to mTg stimulation remained at background levels (Fig. 1A). Cultured hTg-primed cells did not secrete significant amounts of IL-4 or IL-10 in response to either hTg or mTg (data not shown). We next used BM-derived DC, containing 53% CD86⁺ DC (Fig. 1B), to present mTg to hTg-primed T cells. While hTg-pulsed DC induced proliferation of hTg-primed splenocytes as expected, no response was observed to mTg-pulsed DC (Fig. 1C).

To determine if the pathogenic, hTg-primed T cells were recognizing any mTg epitopes present only in the mouse thyroid, we incubated hTg-primed splenocytes with disrupted normal thyroid follicles, as well as with control hTg and mTg. The hTg-primed cells did not respond to increasing concentrations of mouse thyroid follicles, nor to mTg added to SC aliquots (Fig. 1D). Thus, different assays for mTg reactivity did not uncover any response from hTg-primed cells to mTg.

3.2. Identification of an mTg-derived peptide immunogenic for E⁺B10.Ab⁰ (A⁻E⁺) mice

Since hTg-primed cells from A⁻E⁺ mice did not respond to mTg-pulsed DC, nor to thyroid follicular Ag, we searched for mTg epitopes that could be presented by H2E^b molecules. We used the three hTg peptides known to be naturally processed and presented by H2E^b as a basis for synthesizing corresponding mTg peptides with immunogenic potential (Fig. 2A). While none of the hTg-derived peptides constituted an immunodominant epitope, each was recognized by hTg-primed A⁻E⁺ T cells, and hTg410, and to a lesser extent hTg2344, were pathogenic, as they activated hTg-primed splenocytes for thyroiditis transfer to naive animals [9]. Each mTg peptide had at least 2 of 4 predicted anchor residues for H2E^b [19,20], and were designated mTg409, mTg1241, and mTg2342.

First, we tested primed cells from hTg-immunized animals to determine if the cells recognized and responded to any mTg peptide. No significant proliferative response was observed (data not shown). Next, the mTg peptides were used to immunize mice directly. As seen in Fig. 2B, only mTg409 was immunogenic for A⁻E⁺ mice, generating primed cells specific for mTg409. Moreover, this peptide was H2E^b-restricted, as an anti-E^b mAb significantly reduced mTg409-specific proliferation (Fig. 2C). This peptide also induced a weak Ab response (Fig. 3B) and was selected for further study.

3.3. T cells and Abs primed to the pathogenic peptide hTg410 cross-react with self peptide mTg409

Peptide hTg410 was previously found to be pathogenic in the A⁻E⁺ mouse; not only did it activate splenocytes to transfer thyroiditis to naive mice, it also induced mild thyroiditis upon direct immunization [9]. Since the corresponding mouse peptide, mTg409, was found to be immunogenic, we tested whether T cells primed to either peptide could cross-react with the corresponding peptide. After immunization with either mTg409 or hTg410, SC primed to mTg409 cross-reacted with hTg410 (Fig. 3A), and reciprocally, hTg410-primed cells cross-reacted with mTg409 (Fig. 3C). However, only Abs raised to hTg410 cross-reacted with mTg409 (Fig. 3D), while mTg409 immunization induced only a mild Ab response in some animals, with no cross-reactivity to hTg410 (Fig. 3B).

3.4. Peptide mTg409 is pathogenic in E⁺B10.Ab⁰ (A⁻E⁺) mice

We next determined if mTg409, like the corresponding hTg peptide, hTg410, is thyroiditogenic in A⁻E⁺ mice. hTg410 is a naturally processed epitope as it stimulates hTg-primed cells, but we did not observe any proliferation of hTg-primed cells to mTg409, and therefore could not

test if mTg409 could sufficiently activate hTg-primed cells necessary for transferring thyroiditis to naive animals. Instead, we directly immunized mice with mTg409, and observed 23% thyroiditis incidence (Fig. 3E). When SC from such mice were expanded with mTg409 for 3 days *in vitro*, and then $6-7 \times 10^7$ activated cells transferred to irradiated mice, 55% of the recipient animals developed thyroiditis (Fig. 3E). Furthermore, depletion of Tregs with CD25 mAb prior to immunization enhanced EAT induction by mTg409, increasing the incidence from 23 to 53% (Fig. 3E). Prior depletion of Tregs also led to enhanced mTg409 Ab response, which still did not cross-react with hTg410 (data not shown).

