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Tolerance to autoimmune thyroiditis: $CD4^+CD25^+$ regulatory T cells influence susceptibility but do not supersede MHC class II restriction

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1. ABSTRACT

Murine experimental autoimmune thyroiditis (EAT), a model of Hashimoto's thyroiditis, has served for more than three decades as a prototypical model of T cell-mediated autoimmunity. Early investigations demonstrated a clear correlation between genetic factors, particularly the *H2A* locus of the MHC class II region, and susceptibility to autoimmune thyroiditis. Early studies also demonstrated that susceptibility to EAT induction could be modulated by manipulation of circulating levels of thyroglobulin (Tg), the principal thyroid antigen, resulting in the strengthening of self-tolerance. This antigen-specific induced tolerance is mediated by thymus-derived cells, and subsequent investigations revealed that the suppressive function is located in the $CD4^+CD25^+$ T cell subset, similar to findings in other models. We have demonstrated that these $CD4^+CD25^+$ regulatory T cells (Treg) influence susceptibility to thyroiditis in naive, as well as mTg-tolerized mice. Here, we describe the influence of both Treg and MHC class II haplotype, independently, as well in combination, and describe our recent utilization of MHC class II transgenic mice to directly compare the extent of their influences.

2. INTRODUCTION

Murine experimental autoimmune thyroiditis (EAT) is a model of Hashimoto's thyroiditis (HT), an inflammatory autoimmune disease that results in hypothyroidism secondary to destruction by mononuclear cell infiltrate, and is one of the first diseases with defined autoimmune origins (1, 2). For the past three decades, EAT has functioned as a prototypical model in studies of T cell-mediated, organ-specific autoimmunity. Early studies on EAT revealed the correlation of major histocompatibility complex (MHC) class II alleles with susceptibility (3-5), and demonstrated the presence of autoreactive T cells responsive to syngeneic thyroglobulin (Tg) (6), the primary thyroid autoantigen (7, 8). Thus, EAT can be induced by repeated doses of syngeneic Tg, 16 times over a 4-week period without adjuvant (6), or two doses with either complete Freund's adjuvant (CFA) or bacterial lipopolysaccharide (LPS) (9). Similar to patients with HT, the animals exhibit mononuclear cell infiltration, autoantibody production and T cell proliferative response to Tg. Early investigations also demonstrated the ability to induce resistance to subsequent EAT induction in susceptible mice by elevating the circulatory levels of

mouse (m)Tg, either by intravenous administration of exogenous mTg (10) or through stimulated release of endogenous mTg by infusion of thyroid-stimulating hormone (11). The heretofore unknown role of circulatory mTg thus acquired a possible function in maintaining self tolerance (10).

Induced EAT tolerance is antigen-specific, and requires a critical period of 3 days for development, and once established is long-lasting (10-12). Furthermore, tolerance is transferable by thymus-derived cells (10), mediated by $CD4^+$ T cells (13), and subsequent studies have narrowed the suppressive function to the $CD4^+CD25^+$ T cell subset (14). To verify directly the presence of $CD4^+CD25^+$ Treg in naive mice capable of suppressing the activity of thyroiditogenic T cells, we utilized MHC class II transgenic models to compare the influence of both MHC class II haplotype and Treg activity on susceptibility to EAT (15). Here, we present findings related to the influence of both MHC class II and Treg, and discuss the contributions of each to influence the development of autoimmune thyroiditis.

3. INFLUENCE OF $CD4^+CD25^+$ REGULATORY T CELLS ON EAT SUSCEPTIBILITY

3.1. Antigen-Specific Induction of EAT Tolerance

Early studies in EAT demonstrated a strong influence of genetic factors on susceptibility to thyroiditis, as differences in susceptibility to EAT induction between mouse strains can be segregated by MHC class II *H2A* gene alleles (3, 4). However, it is important to note that genetically susceptible mice do not spontaneously develop autoimmune thyroiditis, even though not all self-reactive T cells are deleted in the thymus and T cells that respond to self antigen can be found in the circulation of normal mice, free of autoimmune disease (6). These autoreactive T cells do not normally cause autoimmunity, despite continual encounter with autoantigen, unless provoked by administration of Tg and adjuvant such as LPS or CFA (9, 16), repeated doses of Tg (6), or prolonged external stimuli (NaI), as in the induction of thyroiditis in NOD.$H2^{h4}$ mice on the autoimmune-prone NOD background (17, 18). These observations strongly suggest that some mechanism exists in the periphery to suppress their potential autoreactivity.

