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Accessibility
Transgenically Induced GAD Tolerance Curtails the Development of Early β-Cell Autoreactivities but Causes the Subsequent Development of Supernormal Autoreactivities to Other β-Cell Antigens

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OBJECTIVE—To study how tolerance to GAD65 affects the development of autoimmunity to other β-cell autoantigens (β-CAAs) in GAD65-transgenic (GAD-tg) NOD mice.

RESEARCH DESIGN AND METHODS—We used ELISPOT to characterize the frequency and functional phenotype of T-cell responses to GAD65 and other β-CAAs at different ages in GAD-tg mice and their NOD mouse littermates.

RESULTS—In young GAD-tg mice, Th1 responses to GAD65’s dominant determinants were 13–18% of those in young NOD mice. This coincided with a great reduction in Th1 responses to other β-CAAs. Evidently, GAD65-reactive T-cells are important for activating and/or expanding early autoactivities in NOD mice. As GAD-tg mice aged, their T-cell responses to GAD65 remained low, but they developed supernormal splenic and pancreatic lymph node T-cell autoimmunity to other β-CAAs. Apparently, the elimination/impairment of many GAD65-reactive T-cells allowed other β-CAA–reactive T-cells to eventually expand to a greater extent, perhaps by reducing competition for antigen-presenting cells, or homeostatic proliferation in the target tissue, which may explain the GAD-tg mouse’s usual disease incidence.

CONCLUSIONS—Transgenically induced reduction of GAD65 autoreactivity curtailed the development of early T-cell responses to other β-CAAs. However, later in life, β-CAA–reactive T-cells expanded to supernormal levels. These data suggest that early β-cell autoreactivities are mutually dependent for support to activate and expand, while later in the disease process, autoantigen-specific T-cell pools can expand autonomously. These findings have implications for understanding type 1 diabetes immunopathogenesis and for designing antigen-based immunotherapeutics. Diabetes 58:2843–2850, 2009

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See accompanying commentary, p. 2729.
elimination of GAD65-reactive T-cells by negative selection or to their functional impairment. High levels of GAD65 presentation by APCs could have also promoted antigen-specific Th2 responses. Additionally, previous studies have observed that induction of tolerance to an autoantigen’s dominant determinants can enhance autoreactivity to its subdominant and cryptic determinants (15–17). Such neoautoactivities would have been difficult to detect by testing for T-cell responses to whole GAD65 (14), since subdominant determinants are not well presented from whole antigen and T-cell responses to cryptic determinants can only be detected using short synthetic peptides containing the determinant (18). Therefore, questions remain concerning the dynamics and specificity of spontaneous T-cell responses to GAD65 in GAD-tg mice.

We were also interested in whether tolerance to GAD65 affected the development of T-cell autoreactivities to other β-CAAs. If autoimmunity to GAD65 was important in type 1 diabetes development in NOD mice, elimination of GAD65-reactive T-cells should impair the development and expansion of T-cell autoreactivities to other β-CAAs. However, the observation that GAD-tg mice develop type 1 diabetes with the same kinetics as NOD mice suggests that the elimination of GAD65-reactive T-cells may have had little impact on the development of T-cell responses to other β-CAAs.

We characterized spontaneous T-cell responses to whole GAD65, to peptides containing GAD65’s dominant and cryptic determinants, and to other major β-CAAs at early and late stages of the autoimmune process in GAD-tg and wild-type NOD mice. We found that spontaneous Th1 responses to GAD65 and its dominant determinants were greatly reduced, but still present, in GAD-tg mice, without shifting of GAD65 autoreactivity toward the Th2 phenotype or toward GAD65’s cryptic determinants. Interestingly, young GAD-tg mice had significantly decreased T-cell autoreactivities to other β-CAAs, indicating that GAD65 autoreactivity plays a role in the early activation/expansion of other β-CAA–reactive T-cells. Surprisingly, although older GAD-tg mice continued to have low levels of T-cell responses to GAD65, they had supernormal spontaneous splenic and pancreatic lymph node (PLN) Th1 cell responses to other β-CAAs, suggesting that deletion/inactivation of many GAD65-reactive T-cells provided an opportunity for other β-CAA–reactive T-cells to eventually expand to a greater extent. We discuss the implications of these observations for understanding the type 1 diabetes disease process and for designing antigen-based therapies.