3.5. Peptide mTg179 is pathogenic in E^bB10.Ab⁰ (A⁻E⁺) mice

As shown in Fig. 2A above, mTg 409, the most pathogenic mTg peptide for A⁻E⁺ mice, contained 4/4 anchor residues for E^b, as did the corresponding pathogenic hTg peptide. To delineate additional pathogenic mTg-derived peptides, we searched for mTg peptides containing 4/4 predicted anchor residues and homologous to hTg peptides containing 4/4 predicted anchor residues. We selected one such peptide, mTg179, which contained 4/4 predicted anchor residues for E^b and which was homologous to peptide hTg179. Peptide hTg179 was previously shown to be weakly pathogenic in DR3-transgenic mice [13], as well as a binder for H2A^k and H2E^k molecules [21]. The sequences of the mTg and hTg peptides are compared in Fig. 4A. Of particular note is that, in addition to each peptide containing 4/4 predicted binders for E^b, all residues except 2/4 anchor residues are identical. Similar to pathogenic mTg409, both mTg179 and hTg179 were immunogenic for A⁻E⁺ mice, and mTg179-primed T cells cross-reacted with hTg179 (data not shown). In addition, Abs raised to either mTg179 or hTg179 recognized the corresponding peptides, as well as intact mTg and hTg (data not shown). To determine if we had identified a second pair of corresponding Tg epitopes thyroiditogenic for A⁻E⁺ mice, animals were first depleted of Tregs and immunized with either mTg179 or hTg179. mTg179 immunization resulted in thyroiditis in 5/6 animals (83%), with infiltration encompassing up to 70% of the thyroid, and hTg179 immunization led to thyroiditis in 4/6 mice (67%), with infiltration of up to 50% of the gland (Fig. 4B). Representative thyroid sections from mTg179 and hTg179-immunized animals are shown in Fig. 4C.

4. Discussion

The transgenic E^bB10.Ab⁰ (A⁻E⁺) mouse is unique among all studied mouse strains in that induction of EAT is possible only with heterologous, and not self, Tg [5]. Depletion of Tregs before immunization with mTg, which renders traditionally resistant strains, e.g. B10 [7] and BALB/c [22], permissive for EAT induction, does not alter the profound tolerance to mTg in this A⁻E⁺ strain [7]. Since several attempts to detect a response to mTg in this mouse had been unsuccessful, it was necessary to account for how a pathogenic response directed only to hTg-specific epitopes could induce tissue destruction encompassing up to 80% of the mouse thyroid target [5,7]. We first verified our previous findings that pathogenic, hTg-primed cells did not recognize mTg, using more sensitive assays of T cell reactivity, such as direct stimulation with thyroid follicles as well as IFN- γ and IL-17 production measured by ELISA. We found that IL-17 production, indicative of a Th17 response important in other autoimmune disease models [18], and production of the Th1 cytokine INF- γ were induced upon immunization with hTg, but that that neither was secreted by hTg-primed cells in response to self mTg (Fig. 1A). Instead, we devised a strategy to determine if a response to specific mTg epitopes could be detected, as mTg and hTg are 72% homologous [17]. Using three hTg-derived T cell epitopes as templates, we identified an epitope derived from mTg, mTg409, which was recognized by T cells primed to a pathogenic, hTg-derived epitope, hTg410 [9]. Furthermore, the mouse epitope was itself thyroiditogenic. We also identified a second thyroiditogenic mTg epitope, mTg179.

The identification of these mTg-derived epitopes is the first report of mTg epitopes being presented by H2E^b.

While mTg409-primed cells did not respond to native mTg *in vitro*, it is possible that mTg409 is only processed under circumstances or by cells different from splenic APCs or BM-derived DC that we tested for *in vitro* proliferation assays. Alternatively, mTg409 may not be as efficiently processed from mTg as hTg410 is from hTg, as stimulation with disrupted thyroid follicles did not result in a proliferative response of hTg-primed T cells (Fig. 1). However, it is clear that this epitope is presented *in vivo* in the mouse thyroid, since direct immunization with mTg409 resulted in mild thyroiditis. It is therefore very likely that hTg-primed T cells recognizing the hTg410 homologue of mTg409 responded to mTg409 in the mouse thyroid to initiate thyroid destruction. Since hTg410 is naturally processed from hTg immunization in that hTg-primed T cells are activated by hTg410 *in vitro* [9], mTg409 represents one of the epitopes targeted by hTg-primed T cells. The identification of a second mTg epitope capable of inducing thyroiditis in this A^uE⁺ strain, mTg179, as well as its corresponding hTg epitope, hTg179, further underscores the idea that pathogenic, hTg-primed cells are capable of recognizing specific epitopes on mTg.