Early investigations in EAT demonstrated that this natural tolerance can be strengthened to inhibit subsequent thyroiditis induction with Tg and adjuvant, resulting in decreased mononuclear cell infiltration and destruction of the thyroid, autoantibody production, and *in vitro* T cell response to mTg, the three parameters by which EAT tolerance is assessed (10, 11). EAT tolerance is inducible by increasing the circulating levels of mTg by four means: intravenous administration of two large (100μg) doses of mTg 7 days apart (10), 100μg mTg subdivided into 10 daily doses (11), 20μg LPS given 24 hours prior to two smaller 20μg doses of mTg to inhibit clearance by the reticuloendothelial system (19), or stimulation of the release of endogenous mTg by infusion of thyroid-stimulating hormone (TSH) via an implanted osmotic pump (11). All of these protocols resulted in the elevation of circulatory mTg levels for a critical period of 2-3 days prior to EAT induction (10, 11). The tolerance induced is antigen-specific, as administration of an irrelevant self antigen, liver extract, had no protective effect on subsequent induction of thyroiditis (10). Furthermore, tolerance between heterologous Tgs is also limited; tolerance to human (h)Tg-induced thyroiditis can be induced with hTg pretreatment (20), but hTg pretreatment does not protect against EAT induction with mTg, presumably due to the presence of species-unique epitopes and is MHC class II haplotype-dependent (21).

The exact pathway of activation of the cells responsible for mediating tolerance following mTg pretreatment is as yet undefined. The relatively short interval required for the establishment of tolerance suggests that it is the result of an activation and/or expansion of a pre-existing cell population (10, 12). The context of the tolerogenic stimulus is critical. Administration of proinflammatory signals, such as polyadenylic-polyuridylic acid complex (poly A:U) (10), IL-1 (22), or IL-12 (23) at the time of tolerance induction, inhibits tolerance development even with deaggregated mTg, likely due to a change in the tolerogenic signal, resulting in activation of thyroiditogenic T cells. The importance of the context of the tolerogenic signal has led us to hypothesize that costimulatory signals are critical to the development of EAT tolerance. This influence of costimulatory signals has been shown by the findings that *in vivo* administration of mAbs to tumor necrosis factor receptor family members, CD137 (4-1BB) (14) and glucocorticoid-induced tumor necrosis factor receptor (GITR) (unpublished data), at the time of tolerance induction prevents the development of EAT tolerance.

Other models of induced tolerance have reported a critical role for CTLA-4 in tolerance, including foreign antigens, transplant rejection and colitis models, suggesting that B7-CTLA-4 interactions are involved in the induction of peripheral tolerance, possibly through the activation of $CD4^+CD25^+$ Treg (24-27). We have recently observed that *in vivo* blockade of CTLA-4 by administration of repeated 1mg doses of CTLA-4 mAb during the window of tolerance induction inhibits EAT tolerance development (unpublished data). This requirement for costimulation through CTLA-4 is specific, as blockade of CD40L or CD28 by *in vivo* administration of mAbs has no effect on the induction of tolerance [(23), unpublished data]. Previous reports in other models have suggested that CTLA-4 mAb is capable of inducing tolerance by the induction of anergy (24, 28), though that would not seem to play a role in EAT tolerance, as induced tolerance is an active, rather than a passive, phenomenon, and is transferable with T cells from tolerized mice (10, 12). An alternative explanation for CTLA-4 would involve its role in negative signaling on effector T cells to down-regulate its response. We examined this possibility by administering CTLA-4 mAb during challenge of mice with established tolerance. No effect on protection against thyroiditis development was observed, suggesting little involvement in the activation or function of thyroiditogenic T cells

(unpublished data). This finding differs from reports of others, where blockade of CTLA-4 inhibited the suppressive capability of Treg (24, 25, 27, 29, 30). It is also in contrast to the observation that CD40L and CD28 mAbs interfere with EAT induction by inhibiting the thyroiditogenic T cells (23). These data further support the hypothesis of EAT tolerance resulting from the activation of a pre-existing regulatory T cell population.

