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Research Article

Autoimmunity

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TGF- β signaling is required for the function of insulin-reactive T regulatory cells

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We have previously isolated insulin-reactive Tregs from diabetic NOD mice designated 2H6, from which TCR transgenic mice were generated. The T cells from these 2H6 transgenic mice recognize insulin but have suppressive properties in vitro. They protect NOD mice in vivo from spontaneous development of diabetes and adoptive transfer of disease caused by polyclonal diabetogenic spleen cells as well as the highly diabetogenic monoclonal BDC2.5 TCR transgenic T cells that recognize an islet granule antigen. Using cells from both NOD and BDC2.5 mice that express a dominant-negative TGF- β receptor type II (TGF- β DNRII), we show that 2H6 T cells protected from disease by producing TGF- β and that the ability of the target diabetogenic T cells to respond to TGF- β was crucial. We further demonstrate that TGF- β signaling in 2H6 cells was important for their protective properties, as 2H6 cells were unable to protect from adoptive transfer-induced diabetes if they were unable to respond to TGF- β . Thus, our data demonstrate that insulin-specific regulatory cells protect from diabetes by virtue of their production of TGF- β 1 that acts in an autocrine manner to maintain their regulatory function and acts in a paracrine manner on the target cells.

Introduction

Insulin is an important autoantigen in human type 1 diabetes mellitus (T1D). This is supported by the following findings: (a) a gene linked to T1D that controls expression of insulin in the thymus and the pancreas is located in the VNTR region of the insulin promoter (1); (b) the level of insulin expression in the thymus influences genetic susceptibility to T1D (2, 3), presumably by regulating the selection of insulin-specific T cells; (c) anti-insulin antibodies are frequently present in young prediabetic and diabetic patients (4); and (d) a subset analysis of the large Diabetes Prevention Trial-1 has indicated that oral insulin may protect high-risk subjects (5, 6). In the NOD mouse, many islet-reactive T cells invading the islet are insulin specific (7, 8), and, most importantly, insulin-reactive T cells are capable of adoptively transferring diabetes in NOD mice (7, 9). These cells appear to recognize insulin B chain in the region of peptide 9–23 (9, 10). Interestingly, insulin B chain 9–23 peptide also stimulates peripheral blood T cell responses in newly diagnosed and high-risk patients (11). The importance of insulin as an autoantigen is further underscored by data demonstrating that insulin injections protect NOD mice from developing autoimmune diabetes (12, 13). Subsequent data demonstrating that oral insulin or metabolically inactive insulin B chain and insulin B chain peptide 9–23 injections exert similar effects provide strong evidence that insulin therapy in mice does not act metabolically on the β cell but rather induces a regulatory immune response (14–16).

There are a number of different types of regulatory cells. Naturally arising CD4⁺CD25⁺ T cells in the thymus are released to the periphery. These cells express the inhibitory molecule CTL-associated anti-

gen 4 (CTLA-4) and the forkhead transcription factor FoxP3, and they are responsible for controlling physiological and pathological immune responses (17). These suppressive T cells function through a variety of mechanisms, which include direct contact as well as production of the inhibitory cytokines IL-10 and TGF- β . Separate subsets of Tregs can be induced by antigen stimulation in vivo. Th3 cells, which produce TGF- β , are stimulated by the oral administration of whole proteins (18). More recently, it was shown that the IL-10-secreting type 1 Treg (Tr1) subset of cells can be induced by nasal administration of short peptides (19, 20). In addition, whether regulatory cells of known antigen specificity are able to inhibit cells with the same antigen specificity or whether bystander suppression can occur has varied with the system under study (18, 21, 22).

There is substantial evidence for a regulatory component to the immune response in T1D. In NOD mice, progression to overt diabetes is gradual rather than acute. Similarly, in humans, overt diabetes may require years to become apparent after the appearance of islet cell antibodies, implying that the autoimmune response is downregulated. A number of studies suggest that insulin is capable of generating a diabetes-protective immune response. NOD mice, given oral insulin, generate Tregs (i.e., Th2 or Th3 cells) capable of producing IL-4 or TGF- β (14, 15, 23). We have previously isolated, from pancreatic lymph node (PLN) cells of a diabetic NOD mouse, a cloned T cell line (24), designated 2H6, that recognizes insulin (specifically B chain peptide 12–25 or 9–23), secretes IFN- γ and TGF- β , and has a striking ability to block both the adoptive transfer of diabetes in NOD.scid recipients and the spontaneous development of diabetes in NOD mice. The specificity of the regulatory clone for insulin provides it with a means of targeting the islet, thereby allowing it to direct its secretory product TGF- β , locally, at diabetogenic cells reaching the islet.

We have now generated 2H6 TCR transgenic NOD mouse lines. Here we report the generation and characterization of this TCR transgenic NOD mouse. Like the parental cells, transgenic 2H6

Nonstandard abbreviations used: CTLA-4, CTL-associated antigen 4; CY, cyclophosphamide; GAD, glutamic acid decarboxylase; PLN, pancreatic lymph node; T1D, type 1 diabetes mellitus; TGF- β DNRII, dominant-negative TGF- β receptor type II.

Conflict of interest: The authors have declared that no conflict of interest exists.

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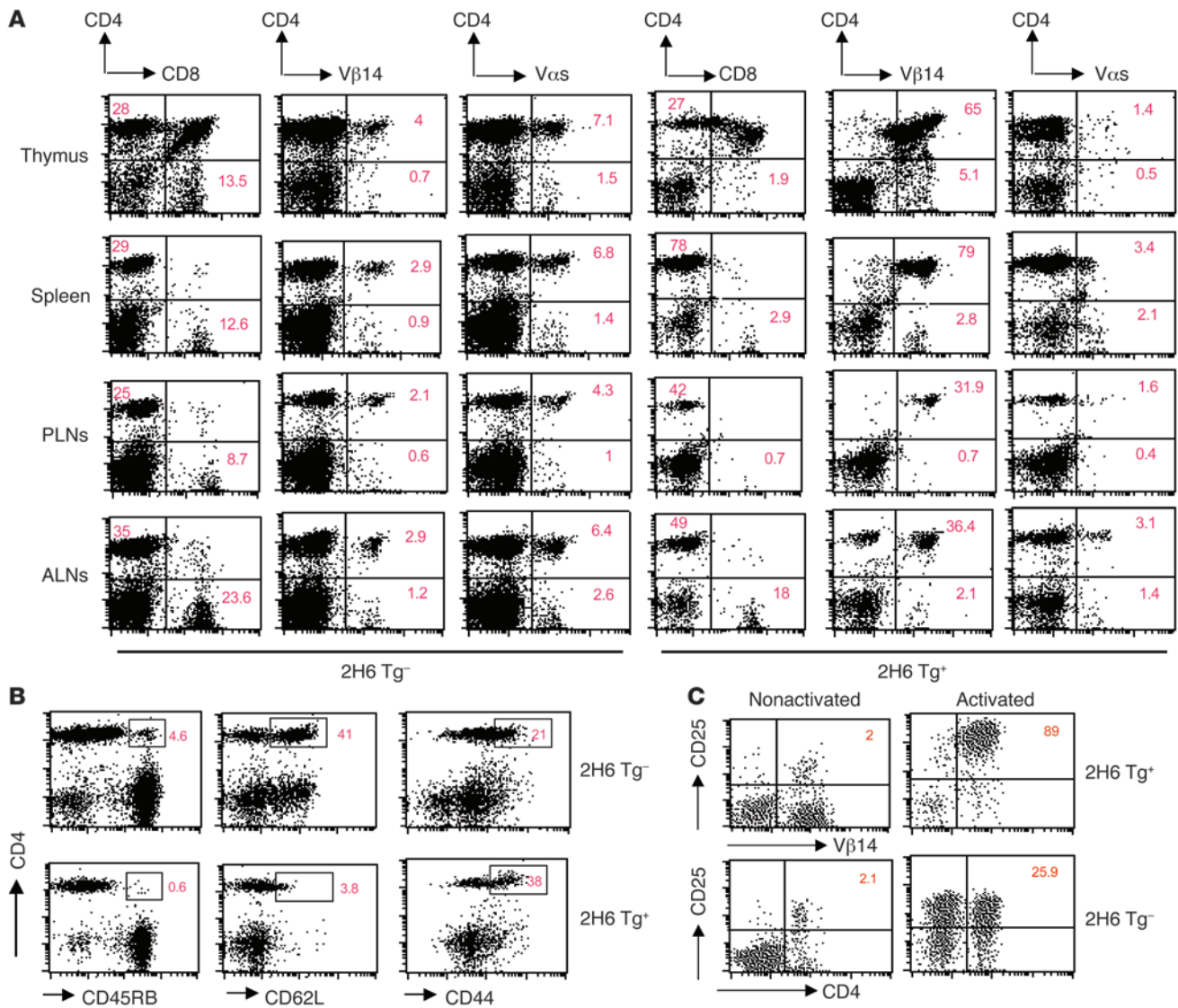


