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14. ABSTRACT In this study we investigated the tolerance mechanisms of high and low avidity T cells reactive to the diabetes autoantigen glutamic acid decarboxylase 65 (GAD65) and their potential role in type 1 diabetes pathogenesis. In diabetes-associated DR4 HLA humanized mice, transgenically expressing GAD65-reactive human T cells, we found that high avidity, but not low avidity, T cells can migrate to the islets and mediate a loss in pancreatic islet function. Interestingly, low avidity GAD65 T cells generate the suppressive cytokine IL-10 and have a naïve phenotype in contrast with the phenotype of high avidity T cells, which make only IFNγ and are activated in the periphery. While both T cell receptors undergo deletional central tolerance, peripheral tolerance in high avidity T cells occurs through activation-induced cell death (AICD). In low avidity T cells it may occur through the generation of IL-10-producing Tr1 cells. These findings suggest that, within a single antigen-specific polyclonal T cell pool, both high avidity islet-infiltrating cells and potentially regulatory T cells may coexist and that antigen-specific T cells targeting the whole pool may have negative consequences.

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Autoimmunity, diabetes, T cells, tolerance

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Humanized in vivo Model for Autoimmune Diabetes
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Research Technical Report—Final

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the loss of insulin secretion from pancreatic islets. A strong HLA (both DR and DQ) association suggests that the adaptive T cell arm of the immune system plays a dominant role in disease pathogenesis. Putative T cell autoantigens involved in disease pathogenesis are correlative and definitive proof of specific target antigens directly involved in disease pathogenesis, along with particular phenotypic T cell types lacking. Through the course of this study, we have used humanized DR0401 (DR4) mice and demonstrated that:

- high avidity T cells reactive to glutamic acid decarboxylase 65 (GAD65) under specific conditions can migrate to pancreatic islets and mediate impaired islet function; and
- differences in T cell receptor (TcR) avidity (with the use of high avidity [164] and low avidity [4.13] TcR transgenic mice) to the self antigen GAD65 dictates the type and extent of central and peripheral tolerance mediated by the immune system.

BODY

Task 1. Evaluate diabetes progression in the DR4/TCR models

a. Cross the DR4/TcR transgenics onto the DR4/RIP-B7 transgenics for an accelerated disease course

We crossed DR0401/GAD 164β chain only, TCR mice (DR4/164β mice) onto DR4/RIP-B7 mice to generate DR4/RIP-B7/164β mice. A cohort of these mice was monitored for hyperglycemia over a 52-week period, a period in which approximately 25% of DR4/RIP-B7 mice become spontaneously diabetic (average age of onset ~40 weeks)[1]. None of the nice became hyperglycemic over the 52-week period (see 2009 annual report). In an alternative approach to ask whether GAD-responsive T cells could precipitate diabetes pathogenesis, we transferred 164αβ GAD-responsive splenocytes from DR4/164 αβ mice (containing both α and β chains of the human 164 T cell) or non-TcR splenocytes (wildtype) into 6-10-week-old non-lethally irradiated diabetes-prone DR4/RIP-B7 mice. As shown in Figure 1, none of these mice became hyperglycemic over the 18-week period of blood glucose monitoring. Histological examination of pancreatic tissue did not show evidence of an islet infiltrate.

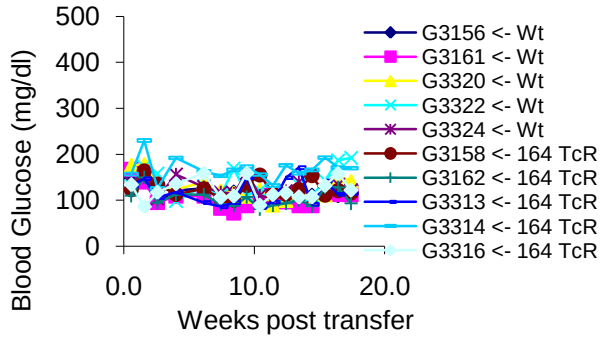

Figure 1. Blood glucose in DR0401/B7 mice that received either 164 αβ or wildtype splenocytes

b. Inject TLR agonists and/or anti-CD40 agonist for further disease acceleration

We treated low avidity DR4/4.13 αβ mice with a CD40L agonist (proprietary drug JFPH; personal communication with Dr. Steve Miller, Northwestern University), which is reported to accelerate experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis). Our reasoning was that, as T cells in DR4/4.13αβ mice have a naïve phenotype, activating them would lead to islet migration, which is seen spontaneously in DR4/164αβ mice, which have a T cell-activated phenotype[2] (Figure 4 from Reference 2 and Appendix Manuscript 1). DR4/4.13αβ mice were treated with CD40L agonist (JFPH), with and without GAD65 555-567 immunization, and blood glucose was monitored for 12 weeks after intraperitoneal injections of the drug (Figure 4 below). JFPH-treated mice, with or without GAD65 peptide immunization, did not exhibit blood glucose value different from control-treated animals. Histological examination of the pancreatic tissue also did not reveal any insulitis.

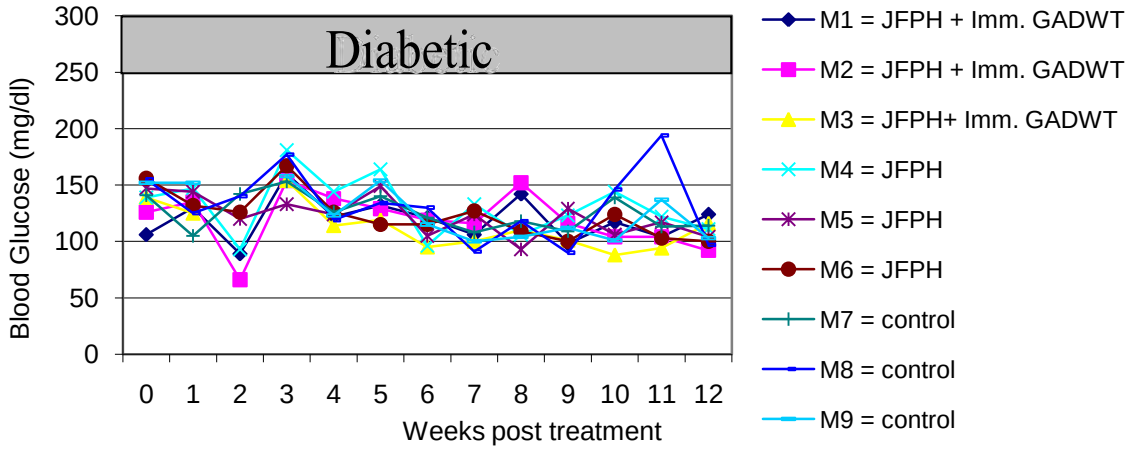

Figure 4. Treatment of DR4/4.13 mice with CD40L agonist JFPH. *(from Gebe et al., 2008)*

c. Inject low-dose streptozotocin to initiate beta cell damage, testing the impact of antigen release in situ

Low dose of streptozotocin (STZ, a beta cell toxin) can accelerate diabetes in NOD mice[3]. To initiate beta cell damage and release islet antigens, we treated wildtype DR4, high avidity DR4/164αβ TcR, and low avidity DR4/4.13αβ TcR mice with low dose STZ (3 x 40 mg/kg). A group of mice was treated with high dose STZ as positive controls for STZ's effectiveness at inducing beta cell damage (all mice became diabetic within one week). Low dose STZ-treated mice were monitored for blood glucose over 10 weeks for signs of hyperglycemia. As shown in Figure 2, none of the low dose STZ-treated animals became hyperglycemic, and, surprisingly, both high avidity GAD 164αβ and low avidity GAD 4.13 TcR transgenic mice demonstrated a statistically significant maintenance of lower blood glucose values compared with non-TcR transgenic (DR4) control mice (Figure 3).

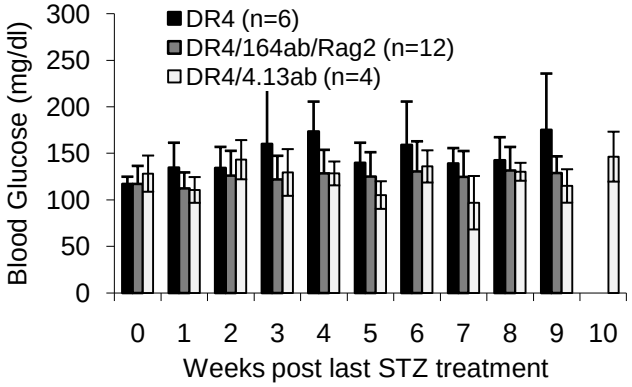

Figure 2. Blood glucose in DR4, DR4/164ab/Rag2, and DR4/4.13αβ mice treated with low dose streptozotocin.