3.2. Characteristics of $CD4^+CD25^+$ Treg in EAT Tolerance

Tolerance to EAT is a cell-mediated phenomenon, as indicated by early observations that induced tolerance can be transferred by splenocytes. This transfer is dependent on the presence of $Thy1^+$ cells, indicating that a T cell population is responsible for mediating EAT tolerance (10). Once established, EAT tolerance is long lasting, with protection against EAT induction for at least 73 days (12), suggesting persistence of these cells in the periphery following tolerance induction. The idea of a T cell subset influencing thyroiditis development exists in untreated mice has been suggested by early observations that thyroiditis can develop spontaneously in thymectomized rodents with or without sublethal irradiation (31, 32). Moreover, mice that are typically poor responders to EAT induction develop significantly more severe thyroiditis following pretreatment with cyclophosphamide (33). These observations suggest the presence of a naturally-existing cell population influencing susceptibility to thyroiditis.

Definition of the T cells mediating tolerance to EAT is further narrowed by the observations that depletion of $CD4^+$ T cells eliminated protection against EAT induction in mice with established tolerance (13). Splenocytes from tolerized mice were capable of transferring protection against EAT (10, 12). The role of CD4 in EAT tolerance was further defined by the finding that administration of a non-depleting CD4 mAb which modulates CD4 expression at the time of challenge interferes with protection against thyroiditis induction, while having no effect on tolerance induction (34). Furthermore, for regulatory $CD4^+$ T cells to mediate their protective effect, they must have prior exposure to antigen, as $CD4^+$ T cells from normal mice (13) or from rats with ablated thyroids do not protect mice or athymic rats respectively from the development of thyroiditis (35), reinforcing the requirements for both antigen specificity and subsequent activation and/or expansion of regulatory T cells mediating tolerance. Thus, T cells mediating tolerance require activation through their TCR and CD4 co-receptor to exert their protective effect (12, 34).

It was clear from early studies that regulatory (suppressor) T cells belong to a subset of $CD4^+$ T cells distinct from thyroiditogenic T cells, but specific markers for T cells with regulatory function have been difficult to delineate. Initially, suppressor T cells were mapped to a region of the murine MHC designated the I-J region, and characterized by expression of an undefined I-J determinant (36, 37). Regulatory $CD4^+$ T cells in EAT tolerance were also observed to express the I-J determinant, as elimination of $I\text{-}J^+$ cells by Ab raised in $I\text{-}J^-$ mice and complement eliminated the ability of splenocytes from tolerized mice to transfer tolerance, although *in vivo* I-J mAb treatment had little effect (12). Subsequent difficulties in mapping the *I-J* locus or determining the structure of the I-J determinant led to questions regarding its existence, the validity of I-J as a marker for Treg, and the utility of the I-J determinant in defining $CD4^+$ regulatory T cell subset mediating EAT tolerance.

More recently, CD25, the alpha chain of the IL-2R, expressed on activated T cells, B cells, macrophages and dendritic cells, has been used in several models of autoimmunity as a useful marker for Treg ever since the initial description of the importance of a $CD4^+$ T cell subset constitutively expressing CD25 in inhibiting the development of multi-organ inflammatory autoimmune disease, including thyroiditis, when co-transferred with $CD4^+CD25^-$ T cells into nude mice (38). We have also shown that the $CD4^+$ T cells responsible for mediating induced EAT tolerance are $CD25^+$; *in vivo* depletion of $CD25^+$ cells by CD25 mAb in CBA/J ($H2^k$) mice with established tolerance eliminates $CD4^+$ T cell-mediated protection against subsequent EAT induction [Figure 1, (14)]. The observation of an induced population of $CD4^+CD25^+$ T cells capable of inhibiting the development of thyroiditis *in vivo* has recently been corroborated by the adoptive transfer of antigen-specific $CD4^+CD25^+$ Treg generated *in vitro* by culture of immature dendritic cells with mTg in the presence of TNF-alpha (39) or by *in vivo* administration of GM-CSF prior to EAT induction (40), leading to suppression of thyroiditis development. It remains to be seen whether these $CD4^+CD25^+$ Treg generated function similarly as $CD4^+CD25^+$ Treg generated *in vivo* during EAT tolerance induction.

3.3. Naturally-Existing $CD4^+CD25^+$ Treg in Self Tolerance

In addition to describing the mediators of induced EAT tolerance as $CD4^+CD25^+$ T cells, we have also demonstrated the existence of $CD4^+CD25^+$ Treg that inhibit the development of EAT in naive mice. Depletion of $CD25^+$ T cells lowers the threshold for thyroiditis induction in CBA mice, resulting in an increase in both incidence and severity of thyroiditis induced by repeated 20μg doses of mTg without adjuvant (41), and enables thyroiditis induction in otherwise EAT-resistant BALB/c ($H2^d$) mice (42, 43) and B10 ($H2^b$) mice [Figure 1, (15)]. Thus, a similar population of $CD4^+CD25^+$ Treg that inhibit EAT induction exist in both tolerized as well as naive mice. Furthermore, depletion of $CD4^+CD25^+$ T cells prior to tolerance induction precludes the establishment of tolerance (41). The presence of a naturally-existing, phenotypically similar population of Treg, whose presence is required for the subsequent induction of tolerance, supports our hypothesis that induced tolerance is a result of strengthening a naturally-existing regulatory T cell population by an antigen-specific activation or expansion (13).