Figure 1

2H6 TCR transgene expression and phenotypic analysis of the transgenic mice. **(A)** Expression of 2H6 TCR transgene. Thymocytes, splenocytes, and lymphocytes isolated from pancreatic and axillary lymph nodes (PLNs) and ALNs, respectively) of 2H6 TCR transgenic (line 50) and nontransgenic NOD mice were stained with anti-Vβ14, anti-CD4, anti-CD8, and a cocktail of anti-Vα mAbs and analyzed by flow cytometry. The numbers in quadrants represent the percentage of positive cells among the total cells analyzed. **(B)** 2H6 transgenic T cells express an effector/memory-like phenotype. Lymphocytes isolated from spleen of nontransgenic (top panel) and 2H6 TCR transgenic (bottom panel) NOD mice were stained with CD4, CD45RB, CD62L, and CD44. The cells were then analyzed by flow cytometry. **(C)** CD25 expression of splenic CD4⁺ T cells in 2H6 TCR transgene-positive and -negative NOD mice. Splenocytes were harvested from both types of mice and stained with CD4 and CD25 with and without anti-CD3 stimulation. The numbers in the top right quadrants represent the percentage of positive cells among the total cells analyzed.

cells also secrete a notable amount of IFN-γ and TGF-β upon TCR ligation. NOD mice expressing the 2H6 TCR transgene are completely protected from diabetes development. Furthermore, cells from the transgenic mouse can differentiate into potent regulatory cells that have the ability to protect NOD mice from attack by spleen cells from diabetic mice as well as the highly diabetogenic cells from the BDC2.5 transgenic mouse. This unique regulatory TCR transgenic model will not only facilitate our understanding of the mechanisms of how Tregs work but also facilitate the design of better therapeutic strategies to restrain autoimmunity effectively.

Results

2H6 TCR transgenic NOD mice express 2H6 TCR. Three 2H6 Treg TCR transgene-positive founders were generated, and all showed germline transmission of the transgene. The expression of the 2H6 TCR transgene was screened for by genomic PCR using 2H6-specific Vα/Jα and Vβ/Jβ primers and confirmed by flow cytometry using mAb against Vβ14. Over 97% of CD4⁺ T cells expressed Vβ14 in transgene-positive mice, whereas only 5–7% of CD4⁺ T cells stained positively for the endogenous Vβ14 TCR in transgene-negative control mice (Figure 1A). There was no difference in the expression of transgenes in different lines detected by mAb against Vβ14