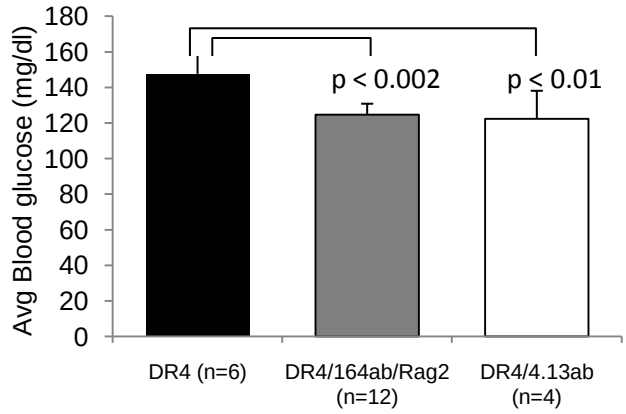

Figure 3. Difference in average blood glucose values between low dose STZ-treated DR4,

d. Transfer spleen and LN cells from DR4/TCR transgenic donors, activated in vitro, after labeling with CSFE, to track their in vivo course

The generation of IL-10 from a subset of 4.13αβ T cells upon GAD65 stimulation suggested that they may be Tr1-type cells and regulatory to T cell proliferation. It could explain why these cells did not have an activated phenotype in the periphery, in contrast to 164αβ cells. We transferred CFSE-labeled 4.13αβ and wildtype splenocytes into DR4/Rag2-/- mice and monitored cell expansion in an immune-deficient host.

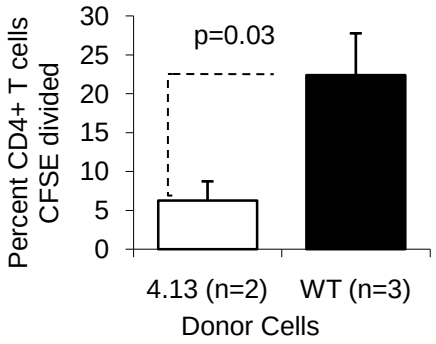

Figure 4. Day 4 cell division of CD4+ 4.13ab CFSE-labeled cells transferred into DR4/Rag2-/- mice

CFSE cell division was measured after 4 days. As shown in Figure 4, the transferred 4.13αβ cells divided to a lesser extent, compared with wildtype CD4+ T cells. This finding supported a regulatory role for self-antigen-reactive low avidity 4.13αβ T cells.

e. Deplete donor cells of the CD4+CD25+ regulatory population prior to transfer; alternatively, supplement donor cells with enriched CD4+CD25+ cells from avidity-selected TcR transgenic donors

Peripheral CD4+ T cells from DR4/164αβ mice exhibited a CD4+/CD25+ population, indicative of a regulatory T cell (Treg) population[4] (Figure 4b from Reference 4 and in Appendix Manuscript 2). To test whether regulatory T cells in DR4/164αβ TcR mice were preventing the potential of GAD65-responsive T cells in inducing diabetes, we depleted CD4+/CD25+ T cells from the spleens of DR4/164αβ TcR mice and transferred them into DR4/Rag2 mice. Mice were then monitored for blood glucose for signs of diabetes. As there was the potential for inducing colitis because of the absence of CD4+/CD25+ cells[5], we monitored the mice for weight loss (indicative of colitis) and blood hyperglycemia (marker for diabetes). Unfortunately, the mice lost 20% of their weight and had to be sacrificed before we saw any evidence of diabetes (hyperglycemia). Therefore, we could not determine if GAD-specific Tregs were preventing GAD TcR T cells from inducing diabetes. Pancreatic islet histology was normal at the time of colitis. In an alternate approach to address Tregs in these mice, we crossed DR4/164αβ mice onto FOXP3 promoter-driven GFP mice. This approach allowed us to sort CD4+/Foxp3+ (Treg) and CD4+/Foxp3- (T effector) cells and determine whether Tregs in DR4/164αβ mice harbor GAD Ag-specific Tregs. In an in vitro suppression assay, we found that, while CD3-stimulated Tregs in DR4/164αβ mice functionally inhibit a CD3-stimulated effector T cell response, we did not find evidence for a GAD65 peptide-specific Treg population as they did not inhibit a GAD65 555-567 effector T cell response (CD4+Foxp3-) cells (Figure 5).

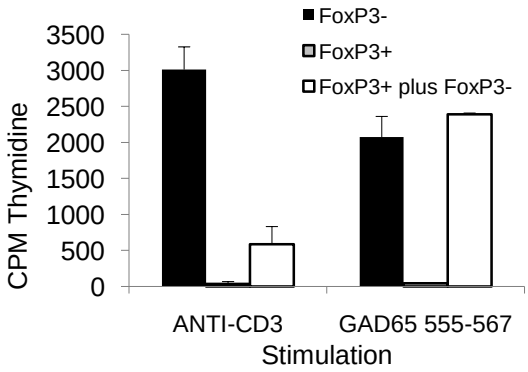

Figure 5. T cell suppression assay using CD4+ T cells from DR4/164ab/Foxp3-GFP mice sorting on CD4+/Foxp3+ and CD4+/Foxp3-

Furthermore, we found little evidence for CD4+/Foxp3+ cells in high avidity TcR DR4/164αβ nor low avidity TcR DR4/4.13αβ mice on a Rag2 background where only the transgenic TcR was expressed[4] (Figure 4C in Reference 4 and Appendix Manuscript 2).

f. In all cases, monitor immunohistochemistry of pancreatic islets, using staining for CD4, FOXP3, and insulin, as well as standard H&E. In all cases, monitor blood glucose and intraperitoneal glucose tolerance test at regular intervals

DR4/164αβ and DR4/4.13αβ mice on a Rag2-sufficient background do not develop spontaneous diabetes nor did they show evidence of an islet infiltrate (data not shown). As both mouse models express endogenous TcR Vα genes[4] (Figure 1 in Reference 4 and Appendix Manuscript 1), we cross these mice onto a Rag2-/- background to limit TcR expression to a single TcR. We observed that high avidity DR4/164αβ/Rag2-/- mice[2] (Figure 5 in Reference 2), but not low avidity DR4/4.13αβ/Rag2-/- mice, nor DR4/Rag2-/- mice (data not shown), exhibited a CD4+ islet infiltrate starting at about 20 weeks of age. Furthermore, the islet infiltrate correlated with a loss of insulin staining[2] (Figure 6 in Reference 2 and Appendix Manuscript 1), suggesting a loss in islet function, which was confirmed as an impaired response to an intraperitoneal glucose tolerance test[2] (Figure 7 in Reference 2 and Appendix Manuscript 1). These data suggest that a GAD65 555-567 specific T cell response can lead to impaired islet function and loss of detectable islet insulin. These mice on a relatively non-autoimmune prone C57Bl/6 background never became hyperglycemic, even when mice were monitored for 40 weeks (see Discussion in Manuscript 1).

Task 2. Evaluate tetramer profiles and disease progression in the TCR 164b model

Our previous work in humanized DR4 mice demonstrated that human tetramers can be used in a flow cytometric setting to identify antigen-specific T cell responses generated by immunization[6]. As both human T cells, 4.13 and 164, bound human DR4-GAD65 555-567 tetramers[7,8], we envisioned no problems with tetramer binding to 164 and 4.13 TcR, when expressed as transgenes in DR4 mice. However, while T cells from DR4/164αβ and DR4/4.13αβ mice are responsive to GAD65 555-567[4], they do not bind DR4 tetramers containing GAD65 555-567 (data not shown). We now speculate that this may be in part a result of the mismatch between mouse CD4 on the murine T cells and its binding to the all human beta 2 domain of the DR4 tetramer. In the mouse this is not an issue, as the MHC is a chimeric molecule consisting of human α1 and β1 and mouse α2 and β2 domains. Evidently, this mismatch does not prevent (all human sequence) tetramer binding to very high avidity T cells (foreign antigen responsive) but may for the anticipated, generally lower avidity spectrum of self-antigen responsive T cells. The issue is not one of biofunctionality of the all human tetramer with mouse T cells, as non-tetramer-binding GAD65 555-567-responsive hybridomas[6] generated in DR4 mice can be stimulated with all human sequence tetramers (data not shown). We were also unable to detect GAD65 555-567-specific tetramer-binding to T cells from GAD65 peptide-immunized

DR4/164β mice. The inability to use tetramers to characterize and follow 164αβ and 4.13αβ murine T cells led to alternative approaches in several of our proposed tasks.

a. *Measure insulitis and glycemia in the DR4/164β transgenics, while monitoring peripheral blood, LN, and spleen cells for DR4-GAD tetramer-binding profiles*

We monitored blood glucose in DR4/164β (TcR β chain only) mice (8 female and 4 male) weekly up to 52 weeks (see 2008 annual progress report). None of the mice displayed hyperglycemic events indicative of diabetes. Histological examination of pancreata was normal. We conclude that DR4/164β-only mice are not spontaneously diabetes prone over this period.

b. *Measure cytokines and TCR alpha utilization in the GAD-responsive T cells, sorting for tetramer+ and activation profiles; compare high tetramer binding cells with low avidity cells*

Our most recently published work with DR4/164αβ and DR4/4.13αβ mice[4] (Appendix Manuscript 2) compares and contrasts the differences in central and peripheral tolerance to these two autoreactive T cells, including thymic selection (Figure 1), peripheral expression of human TcR transgenes (Figure 2), antigen responsiveness, and cytokine profile (Figures 2C and 5).

c. *Monitor disease progression comparing tetramer binding with the same parameters as (f) above; perform this task in the presence of the disease accelerants selected for activity in Task 1*

As noted, this task was changed because of lack of tetramer binding, so we used cytokine profiling instead.

d. *Determine if CD4+CD25+ GAD-specific regulatory T cells are derived from the low or high avidity end of the tetramer-binding spectrum by flow sorting, using tetramers and FOXP3 markers*

As shown above for Task 1e, DR4/164αβ mice did not generate detectable levels of tetramer-binding GAD65-specific CD4+/CD25+ (Foxp3+) regulatory T cells.