While heightened *in vivo* function of $CD4^+CD25^+$ Treg is readily observed after inducing resistance to EAT, we have yet to observe a quantitative difference in the

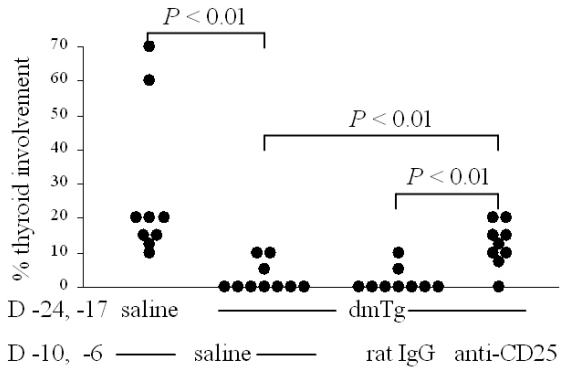

Figure 1. *In vivo* depletion of $CD4^+CD25^+$ T cells in mice with established tolerance abrogates protection against EAT. Mice were given iv 100 μg dmTg on days -24 and –17, followed iv by two 1 mg doses of anti-CD25 mAb or rat IgG on days -14 and -10. Depletion of $CD4^+CD25^+$ T cells in PBL was observed by FACS on day –4. EAT was induced iv with 40 μg mTg plus 20 μg LPS 3 h later on days 0 and 7, and the mice were killed on day 28. Graph represents mononuclear cell infiltration of thyroids of individual mice (50-60 histological sections per thyroid). Reproduced with permission from Morris, *et al.* (14).

$CD4^+CD25^+$ T cell population. There is no measurable increase in the size of the $CD4^+CD25^+$ T cell subset, nor other surface markers associated with regulatory T cell activity by flow cytometric analysis (14). Titration of $CD4^+CD25^+$ Treg from naive and tolerized mice has not revealed any marked difference in the *in vitro* suppressive activity between naive and tolerized cell populations (unpublished data). This is likely due to the absolute number of cells that have been affected; EAT tolerance is antigen specific (10), and only a small number of $CD4^+CD25^+$ T cells actually undergo activation and/or expansion. The suppressive capacity of Treg is likely exaggerated in *in vitro* co-culture assays, possibly due to increased encounter between Treg and thyroiditogenic T cells. *In vitro* assays from other models have suggested that suppression by Treg is mediated through a cell-cell contact-dependent mechanism (44-46). However, whether antigen-specific Treg operate similarly *in vivo* has not been demonstrated. If indeed cell-cell contact is important, then Treg from tolerized mice, still a small number, must have increased migratory capacity to reach the Tg-autoreactive T cells in order to mediate the strong resistance to challenge. It would be of interest to determine the comparative *in vivo* suppressive capacity of $CD4^+CD25^+$ Treg from naive and tolerized mice by adoptive transfer studies. This type of comparison has been shown in transplant studies wherein Treg from naive mice were compared with Treg expanded *in vitro* by mixed lymphocyte reaction (47). The interesting role of lymphocyte trafficking in Treg function is yet to be examined.

Currently, we are examining a more specific regulatory T cell marker, the expression of *foxp3*, a gene encoding the forkhead transcription factor, Scurfin, important in the development of $CD4^+CD25^+$ Treg in other models (48-51). Scurfin is indeed expressed in a subset of $CD4^+CD25^+$ T cells from mTg-tolerized mice [(39), unpublished data], and comparison of *foxp3* expression levels between naive and tolerized mice by both mRNA level and flow cytometric analysis is in progress.