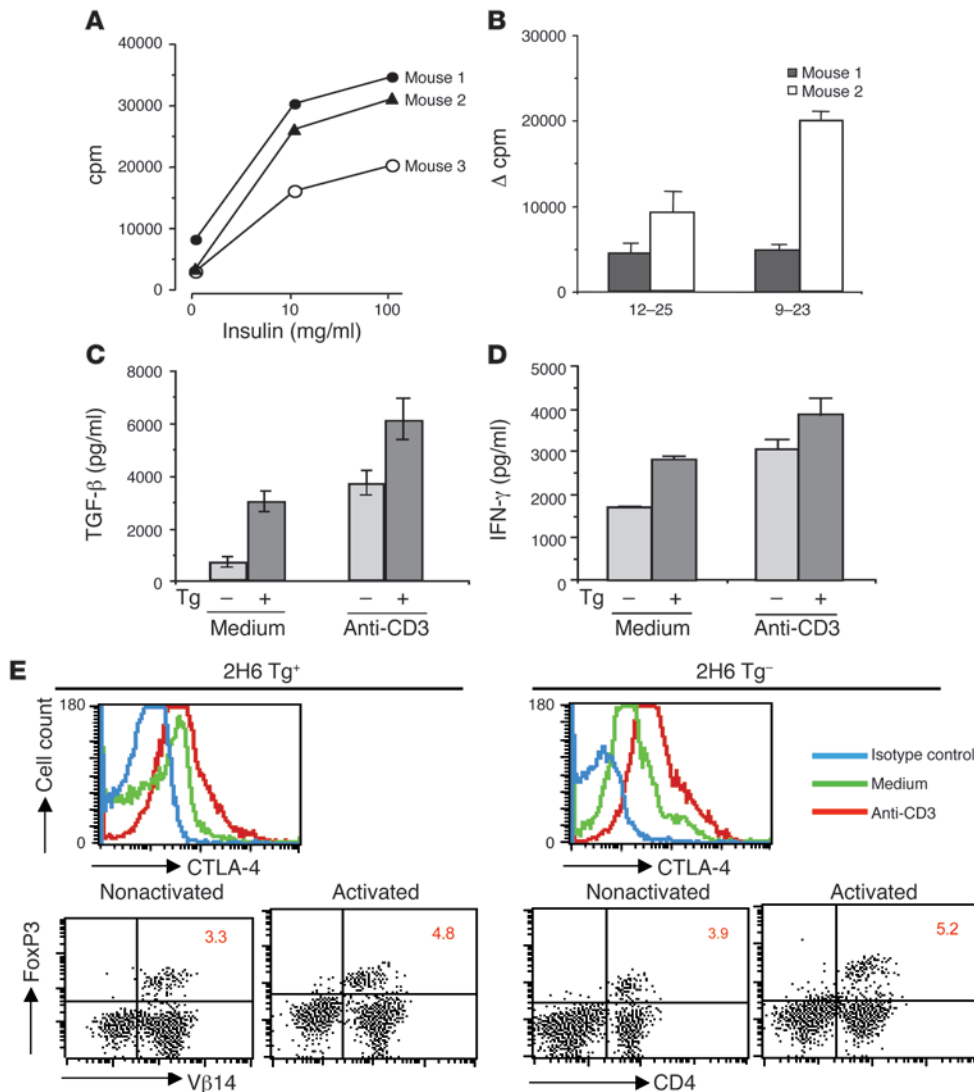


Figure 2

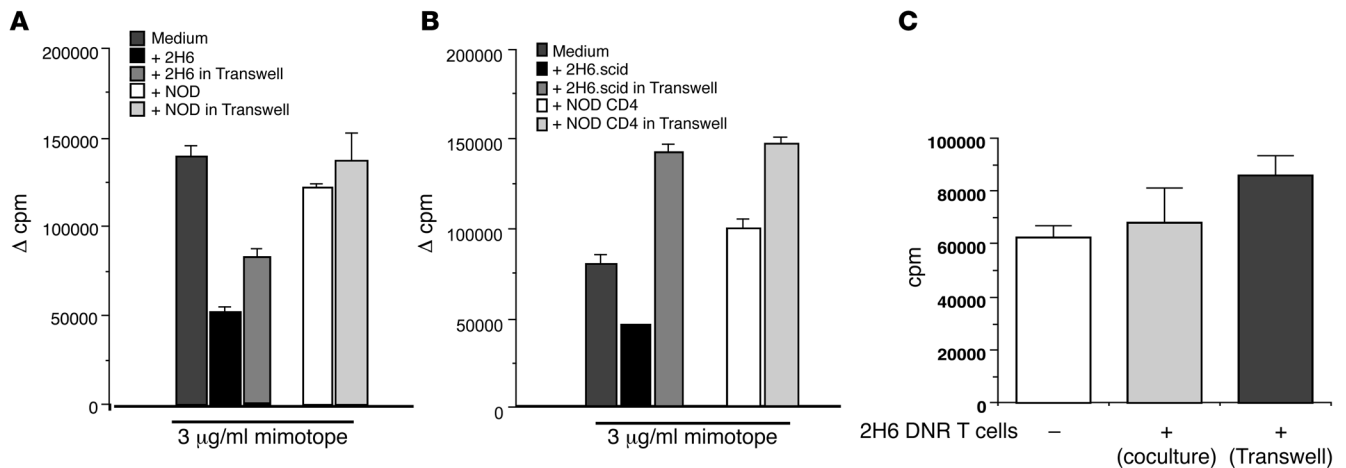
Insulin reactivity, cytokine profile, and CTLA-4 and FoxP3 expression of 2H6 TCR transgenic T cells. (A) Insulin reactivity of 2H6 TCR transgenic T cells. Splenocytes from 3 randomly selected 2H6 TCR transgenic mice (1 from line 22 and 2 from line 50) were used in the proliferation assay. Cells (10^5 per well) were cultured in Click's medium for 72 hours (in triplicate). ^3H -thymidine was added for the last 16–18 hours. (B) The reactivity to insulin B chain peptide 12–25 or 9–23 was also analyzed using the same protocol as in A. Results are illustrated as Δ cpm = proliferation with peptide – proliferation with medium. (C and D) TGF- β (C) and IFN- γ (D) production from cells of transgene-negative and -positive NOD mice. Splenocytes (10^5 cells per well) from both types of mice were cultured in Click's medium in the presence or absence of anti-CD3 (1:100 dilution of 2C11 hybridoma supernatant) for 48 hours (in triplicate). The culture supernatants were collected. IFN- γ and TGF- β contents were measured with an IFN- γ ELISA kit (BD Biosciences) and a TGF- β ELISA kit (R&D Systems). (E) Expression of CTLA-4 and FoxP3 in transgene-positive and -negative NOD mice. Splenocytes (10^6) from both types of mice were stained for intracellular CTLA-4 and FoxP3 expression with or without TCR stimulation (anti-CD3). Monoclonal antibodies against CTLA-4 and FoxP3 were purchased from BD Biosciences and eBioscience, respectively. The staining was carried out according to the manufacturers' protocols. The numbers in the top right quadrants represent the percentage of positive cells among the total cells analyzed.

(Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI27030DS1). As expected, the expression of the 2H6 TCR transgene blocked CD8⁺ T cell development, and there was no difference in the inhibition of CD8⁺ T cell expression among the 3 transgenic lines (Supplemental Figure 1A). Similarly, there was, notably, no T cell deletion observed

in any of the transgenic lines in the thymus and periphery in spite of the insulin specificity of the TCR. The total cell numbers in both thymus and spleen of the 2H6 transgenic mice were comparable to those of transgene-negative littermates (Supplemental Figure 1B). This suggests that there was no obvious deletion of self-antigen-reactive 2H6 T cells in the central and peripheral lymphoid organs. The expression of nontransgenic V α genes was determined by staining with a cocktail of commercially available mAbs against V α 2, V α 3.2, V α 8.3, and V α 11 (V α 11.1 and 11.2). It appears that 2H6 transgene expression somewhat suppressed the endogenous V α gene expression, as many 2H6 mice had a reduced number of V α 2, V α 3.2, V α 8.3, V α 11.1, and V α 11.2 compared with the transgene-negative littermates (Figure 1A). It is interesting that the reduction of endogenous V α -expressing T cells was greater in thymus and PLNs of 2H6 mice (Figure 1A). There was no obvious endogenous V β gene usage when a cocktail of mAbs against V β 2, V β 3, V β 4, V β 5, V β 6, V β 7, V β 8, V β 10, V β 11, V β 12, and V β 17 was used (data not shown). Again, no difference was observed among the 3 lines in the expression of either endogenous V α or V β genes (data not shown). Further characterization of the transgenic mice therefore focused on only 2 of the 3 founder lines (line 22 and line 50).

Phenotype, insulin specificity, and cytokine profile of 2H6 TCR transgenic T cells. Phenotypic analysis of 2H6 transgenic and nontransgenic NOD mice revealed that most of the 2H6 T cells did not express a genuinely naive phenotype (CD44^{low}, CD45RB^{high},

and CD62L^{high}). Instead, 2H6 transgenic T cells, when compared with T cells from nontransgenic NOD mice (Figure 1B, top panel), showed CD44^{high/medium}, CD45RB^{medium/low}, and CD62L^{low}, a phenotype that more resembles that of effector/memory cells (Figure 1B, bottom panel). It is interesting that transgenic 2H6 T cells expressed little CD25 (Figure 1C), a marker for a subset of Tregs,

**Figure 3**

2H6 contact-dependent and -independent suppression of BDC2.5 cells in response to the mimotope peptide. (A and B) Splenocytes from BDC2.5 transgenic NOD mice were cultured with the mimotope peptide in the presence or absence of 2H6 cells. The ratio of BDC2.5 cells to 2H6 cells was 2:1. The same culture was also performed with 2H6 cells in Transwells (0.4 μm) to prevent the cell contact between 2H6 and BDC2.5 cells without preventing the flow of soluble factor(s). Splenocytes from 2H6 transgene-negative NOD mice were used as controls in the coculture system at the same ratio. A represents 1 of 3 experiments. To confirm that suppression was indeed mediated by 2H6 cells, the experiments were also repeated using 2H6 cells from 2H6.scid mice and purified CD4⁺ T cells from NOD mice as controls at the same ratio as in A. B shows 1 of the 2 such experiments. Results are illustrated as Δcpm = proliferation with mimotope peptide – proliferation with medium for each of the conditions. (C) The suppression by 2H6 cells is completely abolished if 2H6 cells express a dominant-negative TGF-β receptor (TGF-βDNRII) (designated 2H6 DNR T cells). The abolition was seen in both coculture and Transwell culture conditions. C represents 1 of 2 experiments, and the ratio of BDC2.5 T cells to 2H6 DNR cells was also 2:1 as for the experiment shown in A and B.

unlike the parental 2H6 clone cells (data not shown). However, when 2H6 transgenic T cells were stimulated *in vitro* with anti-CD3, they all became CD25⁺ (Figure 1C). This suggests that the expression of CD25 on the parental 2H6 T cell clone was the result of long-term *in vitro* culture in the presence of IL-2.

To investigate whether transgenic 2H6 T cells were functional and retained the antigen specificity of their parental clone cells, we performed *in vitro* proliferation assays. As shown in Figure 2A, splenocytes from randomly selected transgenic mice (5–7 weeks, 1 from line 22 and 2 from line 50) exhibited insulin reactivity in a dose-dependent manner. Like the parental cloned cells, 2H6 transgenic T cells also responded to insulin B chain peptides 12–25 and 9–23 (Figure 2B). Many mice also expressed noticeable self-reactivity, *i.e.*, producing a high background in proliferation assays. This was not abolished even when the cells were cultured in serum-free media, AIM V (Invitrogen Corp.) and X-Vivo (Cambrex; data not shown). To investigate whether this high background was due to the low insulin content in the media, in particular in the FCS, we performed an ultrasensitive immunoassay for insulin (Linco Research Inc.). No insulin could be detected in the Click's medium without FCS, and the commonly used Click's medium with 5% FCS contains 3 μU/ml of insulin (which was equivalent to 1 μg/ml insulin protein). However, insulin could not be detected in the serum-free media, whereas 2H6 cells expressed a similar high background when cultured in these media (data not shown). The autoreactivity was also evident when splenocytes from 2H6 NOD.scid mice were tested (data not shown). These splenocytes contain 2H6 T cells, splenic dendritic cells, and macrophages but are devoid of B cells. To prove that the autoreactivity of 2H6 cells is related to responses to a self-antigen other than insulin, we performed blocking experiments in which 2H6 cells (from both 2H6 NOD and 2H6 NOD.scid mice) were cul-

tured in the serum-free medium X-Vivo, which had undetectable insulin in the presence or absence of mAbs against I-A^{s7} (10.2.16), I-A^b (25-9-17), CD4 (GK1.5), and control IgG (Pierce Biotechnology). The blocking results indicate that CD4 plays a critical role in the autoreactivity, as anti-CD4 completely blocked the autoreactivity and partial inhibition was also seen with anti-I-A^{s7} (Supplemental Figure 2). However, anti-I-A^b mAb or control IgG did not show any inhibitory effect on the autoreactivity. These data strongly suggest that, in addition to insulin specificity, 2H6 cells are also reactive to an unidentified self-antigen expressed on NOD APCs.