Task 3. Optimize the translational potential of the tetramer and disease profiles

a. *Compare peripheral blood tetramer profiles and T cell phenotypes with simultaneous LN and spleen profiles, at different stages of disease progression*

There was no detectable peripheral blood tetramer profile found.

b. *Elute lymphocytes from infiltrated pancreatic islets and evaluate for tetramer binding and FOXP3 staining, at different stages of disease progression*

Because of lack of tetramer binding, we substituted TcR analysis.

c. *Create a standardized, multi-parameter analysis protocol combining tetramer staining with the most informative T cell markers (determined in the above tasks), which is suitable for analysis of peripheral blood-derived lymphocytes*

As noted, we substituted cytokine and TcR avidity analysis because of the lack of tetramer staining.

KEY RESEARCH ACCOMPLISHMENTS

- Two peer-reviewed published manuscripts
- High avidity GAD65 555-567-reactive T cells can escape from a strongly negatively selecting environment in the thymus and populate the periphery in humanized DR4 mice (DR4/164 mice). Peripherally, 164αβ T cells have a pro-inflammatory phenotype and are not regulatory-type cells

- The mechanism of tolerance to high avidity GAD65-reactive T cells in 164 mice is predominantly through deletion, skewing to a $CD8^+$ phenotype and down-modulation of the TcR. Peripheral tolerance is mediated by apoptosis via activation-induced cell death.

- High avidity GAD65 555-567-reactive T cells can mediate a loss in pancreatic beta cell function and is strong evidence that GAD65 is an autoantigen capable of mediating beta cell damage in humanized DR4 mice.

- Low avidity 4.13 T cells are less negatively selected in comparison with 164 T cells, and peripheral tolerance to the self-antigen GAD65 may be mediated by the differentiation of a portion of these T cells into a Tr1 (IL-10-secreting) regulatory pathway. This hypothesis is currently being tested by crossing 4.13 mice onto IL-10 knockout mice.

- The generation of potentially regulatory IL-10-secreting peripheral 4.13 T cells in 4.13 TcR transgenic mice is a post-thymic, peripheral, differentiating event, as mature CD4+/CD8- thymocytes do not secrete IL-10 upon autoantigenic stimulation.

- In contrast to 164 mice on a Rag2-/- background, 4.13 mice on a Rag2-/- background do not exhibit islet infiltrates.

REPORTABLE OUTCOMES—MANUSCRIPT

- **Oral Presentation**
  2007 FASEB Summer Conference on Autoimmunity, July 14-19, 2007, Vermont Academy, Saxtons River, VT, Title: Two Humanized HLA-DR4 GAD65 TCR Transgenic Mouse Lines with Similar T Cell Receptors Model Different Autoimmune Tolerance Mechanisms

- **Published manuscripts**

1. Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice. *J Autoimmun* 2008; 30:197-206. PMCID: PMC2440666.

2. Gebe JA, Yue BB, Unrath KA, Falk BA, Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol* 2009; 183:59-65. PMC2811410.

- **Funding applied for based on this work**
  Unfunded submission of National Institutes of Health R21 application, October 2009

CONCLUSION

Using two MHC-humanized DR4 mice expressing GAD65 555-567-autoreactive CD4 T cell-derived TcR cloned from a human diabetic[7] and diabetes-at-risk individuals[8], we addressed the potential contribution of GAD65 T cell reactivity to type 1 diabetes pathogenesis and the tolerance mechanism of self-reactive T cells as a function of TcR-MHC avidity. TcR transgenic mice were generated on a relatively non-autoimmune prone C57Bl/6 background to: 1. generate an alternative to the NOD mouse model of diabetes; and 2. generate a humanized model of type 1 diabetes. We observed that these two TcR, with identical Vα and Vβ germ line sequences that only differ in their junctional CDR3 regions, exhibit distinct methods of self-antigen T cell tolerance. Self-antigen T cell tolerance in the high avidity 164 TcR mice encompasses a strong central tolerance through negative selection in the thymus with a CD4-to-CD8 skewing of single, positive transgenic T cells. Presumably, the skewing to CD8+/CD4- lowers the avidity of the class II interaction by eliminating the CD4-MHC β2 domain interaction and thus enables escape of high avidity T cells.

Interestingly, CD8+/CD4- T cells in the periphery are GAD65 antigen-responsive, albeit at a lower functional avidity compared with CD4+/CD8- 164 cells. Low avidity 4.13 thymocytes are less negatively selected against in the thymus and do not exhibit a skewing toward a CD4-/CD8+ phenotype. Peripheral tolerance in 164αβ mice is also mediated by an AICD mechanism, which correlates with activated caspase 3 expression. Unlike the activated phenotype of peripheral 164αβ T cells, peripheral 4.13αβ T cells have a naïve phenotype. Upon in vitro activation 164αβ T cells generate IFNγ and little or no IL-2, IL-4, IL-5, IL-10, or TNFα, whereas activated 4.13αβ T cells generate IFNγ and IL-10 and little or no IL-2, IL-4, IL-5, or TNFα. Intracellular cytokine staining of activated 4.13αβ T cells demonstrates the existence of both IL-10+/IFNγ- or IL-10-/IFNγ+ cells. The generation of a population of IL-10-producing cells, an immune suppressive cytokine[9], suggests that these may be keeping 4.13αβ T cells from activation, which is in stark contrast to the activated T cells observed in 164αβ mice. Crosses onto IL-10 knockout mice and mixed bone marrow chimeras between 164αβ and 4.13αβ mice could determine whether the Tr1-

like 4.13αβ T cells are regulatory. Our preliminary data (2009 progress report) suggest that the absence of IL-10 in 4.13αβ mice is regulatory to T cell activation.

In investigating the role of GAD65-responsive T cells in the pathogenesis of diabetes, we found that high avidity 164αβ, but not low avidity 4.13αβ, T cells are capable of infiltrating the islets. Whereas a loss in islet function was confirmed (a loss in immunofluorescent insulin staining and an impaired glucose tolerance test), these mice did not succumb to hyperglycemic diabetes. We think it is, in part, due to the relatively non-autoimmune susceptible C57Bl/6 background. In addition, the islet infiltrate only appeared when crossed onto a Rag2-/- background (^{2}) or TcR Ca-/- mice (data not shown). Low TcR avidity DR4/4.13αβ/Rag2-/- mice did not show signs of islet infiltration. Our finding of islet insulitis in DR4/164αβ/Rag2-/- mice does support a role of T cell response to GAD65 in the pathogenesis of diabetes, but as a single antigen response it is not sufficient to induce diabetes in B6 background mice. The reason that insulitis was not observed in DR4/4.13αβ/Rag2-/- mice may be due to the lower avidity of the TcR or possible in vivo production of IL-10, which was observed upon ex vivo activation. IL-10 is known to have immunoregulatory activities[10]. We did find in our preliminary data on DR4/4.13αβ mice on an IL-10 knockout background (2009 annual report) that 4.13αβ T cells are more activated.

The findings in this study could have implications for antigen-specific therapies aimed at targeting T cells as well as immune monitoring. Our study suggests that subpopulations within an autoreactive T pool may contain both undesired effector (those capable of islet infiltration) and desired regulatory phenotypes, so that selective targeting based on phenotypic properties as described may be necessary.

Existing DOD research relating to diabetes emphasizes technologies that improve disease management, such as continuous glucose monitoring, non-invasive testing, insulin pumps, and better means to monitor and treat complications. Our focus in this project was to analyze the underlying causes of T1D, namely, the autoimmune cellular response against pancreatic islet antigens, to help develop potential biomarkers in order to improve and speed up the outcomes from therapies directed at the immune system—increasingly the target of newer clinical trials for this disease. Suitable biomarkers that detect and interpret the T cell autoimmune function are sorely needed. This project advances the field by classifying the T cell biomarkers based on avidity properties, which are associated with in vivo functional differences. Because this work was done in a humanized mouse model, the principal observations—cytokine differences, peripheral tolerance deletion and regulation mechanisms, non-progressive insulitis—should be transferrable to the clinical trial context. Such clinical follow-on studies are currently in the planning stages.

REFERENCES

1. Gebe JA, Unrath KA, Falk BA, Ito K, Wen L, Daniels LT, Lernmark A, Nepom GT. Age-dependent loss of tolerance to an immunodominant epitope of glutamic acid decarboxylase in diabetic-prone RIP-B7/DR4 mice. *Clin Immunol* 121:294-304, 2006.

2. Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice. *J Autoimmun* 30:197-206, 2008.

