

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 autoreactivities 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.

The role of T-cell autoreactivity to the 65-kDa form of GAD (GAD65) in the etiology of type 1 diabetes in nonobese diabetic (NOD) mice has been much debated, in large part due to divergent results from different studies of GAD65-tolerized mice. The early induction of passive tolerance to GAD65 by intravenous antigen injection of 3- to 4-week-old NOD mice prevented the development of spontaneous T-cell autoimmunity to β -CAAs, insulinitis, and type 1 diabetes (1). Intrathymic injection of GAD65 at 3–4 weeks of age also prevented type 1 diabetes in NOD mice (2,3), and intrathymic administration of different GAD65 peptides retarded or accelerated disease onset in NOD mice, depending on the peptide (3,4).

Using a transgenic approach, Bækkeskov and colleagues (5) generated GAD65-deficient NOD mice and observed that these mice had the usual incidence of type 1 diabetes. This result is difficult to interpret because mouse β -cells express both GAD65 and GAD67 (6), which share great amino acid sequence identity (7), and T cross-reactivity occurs between GAD65 and GAD67 (8,9). In addition, eliminating GAD65 expression may have 1) changed β -cell metabolism, since the GABA produced by GAD65 can enter the Krebs cycle, or 2) reduced β -cell secretion of GABA, which could have promoted disease progression, since T-cells possess GABA receptors and GABA inhibits type 1 diabetes in NOD mice (10,11). In another transgenic study, Yoon et al. (12) found that antisense-mediated reduction of both GAD65 and GAD67 specifically in the β -cells of NOD mice prevented type 1 diabetes and protected islet grafts from destruction in newly diabetic NOD mice. Hayday and colleagues (13) used a major histocompatibility complex class I (MHC I) promoter to widely express GAD65 in newborn and adult NOD mice. This, however, did not establish GAD65 tolerance, and there was no change in disease incidence. Our recent studies have shown that GAD65-transgenic mice (GAD-tg mice), which have an MHC class II (MHCII) promoter construct driving GAD65 expression in APCs, developed great tolerance to GAD65, but their type 1 diabetes incidence was similar to that of wild-type NOD mice (14). Hence, the impact of tolerance to GAD65 on the development of T-cell autoimmunity and type 1 diabetes in NOD mice remains controversial.

We were interested in whether GAD65 tolerance in the GAD-tg mice (14) altered the specificity, frequency, functional phenotype, or timing of autoimmune responses. Theoretically, the MHCII promoter-driven expression of GAD65 in their professional APCs should lead to the

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 neoautoreactivities 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.

RESEARCH DESIGN AND METHODS

GAD-tg mice were generated by microinjecting NOD mouse oocytes with a DNA construct consisting of an MHCII enhancer/invariant chain promoter linked to a mouse GAD65 cDNA (14). The GAD-tg mice were bred with NOD mice (Jackson Labs), producing GAD-tg offspring that were heterozygous for the transgene or that were nontransgenic NOD mice. The presence of the transgene was detected by PCR analysis of tail DNA (14). Mice were maintained under specific pathogen-free conditions.

Antigens. Mouse GAD65 was purified as previously described (1). The autoantigenic and immunodominant GAD65 peptides that were tested included GAD(524–543) (also termed GADp35; SRLSKVAPVIKARMEYGT), GAD(217–236) (also termed GADp15; EYVTLKKMREIIGWPGGSGD) (1,19,20), and GAD(206–220) (TYEIAPVFVLEYYT) (21). Two peptides containing “absolute” GAD65 cryptic determinants, which are immunogenic but ignored by the spontaneous autoimmune response in NOD mice, were also tested: GAD(260–279) (also known as GADp18, PEVKEKGMAALPRLIAFTSE) and GAD(398–420) (also known as GADp27, VPLQCSALLVREELMONCNG) (22). Peptides from other key β -cell autoantigens included the immuno-

dominant peptide of the 65-kDa heat shock protein, termed HSPp277 (VLGGGALLRCIPALDSLTPANED) (23) and insulin B-chain (Sigma) (22–26). An immunogenic hen egg lysozyme (HEL) peptide (11–25), AMKRHGLDNYR-GYSL (27), was used as a control foreign peptide. Mouse myelin basic protein (MBP) (Sigma) was further purified as previously described (28) and used as a control self-antigen not involved in the autoimmune response. All peptides were synthesized by Bio-Synthesis (Lewisville, TX) at $\geq 95\%$ purity. Islet lysate was prepared by brief sonication of isolated islets from NOD.scid mice.