In response to insulin or anti-CD3 stimulation, transgenic 2H6 T cells produced a considerable amount of IL-2 (data not shown), TGF-β, and IFN-γ (Figure 2, C and D), a cytokine profile similar to that of their parental clone (24). It is noteworthy that 2H6 transgenic T cells secrete a large amount of TGF-β and IFN-γ even without stimulation. The fact that 2H6 cells are not genuinely naive and are also autoreactive could contribute to this.

CTLA-4, a negative regulator of T cell response, has been reported to be associated with TGF-β-producing Tregs (25). However, the expression of CTLA-4 on 2H6 T cells did not differ from that found on CD4⁺ T cells from transgene-negative littermates (Figure 2E). We also analyzed intracellular FoxP3 expression in 2H6 cells and in CD4⁺ T cells of transgene-negative NOD mice. It has recently been reported that expression of FoxP3 is also associated with TGF-β-producing Tregs (26). The numbers of FoxP3⁺ 2H6 cells were the same as those of FoxP3⁺ CD4⁺ T cells of nontransgenic NOD mice, a similarity that resembles that found in the expression of CTLA-4 (Figure 2E).

2H6 cells inhibit BDC2.5 cells in response to a mimotope through a mainly contact-dependent mechanism. BDC2.5 T cells recognize an as-yet unidentified granule antigen (27). However, recently a peptide

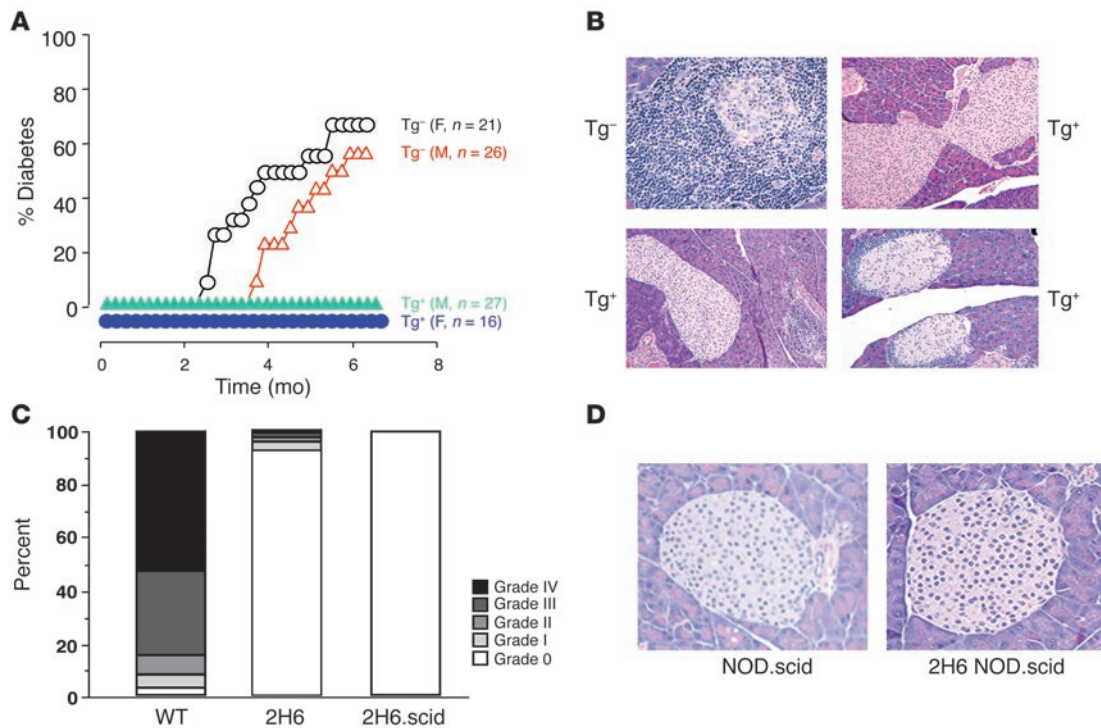
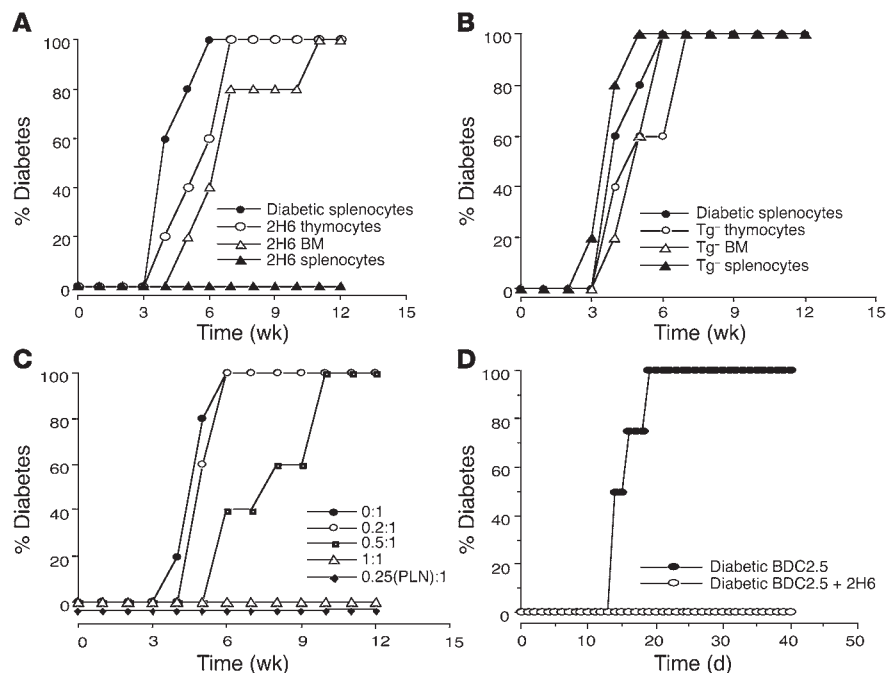


Figure 4 Natural history of diabetes development and islet histology. **(A)** Incidence of diabetes. 2H6 TCR transgene-positive and -negative NOD mice (both sexes) were observed for diabetes development over 7 months. All the mice were monitored for glycosuria weekly, and diabetes was confirmed by blood glucose measurement (>13.9 mmol/l). **(B)** Histology. Paraffin sections of pancreas were made, stained with H&E, and examined for insulinitis and/or islet destruction. The magnification for the transgene-negative section is $\times 100$. The magnification for the transgene-positive sections is $\times 40$. **(C)** Insulinitis score. One hundred to 240 islets were analyzed microscopically for insulinitis and scored as grade 0 (clean islets, no infiltration), grade I (peri-insulinitis), grade II (mild insulinitis), grade III (severe insulinitis), and grade IV (complete destruction by infiltrating cells). **(D)** Histology. Paraffin sections of pancreas from 2H6.scid mice were prepared and stained with H&E. The sections were compared with nontransgenic NOD.scid islets. The magnification is $\times 100$.