3. Gerling IC, Friedman H, Greiner DL, Shultz LD, Leiter EH. Multiple low-dose streptozocin-induced diabetes in NOD-scid/scid mice in the absence of functional lymphocytes. *Diabetes* 43:433-40, 1994.

4. Gebe JA, Yue BB, Unrath KA, Falk BA, Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol* 183:59-65, 2009.

5. Foussat A, Cottrez F, Brun V, Fournier N, Breittmayer JP, Groux H. A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation. *J Immunol* 171:5018-26, 2003.

6. Gebe JA, Falk BA, Rock KA, Kochik SA, Heninger AK, Reijonen H, Kwok WW, Nepom GT. Low-avidity recognition by CD4+ T cells directed to self-antigens. *Eur J Immunol* 33:1409-17, 2003.

7. Reijonen H, Novak EJ, Kochik S, Heninger A, Liu A, Kwok W, Njepom G. Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects. *Diabetes* 51:1375-82, 2002.

8. Reijonen H, Mallone R, Heninger AK, Laughlin EM, Kochik SA, Falk B, Kwok WW, Greenbaum C, Nepom GT. GAD65-specific CD4+ T-cells with high antigen avidity are prevalent in peripheral blood of patients with type 1 diabetes. *Diabetes* 53:1987-94, 2004.

9. Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* 161:3299-306, 1998.

10. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, Kronenberg M. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 10:1178-84, 2009.

APPENDICES

- Manuscript 1: Gebe JA, Unrath KA, Yue BB, Miyake T, Falk BA, Nepom GT. Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice. J Autoimmun 30:197-206, 2008.

- Manuscript 2: Gebe JA, Yue BB, Unrath KA, Falk BA, Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. J Immunol 183:59-65, 2009.

- Supplementary Figures S1, S2, and S3

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Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice

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Abstract

A human T-cell receptor (TcR) derived from an autoreactive T-cell specific for GAD65, from a subject at high risk for autoimmune diabetes, was introduced into HLA-DR4 transgenic mice. The source of TcR was a $CD4^+$ T_H1^+ T-cell clone which responded to an immunodominant epitope of the human islet protein GAD65, an epitope shared with both GAD65 and GAD67 in the mouse. The resulting HLA-DR4/GAD-TcR transgenic mice on a $Rag2^{o/o}$/$I\text{-}Ab^{o/o}$/B6 background exhibited a $CD4^+$ infiltrate into pancreatic islets that correlated with a loss of insulin in infiltrated islets. These mice also exhibited a subclinical impaired tolerance to exogenously fed glucose as assayed by an intraperitoneal glucose tolerance test. T cells containing the GAD65/67 (555–567) responsive TcR undergo strong negative selection as evidenced by a 10-fold lower thymocyte cellularity compared to non-TcR transgenic mice, and clonotype peripheral T cells represented approximately 1% of $CD4^+$ T cells in Rag2 sufficient mice. Upon *in vitro* stimulation, GAD65/67 555–567 responsive T cells secrete interferon-γ, minimal interleukin (IL)-2 and tumor necrosis factor-α, and no IL-4, IL-5, IL-10, or IL-17, consistent with a T_H1 profile. These data demonstrate that $CD4^+$ T cells specific for a naturally processed epitope within GAD can specifically home to pancreatic islets and lead to impaired islet β-cell function in diabetes-associated HLA-DR4 transgenic mice on the relatively non-autoimmune C57BL/6 background. The relatively slow progression and patchy insulitis are reminiscent of the chronic pre-clinical phase similar to a majority of human at-risk subjects, and models these indolent features of human T1D.

Keywords: Insulitis; Human T-cell receptor; HLA transgenic mouse; GAD

1. Introduction

The human HLA-DQB1*0302 and -DRB1*04 gene products are strongly associated with autoimmune diabetes, and are also powerful susceptibility genes predisposing to diabetes when expressed as transgenes in the absence of endogenous class II ($I\text{-}Ab^{o/o}$) in the relatively non-autoimmune prone C57BL/6 mouse [1–3]. We recently described an age-dependent spontaneous loss of tolerance to an epitope within a naturally processed region of the diabetes autoantigen GAD65 (GAD65 555–567) in the presence of the autoimmune accelerator RIP-B7 in these diabetes prone DR4 transgenic mice. The loss of tolerance to GAD65 555–567 precedes overt hyperglycemia and is associated with a loss in glucose tolerance evidenced by an intraperitoneal glucose tolerance test [2,4].

Previous studies using immunization with putative human autoantigens (primarily GAD and insulin) in HLA transgenic mice have been used to identify, correlate, and confirm human T-cell reactive antigenic epitopes that may be targets for autoreactive T cells [5–8]. The spontaneous islet autoimmunity in the B7/DR4 C57BL/6 mouse, however, offers the opportunity

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to explore mechanisms of T-cell selection and autoreactivity in an unimmunized context. Glutamic acid decarboxylase exists in GAD65 and GAD67 isoforms, with GAD65 the predominant expressed form in human islets and GAD67 in murine islets [9]. It is important to note that GAD65 (555–567) is an ideal epitope for translational studies, as this epitope sequence is identical in all forms of mouse and human GAD (65 and 67). While cellular and humoral reactivity to glutamic acid decarboxylase 65 (GAD65) is readily detected in human T1D and diabetes-at-risk subjects [10–12], its direct role in the pathogenesis leading to islet insulin-producing β-cell destruction in the human disease is still uncertain. Antibodies to GAD65 are one of three serum antibody markers used in determining susceptibility to T1D in genetically predisposed individuals and imply a temporal relationship between immune reactivity to GAD65 and progression to human diabetes [13,14].

The common murine model for T1D, the NOD mouse, only partially recapitulates this pattern. GAD-mediated tolerogenic protection of diabetes in NOD mice can be afforded by intrathymic injection of GAD protein [15–17], inoculation with GAD65 encoding vaccinia virus [18], rat insulin promoter driven GAD65 [19], and antisense expression of GAD [20]. On the other hand MHC class I promoter-driven expression of GAD65 was shown to exacerbate disease [21]. In T-cell directed studies, a GAD65 responsive cell line has been shown to induce diabetes in NOD.*scid* mice [22] and recent evidence indicates that GAD epitopes are capable of stimulating diabetes-inducing BDC2.5 T cells and cause diabetes in transfer studies [23,24]. However, a protective role of cellular reactivity to GAD65 was shown to delay diabetes when interferon (IFN)-γ and interleukin (IL)-10 producing GAD65 responsive T cells were transferred in NOD mice from either T-cell transgenic mice [25,26], a T-cell clone [27] or a T-cell line [28]. Unaltered diabetes progression in NOD mice has also been observed in retrogenic expression of other I-Ag7-restricted GAD T-cell receptors [29].

In order to assess the characteristics of anti-islet T-cell specificities which are prevalent in human T1D we have transgenically expressed a GAD65 (555–567) responsive human T-cell receptor (TcR), derived from a diabetes at-risk individual, in DR4 transgenic mice on the C57BL/6 background. We report here that T cells in DR4 mice expressing TcR transgenes specific for GAD65 are strongly negatively selected in the thymus and are limited in numbers in the periphery organs. The percentage of $FoxP3^+$ cells among $CD4^+$ T cells in peripheral organs is about 3-fold greater than non-TcR transgenic mice. TcR transgenic mice were normoglycemic to 40 weeks of age, possibly related to the increase in selected regulatory components. Notably, mice transgenic for this TcR on a Rag2 deficient background are also normal for blood glucose but do exhibit insulitis at around 25 weeks of age that is correlated with a loss in glucose tolerance in an intraperitoneal glucose tolerance test and a loss in immunoreactive insulin in infiltrated islets. Thus, an anti-GAD T-cell specificity associated with human T1D is sufficient to elicit insulitis and impair glucose tolerance in a HLA-transgenic murine model, even in the context of the relatively autoimmune disease-resistant C57BL/6 background. In contrast to the autoimmune-prone NOD model, disease is indolent, insulitis is patchy, and lack of progression to overt diabetes is associated with evidence of T-cell regulation, features which may correspond to a large segment of the human T1D and at-risk population.

2. Research design and methods

2.1. Mice

DR0404-IE mice (DR4) were generated as previously described [2]. These C57BL/6 I-$Ab^{o/o}$ mice express a human-mouse chimeric class II molecule in which the TcR interacting and peptide binding domains of mouse I-E (domains α1 and β1, exon 2 in both genes) have been replaced with the α1 and β1 domains from DRA1*0101 and DRB1*0404 respectively. Retention of the murine α2 and β2 domains allows for the cognate murine CD4-murine MHC interaction [30].

The GAD65 (555–567) responsive human $CD4^+$Vα12.1/Vβ5.1 T-cell clone 164 was cloned from an HLA DR4 diabetes at-risk individual as previously described [12]. Human-mouse chimeric TcR transgenes were constructed by subcloning PCR amplified regions encoding rearranged VαJα and VβDβJβ domains from the human clone derived TcR sequences into pTαcass and pTβcass TcR transgenic vectors, respectively [31]. TcR transgenic vectors pTαcass and pTβcass contain the natural mouse TcR α and β promoter/enhancer elements and mouse Cα and Cβ constant region, respectively. DNA injection into C57BL/6/I-$Ab^{o/o}$ mouse embryos was performed at the University of Washington (Seattle, WA) in the Comparative Medicine animal facility. Founder mice containing the GAD65 TcR transgene were then crossed onto DR0404-IE mice to generate DR4/164 mice. Additional crosses were made onto Rag2 KO mice.