**RESULTS**

GAD-tg and their nontransgenic NOD mouse littermates have similar incidence of type 1 diabetes in our colonies. Because the environment can affect type 1 diabetes incidence in NOD mice, we examined the kinetics of type 1 diabetes development in the newly established GAD-tg mouse colony at the University of California Los Angeles. As in the previous study of GAD-tg mice (14), we observed that GAD-tg mice and their nontransgenic NOD mouse littermates developed type 1 diabetes at similar rates (Fig. 1), although the onset of type 1 diabetes was somewhat delayed in our mice relative to the previous study.

GAD-tg mice develop spontaneous and weak Th1 responses to GAD65. We analyzed the functional phenotype and frequency of antigen-specific autoreactive T-cell responses to GAD65 in GAD-tg mice and their NOD littermates directly ex vivo by an ELISPOT assay. Previous studies have shown that Th1 responses to GAD65 are detected in the spleen of 3- to 4-week-old NOD mice, and T-cell autoimmunity to insulin B-chain and HSPp27 become detectable after 6–8 weeks of age (1,2,29). We therefore examined T-cell responses to these autoantigens in 8-week-old GAD-tg mice, by which time T-cell responses to GAD, HSPp27, and insulin B-chain should have been well established. We detected an average frequency of 159/10^6 IFN-γ–secreting splenic mononuclear cells T-cells reactive to whole GAD65 in NOD mice (Fig. 2A), consistent with previous studies (1,20,29). In GAD-tg mice, the frequency of reactive IFN-γ–secreting T-cells was reduced to 12/10^6 (8% of that in age-matched NOD mice) (Fig. 2A), similar to the previous assessment of...
their tolerance to GAD65 using GAD65 immunization and recall response testing (14).

We did not detect any IL-4– or IL-5–secreting splenic T-cells responding to GAD65 in NOD mice, consistent with previous studies (20,29); we also did not detect any IL-4 or IL-5 responses in the spleens of GAD-tg mice (data not shown). These data suggest that expression of GAD65 in professional APCs did not shift the functional phenotype of spontaneous T-cell autoreactivities to GAD65.

Characterization of autoreactivity to dominant and cryptic GAD65 determinants in GAD-tg mice. GAD65 expression by professional APCs in GAD-tg mice should enforce passive tolerance to the dominant determinants of GAD65 that are well presented after antigen processing. Previous studies have shown that induction of passive tolerance to a dominant determinant of a β-cell autoantigen can enhance T-cell responses to its subdominant and cryptic determinants (15), which can be detected by testing T-cell responses to antigenic peptides, since the determinants are poorly presented from whole protein (18).

We tested whether expression of GAD65 in professional APCs modulated spontaneous T-cell responses to GAD65 antigenic peptides containing dominant determinants GAD(524–543), GAD(217–236), and GAD(206–220) (1,20,21,29,34) and two “absolute” cryptic determinants GAD(260–279) and GAD(398–420), which are immunogenic but ignored by spontaneous autoimmune responses in NOD mice (22). We found that the frequency of IFN-γ–secreting T-cells recognizing GAD(524–543), GAD(206–220), and GAD(217–236) in 8-week-old GAD-tg mice were reduced to 14, 13, and 18%, respectively, of that detected in the spleens of NOD mouse littermates (Fig. 2B). It is likely that some T-cell responses also remain to many other GAD65 autoantigenic determinants that we did not test.

We did not detect IL-4 and IL-5 responses to the GAD65 peptides (data not shown), again suggesting that increased presentation of the dominant/subdominant determinants did not alter the functional phenotype of their cognate effectors. We also did not detect T-cell responses to GAD65 cryptic determinants in NOD mice, consistent with previous observations (22), nor in GAD-tg mice (Fig. 2B). Thus, in contrast to observations in studies of other β-cell autoantigens (35), induction of passive tolerance to GAD65’s dominant determinants did not enhance T-cell autoimmunity to its cryptic determinants in our experimental system.