In a previous report [5], we raised the possibility that the total lack of response to mTg in this strain might be related to deficient positive selection of mTg-specific T cells, due to the absence of H2A molecules serving this role in conventional strains, or to the inability of H2E^b to present mTg-derived epitopes. We demonstrated here that indeed T cells specific for at least two mTg-derived epitopes, mTg179 and mTg409, are positively selected, and that these epitopes are presented by H2E^b without the need for A^b expression. Thus, it appears more likely that the profound tolerance to mTg is related to the deletion of mTg-specific autoreactive T cells by H2E^b molecules, similar to its efficient role in deleting various T cells self-reactive to *mtv*-encoded superantigens, such as V β 5⁺ and 11⁺ T cells [23]. However, as discussed previously, it is unlikely that these two subsets of T cells contain all the mTg-specific clones, since V β 8⁺ cells from this strain contain thyroiditogenic clones detectable after hTg immunization [8], which presumably recognize mTg-derived epitopes presented in the mouse thyroid. Moreover, we have also shown that the T cell repertoire is highly flexible, as vast alterations in TCR V β usage in susceptible strains with the appropriate class II molecules do not alter thyroiditis induction [24].

Instead, we hypothesize that the H2E-mediated deletion of mTg-specific clones must include T cells with a broader range of affinity for mTg than those deleted by H2A molecules, such that only very low-affinity mTg-reactive T cells, refractory to stimulation with intact mTg, remain. This deletion must be quite efficient, since it apparently extends to mTg-autoreactive T cells that normally provide help to B cells in H2A-expressing strains, and results in the absence of mTg Ab production in mTg-immunized mice [5]. This deletion of mTg-specific T helper cells is reflected by the inability of mTg409 to induce an Ab response comparable to hTg410 (Figs. 3B, D). Transgenic *H2E* expression has been shown to reduce disease severity in autoimmune diabetes, myasthenia gravis and thyroiditis [2–4], suggesting that efficient deletion of self-reactive T cells is an important function of H2E molecules. Continuing exposure to circulatory mTg after T cell clones reach the periphery perpetuates this profound tolerance, further enhancing self resistance to mTg. As shown recently, the Tregs in this strain are H2E-restricted; treatment with CD25 mAb exacerbated hTg-induced thyroiditis without abrogating the tolerant status to mTg [7]. In the current study, the H2E-restricted T cell reactivity to mTg409 was also enhanced by Treg depletion, with increases in both the incidence and severity of EAT (Fig. 3E).

Clearly, the strong tolerance to mTg, resulting from a combination of efficient clonal deletion and continuing peripheral tolerance, is not easily broken. Our studies show that the remaining

low-affinity, mTg-reactive clones can only be activated by molecular mimicry provided by heterologous Tg epitopes with high homology to the corresponding mTg epitopes. This high homology appears to be associated with the number of predicted H2E^b anchor residues of the corresponding peptides. Of the three mTg epitopes derived according to corresponding immunogenic hTg peptides, only mTg 409, but not mTg1241 or mTg2342, is immunogenic, despite lower amino acid identity (7/15), compared to mTg1241 and mTg2342 (10/15 and 12/16, respectively) (Fig. 2A). mTg409 and hTg410 both contain 4/4 predicted anchor residues, even though those amino acids are not necessarily identical, while those within mTg1241 and mTg2342 drop to 3/4 and 2/4 predicted anchor residues, respectively. In another report, the difference between a nonimmunogenic hTg-derived epitope with 15/19 amino acid identity to the corresponding immunogenic peptide from rat Tg in an SJL (*H2A^a*) mouse [25] may also be due to the reduction of one anchor residue, resulting in a binding score below the cutoff as determined by RANKPEP [26]. The ability to be presented by class II molecules via anchor residues, an important characteristic of immunogenicity for T cell epitopes, is particularly critical in this A⁺E⁺ strain, in which self reactivity has been reduced to a minimum. Only when both immunizing antigen and target self antigen contain epitopes with maximal anchor residue affinity for H2E^b, accompanied by inflammatory responses stimulated by hTg or pTg epitopes foreign to the mouse, can peptides such as hTg410 and hTg 179 act as molecular mimics strong enough to activate the low-affinity, mTg-autoreactive T cells to induce thyroid destruction.