Blood glucose was performed via saphenous veins bleeds using a One-Touch FastTake glucometer (LifeScan, Milpitas, CA). All animal work was approved by the Benaroya Research Institute (BRI) Animal Care and Use Committee (ACUC) and animals were housed in the BRI AAALAC-accredited animal facility. For intraperitoneal glucose tolerance tests (IPGTT) mice were fasted (given water only) for 6 h. At the end of 6 h mice were injected intraperitoneal with 1.0 mg/ml D-glucose (stock solution in PBS) at a dose of 1 g/kg body weight. Saphenous blood glucose readings were taken at 0, 15, 30, and 60 min time points post injection.

2.2. Tissue processing and flow cytometry

Thymus, spleen, and lymph node tissues were processed into single cell suspensions by gently pressing through 0.40 μm cell strainers (BD-Falcon REF 352340, Bedford, MA) using the rubber end of a 1 ml tuberculin syringe in DMEM-10 media (DMEM cat. #11965-092; Gibco, Rockville MD) supplemented with 10% FBS (Hyclone, Logan, UT), 100 μg/ml penicillin, 100 U/ml streptomycin, 5 μM βme, 2 mM glutamine and 1 mM sodium pyruvate (Gibco, Rockville

MD). Cell suspensions were centrifuged at 200 × *g* for 10 min and resuspended in DMEM-10 media. Splenic RBC were lysed using ACK lysis buffer [32] for 5 min at 37 °C at which time ~25 ml of media was added and cells spun down (200 × *g*). The following chromophore-labeled antibodies were used in flow cytometric analysis: anti-mouse CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD62L (Mel-14), CD44 (IM7, BD-Pharmingen, San Jose, CA), anti-human Vβ5.1-PE (clone IMMU 157 Immunotech-Coulter, Miami, FL) and Vα12.1-FITC (clone 6D6; Endogen Woburn, MA). FACS samples were stained in media on ice for 45 min, washed once, and resuspended in FACS stain buffer (PBS containing 1% FBS, 0.1% Na-azide) before being run on a FACSCaliber or LSR II flow cytometer (Becton Dickinson). Internal staining of cells for FoxP3 was performed using eBioscience kit (FJK.16a Ab, San Diego, CA) according to the manufacturer's instructions.

Pancreatic tissues were in either: (1) fixed in phosphate-buffered formalin prior to paraffin embedding for H&E staining or insulin staining or (2) frozen in Tissue-Tek OCT embedding media (Sakura Finetek, Torrance, CA) for immunofluorescence. For immunofluorescence staining of frozen tissues, 6 μM tissue slices were fixed for 10 min in 4 °C acetone and either air dried and stored at −20 °C or stained directly. Frozen tissues section were blocked, stained, and washed in PBS containing 0.1%NaN_3/1%FBS/2% horse serum. The following antibodies were used at 1:100 dilution: CD4-Alexa-fluor 488 (MCD0420), CD8-Alexa-fluor 488 (MCD0820), control Alexa-fluor 488 (R2a20, Caltag, Burlingame, CA). Islet insulin was detected with primary guinea-pig polyclonal anti-insulin (1:100 dilution, Abcam Ab7842-500, Cambridge, MA) and a secondary goat anti-guinea-pig Alexa-fluor 568 (1:100 dilution, Molecular Probes, Eugene, OR). Immunofluorescence was detected on a Leica DM IRB microscope. For islet infiltrate scoring, at least 8 islets were viewed for each mouse and H&E stained islets we scored as follows: 0, no infiltrate; 1, less than 33% infiltrated; 2, less than 66% infiltrated; and 3, greater than 66% infiltrated.

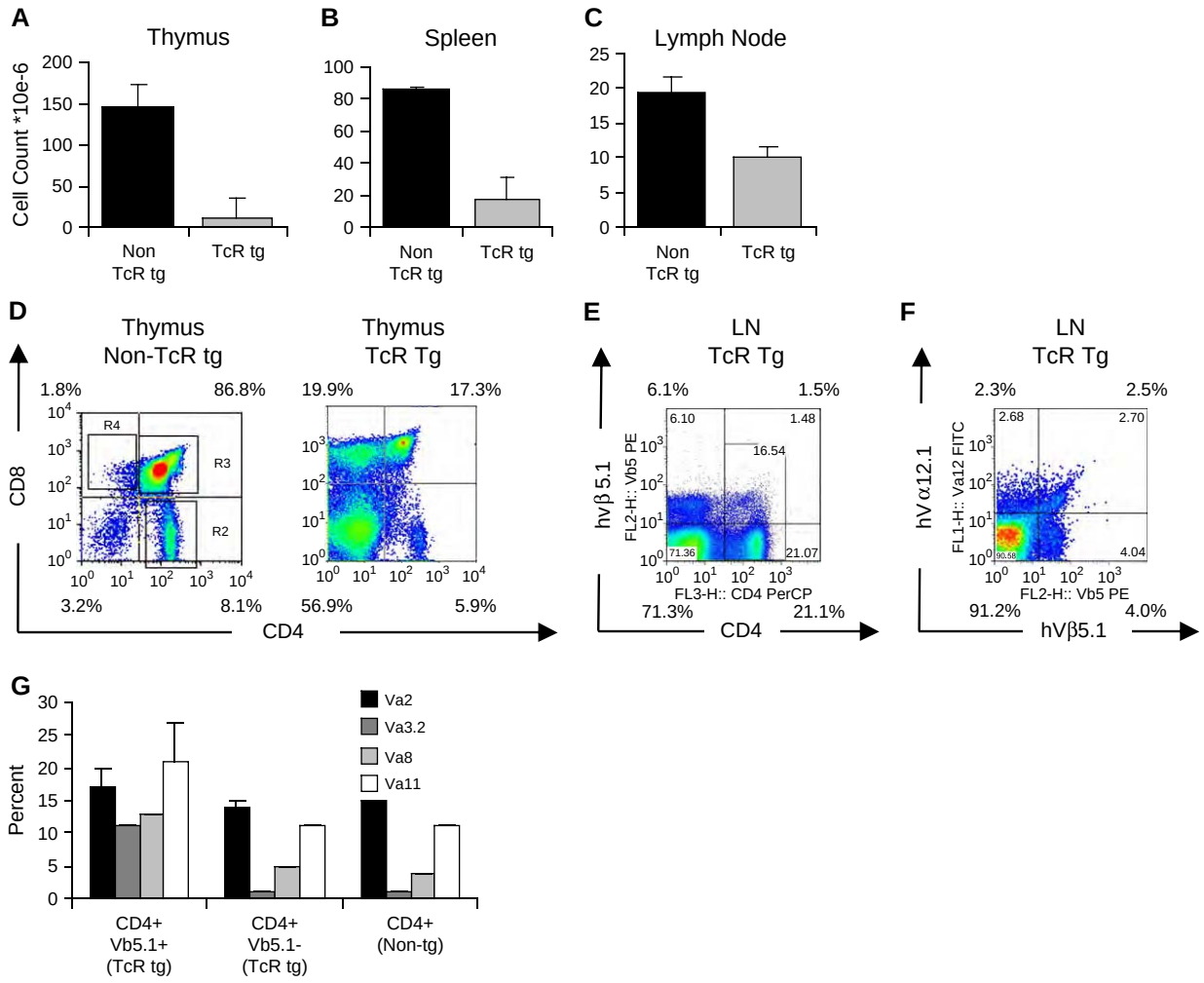

Fig. 1. Tissue cellularity and GAD TcR expression in DR4/164 GAD65 555–567 specific TcR transgenic mice. Cellularity for Thymus (A), Spleen (B), and Lymph nodes (C) represent the average counts ($n = 3$ mice each) for Non TcR transgenic (Non TcR tg) and TcR transgenic mice (TcR tg) at 8 weeks of age. CD4 vs. CD8 profiles in thymus of DR4 and DR4/164 GAD TcR mice (D). Expression of human TcR Vβ5.1 transgene on $CD4^+$ lymph node cells from the DR4/164 mouse is shown (E) along with clonotypic expression of the Vα12.1/Vβ5.1 TcR on CD4-gated lymph node cells (F). $CD4^+$ human $V\beta5.1^+$ transgenic T cells express an increase in the use of endogenous mouse TcR Vα compared to $V\beta5.1^-$ T cells (G).