mimotope has been used to stimulate BDC2.5 T cells in vitro (28). To investigate whether 2H6 cells also suppress a non-insulin-specific diabetogenic T cell response, we stimulated BDC2.5 T cells with the mimotope peptide in the presence or absence of the 2H6 cells. To dissect whether the 2H6 effect was mediated by direct cell-cell contact or through soluble factors, such as TGF- β , we also performed coculture experiments in which 2H6 cells were cultured either together or in a Transwell with BDC2.5 cells in the presence or absence of mimotope. Figure 3A shows that 2H6 cells markedly suppressed BDC2.5 cells in response to the mimotope when the cells were cultured together (64%). However, cells from 2H6 mice also suppressed the proliferation of BDC2.5 cells when they were not in direct cell contact (38%). In contrast, cells from 2H6 transgene-negative NOD mice did not obviously inhibit the proliferation of BDC2.5 T cells in response to the mimotope (Figure 3A). Similar contact inhibition results were obtained when 2H6 cells from transgenic NOD.scid mice were used (Figure 3B). It is interesting that, when tested in Transwells, 2H6 NOD.scid cells lost the inhibitory effect, suggesting that the mild inhibition seen in Transwells (Figure 3A) when cells from non-scid 2H6 NOD mice were used might be mediated by either non-T cells, such as APCs, or non-2H6 transgenic T cells. Regardless, cell-cell contact appears to play a major role in the inhibition mediated by 2H6 cells. To further confirm this contact-dependent suppression, we performed 2 additional sets of experiments. In the first set, we cultured BDC2.5

T cells with the mimotope peptide in the presence or absence of the 2H6 cells with or without mAbs against TGF- β (R&D Systems) or control IgG (Pierce Biotechnology). Anti-TGF- β partially reversed the suppression, and control IgG had little effect (Supplemental Figure 3). This suggests that although the mechanism of the suppression is not totally dependent on soluble cytokines, TGF- β may be important for the suppression. In the second set of experiments, we cultured BDC2.5 T cells with the mimotope peptide in the presence or absence of the 2H6 cells expressing a dominant-negative form of TGF- β receptor type II (TGF- β DNRII). Therefore, these 2H6 T cells were not able to bind TGF- β and trigger the signaling transduction in the cells. In this culture system, 2H6 TGF- β DNRII T cells were either in direct cell contact with BDC2.5 T cells (coculture) or separated from BDC2.5 T cells (Transwell). As shown in Figure 3C, the lack of TGF- β receptor on 2H6 cells resulted in loss of their ability to suppress BDC2.5 T cells in coculture. However, the lack of TGF- β receptor did not cause 2H6 cells to lose the ability to produce TGF- β (data not shown). These data indicate that the signaling induced by TGF- β upon binding the TGF- β receptor is important for the suppression mediated by 2H6 T cells.

Inhibition of diabetes development in 2H6 TCR transgenic NOD mice. The natural history of spontaneous diabetes was studied in 2H6 transgene-positive and -negative littermates. By 30 weeks of age, the majority of transgene-negative mice (both males and females) had developed autoimmune diabetes, whereas none of the 2H6

**Figure 5**

Adoptive transfer of diabetes. (A) Diabetic splenocytes (10^7 per recipient) from transgene-negative NOD mice were transferred alone (filled circles, $n = 5$) or together with an equal number of 2H6 transgene-positive thymocytes (open circles, $n = 5$), BM cells (open triangles, $n = 5$), or splenocytes (filled triangles, $n = 5$). (B) This graph shows the control groups of the experiment in A. Diabetic splenocytes (10^7 per recipient) from transgene-negative NOD mice were transferred alone (filled circles, $n = 5$) or together with an equal number of 2H6 transgene-negative thymocytes (open circles, $n = 5$), BM cells (open triangles, $n = 5$), or splenocytes (filled triangles, $n = 5$). (C) Titration of the protective effect of 2H6 transgene-positive splenocytes and cells from PLNs. Diabetic splenocytes (10^7 per recipient) from transgene-negative NOD mice were transferred alone (filled circles, $n = 5$) or together with 2H6 transgene-positive splenocytes or PLN cells at different ratios: 2H6 cells/diabetic cells: 0.2:1 (open circles, $n = 5$), 0.5:1 (filled squares, $n = 5$), 1:1 (open triangles, $n = 6$); PLN cells/diabetic cells: 0.25:1 (filled diamonds, $n = 4$). (D) Diabetic splenocytes (10^7 per recipient) from BDC2.5 NOD mice were transferred alone (filled circles, $n = 4$) or together with an equal number of 2H6 transgene-positive splenocytes (open circles, $n = 5$). All the mice (A–D) were monitored for glycosuria weekly, and the experiments were terminated as indicated unless the mice developed diabetes, which was confirmed by blood glucose measurement (>13.9 mmol/l).

mice (of both sexes) became diabetic by 7 months (Figure 4A) or even 10 months of age (data not shown). Since there was no difference in any of the phenotypic parameters studied between the transgenic lines, Figure 4A represents the pooled data from lines 22 and 50 and their transgene-negative littermates. In accord with the lack of diabetes development, islet pathology of 2H6 transgenic mice also showed either no islet infiltration or mild peri-insulinitis (Figure 4B). In sharp contrast, heavy lymphocytic infiltration could be seen in transgene-negative mice (before and after diabetes onset) as predicted (Figure 4B). We also analyzed the insulinitis score, and the results correlated with the disease expression clinically (Figure 4C).

Elimination of endogenous Tregs does not affect the tolerance established by 2H6 T cells. The diabetes-protective phenotype described above was derived from the studies using the 2H6 transgenic NOD mice. It is possible that endogenous Tregs that were not of the 2H6 specificity may have contributed to protection from diabetes in 2H6 TCR transgenic mice as seen in the case of diabetogenic

BDC2.5 TCR transgenic mice (29) or pathogenic myelin basic protein-specific TCR transgenic mice (30). To elucidate whether this was also true for 2H6 mice, we bred 2H6 transgenic mice to NOD.scid mice to eliminate the other endogenous potentially regulatory T cells. The development and phenotype of 2H6 transgenic T cells from 2H6.scid mice were very similar to those seen in the 2H6 cells on the non-scid background (Supplemental Figure 4A), although the cellularity in the peripheral lymphoid organs of 2H6 NOD.scid mice was markedly lower than that in 2H6 NOD mice because of B cell deficiency (Supplemental Figure 4B). Diabetes development was studied in long-term (30 weeks) natural history experiments. Unlike the results obtained using pathogenic TCR transgenic mouse models, the elimination of other potential endogenous Tregs in 2H6 transgenic mice did not alter the disease-protected phenotype, as 2H6.scid mice were also diabetes free ($n = 20$, 11 females and 9 males). In addition, islet β cell destruction was not found in any of the mice studied (Figure 4, C and D). It is noteworthy that some 2H6 transgenic NOD mice expressed various degrees of insulinitis whereas 2H6.scid mice were completely free of insulinitis. This result also indicates that diabetes protection seen in transgenic NOD mice is due to the nature of 2H6 T cells and not to the presence of endogenous potentially regulatory T cells expressing a nontransgenic TCR.

2H6 transgenic mice are resistant to cyclophosphamide-induced diabetes. Cyclophosphamide (CY), an immunosuppressive drug, is thought to specifically eradicate immunoregulatory cells and accelerate the development of autoimmune diabetes (31). To test whether CY treatment could promote diabetes in 2H6 mice, we injected CY (200 mg/kg) into 2H6 mice and their transgene-negative littermates (6–8 weeks of age). All the mice treated with CY had transient mild weight loss, approximately 2 weeks after the injection, possibly due to side effects of the drug, such as anorexia. Six of 8 transgene-negative control littermates developed accelerated diabetes within 4 weeks after CY treatment, whereas none of the six 2H6 mice had developed the disease (Supplemental Figure 5) when the experiment was terminated (24 weeks after the treatment).