HLA-DR molecules, especially HLA-DR3, have been associated with Hashimoto's thyroiditis, and our laboratory has previously used a DR3-binding algorithm and humanized, DR3-transgenic mice to delineate DR3-binding hTg peptides involved in thyroiditis [13]. As H2E is homologous to human HLA-DR, the successful identification of mTg-derived peptides presented by an H2E class II molecule opens the possibility of comparing the roles of H2E and HLA-DR molecules in autoimmune thyroiditis. Interestingly, a 20-mer hTg peptide (p2340), which contains four N-terminal amino acids additional to a peptide (hTg2344) pathogenic in our A⁺E⁺ strain [9], has been reported to bind to H2E^k [27], as well as HLA-DR3 [28]. Similarly, we report here that an hTg-derived peptide, hTg179, weakly pathogenic in DR3-transgenic mice [13], is immunogenic in A⁺E⁺ mice. This hTg-derived epitope appears to be a promiscuous class II-binder; in addition to HLA-DR3 and H2E^b, it has also been reported to bind to H2A^k and H2E^k molecules [21]. The corresponding mTg peptide, mTg179, contains four anchor residues for H2E^b, as does hTg179, and is immunogenic and pathogenic. The identification of this second mTg-epitope presented in the context of H2E^b demonstrates that even with efficient deletion of mTg autoreactivity by H2E, strong molecular mimics from multiple heterologous Tg epitopes can provoke vigorous responses to self, accounting for the up to 80% thyroid destruction observed in the *H2E*-transgenic model.

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References

1. Beisel KW, David CS, Giraldo AA, Kong YM, Rose NR. Regulation of experimental autoimmune thyroiditis: mapping of susceptibility to the *I-A* subregion of the mouse *H-2*. Immunogenetics 1982;15:427–431. [PubMed: 6804377]
2. Nishimoto H, Kikutani H, Yamamura K, Kishimoto T. Prevention of autoimmune insulinitis by expression of I-E molecules in NOD mice. Nature 1987;328:432–434. [PubMed: 3302721]
3. Christadoss P, David CS, Shenoy M, Keve S. Ea^k transgene in B10 mice suppresses the development of myasthenia gravis. Immunogenetics 1990;31:241–244. [PubMed: 2109730]