Preparation of mononuclear cells. Splenic mononuclear cells were prepared as in our past studies (29). PLN mononuclear cells were prepared as previously described (30,31). Briefly, pancreata from individual NOD or GAD-tg mice at the indicated ages were removed and digested with collagenase and DNase I. Pancreatic lymph nodes were obtained by hand picking. Mononuclear cells were isolated and counted, and only preparations with $>95\%$ viability were used.

ELISPOT analysis. The frequency of antigen-specific interferon (IFN)- γ -, interleukin (IL)-4-, and IL-5-secreting T-cells was determined using a modified ELISPOT technique and optimal antigen concentrations as previously described (29,32,33). Briefly, 10^6 splenic mononuclear cells or 10^4 PLN mononuclear cells from individual mice together with 10^6 irradiated (3,000 rads) splenic mononuclear cells were added to individual wells in duplicate in an ELISPOT plate that had been coated with cytokine capture antibodies and incubated with peptide (20 $\mu\text{mol/l}$), GAD65 (100 μg), or islet lysate (from ~ 40 islets) overnight for IFN- γ or 40 h for IL-4 and IL-5 detection. After washing, biotinylated detection antibodies were added and the plates were incubated at 4°C overnight. Bound secondary antibodies were visualized using horseradish peroxidase-streptavidin (DAKO) and 3-amino-9-ethylcarbazole. Antibodies R4-6A2/XMG 1.2-biotin, 11B11/BVD6-24G2-biotin, and TRFK5/TRFK4-biotin (PharMingen) were used for capture and detection of IFN- γ , IL-4, and IL-5, respectively. All assays were done in duplicate or triplicate, and data are from two to three independent experiments.

Diabetes assessment. After 12 weeks of age, GAD-tg and control NOD mice were monitored weekly for the development of diabetes by testing their blood glucose levels. Two consecutive levels of blood glucose >250 mg/dl was considered onset of diabetes.

Statistical analysis. Data are presented as means \pm SEM. The difference between GAD-tg and wild-type NOD mice was statistically analyzed by Student's *t* test. A *P* value <0.05 was considered statistically significant.

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 HSPp277 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, HSPp277, 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 splenic GAD65-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

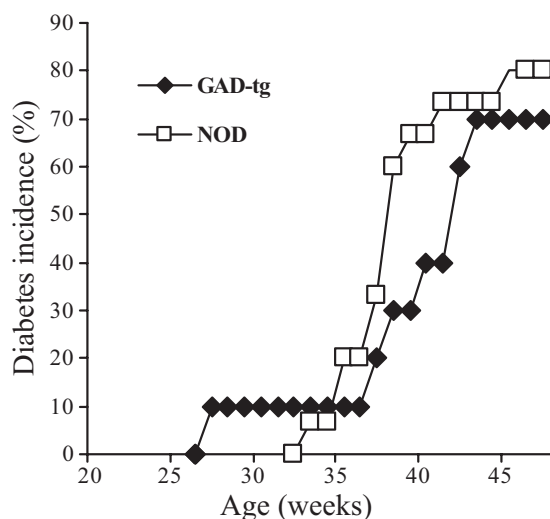


FIG. 1. GAD-tg and nontransgenic NOD mouse littermates have similar incidence of diabetes in our colonies at the University of California Los Angeles. GAD-tg mice, $n = 10$; NOD mice, $n = 11$.