Inhibition of diabetes development in adoptive transfer by 2H6 transgenic cells. To study whether the inhibition of diabetes development in 2H6 mice was mediated by immune regulation or was simply due to the limited T cell repertoire of these mice, we performed a series of adoptive transfer experiments. Diabetic NOD splenocytes were adoptively transferred i.v. into NOD.scid mice, alone or together with lymphocytes from spleen, PLNs, thymus, and BM of 2H6 mice. Diabetes development was monitored for

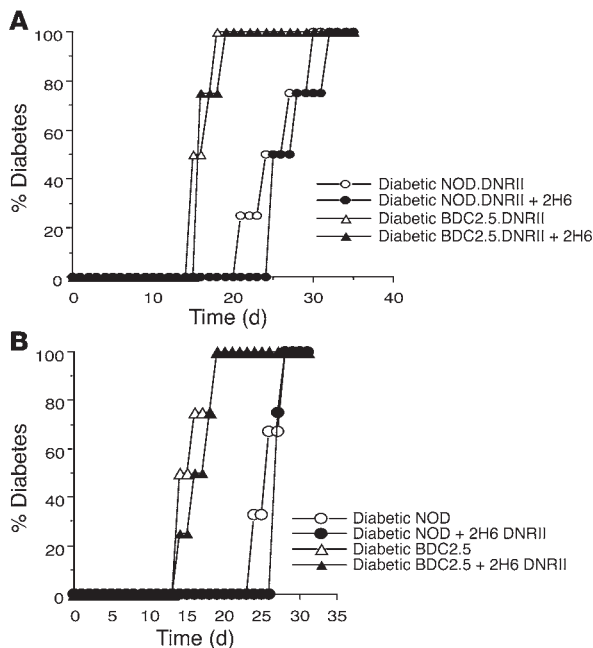


Figure 6

Adoptive transfer of diabetogenic or protective cells deficient in TGF- β receptor. **(A)** Splenocytes from diabetic TGF- β DNRII NOD (open circles) or diabetic TGF- β DNRII BDC2.5 NOD (open triangles) mice were transferred into NOD.scid recipients (10^7 per recipient) alone ($n = 5$ for each group) or together with an equal number of 2H6 NOD splenocytes (filled circles and filled triangles, $n = 5$ for each group). All the mice were monitored for glycosuria weekly, and the experiments were terminated when the mice developed diabetes. **(B)** Diabetic spleen cells from either NOD (open circles) or BDC2.5 NOD (open triangles) mice were transferred into NOD.scid recipients (10^7 per recipient) alone ($n = 4$ for each group) or together with an equal number of TGF- β DNRII-deficient 2H6 NOD cells (2H6 DNRII, filled circles and filled triangles, $n = 5$ for each group). All the mice were monitored for glycosuria weekly and the experiments were terminated when the mice developed diabetes.

15 weeks after adoptive transfer. It is interesting that, at the indicated cell ratios, lymphocytes from spleen or PLNs of 2H6 mice protected the recipients from diabetes development induced by diabetogenic cells whereas 2H6 thymocytes or BM did not protect from disease (Figure 5, A and B). To exclude the possibility of inefficient disease protection owing to the influence of immature thymocytes, we purified CD4 single-positive thymocytes from 2H6 TCR transgenic mice and infused these cells together with diabetic splenocytes harvested from nontransgenic diabetic mice. Like total thymocyte transfer, purified CD4 single-positive 2H6 thymocytes did not protect from diabetes in co-adoptive transfer (data not shown). Our data suggest that the protective properties develop in the periphery. The PLN cells provided the strongest protection, as 100% disease inhibition could be achieved at the ratio of approximately 0.25 PLN cells to 1 diabetic spleen cell, whereas full protection could be obtained only at a 1:1 ratio when 2H6 splenocytes were used (Figure 5C). The number of 2H6 cells (double positive for TCR V β 14 and CD4) in spleen and PLNs of 2H6 mice was comparable ($34.86\% \pm 8.55\%$ and $43.82\% \pm 14.8\%$, respectively, $n = 3$). The suppressive potency of PLNs was further confirmed by in vitro suppression assay using proliferation of BDC2.5 T cells as a read-out. As shown in Supplemental Figure 6, 2H6 cells from PLNs completely suppressed the proliferation of BDC2.5 T cells in response to mimotope, whereas PLN cells from 2H6-negative NOD mice had no inhibitory effect. In comparison with Figure 3B, where there was only 50% suppression when 2H6 NOD.scid spleen cells were used, 2H6 cells from PLNs expressed the strongest suppression (100%). It is noteworthy that PLNs expressed the lowest number of endogenous T cells (Figure 1A) but showed the highest protective ability compared with splenocytes; therefore, it is unlikely that the T cells carrying endogenous TCRs contributed to disease inhibition.

2H6 transgenic cells prevent diabetes development induced by BDC2.5 T cells. Diabetic spleen cells contain polyclonal diabetogenic T cells. The disease protection by 2H6 cells shown above suggests that the protection is not directed solely to insulin-specific T

cells. To further test this hypothesis, we used antigen-specific (but not insulin-specific) diabetogenic T cells from the BDC2.5 transgenic mouse. Diabetic BDC2.5 TCR transgenic spleen cells were adoptively transferred into NOD.scid recipients either alone or together with 2H6 TCR transgenic spleen cells at a 1:1 ratio. Like the NOD.scid mice that received adoptively transferred diabetogenic NOD spleen cells and 2H6 cells, NOD.scid mice ($n = 5$) that received coinjection of 2H6 and BDC2.5 cells were diabetes free. The mice that were injected with diabetic BDC2.5 TCR transgenic spleen cells alone developed the disease within 2 weeks after transfer ($n = 4$; Figure 5D).

2H6 transgenic T cells suppress diabetes development in wild-type NOD mice. NOD mice expressing 2H6 transgene were completely protected from diabetes development (Figure 4), and 2H6 transgenic T cells were also able to suppress diabetes induced by diabetic splenocytes in NOD.scid recipients (Figure 5). We next sought to test whether 2H6 transgenic T cells could protect from diabetes in immune-competent wild-type NOD mice. Twenty 4- to 5-week-old female NOD mice were infused i.v. with either 2H6 transgene-positive ($n = 10$) or transgene-negative ($n = 10$) splenocytes (10^7) weekly for 5 weeks. Diabetes development was then observed. The fresh ex vivo 2H6 T cells from transgenic mice protected 50% of wild-type NOD mice from diabetes (Supplemental Figure 7).

Signaling of TGF- β is important for the immune protection by 2H6 T cells. T cells from the 2H6 TCR transgenic mice showed suppressive properties and produced more TGF- β upon activation compared with those from the transgene-negative mice. To test whether 2H6 T cells are dependent on TGF- β signaling for their regulatory properties, we generated TGF- β DNRII NOD mice and TGF- β DNRII BDC2.5 NOD mice. T cells from these mice cannot respond to TGF- β because of the defect in the receptor (32). TGF- β DNRII NOD and TGF- β DNRII BDC2.5 NOD mice develop somewhat accelerated diabetes compared with their wild-type counterparts, NOD and BDC2.5 NOD mice, respectively (M.O. Li and R.A. Flavell, unpublished data). We performed a series of adoptive transfer experiments in NOD.scid mice using diabetic TGF- β DNRII NOD or diabetic TGF- β DNRII BDC2.5 NOD spleen cells in the presence or absence of 2H6 cells. As shown in Figure 6A, 2H6 cells were unable to protect the NOD.scid recipients from diabetes development when the diabetogenic cells (from either TGF- β DNRII NOD or TGF- β DNRII BDC2.5 NOD donors) lost the ability to respond to TGF- β . This strongly suggests that diabetes protection by 2H6 T cells is mediated by the signaling of TGF- β .