Autoreactivities to other β-cell autoantigens are reduced in young GAD-tg mice. We next examined the impact of reduced GAD65 autoimmunity on the development of T-cell autoreactivities to the immunodominant determinants of other β-CAAs, namely, insulin B-chain and HSPp277, in young GAD-tg mice. While we detected frequent splenic IFN-γ–secreting T-cell responses to insulin
B-chain and HSPp277 in 8-week-old NOD mice, Th1 responses to insulin B-chain and HSPp277 in the spleen and PLN of age-matched GAD-tg mice were significantly reduced. For example, splenic T-cell responses to HSPp277 and insulin B-chain were 14 and 56% of those, respectively, in NOD mice (P < 0.01 for both, Fig. 3A and B). Moreover, splenic and PLN responses to islet lystate were also significantly reduced (P < 0.05 for both). The responses to islet lystate were not as greatly reduced as those to HSPp277 and insulin B-chain. This may be because the islet lystate is comprised of thousands of whole proteins each at different concentrations such that β-CAAs are not at their optimal concentrations for ELISPOT detection. In contrast, the insulin B-chain and HSPp277 peptides contain a major autoantigenic determinant, are readily presented, and are at an optimal concentration in the ELISPOT assay, providing a more sensitive readout. We did not detect IL-4– and IL-5–secreting splenic T-cells responding to GAD, insulin B-chain, or HSPp277, as in previous studies (28,29) (data not shown) in either strain of mouse. As expected, T-cell responses to control MBP were at background levels in the spleen and PLN. We did not test even younger GAD-tg mice for T-cell responses to insulin B-chain and HSPp277, as previous studies have shown that these responses first arise in NOD mouse spleen at 6–8 weeks of age (1,2,20). Thus, induced passive tolerance to GAD65 curtailed the early activation and expansion of autoreactive Th1 cells responding to other β-CAAs in GAD-tg mice. The reduced T-cell autoreactivities to β-CAAs in young GAD-tg mice suggests that at early stages of the disease process, activated β-CAA–reactive T-cells are interdependent for support to activate and expand.

Enhanced T-cell autoreactivities to other β-CAAs in older GAD-tg mice. The significantly reduced T-cell autoimmunity to β-CAAs in young GAD-tg mice was enigmatic, since GAD-tg mice develop type 1 diabetes at the same rate as NOD mice. We therefore examined T-cell autoreactivities to β-CAAs at later stages of the disease process in GAD-tg mice. We found that at 12 weeks of age, the frequency of IFN-γ–secreting T-cells responding to GAD65 in the spleen and PLN remained at low levels (Fig. 4A and B). However, in contrast to the reduced levels of T-cell autoreactivities to HSPp277 and insulin B-chain in young GAD-tg mice, we observed significantly higher levels of T-cell autoreactivity to HSPp277 and insulin B-chain in GAD-tg mice. At 12 weeks of age, splenic T-cells responding to HSPp277 and insulin B-chain in GAD-tg mice were on average 137 and 121% more frequent, respectively, than in their NOD mouse littermates (P < 0.05 for both) (Fig. 4A). Significantly greater Th1 responses to insulin B-chain and HSPp277 were also observed in the spleen of 16-week-old GAD-tg mice (P < 0.01 and 0.05, respectively) (Fig. 4C). Therefore, induction of tolerance to GAD65 led to supernormal frequencies of β-CAA–reactive T-cells in the spleen of older GAD-tg mice.