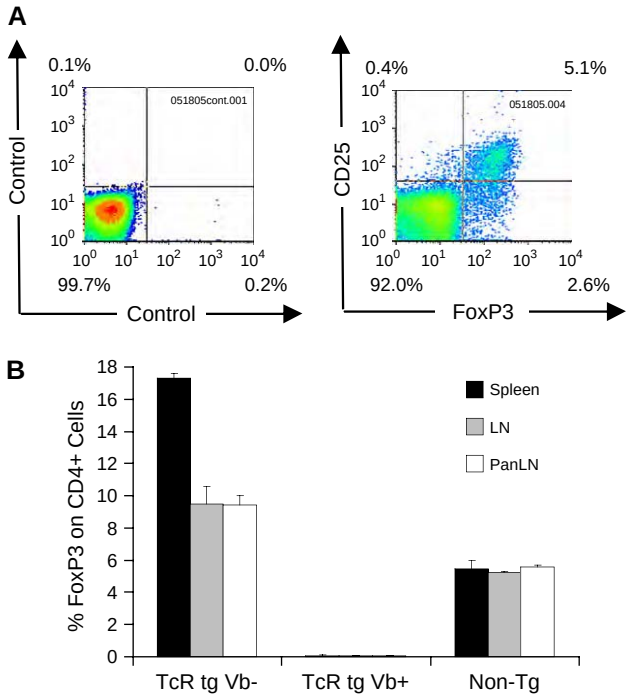

Fig. 2. Intracellular FoxP3 expression on $CD4^+$ T cells in GAD65 555–567 TcR transgenic and non-transgenic DR4 mice. FoxP3 expression in lymph node cells from DR4/164 TcR transgenic mice showing a correlation of FoxP3 expression with CD25 expression on $CD4^+$ gated cells (A). Average percentages from 3 mice (8 weeks of age) of $CD4^+$ $FoxP3^+$ expressing cells of all $CD4^+$ cells (B). Percentages are shown for $CD4^+/V\beta5.1^+/FoxP3^+$ (Tg $V\beta^+$) and $CD4^+/V\beta5.1^-/FoxP3^+$ (Tg$V\beta^-$) for DR4/164 GAD-TcR mice and for non-TcR transgenic mice (Non-tg).

2.3. Proliferation assays

Single cell suspensions of lymph node cells (LNC) from inguinal, mesenteric and brachial lymph nodes and spleen cells were prepared by gently pressing through 0.4 μm nylon cell strainers (BD-Falcon REF. 352340, Bedford, MA) in Hanks buffer (Gibco, Rockville, MD) and spun down (1000 rpm, 200 × *g*). Splenic RBC were lysed using ACK lysis buffer [32] for 5 min at 37 °C at which time ~25 ml of media was added and cells spun down (200 × *g*). Splenocytes were resuspended in DMEM-10 (DMEM cat. #11965-092; Gibco) supplemented with 10% FBS (Hyclone), 10 μg/ml penicillin, 100 U/ml streptomycin, 50 μM βme, 2 mM glutamine and 1 mM sodium pyruvate (Gibco). In lymph node proliferation assays 1e5 lymph node cells were cultured with 2e5 3000 Rad Cs-g irradiated splenocytes. Supernatants for cytokine analysis were taken (50 μl) at 48 h and μCi/well of [^{3}H]thymidine was added at 72 h. Thymidine incorporation was assayed at 96 h using a liquid scintillation counter analyzed on a scintillation counter (Wallac–Perkin/Elmer Life Sciences, Boston, MA) at 96 h. Splenocyte responses were measured in the same manner using 5×10^5 splenocytes per well.

2.4. Cytokine analysis

Cytokines IL-2, IL-4, IL-5, tumor necrosis factor (TNF)-α, and IFN-γ were assayed using a Mouse Th1/Th2 Cytokine CBA kit (BD Bioscience, San Diego, CA, cat. #551287). IL-10 was assayed using a BD OptEIA mouse IL-10 Elisa Set (BD Bioscience, cat. #555252) and IL-17A was assayed using an IL-17A ELISA kit (eBioscience, San Diego, CA, cat. #88-7147-22). Supernatants from triplicate proliferation wells (50 μl/well) were combined for cytokine analysis with 50 μl used for CBA analysis and 50 μl each for IL-10 and IL-17A ELISA.

3. Results

GAD65 (555–567) is a minimal stimulating epitope within a naturally processed immunodominant epitope (GAD65 552–572) within the diabetes autoantigen GAD65 [7,33]. In studying T1D in human diabetes-correlated HLA transgenic mice, the MHC DR4-binding GAD65 (555–567) epitope is an autologous antigen as mouse and human sequences in GAD65 and GAD67 are all identical [34]. A human $CD4^+V\alpha12.1/V\beta5.1$ TcR (Arden nomenclature [35]) T-cell clone (164) responsive to GAD65 (555–567) was derived from PBMC of an autoantibody-positive diabetes at-risk individual by in vitro stimulation with a GAD65 (555–567) superagonist APL peptide and single cell sorted from a $CD4^{Hi}/CD25^+$ activated population [12]. 164 TcR transgenic mice were generated using

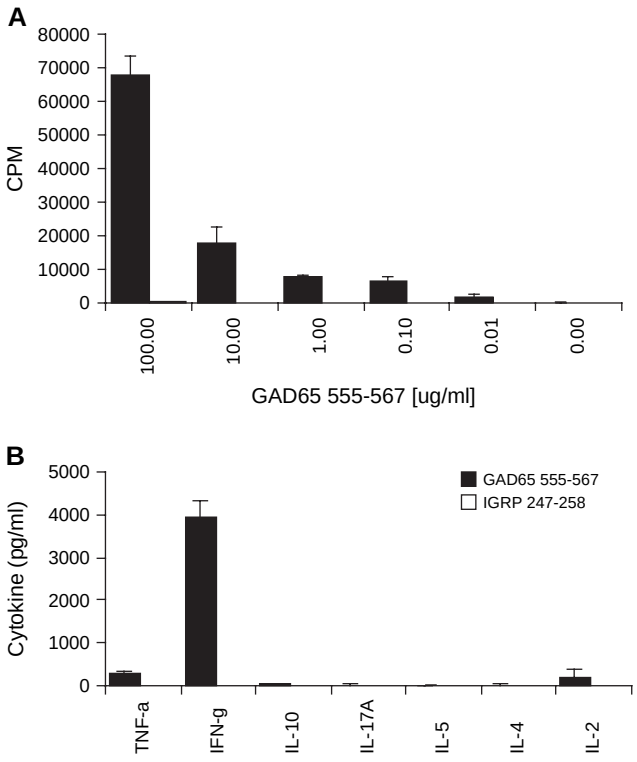

Fig. 3. Antigen-specific dose–response and cytokine analysis of in vitro stimulated 164 GAD TcR T cells. Splenocytes from Dw14/164/Rag2$^{o/o}$ mice (2e5) were stimulated with increasing amount of GAD65 555–567 or control IGRP 247-258 peptide for 96 h. [^{3}H]Thymidine was added at 72 h and incorporation was assayed by scintillation counting (A). Cytokines (pg/ml) from supernatants from proliferation assays (at 100 μg/ml antigen) taken at 72 h (B). The experiment was repeated 3 times with similar results.

murine TcR cassettes pTαcass and pTβcass [31] in which the variable regions of the mouse TcR were substituted with the human sequences from the 164 human clone TcR. Purified DNA was microinjected directly into C57BL/6 embryos. TcR positive founder mice were crossed onto (I-Ab$^{o/o}$) C57BL/6 DR4 HLA transgenic mice.

3.1. Thymic and peripheral cellularity in GAD65 555–567 responsive TcR transgenic mice

Thymocyte cellularity in DR4/164 TcR transgenic mice is severely reduced compared with non-TcR mice, indicating a threshold in negative selection has been crossed in selection of the 164 TcR. (Fig. 1A). In wild type DR4 mice the CD4:CD8 single positive ratio in the thymus is approximately 4:1, the CD4:CD8 ratio in the TcR negative selecting DR4/164 mouse is 1:3.4 and the percentage of CD8 single positive cells in the thymus is ~20% or nearly 10-fold above that seen in wild type DR4 mice (Fig. 1D). A similar type of CD8 skewing has been observed on other self antigen specific TcR transgenic mice [36–38]. The presence of the GAD65 (555–567) specific TcR transgenes in DR4 mice results in an increase in the thymic CD4$^-$/CD8$^-$ population from less than 5% in wild-type DR4 mice to nearly 60% in DR4/164 mice (Fig. 1D).

A result of the extensive negative selection in the thymus of DR4/164 mice is reflected in the peripheral organs where both splenic and lymph node cellularity are well below non-TcR DR4 mouse levels (Fig. 1B and C). In the peripheral lymph nodes the expression of the Vβ5.1 transgene is found on about 6% of CD4$^+$ T cells (Fig. 1E) and these CD4$^+$/Vβ5.1$^+$ T cells display an increase usage of endogenous Vα TcR compared to CD4$^+$/Vβ5.1$^-$ cells (Fig. 1G). The percentage of peripheral CD4$^+$ T cells expressing the clonotypic Vα12.1/Vβ5.1 transgene is around 2% (Fig. 1F).