TGF- β signaling on 2H6 T cells is also important for the immune protection. To test whether the signaling of TGF- β regulates the function of 2H6 T cells *in vivo*, we then generated TGF- β DNRII 2H6 TCR double-transgenic NOD mice. The loss of the TGF- β receptor did not render 2H6 mice susceptible to diabetes; *i.e.*, TGF- β DNRII 2H6 mice were diabetes free (data not shown). However, it is interesting that 2H6 T cells without TGF- β receptor were no longer able to protect against diabetes development in the adoptive transfer model shown in Figure 6B. Although TGF- β DNRII 2H6 TCR cells did not become pathogenic T cells in the absence of TGF- β action, this result indicates that TGF- β signaling is important for the suppressive effect of 2H6 T cells on other effector cells, as shown both *in vitro* (Figure 3C) and *in vivo* (Figure 6B).

Discussion

In this study we report the generation and characterization of an insulin-reactive T regulatory TCR transgenic NOD mouse in which autoimmune diabetes mellitus development is completely prevented in both spontaneous and adoptive transfer model systems. The protection is mediated by the signaling of TGF- β in both paracrine and autocrine fashion.

T1D onset is the result of ultimate failure in immune tolerance to islet autoantigens. Proinsulin/insulin, glutamic acid decarboxylase (GAD), and a tyrosine phosphatase-like protein known as IA-2 are major islet autoantigens targeted by both T cell- and B cell-mediated autoimmune responses (33). Recently, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) has also been identified as a target for diabetogenic CD8⁺ T cells (34). Insulin, a key hormone for survival, is produced exclusively by pancreatic β cells in large quantities. Insulin is a major autoantigen in the disease (35, 36) and not only appears, however, to promote diabetes development but also has the capacity to generate regulatory cells as well. To better understand how such regulatory cells might develop, we generated an insulin-reactive, regulatory TCR transgenic NOD mouse – the 2H6 NOD mouse. It is noteworthy that most, if not all, CD4⁺ T cells from TCR transgenic mouse strains have a pathogenic phenotype in autoimmune diabetes and other autoimmune disease models (30, 37–39). Tarbell *et al.* have recently reported a TCR transgenic NOD mouse in which the T cells are specific for a GAD peptide. However, the mouse does not develop diabetes in spite of the fact that its lymphocytes proliferate and make IFN- γ , IL-2, TNF- α , and IL-10 when stimulated *in vitro* with GAD65 peptide (40). Apart from the antigen specificity, the biological properties of the original T cell clone that was used to generate this transgenic mouse are unknown, as the TCR was cloned from a T cell hybridoma. Here, we describe the generation and characterization of a bona fide T regulatory TCR transgenic mouse derived from a known insulin-reactive T regulatory clone. The importance of this regulatory TCR transgenic mouse is that it is a unique tool that will permit the elucidation of the role for autoantigen-reactive and TGF- β -producing Tregs in autoimmune diabetes and for similar Tregs in other autoimmune conditions.

There are 3 isoforms of TGF- β – β 1, β 2, and β 3 – that have overlapping and distinct biological functions. TGF- β 1 is the most studied isoform in immunology and is also the isoform produced by 2H6 T cells. TGF- β 1-deficient mice express a severe inflammatory and autoimmune phenotype in multiple organs (41). TGF- β receptor II is the high-affinity receptor for TGF- β 1, and mice deficient in TGF- β receptor II specifically in T cells showed a phenotype very similar to that of systemic TGF- β 1-deficient mice (32). This suggests that

TGF- β 1 plays an important role in immune tolerance, especially mediated by T cells. Thus, TGF- β 1 is generally considered to be an immune regulatory cytokine with strong therapeutic potential for treatment of human autoimmune or chronic inflammatory diseases. In several animal models of human autoimmune disease, including T1D, administration of TGF- β 1 has demonstrated promising therapeutic applications (42–44). Furthermore, induction and/or enhancement of endogenous TGF- β 1 production, such as through oral tolerance, also produced a striking inhibitory effect on chronic inflammatory and autoimmune diseases, including T1D (14, 45, 46). It has been reported that TGF- β 1 exerts immune regulatory function through cell contact-dependent or -independent mechanisms (47, 48). It is not clear which of these pathways 2H6 T cells use to mediate their islet-specific immune tolerance *in vivo*, and our *in vitro* data suggest that cell contact-dependent mechanisms may play a major role. However, it is clear that regulation within the immune system is possible, and if manipulation can increase regulatory cell activity, then this has important implications for both prevention and treatment of autoimmune diseases; a recent study has provided evidence for proof of this principle (49).

Many insulin-reactive CD4⁺ T cells in the NOD mouse recognize the insulin B chain peptide 9–23 and are diabetogenic, *i.e.*, capable of adoptively transferring diabetes (9, 10). It is interesting that a very potent diabetogenic CD8⁺ T cell clone, derived from a young NOD mouse, also recognizes insulin B chain 15–23 (7). Those studies suggested that T cells reactive to this region of the insulin B chain are mostly pathogenic. It is intriguing, therefore, that 2H6 T cells recognize insulin B chain peptides 12–25 and 9–23 (24), a region similar to that recognized by the insulin-reactive pathogenic T cells. In addition, 2H6 T cells share the same J α sequence, KLTFGKGT, with diabetogenic CD4⁺ T cell clones (9, 10), and the same J β sequence, YFGSGTRLTVL, with a diabetogenic CD8⁺ T cell clone (7). However, unlike those pathogenic insulin-reactive T cells, 2H6 T cells are diabetes protective and express a Treg phenotype, and the phenotype of 2H6 TCR transgenic T cells mirrors the parental cloned 2H6 cells. Our data provide evidence that, firstly, Tregs can be antigen specific; and secondly, both pathogenic and regulatory T cells can recognize a similar, if not identical, antigenic region. This is important as the information may have an impact on the design of antigen-based immunotherapy for the disease.

Insulin- or proinsulin-reactive T cells have also been found in patients with T1D (11). However, the *in vivo* biological function of those T cells is not known. Interestingly, some of the insulin- or proinsulin-reactive T cells found in patients with T1D showed an IL-4- and/or IL-10-producing profile (50, 51), implying protective or regulatory properties. This suggests that the autoimmune response may be downregulated. The PLN origin of 2H6 cells and their isolation from a diabetic mouse indicate that Tregs are present even when pathogenic T cells have destroyed most of the β cells (24). 2H6 TCR transgenic NOD mice are completely protected from development of diabetes. 2H6 transgenic cells also blocked the disease induced by diabetic spleen cells in the adoptive transfer model. Moreover, 2H6 TCR transgenic cells were able to inhibit diabetes development in an accelerated disease model. It is interesting that the protection is more likely mediated by peripheral 2H6 cells, as neither 2H6 thymocytes nor 2H6 BM provided disease protection.

There is no single marker that identifies a population of Tregs. Tregs are a heterogeneous group of cells. However, naturally occurring Tregs found in both humans and mice are commonly CD4⁺CD25⁺ and express FoxP3. 2H6 transgenic T cells do not



express CD25 *ex vivo*. However, almost all the 2H6 transgenic T cells become CD25⁺ after stimulation *in vitro*. Thus, CD25 expression on 2H6 cells is an activation marker, not the signature of conventional Tregs. FoxP3, highly expressed in CD4⁺CD25⁺ cells, has recently been considered as a master transcription factor for Tregs (17, 52). FoxP3 has also been reported to be induced by TGF- β (26). It is interesting that 2H6 cells express very little FoxP3 even after anti-CD3 stimulation, which induces a large amount of TGF- β production by 2H6 cells. This suggests that the immune regulation, in particular diabetes protection mediated by 2H6 cells, is not associated with FoxP3. It is possible that the 2H6 cell represents a nonclassical naturally occurring Treg. It is also conceivable that 2H6 cells represent a novel type of Treg that might have developed in the periphery and has an important immune regulatory role at the site of the immune response, given that the 2H6 T cell clone was derived from diabetic PLN cells after adoptive transfer (24).