Importantly, T-cell autoimmunity to other β-CAAs was also expanded in the PLN of GAD-tg mice. At 12 weeks of age, PLN T-cell responses against insulin B-chain had expanded to a greater extent than observed in the spleen and were on average 202% more frequent than those in the PLN of their NOD mouse littermates (P < 0.01) (Fig. 4D). The T-cell responses against HSPp277 in the PLN population of GAD-tg mice were increased by 131% (P < 0.01). At 16 weeks of age, supernormal T-cell responses were again observed in the PLN, with responses to insulin B-chain and HSPp277 166 and 133%, respectively, greater than those in NOD mice (P < 0.01 and 0.05, respectively) (Fig. 4D). We did not detect IL-4 and IL-5 T-cell responses to these β-CAAs (data not shown). Thus, while partial GAD65 tolerization initially impaired the activation and expansion of autoreactive T-cells recognizing other β-CAAs, it led to supernormal T-cell autoreactivities to other β-CAAs in the spleen and PLN later in the disease process.

We also analyzed spleen and PLN T-cell responses to an islet lysate from NOD.scid mice. While these responses were significantly reduced in 8-week-old GAD-tg mice, in both 12- and 16-week-old GAD-tg mice the frequency of spleen and PLN IFN-γ–secreting T-cell responses to the islet lysate increased to essentially the same level as that in their age-matched NOD mouse littermates (Fig. 4). This may explain why the ectopic GAD65 expression in APCs...
DISCUSSION

Our characterization of T-cell autoreactivities in NOD GAD-tg mice, which ectopically express GAD65 in professional APCs, revealed unexpected changes in the dynamics of their autoimmune responses. We observed that in GAD-tg mice, autoimmune responses to whole GAD65 at 8, 12, and 16 weeks of age were 8–10% of those in age-matched NOD mouse littermates and that both strains of mice had similar rates of type 1 diabetes incidence, consistent with the initial characterization of these mice (14). T-cell responses to peptides containing GAD65 dominant determinants [GAD(524–543), GAD(206–220), and GAD(217–236)] were somewhat stronger, ranging from 13 to 18% of those in age-matched NOD mice. The detection of more frequent Th1 cell responses to synthetic peptides of GAD65 was likely due to circumventing whole antigen processing and using optimal peptide concentrations in our in vitro ELISPOT assays. Since GAD65 is a fairly large protein with many autoantigenic determinants that we did not test, the total remaining T-cell responses to GAD65 determinants in these mice is still substantial. Accordingly, it is not possible to draw conclusions from these mice as to whether GAD65 autoreactivity is required for the pathogenesis of type 1 diabetes. However, the reduced responses to other β-CAAs in young GAD-tg mice demonstrate that GAD65-reactive T-cells play an important role in activating or expanding early β-cell autoreactivities.

In contrast to another study of passive tolerance (15), we did not detect shifting of T-cell autoreactivity to cryptic determinants of GAD65 in GAD-tg mice. We also did not observe the induction of GAD65-specific Th2 responses. Thus, the increased presentation of GAD65 by GAD-tg APCs did not alter the determinant recognition pattern of spontaneous T-cell responses to GAD65 or the T-cell's functional phenotype.

Despite the transgenic expression of GAD65 by professional APCs to enforce passive tolerance to GAD65, some cognate T-cells, presumably those with low avidity, escaped negative selection and were spontaneously activated before the mice were 8 weeks of age. Another study showed that GAD65 expression under the control of an MHC1 promoter was unable to induce tolerance to GAD65 (13). Young preautoimmune NOD mice have a large pool of high-avidity GAD65-reactive precursor T-cells (29). Together, these observations indicate that GAD65 has limited impact on T-cell selection during the development and maturation of NOD mouse T-cells and that even when extraordinary efforts are taken to ectopically express GAD65 in a manner expected to delete/anergize cognate T-cells in transgenic mice, it is difficult to establish com-

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*P < 0.05, **P < 0.01.

FIG. 4. Enhanced autoreactivities to other β-CAAs in older GAD-tg mice. The frequency of T-cell responses to HSPp277, insulin B-chain, MBP, and islet lysate were tested at 12 weeks of age (A and B) and at 16 weeks of age (C and D) in the spleen (A and C) and PLN (B and D) of GAD-tg mice and age-matched NOD mouse littermates. While the frequency of T-cells responding to whole GAD65 remained greatly reduced in GAD-tg mice, splenic T-cell responses to HSPp277 and insulin B-chain were significantly increased at 12 weeks of age, as were PLN T-cell responses. T-cell responses to HSPp277 and insulin B-chain were also supernormal in the spleen and PLN of 16-week-old GAD-tg mice.