3.4. Cytokines in the Function of $CD4^+CD25^+$ Treg

To determine the mechanism by which $CD4^+CD25^+$ Treg mediate their protective effect against thyroiditis development, the role of several cytokines has been examined. The inhibition of autoimmunity as a function of cytokines, such as IL-4, IL-10, or TGF-beta, has been implicated in the suppression of several autoimmune diseases, including thyroiditis (39, 52-56). However, we have been unable to demonstrate a role for these cytokines in EAT tolerance; neutralizing mAbs to IL-4 and IL-10, administered either alone, together, or anti-IL-10 into $IL\text{-}4^{-/-}$ mice, all failed to inhibit either the induction or mediation of tolerance *in vivo* (23). Similarly, neutralizing TGF-beta mAb has no effect on either the induction or mediation of EAT tolerance (unpublished data). Furthermore, *in vitro* culture of $CD4^+CD25^+$ T cells from tolerized mice does not result in measurable production of IL-4, IL-10, or TGF-beta following stimulation with either mTg or Con A, and neutralizing mAbs to IL-4 or IL-10 have no effect on *in vitro* suppression of thyroiditogenic T cells (unpublished data). We have observed, however, an effect of neutralization of TGF-beta, either by a mAb or a specific inhibitor of the TGF-beta receptor, on *in vitro* suppression by $CD4^+CD25^+$ Treg (unpublished data). The lack of detectable TGF-beta in culture supernatants from $CD4^+CD25^+$ Treg and an observed effect of neutralizing TGF-beta on *in vitro* suppression can be reconciled by recent reports describing a membrane-bound form of TGF-beta on the surface of $CD4^+CD25^+$ Treg that is involved in mediating at least part of their suppressive function (57, 58). The discrepancy between the observed effect of neutralization of TGF-beta on $CD4^+CD25^+$ Treg suppression of mTg-reactive T cells *in vitro*, and a lack of measurable effect *in vivo* may be due to an inability to achieve adequate inhibition of TGF-beta systemically, or may represent a difference in the mechanism of suppression *in vivo*.

4. INFLUENCE OF MHC CLASS II GENES ON EAT SUSCEPTIBILITY

The influence of genetic factors on susceptibility to thyroiditis was observed over three decades ago, and differences in susceptibility to EAT induction between mouse strains were noted, and subsequently linked to MHC class II genes (4, 59, 60). The presence of $H2^k$, $H2^q$, or $H2^s$ strongly correlates positively with susceptibility to EAT, while $H2^b$ and $H2^d$ correlate negatively. Further mapping of genetic influence points to *H2A* as the primary locus (3, 5), corroborated by the observation that introduction of an Aa^kAb^k transgene confers EAT susceptibility to an otherwise resistant strain (61). The influence of the other murine class II locus, *H2E*, is less clear. Its presence is not required for susceptibility, although it appears to influence the induction and development of thyroiditis. For example, induction of E^s molecule expression by introducing an

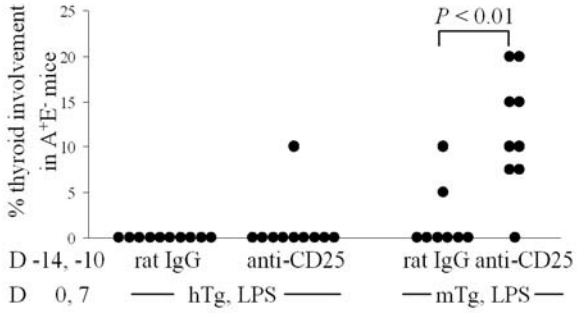

Figure 2. *In vivo* depletion of $CD4^+CD25^+$ T cells enables EAT induction with mTg, but not hTg, in B10 (A^+E^-) mice. Two 1 mg doses of anti-CD25 or polyclonal rat IgG iv were administered to B10 mice on days -14 and -10. Depletion of $CD4^+CD25^+$ T cells in PBL was observed by FACS on day –4. Mice were challenged on days 0 and 7 with 40 µg of either hTg or mTg followed 3 h later by 20 µg LPS, and killed on day 28. Graph represents mononuclear cell infiltration of thyroids of individual mice. Reproduced with permission from Morris *et al.* (15).