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

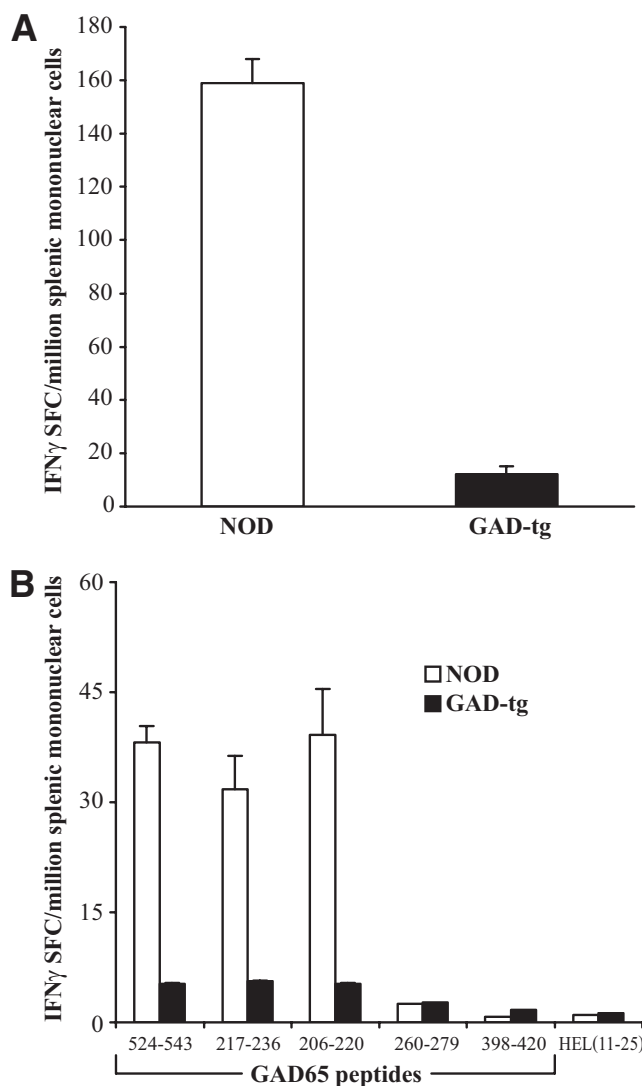


FIG. 2. Reduced Th1 responses to GAD65 and its dominant determinants without spreading of autoreactivity to its cryptic determinants or induction of Th2 responses. **A:** The frequency of IFN- γ , IL-4, and IL-5-secreting T-cells responding to whole GAD65 in the spleen of 8-week-old GAD-tg and nontransgenic NOD mouse littermates was analyzed directly ex vivo by ELISPOT assays. The mean number of IFN- γ -secreting spot-forming cells (SFCs) \pm SEM per million spleen cells is shown. We did not detect any IL-4- or IL-5-secreting splenic T-cells responding to GAD65 in the spleens of NOD or GAD-tg mice (data not shown). **B:** The frequency of IFN- γ -secreting splenic T-cells responding to peptides containing dominant determinants GAD(524–543), GAD(217–236), and GAD(206–220), as well as “absolute” cryptic determinants GAD(260–279) and GAD(398–420), which are immunogenic but ignored by the autoimmune response (22) and control peptide HEL(11–25). We did not detect IL-4 responses to these peptides (data not shown). Data shown are averages from four to five mice individually analyzed in duplicate in two separate experiments. Background averaged one SFC in cultures stimulated with control HEL_{11–25} or medium alone.