Like the parental clone, 2H6 transgenic cells produce a significant amount of TGF- β in response to TCR ligation. It is noteworthy that a high level of TGF- β was found in 2H6 transgenic mice even without stimulation, likely because 2H6 cells are also autoreactive. Increasing evidence suggests that TGF- β is an important mediator of T regulatory function; however, it is not clear how TGF- β mediates the immune regulation, such as in the case of diabetes protection by 2H6 cells. Using the TGF- β DNRII model system, we were able to determine that TGF- β signaling in the target pathogenic cells is critical for the disease suppression. It is interesting that a similar finding was reported very recently for a colitis model (53). The authors showed that TGF- β receptor-deficient CD4⁺CD45RB^{high} T cells escape control by disease-suppressive CD4⁺CD25⁺ T cells *in vivo*. Lack of TGF- β signaling in diabetogenic T cells in our system somewhat enhanced their pathogenicity in both NOD and BDC2.5 NOD mice in spontaneous diabetes development and also allowed them to escape from the immune regulation by Tregs. On the other hand, the lack of TGF- β signaling in the 2H6 Tregs did not change their diabetes-protective properties during the natural history of autoimmune diabetes but disarmed their ability to protect from disease development in the adoptive transfer model system.

In conclusion, our TCR transgenic model has demonstrated the mechanisms by which insulin-specific, nonclassical Tregs inhibit both spontaneous and induced diabetes development. These PLN-derived cells arise naturally and do not share the common features of classical Tregs, such as the expression of CD25 and FoxP3. Instead, they represent more a Th3 phenotype and mediate immune regulation through both the paracrine and the autocrine action of TGF- β . This model system has the potential to elucidate how insulin-specific T cells downregulate islet-specific autoimmunity.

Methods

Generation of 2H6 TCR transgenic NOD mice. The full-length sequence of 2H6 TCR V β 14 was identified as V β 14/D β 1.1/J β 2.3/C β 2. The TCR V α chain gene usage was first tested with 13 pairs of V α primers that recognize V α 1–V α 13. Although the results indicated that more than one V α chain was used in the parental 2H6 cell line, V α 6 was the most dominant detected. Using a subclone of the parental 2H6 cells, V α 6 cDNA was cloned and sequenced. The full-length sequence of 2H6 V α is V α 6/J α 45/C α . TCR α and TCR β transgenic constructs were then generated using genomic DNA of 2H6 TCR and cloned into the plasmid cassette vectors (54) originally provided by D. Mathis (Harvard University, Cambridge, Massachusetts, USA). The biological functions of both α and β constructs were tested by transfection of the 2 con-

structs together with a CD4 construct into 4G4 T lymphoma cells, which are defective in TCR and CD4 expression. The experimental data from the transfectants demonstrated insulin specificity of the reconstituted 2H6 TCR. The constructs were then coinjected into fertilized NOD oocytes directly (Yale Diabetes Endocrinology Research Center Transgenic Core, New Haven, Connecticut, USA), which circumvented the need for time-consuming backcrosses to NOD mice. Offspring were screened for integration of the transgenes by PCR using 2H6 V β /J β - and V α /J α -specific primers. Three transgenic founder mice were identified and used for breeding with NOD mice to establish the transgenic colonies. The use of the animals and the procedures applied in this study was in accordance with the current National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University.

Generation of 2H6 TCR transgenic NOD.scid mice. 2H6.scid mice were generated by breeding of 2H6 TCR NOD mice with NOD.scid mice. The scid mutation was confirmed by absence of B lymphocytes in the 2H6 TCR transgene-positive mice using flow cytometry.

Generation of TGF- β DNRII NOD, TGF- β DNRII BDC2.5, and TGF- β DNRII 2H6 mice. After backcrossing of TGF- β DNRII mice to the NOD genetic background for 7 generations, the TGF- β DNRII NOD mice were bred with BDC2.5 NOD and 2H6 NOD mice. All the transgenes were typed by PCR using genomic DNA isolated from mouse tail biopsies.

Antibodies and reagents. All the mAbs used in this study, unless indicated, were purchased from BD Biosciences – Pharmingen. All the hybridoma supernatants containing different mAbs, used for cell purification and blocking assays, were generously provided by the late Charlie Janeway (Yale University). Magnetic beads conjugated with goat anti-mouse IgG, goat anti-mouse IgM, or goat anti-rat IgG were purchased from QIAGEN. CY was purchased from Sigma-Aldrich. The insulin detection kit was purchased from Linco Research Inc.

Purification of CD4⁺ T cells. After removal of erythrocytes, total splenocytes were incubated with anti-CD8 mAb (supernatant of TIB-105, rat IgG2a) on ice for 30 minutes and washed once with cold Click's medium (Gibco). The cells were then incubated with magnetic beads conjugated with goat anti-mouse IgM/IgG and goat anti-rat IgG (BioMag; QIAGEN) on ice with gentle agitation for 45 minutes. B cells and CD8⁺ cells were removed with a magnetic plate. The purity of the CD4⁺ population using this method is routinely at least 90% by FACS analysis.

Proliferation and blocking assays. Splenocyte T cells (5×10^4 or 2×10^5 per well) were cultured in triplicate in the presence or absence of insulin as antigen for 2H6 cells, mimotope peptide (28) for BDC2.5 cells, or anti-CD3 (2C11) as a pan-T cell stimulator, for 3 days in 96-well plates at 37°C with 5% CO₂. Irradiated (30 Gy) splenocytes, as APCs, were also added to the culture when purified CD4⁺ T cells (5×10^4 per well) were used. A portion of supernatant was collected from each well before pulsing with ³H-thymidine, and the cultures were pulsed with 0.5 μ Ci of ³H-thymidine during the last 16–18 hours of incubation. Proliferation was measured by ³H-thymidine incorporation, after harvest, in a β -plate counter. Some proliferation assays were set up in the presence of a 0.4- μ m membrane Transwell (BD), and other proliferation assays were set up in the presence of blocking antibodies (mAb hybridoma supernatants): anti-CD4 (GK1.5), anti-CD8 (TIB-105), anti-I-A^b (10.2.16), anti-I-A^b (25-9-17), anti-TGF- β (purified; R&D Systems), and control rat IgG (purified; Pierce Biotechnology).

Cytokine analysis. The production of IL-2, IFN- γ , IL-10, and TGF- β was investigated in the culture supernatants obtained as described above. The IL-2 production was analyzed by cytotoxic T lymphoid line assay, and ELISA was used to measure the production of IFN- γ , IL-10, and TGF- β . Monoclonal antibody pairs (purified XMG1.2 and biotin-conjugated XMG1.2) for IFN- γ ELISA were purchased from BD Biosciences – Pharmingen. An OptEIA kit was used for IL-10 detection (BD Bioscience –



Pharming), and a TGF- β kit was used for the detection of activated TGF- β (R&D Systems). Recombinant cytokines (IL-2, IFN- γ , IL-10, and TGF- β) used to generate standard curves in the cytokine assays were either provided with the kits or purchased from Invitrogen Corp.

Natural history of diabetes development. Transgene-negative or -positive NOD mice were observed for diabetes development. The mice were screened for glycosuria weekly for up to 35–40 weeks of age. Diabetes was confirmed by blood glucose (>13.9 mmol/l).

Adoptive transfer experiments. NOD.scid mice (5–7 weeks) were used as recipients in adoptive transfer experiments. Diabetic splenocytes from various donors were injected i.v. (10^7 per mouse) alone or together with 2H6 transgenic cells. The mice were monitored for glycosuria biweekly after adoptive transfer, and the experiments were terminated 15 weeks after transfer unless the mice developed diabetes, which was confirmed by blood glucose (>13.9 mmol/l); these mice were sacrificed immediately. Wild-type NOD mice (4–5 weeks) were also used as recipients in some adoptive transfer experiments, in which the mice received 2H6 cells i.v. at weekly intervals for 5 weeks and were then monitored for diabetes development up to 35 weeks of age.

Histopathology and insulinitis score. Pancreata were fixed in buffered formalin. Paraffin-embedded tissues were sectioned and stained with H&E. Insulinitis was scored under a light microscope.

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