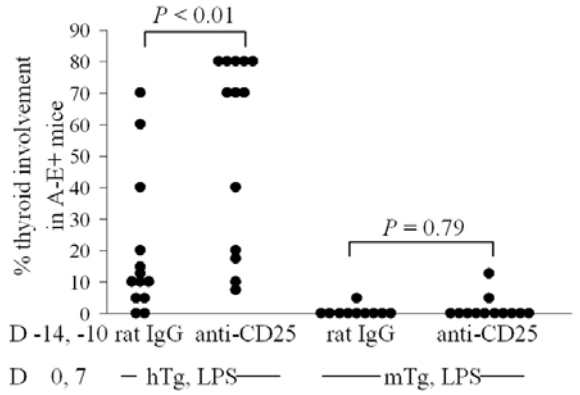

Figure 3. *In vivo* depletion of $CD4^+CD25^+$ T cells enhances EAT induction with hTg, but does not alter restriction against mTg, in E^+ $B10.Ab^0$ (A^-E^+) mice. E^+ $B10.Ab^0$ mice were given two 1 mg doses of anti-CD25 or rat IgG i.v. on days -14 and -10. Depletion of $CD4^+CD25^+$ T cells in PBL was observed by FACS on day –4. Mice were challenged with 40 µg of either hTg or mTg followed 3 h later by 20 µg LPS on days 0 and 7, and the mice were killed on day 28. Graph represents mononuclear cell infiltration of thyroids of individual mice. Reproduced with permission from Morris, *et al.* (15).

H2Ea transgene decreases the severity of thyroiditis inducible in otherwise susceptible B10.S mice (61), while the induction of E^b molecule expression in EAT-resistant, H2A-knockout $B10.Ab^0$ mice confers EAT susceptibility, surprisingly limited to induction with foreign Tgs such as human, porcine or bovine (62). Interestingly, when this novel *H2E*-transgene was introduced into B10 mice expressing the endogenous A^b molecule, the severity of induced thyroiditis was decreased (62). Thus, we can demonstrate reciprocal reduction in thyroiditis severity due to the function of additional *H2A* or *H2E* genes, depending on the Tg being presented.

After the initial demonstration of *H2*-based EAT susceptibility, the link between MHC class II genes and susceptibility to thyroiditis observed in mice was subsequently observed in HT patients, in particular, the association between thyroiditis and *HLA-DR3* (63-67), illustrating the validity of the murine model for investigation of human disease. The link between human MHC class II alleles and autoimmune thyroid disease has been strengthened by the use of transgenic mice expressing human class II molecules. The expression of transgenic HLA-DR3 in EAT-resistant mice, or in mice lacking endogenous class II molecules, positively correlates with autoimmune thyroiditis (68), and permits susceptibility to thyroiditis induction with hTg, as well as human thyroid peroxidase (69), while expression of DR2, DR4 or DQ6 does not (68, 70). A modulating influence between MHC loci was observed, as the expression of DQ8 decreases thyroiditis induced in otherwise susceptible $DR3^+$ mice (71), again mirroring observations in the murine MHC system.

The reciprocal suppressive effect between both murine and human class II alleles may result from a change in the T cell repertoire due to deletion of autoreactive T cells in the thymus, or it may be related to a qualitative change in T cell repertoire and ability to respond to autoantigens. Investigations in other systems where similar suppression between class II molecules has been observed suggest that it is an active mechanism of suppression, and may involve the generation of a distinct subset of regulatory T cells capable of inhibiting the development of autoimmune thyroiditis (72-74).

5. $CD4^+CD25^+$ T CELL REGULATION DOES NOT SUPERSEDE THE REQUIREMENT FOR CORRECT MHC CLASS II PRESENTATION IN EAT SUSCEPTIBILITY

As described above, EAT susceptibility has traditionally been defined by MHC class II haplotype, specifically *H2A* genes, although we have demonstrated that changes in the function of $CD4^+CD25^+$ Treg markedly influence susceptibility. Increasing regulatory T cell function by tolerance induction converts an EAT-susceptible strain (such as $H2^k$) to resistant. On the other hand, eliminating $CD4^+CD25^+$ T cells converts a traditionally resistant strain (such as $H2^b$) to susceptible, albeit not to the degree of a susceptible haplotype, and does not enable thyroiditis induction with hTg, presumably due to an inability of the A^b molecule to properly present hTg epitopes [Figure 2, (15)]. To directly compare the relative influence of T cell regulation and MHC, we utilized the aforementioned *H2E* transgenic model, in which EAT is limited to induction with hTg, while being restricted against mTg (62). $E^+B10.Ab^0$ (A^-E^+) transgenic mice depleted of $CD4^+CD25^+$ T cells *in vivo* showed increased susceptibility to thyroiditis induction with hTg, but remained resistant to EAT induction with mTg [Figure 3,