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

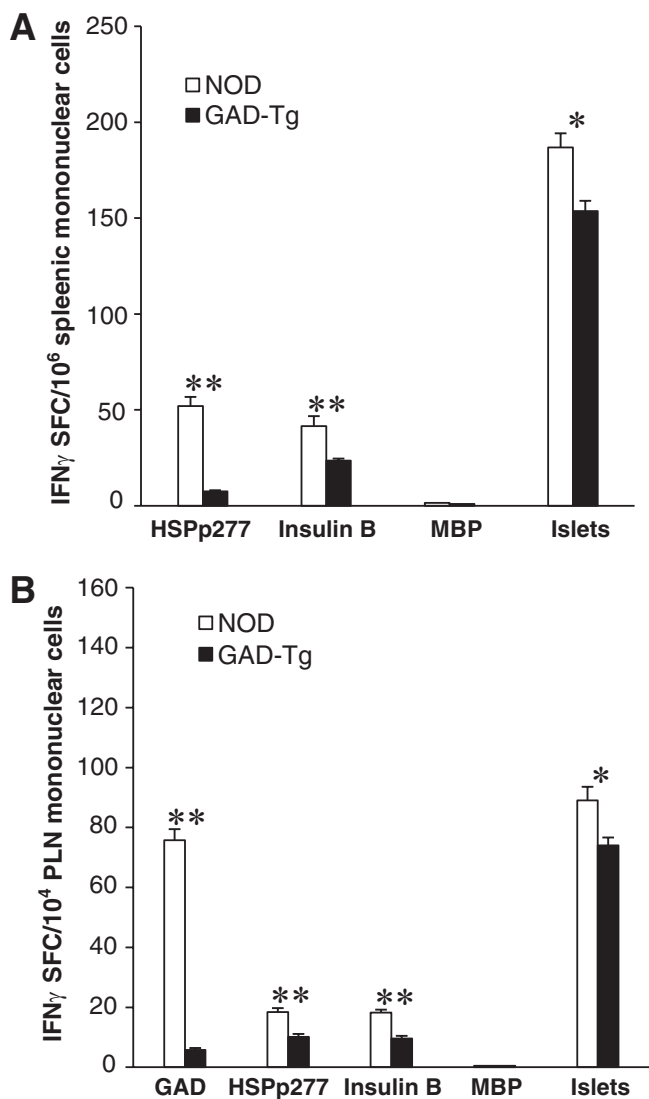


FIG. 3. Autoreactivities to β -CAAs are reduced in young GAD-tg mice. The frequency of splenic (A) IFN- γ -secreting T-cell responses to HSPp277, insulin B-chain, MBP, and islet lysate in 8-week-old GAD-tg mice were reduced relative to their nontransgenic NOD mouse littermates. (B) PLN responses to HSPp277, insulin B-chain, and islet lysate were also significantly reduced in GAD-tg mice. We did not detect IL-4- or IL-5-secreting splenic T-cells responding to these antigens in either strain of mouse (data not shown). No T-cell responses were detected in either organ to control MBP. The mean number of IFN- γ -secreting SFC \pm SEM per 10⁶ spleen cells, or per 10⁴ PLN mononuclear cells, is shown. * $P < 0.05$, ** $P < 0.01$.

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 lysate were also significantly reduced ($P < 0.05$ for both). The responses to islet lysate were not as greatly reduced as those to HSPp277 and insulin B-chain. This may be because the islet lysate 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, provid-

ing 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 older 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. 4B). 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