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*P < 0.05, **P < 0.01.
plete tolerance to GAD65 and prevent the early activation of remaining cognate T-cells.

In NOD mice, high-avidity GAD65-reactive T-cells are spontaneously activated at the earliest stages of the autoimmune process (29). If GAD65-specific T-cell autoimmunity is a minor component of early autoimmune response, the elimination or functional impairment of many GAD65-reactive T-cells in GAD-tg mice should have little impact on their development of T-cell autoreactivities to other β-CAAs. In contrast to this notion, we observed that in the spleen and PLN from 8-week-old GAD-tg mice, spontaneous Th1 responses were reduced not only to whole GAD65 and peptides containing GAD65’s dominant determinants but also to HSPp277 and insulin B-chain. For example, splenic T-cell responses to HSPp277 and insulin B-chain were 14 and 56%, respectively, of those in age-matched NOD mice. It is likely that T-cell autoreactivities were also reduced to other β-CAAs that we did not test. Indeed, T-cell responses to islet lysate were also significantly reduced. Evidently, T-cell responses to GAD65 provide support for the activation and expansion of T-cell autoreactivities to other β-CAAs in young NOD mice. In a more general sense, during the early stages of organ-specific autoimmune disease, nascent Th1 autoreactivities to different target tissue antigens may be highly interdependent for support to further their activation and expansion.

Negative selection purges T-cells that interact too strongly with self-determinants, leaving T-cells that interact with cognate β-CAAs near to their activation threshold (36,37). Accordingly, young preautoimmune NOD mice should have some T-cells that interact with cognate β-CAAs near to their activation threshold (36,37). It is thought that a wave of β-cell apoptosis at about 2 weeks of age leads to the increased presentation of β-CAAs by APCs in the PLN (38,39). This increased antigen presentation should theoretically result in the activation of T-cells that previously interacted with different β-CAAs at just below their activation thresholds. This model predicts that there should be a simultaneous loss of self-tolerance to many different β-CAAs. Recently, an elegant study of insulin-tolerant transgenic NOD mice found that these mice have little or no insulitis and do not develop type 1 diabetes, suggesting that insulin is the primary target autoantigen (40). An alternative explanation, consistent with the observations reported here, is that early T-cell autoreactivities are interdependent for the expansion of effector T-cells. Without the usual support from insulin-reactive T-cells in the insulin-tolerant NOD mice, other β-CAA–reactive effector T-cells that were activated following β-cell remodeling may have succumbed to activation-induced cell death or they were well controlled by Tregs. Indeed, T-cells from insulin-tolerant mice efficiently transfer type 1 diabetes to NOD.scid mice, albeit more slowly, demonstrating that effector T-cells do arise in these mice (40). It remains an open question whether more extensive tolerance to GAD65 would have prevented the expansion of autoimmune responses in GAD-tg NOD mice.

Contrasting with the diminished T-cell autoreactivities to β-CAAs in young GAD-tg mice, we observed supernormal autoreactivities to β-CAAs in older GAD-tg mice. Although Th1 responses to GAD65 and its dominant peptides remained at a low level in older GAD-tg mice, at 12 weeks of age splenic T-cell autoimmunity to HSPp277 and insulin B-chain were 137 and 121%, respectively, and their PLN responses were 131 and 202%, respectively, of those in their NOD mouse littermates. A similar pattern was observed in 16-week-old GAD-tg mice. The increased Th1 autoimmunity to non-GAD65 β-CAAs indicates that these antigens are well presented by GAD-tg APCs, despite their ectopic expression of GAD65.