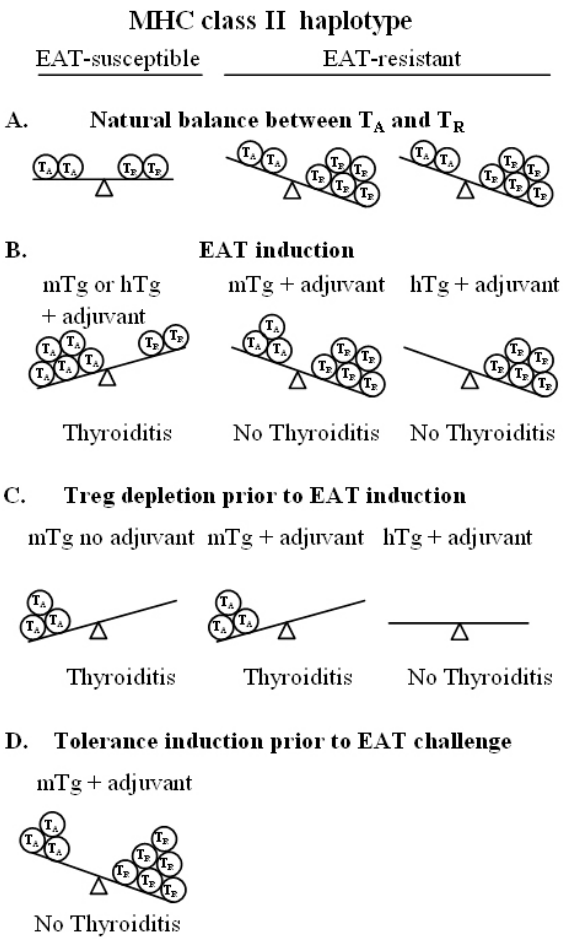

Figure 4. MHC class II genes influence the extent of Treg function on EAT susceptibility as illustrated in different strains. (**A**) Naive traditionally EAT-susceptible CBA ($H2^k$) strain as well as traditionally EAT-resistant B10 ($H2^b$) strain have natural balance between autoreactive T cells (T_A) and Treg (T_R) as neither spontaneously develops thyroiditis. (**B**) Thyroiditis can be induced in CBA mice by activating T_A with either mTg or hTg and adjuvant. B10 mice are resistant to EAT induction by either mTg or hTg and adjuvant. (**C**) Depletion of T_R in CBA mice removes the peripheral inhibition of T_A, enhancing thyroiditis induction by mTg without adjuvant. T_R depletion in B10 mice enables EAT induction with mTg and adjuvant. However, B10 mice remain resistant to EAT induction with hTg, even after Treg depletion, indicating a lack of T_A capable of responding. (**D**) Pretreatment of naive CBA mice with mTg induces tolerance to subsequent EAT induction, probably by activation or expansion of the pre-existing T_R.

(15)]. These findings demonstrate that, while regulatory T cell function influences susceptibility to autoimmune thyroiditis, correct presentation of autoantigen by MHC class II molecules is an absolute, invariant requirement for the development of autoimmune thyroiditis.

The existence of $CD4^+CD25^+$ Treg influencing thyroiditis induction in the context of either $H2A^b$ (B10) (Figure 2) or $H2E^b$ ($E^+B10.Ab^0$) (Figure 3) molecules suggests that they may be responsible for the reciprocal suppressive effect between the two molecules. As reviewed above, $H2E^s$ molecules reduce the severity of thyroiditis encoded by $H2A^s$ in B10.S mice (61), and $H2A^b$ molecules suppress *H2E*-mediated thyroiditis of $E^+B10.Ab^0$ mice (62). The generation of Treg responding to antigen presented in the context of H2E has been proposed as a potential mechanism for suppression in other models of autoimmunity (75, 76). We investigated this possibility by *in vivo* elimination of $CD25^+$ T cells in E^+B10 transgenic mice, which express both the native H2A molecule permissive for mTg-induced EAT, and the transgenic H2E molecule permissive for hTg-induced EAT (15). $CD25^+$ T cell-depleted mice developed more severe disease following induction with either mTg or hTg, suggesting the involvement of $CD4^+CD25^+$ Treg. However, since we cannot yet discriminate between H2A- or H2E-derived Treg, and all $CD25^+$ T cells were depleted *in vivo*, the increase in thyroiditis severity may be a result of decreased function of A^b- or E^b-derived Treg acting independently to inhibit thyroiditis mediated in the same context. Moreover, several other possibilities exist in this model to explain the suppressive effect of the *H2A* genes, such as alteration of the T cell repertoire, potentially including the deletion of autoreactive cells, and qualitative changes in T cell responses due to a combination of T cell epitopes presented in the context of A^b and E^b. Another interesting possibility arises from others' observations that an epitope from the E-beta chain with high affinity for H2A molecules (77) can inhibit collagen-induced arthritis in mice (78), and similar presentation of an HLA-DR-derived peptide by HLA-DQ can suppress the disease in HLA-transgenic mice (79). A similar event may be occurring in E^+B10 mice for EAT, although we have not examined whether the E^b molecule can present A^b-derived peptides, nor whether A^s molecules can present E^s-derived peptides in B10.S mice (61). The potential reciprocal suppressive ability of Treg between MHC *H2A* and *H2E* genes bears further investigation.