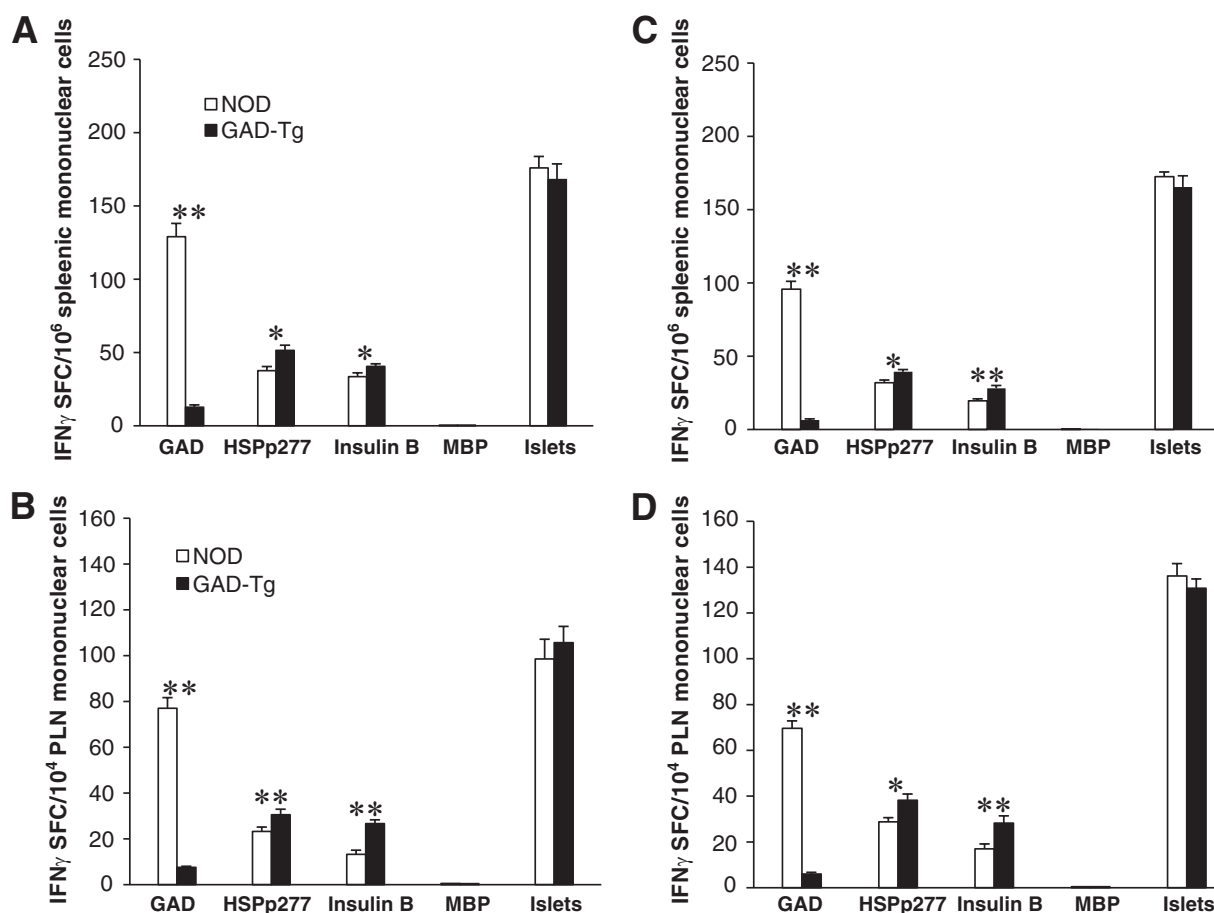


FIG. 4. Enhanced autoreactivities to other β -CAAs in older GAD-tg mice. The frequency of T-cell responses to HSPp277, insulin B-chain, MBP, and islet lysate were tested at 12 weeks of age (A and B) and at 16 weeks of age (C and D) in the spleen (A and C) and PLN (B and D) of GAD-tg mice and age-matched 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. * $P < 0.05$, ** $P < 0.01$.

had no discernable effect on the disease kinetics of GAD-tg mice.

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 MHCI 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-

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 self-antigens at below-activation thresholds (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 insulinitis 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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REFERENCES

- Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann PV. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* 1993;366:69–72
- Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulinitis in non-obese diabetic mice. *Nature* 1993;366:72–75
- Cetkovic-Cvrlje M, Gerling IC, Muir A, Atkinson MA, Elliott JF, Leiter EH. Retardation or acceleration of diabetes in NOD/Lt mice mediated by intrathymic administration of candidate beta-cell antigens. *Diabetes* 1997; 46:1975–1982
- Gerling IC, Atkinson MA, Leiter EH. The thymus as a site for evaluating the potency of candidate beta cell autoantigens in NOD mice. *J Autoimmun* 1994;7:851–858
- Kash SF, Condie BG, Baekkeskov S. Glutamate decarboxylase and GABA in pancreatic islets: lessons from knock-out mice. *Horm Metab Res* 1999;31:340–344
- Kim J, Richter W, Aanstoot HJ, Shi Y, Fu Q, Rajotte R, Warnock G, Baekkeskov S. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. *Diabetes* 1993;42:1799–1808
- Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. *Neuron* 1991;7:91–100
- Elliott JF, Qin HY, Bhatti S, Smith DK, Singh RK, Dillon T, Lauzon J, Singh B. Immunization with the larger isoform of mouse glutamic acid decarboxylase (GAD67) prevents autoimmune diabetes in NOD mice. *Diabetes* 1994;43:1494–1499
- Zechel MA, Elliott JF, Atkinson MA, Singh B. Characterization of novel T-cell epitopes on 65 kDa and 67 kDa glutamic acid decarboxylase relevant in autoimmune responses in NOD mice. *J Autoimmun* 1998;11:83–95
- Tian J, Chau C, Hales TG, Kaufman DL. GABA(A) receptors mediate inhibition of T cell responses. *J Neuroimmunol* 1999;96:21–28
- Tian J, Lu Y, Zhang H, Chau CH, Dang HN, Kaufman DL. Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. *J Immunol* 2004;173:5298–5304
- Yoon JW, Yoon CS, Lim HW, Huang QQ, Kang Y, Pyun KH, Hirasawa K, Sherwin RS, Jun HS. Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells. *Science* 1999;284:1183–1187
- Geng L, Solimena M, Flavell RA, Sherwin RS, Hayday AC. Widespread expression of an autoantigen-GAD65 transgene does not tolerize non-obese diabetic mice and can exacerbate disease. *Proc Natl Acad Sci U S A* 1998;95:10055–10060
- Jaecel E, Klein L, Martin-Orozco N, von Boehmer H. Normal incidence of diabetes in NOD mice tolerant to glutamic acid decarboxylase. *J Exp Med* 2003;197:1635–1644
- Han B, Serra P, Amrani A, Yamanouchi J, Marée AF, Edelstein-Keshet L, Santamaria P. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat Med* 2005;11:645–652
- Harrington CJ, Paez A, Hunkapiller T, Mannikko V, Brabb T, Ahearn M, Beeson C, Goverman J. Differential tolerance is induced in T cells recognizing distinct epitopes of myelin basic protein. *Immunity* 1998;8: 571–580
- Targoni OS, Lehmann PV. Endogenous myelin basic protein inactivates the high avidity T cell repertoire. *J Exp Med* 1998;187:2055–2063
- Sercarz EE, Lehmann PV, Ametani A, Benichou G, Miller A, Moudgil K. Dominance and crypticity of T cell antigenic determinants. *Annu Rev Immunol* 1993;11:729–766
- Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 1994;94:2125–2129
- Kaufman DL, Tisch R, Sarvetnick N, Chatenoud L, Harrison LC, Haskins K, Quinn A, Sercarz E, Singh B, von Herrath M, Wegmann D, Wen L, Zekzer D. Report from the 1st International NOD Mouse T-Cell Workshop and the follow-up mini-workshop. *Diabetes* 2001;50:2459–2463
- Chao CC, McDevitt HO. Identification of immunogenic epitopes of GAD 65 presented by Ag7 in non-obese diabetic mice. *Immunogenetics* 1997;46: 29–34
- Olcott AP, Tian J, Walker V, Dang H, Middleton B, Adorini L, Washburn L, Kaufman DL. Antigen-based therapies using ignored determinants of beta cell antigens can more effectively inhibit late-stage autoimmune disease in diabetes-prone mice. *J Immunol* 2005;175:1991–1999
- Elias D, Reshef T, Birk OS, van der Zee R, Walker MD, Cohen IR. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc Natl Acad Sci U S A* 1991;88:3088–3091
- Muir A, Peck A, Clare-Salzler M, Song YH, Cornelius J, Luchetta R, Krischer J, Maclaren N. Insulin immunization of nonobese diabetic mice induces a protective insulinitis characterized by diminished intraislet interferon-gamma transcription. *J Clin Invest* 1995;95:628–634
- Tisch R, Wang B, Serreze DV. Induction of glutamic acid decarboxylase 65-specific Th2 cells and suppression of autoimmune diabetes at late stages of disease is epitope dependent. *J Immunol* 1999;163:1178–1187
- Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, Kaufman DL. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J Exp Med* 1996;183:1561–1567
- Deng H, Apple R, Clare-Salzler M, Trembleau S, Mathis D, Adorini L, Sercarz E. Determinant capture as a possible mechanism of protection afforded by major histocompatibility complex class II molecules in autoimmune disease. *J Exp Med* 1993;178:1675–1680
- Tian J, Lehmann PV, Kaufman DL. Determinant spreading of T helper 2 (Th2) responses to pancreatic islet autoantigens. *J Exp Med* 1997;186: 2039–2043
- Tian J, Gregori S, Adorini L, Kaufman DL. The frequency of high avidity T cells determines the hierarchy of determinant spreading. *J Immunol* 2001;166:7144–7150
- Clare-Salzler M, Mullen Y. Marked dendritic cell-T cell cluster formation in