3.2. 164 TcR transgenic mice have an increased percentage of FoxP3 positive T regulatory cells

Several other models using TcR transgenic mice have shown an increase in the percentage of FoxP3 expressing cells in the population of T cells responding to antigens which are introduced by transgenesis, and are therefore surrogates for self antigens [39,40]. Consistent with these observations, the percentage of FoxP3 positive cells in the CD4$^+$CD25$^+$ subset of DR4/164 mice is greater in DR4/164 mice compared to non-TcR transgenic DR4 mice (Fig. 2B). However, while an increase in the percentage of CD4$^+$/FoxP3$^+$ cells among the CD4$^+$ subset is observed in inguinal and pancreatic lymph nodes and also in spleen, the increase is only observed in the non-TcR transgenic Vβ5.1$^-$ population and not the Vb5.1$^+$/CD4$^+$ cells.

3.3. 164 TcR T cells are GAD65 responsive and exhibit a T_H1 phenotype

Proliferation and cytokine production of T cells from DR4/164/Rag2$^{o/o}$ mice in response to GAD65 (555–567) and a control peptide derived from diabetes autoantigen islet-specific glucose-6-phosphatase subunit related protein (IGRP 247–258) are shown in Fig. 3. Antigen specific response to GAD65 (555–567) is seen as low as 0.01 μg/ml (6.8 nM, lowest concentration tested) but not to control DR4-binding IGRP (247–258) peptide. A cytokine analysis in response to GAD65 (555–567) stimulation showed that these cells secrete IFN-γ with minimal TNF-α and IL-2 and no IL10, IL-17, IL-4, or IL-5, and is indicative of a T_H1 type cell cytokine profile.

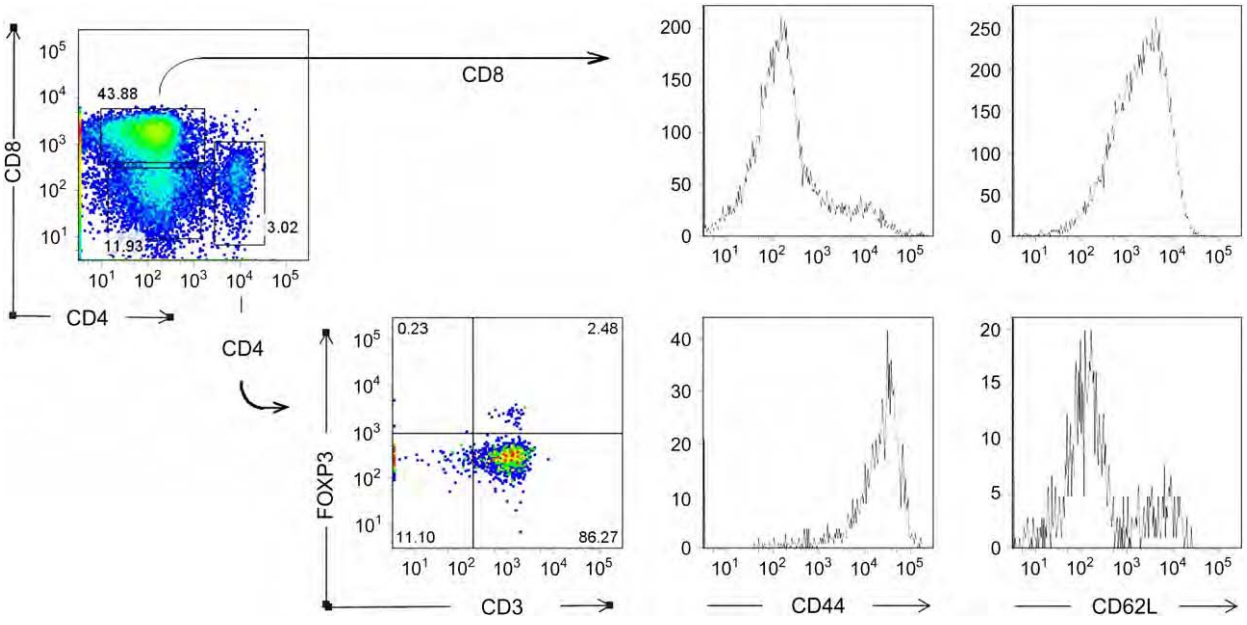

Fig. 4. TcR transgenic CD4$^+$ cells in DR4/164/Rag2$^{o/o}$ mice exhibit an activated phenotype. CD4$^+$ lymph node T cells, but not CD8$^+$ cells from DR4/164/Rag2$^{o/o}$ mice (10 weeks of age) are CD44^{Hi} and CD62L$^-$.

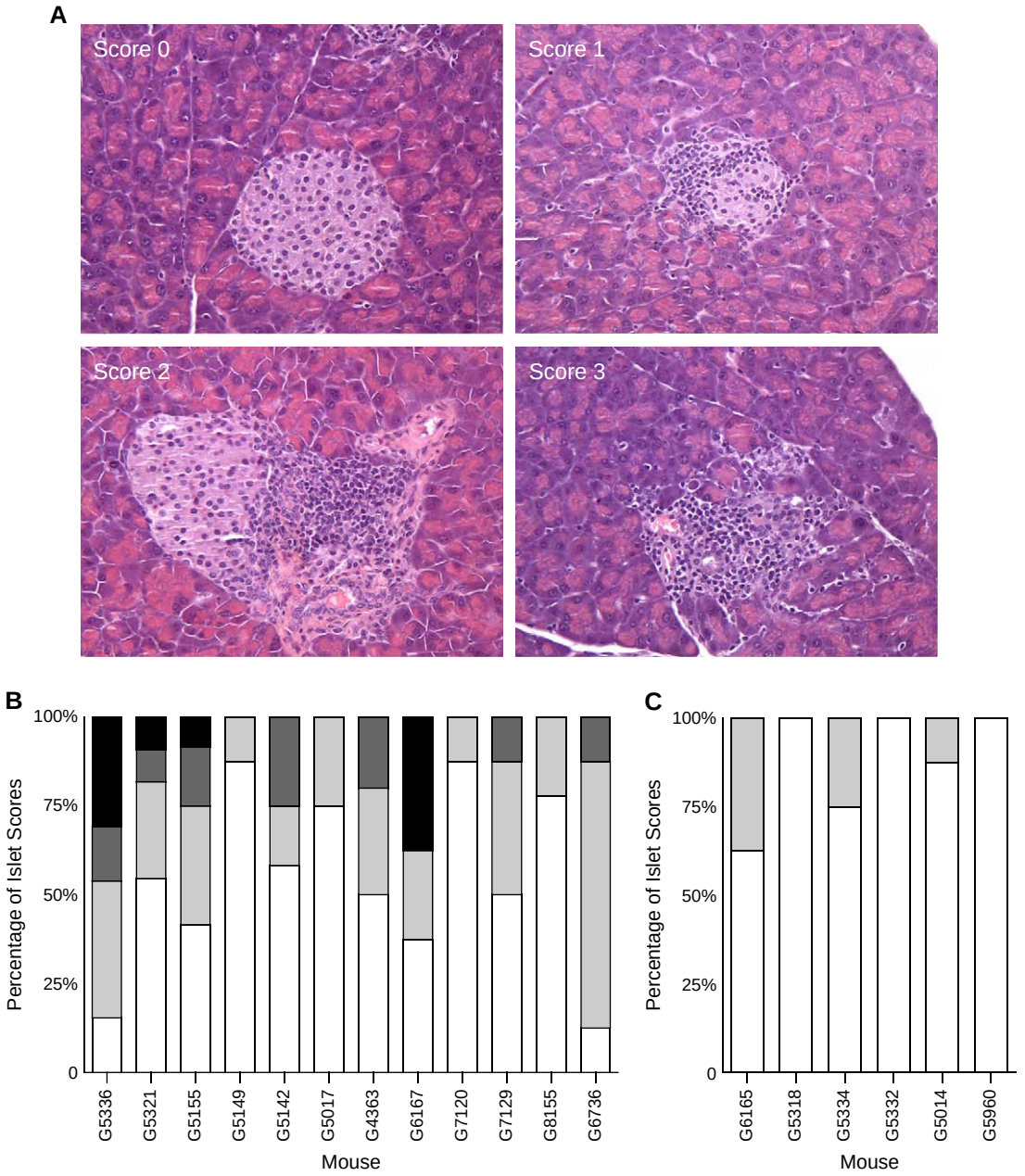

Fig. 5. Islet cell infiltrate in DR4/164/Rag2$^{o/o}$ TcR transgenic mice. H&E staining on formalin fixed paraffin embedded pancreatic tissue from a 28-week-old female DR4/164/Rag2$^{o/o}$ mouse (A). Islets were scored as 0, no infiltrate; 1, less than 33%; 2, less than 66%; 3, greater than 66%. A summary of islet infiltrate scores for 12 female and 6 male DR4/164/Rag2$^{o/o}$ mice is shown in (B) and (C) respectively. Islets were scored as 0, no infiltrate (white bars); 1, less than 33% (light gray bars); 2, less than 66% (dark gray bars); 3, greater than 66% (black bars).

The same cytokine profile is seen in Rag2 sufficient mice (data not shown) but proliferative values and detectable cytokines are lower, likely due to the large percentage of non-clonotypic T cells and FOXP3^{+} cells selected in Rag2 sufficient mice (Figs. 1E and 2B).