The effects of Treg on modulating susceptibility to thyroiditis in both traditionally EAT-susceptible and EAT-resistant strains point to their importance in inhibiting autoimmunity, although the influence of MHC class II and Treg may not be mutually exclusive. The traditional importance of MHC class II haplotype in determining susceptibility to autoimmunity appears multifaceted, as presentation of autoantigen by MHC class II molecules is important not only regarding the stimulation of autoreactive T cells, but also for generation of either autoreactive T cells or Treg in the thymus. It is likely that MHC class II haplotype determines not only the repertoire of autoreactive T cells, but also the extent of peripheral regulation of autoreactive T cells that escape negative selection (Figure 4-A). In a traditionally EAT-susceptible strain such as CBA ($H2^k$), a balance between autoreactive T cells (T_A) and Treg (T_R) normally exists, though thyroiditis can be induced by activating T_A with Tg and adjuvant (Figure 4-B). Removal of Treg lowers the threshold for thyroiditis induction, resulting in an increase in both incidence and severity of thyroiditis induced with repeated doses of Tg without adjuvant (Figure 4-C). Furthermore, Treg activity can be

enhanced by antigen-specific tolerance induction, converting a traditionally susceptible mouse strain to an EAT-resistant strain, again underscoring the influence of Treg on thyroiditis susceptibility (Figure 4-D). Examination of traditionally EAT-resistant B10 ($H2^b$) mice further demonstrates the dominance of Treg, as Treg depletion enables thyroiditis induction with mTg and adjuvant (Figure 4-C). Challenge of B10 mice with hTg and adjuvant reveals the hierarchy of MHC class II haplotype over Treg in determining EAT susceptibility (Figure 4-C). In this case, depletion of Treg still does not permit EAT induction with hTg, indicating a lack of autoreactive T cells capable of responding to stimulation. This hierarchy of MHC over Treg is corroborated by our *H2E* transgenic model where Treg-depleted mice remain unresponsive to EAT induction with mTg. Thus, Treg function cannot supersede the influence of either *H2A* or *H2E* class II genes in generating autoreactive T cells necessary for thyroiditis induction. It remains a challenge to continue examining the differences in Treg generation and function between MHC class II haplotypes as a potential basis for susceptibility to autoimmunity.

6. PERSPECTIVE

Susceptibility to autoimmunity has long been described as a function of class II genes in animal models (4), an association mirrored by human studies (63). More recently, human autoimmune disease has been associated with *CTLA4* (80) and *FOXP3* (81), genes that have been associated with $CD4^+CD25^+$ Treg activity (25, 48, 50, 82, 83). Our data suggest that the influence of class II genes and regulatory T cell-associated genes may not be independent, and that class II genes may influence the development of autoimmunity both by their ability to correctly present autoantigen as well as by their ability to generate $CD4^+CD25^+$ Treg. The ability of class II molecules to generate Treg efficacious in inhibiting autoimmunity likely varies among haplotypes, and may explain the genetic predisposition of certain haplotypes to the development of autoimmunity.

The observations that depletion of $CD4^+CD25^+$ T cells abrogates protection established by antigen inducing tolerance to EAT, and that naive $CD4^+CD25^+$ T cells help maintain a natural barrier against autoimmune thyroid disease indicate that $CD4^+CD25^+$ T cells may be a common mediator for both induced and natural tolerance. Furthermore, the requirement for the presence of $CD4^+CD25^+$ T cells at the time of tolerization with mTg supports the hypothesis that tolerance induction is the result of an activation and/or expansion of an existing population of autoantigen-specific regulatory T cells. This hypothesis is supported by findings in other models, where $CD4^+CD25^+$ Treg have been shown to arise from high-affinity interactions with self peptide, both in the thymus during ontogeny (84-86), and in the periphery (87).

7. ACKNOWLEDGMENTS

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