Why do supernormal responses to other β-CAAs arise in older GAD-tg mice? GAD65 contains many autoantigenic determinants (1,21), and a large pool of high-avidity GAD65-reactive precursor T-cells are present in preautoimmune NOD mice (29). Early in the disease process, when activated autoreactive T-cells are infrequent and the local inflammation is at a low-level, competition for APCs and room for homeostatic proliferation should not be limiting factors. As the disease progresses, autoreactive T-cells become more frequent, and their functional development and activity require antigen presentation and costimulation, such that competition for APCs and space for T-cell expansion may become limiting factors. Conceivably, the elimination/inactivation of many GAD65-reactive T-cells in GAD-tg mice removed many competitors for APCs and provided more room for the expansion of T-cells that recognize other β-CAAs by homeostatic proliferation in the target tissue (41), particularly at late stages of the disease process. The greater opportunity for non–GAD65-reactive T-cells to interact with APCs and to expand may account for the development of supernormal T-cell responses to non-GAD65 autoantigens in 12- and 16-week-old GAD-tg NOD mice. T-cell responses to islet lysate in GAD-tg mice were reduced at 8 weeks of age, but at 12 and 16 weeks of age they increased to the same level as that in age-matched NOD mice. We suspect that the “hole” left by eliminating GAD65-reactive T-cells was filled-in by other autoreactive T-cells, such that there was an increase in the frequency of individual β-CAA–specific T-cell responses without an overall increase in the number of islet lysate-reactive T-cells. The enhanced autoimmunity to non-GAD65 autoantigens, together with the normal frequency of Th1 responses to islet lysate in older GAD-tg mice, may explain why GAD-tg mice develop type 1 diabetes at a rate similar to that of NOD mice.

Our observations suggest that during the autoimmune process, there are two phases of T-cell activation and expansion: an early phase in which T-cells recognizing different β-CAAs are mutually dependent for activation and expansion, followed by a later phase in which an autoantigen-specific T-cell pool can expand autonomously. Early in the autoimmune process, a few T-cells from different β-CAA–reactive T-cell pools activate and collectively generate a weak proinflammatory environment that is barely sufficient to support further naïve T-cell activation and activated T-cell expansion. Elimination or functional impairment of one of the major autoantigen-specific pools during this early phase can significantly decrease inflammation in the local environment and curtail the activation and expansion of other autoreactive T-cells. With disease progression, some autoantigen-specific T-cell pools have expanded to the point that they can continue expansion autonomously via positive feedback mechanisms. Small autoantigen-specific T-cell pools may remain reliant on bystander support. The shift of autoantigen-specific effector T-cells from interdependence to autonomous expansion may be an important feature of the maturing autoimmune response. In the later autonomous phase, elimination/impairment of T-cells recognizing one autoantigen may have little beneficial effect and rather may promote the activation and expansion of other
β-CAA–reactive T-cell pools that have already reached the autonomous phase. We found that inducing passive tolerance to one autoantigen can lead subsequently to enhanced autoreactivities to other target tissue antigens. This suggests that therapeutic strategies based on inducing passive tolerance may not be very effective for T-cell–mediated autoimmune diseases. The induction of long-lived regulatory responses (active tolerance) may be a better therapeutic strategy to control a diverse autoimmune response (rev. in 42). Many different β-CAA–based immunotherapies that induce regulatory responses can prevent type 1 diabetes when administered to young NOD mice but lose efficacy when administered later in the disease process (42–45). Their efficacy in young NOD mice may, in large part, be due to reducing the local inflammation on which β-CAA–reactive T-cells depend for activation and expansion during their mutual dependence phase. In older NOD mice, the ability of some autoantigen-specific T-cell pools to autonomously expand, together with increased proinflammatory bystander effects, makes it more difficult for therapy-induced regulatory T-cells to inhibit disease progression. Moreover, antigen-based therapies prime fewer regulatory responses in older NOD mice because many cognate T-cells have already been activated and committed to the autoimmune response, reducing the pool of naïve T-cells available for priming (46). These coinciding effects may help explain the attenuated efficacy of antigen-based therapeutics as NOD mice mature.

Our studies underscore the dynamic nature of autoreactive T-cell responses and the need to better understand the immunological impact of antigen-based therapies designed to induce passive or active tolerance. Modulating a single autoreactive T-cell pool may have complex and unexpected effects on other autoreactive T-cell responses.

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