4. Kong YM, David CS, Lomo LC, Fuller BE, Motte RW, Giraldo AA. Role of mouse and human class II transgenes in susceptibility to and protection against mouse autoimmune thyroiditis. *Immunogenetics* 1997;46:312–317. [PubMed: 9218533]
5. Wan Q, Shah R, McCormick DJ, Lomo LC, Giraldo AA, David CS, Kong YM. *H2-E* transgenic class II-negative mice can distinguish self from nonself in susceptibility to heterologous thyroglobulins in autoimmune thyroiditis. *Immunogenetics* 1999;50:22–30. [PubMed: 10541803]
6. Nabozny GH, Simon LL, Kong YM. Suppression in experimental autoimmune thyroiditis: the role of unique and shared determinants on mouse thyroglobulin in self-tolerance. *Cell. Immunol* 1990;131:140–149. [PubMed: 1699671]
7. Morris GP, Yan Y, David CS, Kong YM. H2A- and H2E-derived CD4⁺CD25⁺ regulatory T cells: a potential role in reciprocal inhibition by class II genes in autoimmune thyroiditis. *J. Immunol* 2005;174:3111–3116. [PubMed: 15728527]
8. Yan Y, Panos JC, McCormick DJ, Wan Q, Giraldo AA, Brusic V, David CS, Kong YM. Characterization of a novel H2A⁺E⁺ transgenic model susceptible to heterologous but not self thyroglobulin in autoimmune thyroiditis: thyroiditis transfer with V β 8⁺ T cells. *Cell. Immunol* 2001;212:63–70. [PubMed: 11716530]
9. Kong YM, Flynn JC, Wan Q, David CS. *HLA* and *H2* class II transgenic mouse models to study susceptibility and protection in autoimmune thyroid disease. *Autoimmunity* 2003;36:397–404. [PubMed: 14669947]
10. Cosgrove D, Gray D, Dierich A, Kaufman J, Lemeur M, Benoist C, Mathis D. Mice lacking MHC class II molecules. *Cell* 1991;66:1051–1066. [PubMed: 1909605]
11. Le Meur M, Gerlinger P, Benoist C, Mathis D. Correcting an immune-response deficiency by creating *E α* gene transgenic mice. *Nature* 1985;316:38–42. [PubMed: 3925351]
12. Kong, YM. Experimental autoimmune thyroiditis in the mouse. In: Coligan, JE.; Bierer, BE.; Margulies, DH.; Shevach, EM.; Strober, W., editors. *Current Protocols in Immunology*. New York: John Wiley & Sons, Inc.; 2007. p. 15.7.1-15.7.21.
13. Flynn JC, McCormick DJ, Brusic V, Wan Q, Panos JC, Giraldo AA, David CS, Kong YM. Pathogenic human thyroglobulin peptides in HLA-DR3 transgenic mouse model of autoimmune thyroiditis. *Cell. Immunol* 2004;229:79–85. [PubMed: 15474522]
14. Inaba, K.; Swiggard, WJ.; Steinman, RM.; Romani, N.; Schuler, G. Isolation of dendritic cells. In: Coligan, JE.; Kruisbeck, AM.; Margulies, DH.; Shevach, EM.; Strober, W., editors. *Current Protocols in Immunology*. New York: John Wiley & Sons, Inc.; 1998. p. 3.7.1-3.7.15.
15. Creemers P, Rose NR, Kong YM. Experimental autoimmune thyroiditis: in vitro cytotoxic effects of T lymphocytes on thyroid monolayers. *J. Exp. Med* 1983;157:559–571. [PubMed: 6296264]
16. Lerner EA, Matis LA, Janeway CA Jr, Jones PP, Schwartz RH, Murphy DB. Monoclonal antibody against an Ir gene product. *J. Exp. Med* 1980;152:1085–1101. [PubMed: 6158546]
17. Caturegli P, Vidalain PO, Vali M, Aguilera-Galaviz LA, Rose NR. Cloning and characterization of murine thyroglobulin cDNA. *Clin. Immunol. Immunopathol* 1997;85:221–226. [PubMed: 9344706]
18. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu. Rev. Immunol* 2007;25:821–852. [PubMed: 17201677]
19. Schild H, Grüneberg U, Pougialis G, Wallny H-J, Keilholz W, Stevanovic S, Rammensee H-G. Natural ligand motifs of H-2E molecules are allele specific and illustrate homology to HLA-DR molecules. *Inter. Immunol* 1995;7:1957–1965.
20. Rammensee H-G, Bachmann J, Emmerich NPN, Bacher OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 1999;5:213–219. [PubMed: 10602881]
21. Li HS, Jiang HY, Carayanniotis G. Modifying effects of iodine on the immunogenicity of thyroglobulin peptides. *J. Autoimmun* 2007;28:171–176. [PubMed: 17327138]
22. Wei W-Z, Jacob JB, Zielinski JF, Flynn JC, Shim KD, Alsharabi G, Giraldo AA, Kong YM. Concurrent induction of antitumor immunity and autoimmune thyroiditis in CD4⁺CD25⁺ regulatory T cell-depleted mice. *Cancer Res* 2005;65:8471–8478. [PubMed: 16166327]
23. Kotzin BL, Leung DYM, Kappler J, Marrack P. Superantigens and their potential role in human disease. *Adv. Immunol* 1993;54:99–166. [PubMed: 8397479]

24. Lomo LC, Zhang F, McCormick DJ, Giraldo AA, David CS, Kong YM. Flexibility of the thyroiditogenic T cell repertoire for murine autoimmune thyroiditis in CD8-deficient ($\beta 2m^{-/-}$) and T cell receptor $V\beta^c$ congenic mice. *Autoimmunity* 1998;27:127–133. [PubMed: 9609129]
25. Rao VP, Carayanniotis G. Contrasting immunopathogenic properties of highly homologous peptides from rat and human thyroglobulin. *Immunology* 1997;90:244–249. [PubMed: 9135553]
26. Reche PA, Glutting J-P, Zhang H, Reinherz EL. Enhancement to the RANKPEP resource for the prediction of peptide binding to MHC molecules using profiles. *Immunogenetics* 2004;56:405–419. [PubMed: 15349703]
27. Karras E, Carayanniotis G, Lymberi P. Induction of murine thyroiditis by a non dominant E^k -restricted peptide of human thyroglobulin. *Immunology* 2003;108:556–561. [PubMed: 12667218]
28. Karras E, Yang H, Lymberi P, Christadoss P. Human thyroglobulin peptide p2340 induces autoimmune thyroiditis in HLA-DR3 transgenic mice. *J. Autoimmun* 2005;24:291–296. [PubMed: 15927791]