- the pancreatic lymph node of the non-obese diabetic mouse. *Immunology* 1992;76:478–484
31. Hill NJ, Van Gunst K, Sarvetnick N. Th1 and Th2 pancreatic inflammation differentially affects homing of islet-reactive CD4 cells in nonobese diabetic mice. *J Immunol* 2003;170:1649–1658
 32. Forsthuber T, Yip HC, Lehmann PV. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 1996;271:1728–1730
 33. Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Newman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV, Kaufman DL. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 1996;2:1348–1353
 34. Quinn A, McInerney B, Reich EP, Kim O, Jensen KP, Sercarz EE. Regulatory and effector CD4 T cells in nonobese diabetic mice recognize overlapping determinants on glutamic acid decarboxylase and use distinct V beta genes. *J Immunol* 2001;166:2982–2991
 35. Han B, Serra P, Yamanouchi J, Amrani A, Elliott JF, Dickie P, Dilorenzo TP, Santamaria P. Developmental control of CD8 T cell-avidity maturation in autoimmune diabetes. *J Clin Invest* 2005;115:1879–1887
 36. Ashton-Rickardt PG, Tonegawa S. A differential-avidity model for T-cell selection. *Immunol Today* 1994;15:362–366
 37. Lehmann PV, Targoni OS, Forsthuber TG. Shifting T-cell activation thresholds in autoimmunity and determinant spreading. *Immunol Rev* 1998;164:53–61
 38. Höglund P, Mintern J, Waltzinger C, Heath W, Benoist C, Mathis D. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. *J Exp Med* 1999;189:331–339
 39. Turley S, Poirot L, Hattori M, Benoist C, Mathis D. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. *J Exp Med* 2003;198:1527–1537
 40. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature* 2005;435:220–223
 41. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 2004;117:265–277
 42. Tian J, Kaufman DL. Antigen-based therapy for the treatment of type 1 diabetes. *Diabetes* 2009;58, 1939–1946
 43. Tisch R, McDevitt HO. Antigen-specific immunotherapy: is it a real possibility to combat T-cell-mediated autoimmunity? *Proc Natl Acad Sci U S A* 1994;91:437–438
 44. Steinman L. A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: a tale of smart bombs and the infantry. *Proc Natl Acad Sci U S A* 1996;93:2253–2256
 45. Tian J, Olcott A, Hanssen L, Zekzer D, Kaufman DL. Antigen-based immunotherapy for autoimmune disease: from animal models to humans? *Immunol Today* 1999;20:190–195
 46. Tian J, Kaufman DL. Attenuation of inducible Th2 immunity with autoimmune disease progression. *J Immunol* 1998;161:5399–5403