Fig. 1.

hTg-primed SC proliferate only in response to hTg, and not to mouse thyroid antigens. (A) Mice were immunized with hTg-CFA and lymph nodes were removed on Day 11. LNC (6×10^5 cells plus 4×10^5 irradiated SC/well) were cultured with mTg, hTg (40 μ g/ml) or control PPD (20 μ g/ml) for 2 days, and the concentrations of IL-17 and IFN- γ in the supernatants were determined by ELISA and expressed as multiples above background (cells incubated with medium: 1.7 ng/ml for IL-17 and 51 pg/ml for IFN- γ). (B) On day of culture, BM-derived, Tg-pulsed DC were labeled with anti-CD11c and anti-CD86 and analyzed by FACS. (C) Purified hTg-primed SC obtained from mice immunized with hTg + LPS were cultured for 5 days, at the indicated cell number, with Tg-pulsed, BM-derived DC from (B) (3×10^3 DC/well). Proliferation was assessed by [3 H]thymidine uptake. (D) Mice immunized with hTg + LPS were sacrificed on Day 28. SC from hTg-primed mice or from unimmunized mice were cultured with mTg, hTg (40 μ g/ml), or varying amounts of mouse thyroid follicles for 5 days (background cpm $480\text{--}570 \pm 40\text{--}80$), and proliferation was assessed by [3 H]thymidine incorporation.

Fig. 2.

Peptide mTg409 is immunogenic in H2A⁺E⁺ mice, and H2E^b-restricted. (A) mTg sequences of the corresponding hTg peptides are compared. Boxed amino acids indicate predicted H2E^b-binding anchor residues, and lines between amino acids indicate identity. (B) Mice were immunized with the indicated peptide in CFA and lymph nodes were removed on Day 10. LNC (6×10^5 cells plus 4×10^5 irradiated SC/well) were cultured with mTg-derived peptides (10 $\mu\text{g}/\text{ml}$) for 5 days and proliferation was assessed by [^3H]thymidine incorporation (background cpm 2270–2520 $\pm$ 330–420). (C) LNC (6×10^5 cells plus 4×10^5 irradiated SC/well) from mice immunized with mTg409 in CFA were cultured with mTg409 (10 $\mu\text{g}/\text{ml}$) in the presence of anti-E^b (Y-17) mAb for 5 days before proliferation was assessed. The filled bars represent proliferation to mTg409 while the open bars reflect the background proliferation (medium alone or anti-E^b mAb alone, respectively).

Fig. 3.

T cells and Abs from hTg410-immunized mice cross-react with pathogenic epitope mTg409. Mice were immunized with mTg409 (A and B) or hTg410 (C and D) in CFA and spleens were collected between Days 28–35. (A and C) SC were cultured with mTg409 or hTg410 (10 μ g/ml) for 5 days and proliferation was assessed by [3 H]thymidine incorporation (background cpm 2230 ± 270 and 2600 ± 360 , respectively). (B and D) Cross-reactive Ab responses from individual mTg409-immunized or hTg410-immunized mice were measured by ELISA (1:20 dilution). (E) Mice were immunized on Days 0 and 7 with mTg409 in CFA. Thyroiditis was assessed on Day 35 ($\bullet$). Spleen cells were cultured for 3 days with the immunizing peptide and transferred at $6-7 \times 10^7$ cells/500-rads irradiated recipient. Thyroids were evaluated for

inflammation 14 days post adoptive transfer (□). For comparison, a separate group of mice were depleted of Tregs by injection of 1 mg CD25 mAb 14 and 10 days before immunization with mTg409 in CFA. Pathology was assessed on Day 35 (▲).

A

Sequences of corresponding Tg peptides

hTg179	NTTDMMI F DL V H S YN R FPD
mTg179	NTTDMMI F DL I H N YN R FPD

B

Fig. 4. mTg179 is thyroiditogenic in H2A^bE⁺ mice. (A) mTg and hTg sequences at position 179 are compared. Boxed amino acids indicate predicted H2E^b-binding anchor residues, and lines between amino acids indicate identity. (B) Mice were depleted of Tregs by injection of 1 mg CD25 mAb 14 and 10 days before immunization with mTg179 (●) or hTg179 (□) in CFA. Thyroiditis was assessed on Day 35. (C) Representative sections of thyroid destruction from an mTg179-immunized mouse (left panel) and an hTg179-immunized mouse (right panel) with thyroid involvement of 70% and 15%, respectively (original magnification 100x).