3.4. GAD65 555–567 TcR transgenic mice on a Rag2$^{o/o}$ background have insulitis

Blood glucose levels in DR4/164 Rag2 sufficient mice ($n = 20$) were monitored up to 40 weeks of age, and no mice showed overt hyperglycemia. Histological examination of pancreata from DR4/164 Rag2 sufficient mice also appeared normal with no indication of an islet infiltrate (data not shown). To increase the expression of the clonotypic transgenic GAD TcR, DR4/164 mice were crossed onto Rag2 knockout mice to generate DR4/164/Rag2$^{o/o}$ mice. As previously seen for Rag2$^{+/+}$ animals (Fig. 1D), concomitant with the strong negative selection of the 164 TcR in these mice on the Rag2$^{o/o}$ background, a peripheral skewing of the clonotypic TcR towards the CD8^{+} single positive T-cell lineage was observed (Fig. 4). However in contrast to the CD8^{+} T cells, the CD3^{+}/CD4^{+} T cells from DR4/164/Rag2$^{o/o}$ mice display an activated CD44^{Hi}/CD62L^{-}

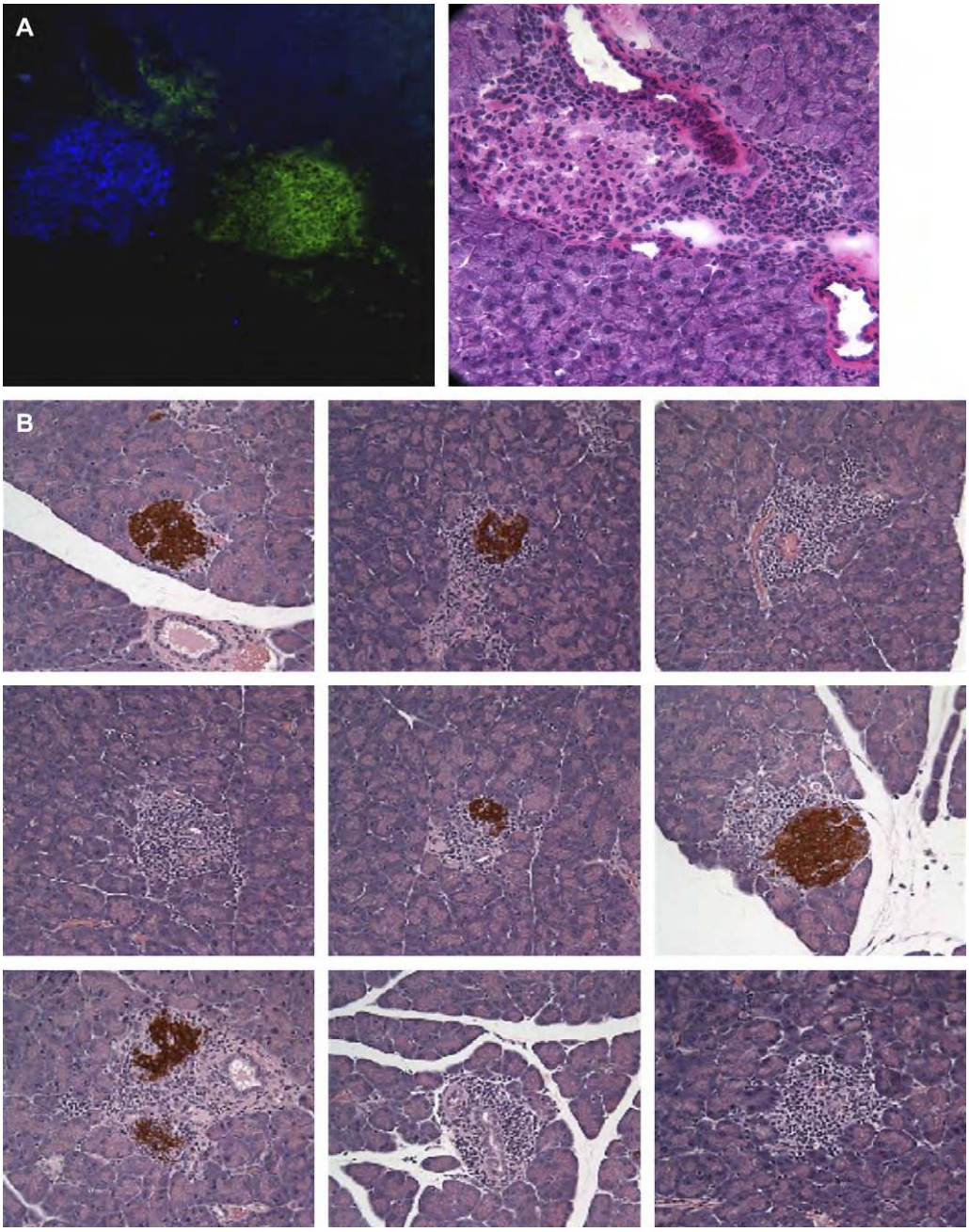

Fig. 6. Detection of $CD4^+$ cells and loss of insulin staining in infiltrated islets from DR4/164/$Rag2^{o/o}$ mice. Immunofluorescence staining for insulin (blue) and CD4 (green) in an infiltrated islet from a 28-week-old female DR4/164/$Rag2^{o/o}$ mouse (A). Immunohistochemistry staining for insulin (brown) in infiltrated islets from a female DR4/164/$Rag2^{o/o}$ mouse (B).

phenotype (Fig. 4). In addition, unlike the lack of FoxP3 expression on $V\beta5.1^+/CD4^+$ T cells in DR4/164/$Rag2^{+/+}$ mice (Fig. 2B), a fraction of peripheral $CD4^+$ cells in DR4/164 $Rag2^{o/o}$ do express FoxP3 (2.5%).

Unlike DR4/164/$Rag2^{+/+}$ mice, DR4/164/$Rag2^{o/o}$ mice exhibit an islet-specific cellular infiltrate into the pancreas beginning at about 25 weeks of age (Fig. 5A). The islet-specific infiltrate is observed primarily in female mice (Fig. 5B and C) and is not seen in other organs (supplemental data S1). Immunofluorescence staining of the islets showed $CD4^+$ staining indicative of the 164 T cells infiltrating into these islets (Fig. 6A). $CD8^+$ cells were not detected in the infiltrated islets (data not shown). Correlating with the cellular infiltrate in DR4/164/$Rag2^{o/o}$ islets is a loss of detectable insulin staining in most, but not all, islets (Fig. 6B). To assay if the loss of insulin staining in T-cell infiltrated islets is reflected in pancreatic function we performed an intra-peritoneal glucose tolerance test (IPGTT) on DR4/164/$Rag2^{o/o}$ mice. As shown in Fig. 7, DR4/164/$Rag2^{o/o}$ mice are impaired in their response to injected glucose at a time when they display a patchy T-cell infiltrate into the islets with loss of immunoreactive insulin in these islets.

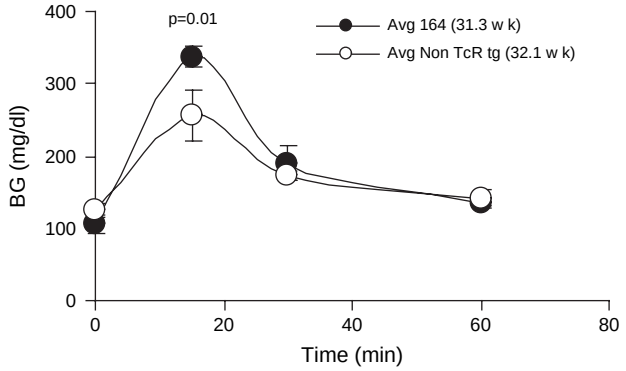

Fig. 7. Impaired response to exogenous glucose in DR4/164/Rag2$^{o/o}$ mice. DR4/164/Rag2$^{o/o}$ (164, filled circles) and DR4/Rag2$^{o/o}$ (Non TcR tg, open circles) mice were fasted for 6 h prior to glucose injection and blood glucose monitoring (see Section 2). Four mice 30 weeks of age were used in each group.

4. Discussion

HLA-DR4 MHC transgenic mice on a relatively non-autoimmune prone C57BL/6 background were evaluated for propensity to autoimmune diabetes by introduction of self-antigen specific GAD65 (555–567) responsive TcR transgenes. The TcR sequence originated from a human T-cell clone (164) derived from a diabetes at-risk individual and was chosen because: (1) the GAD65 (555–567) sequence is identical to both GAD65 and GAD67 forms in both mice and humans with the later form of GAD being the most dominant in the murine pancreas [9], and (2) the minimal stimulating GAD65 (555–567) sequence is within a naturally processed epitope in both mouse and man [7,33]. These 164 transgenic T cells in DR4 mice were strongly negatively selected against and weakly populate the secondary lymphoid organs. Nevertheless, peripheral transgenic T cells are antigen-specific for GAD65 (555–567) and display a T_H1 phenotype by expressing IFN-γ with minimal TNF-α and IL-2 and no IL10, IL-17, IL-4, or IL-5 upon in vitro challenge.

While GAD65 (555–567) TcR transgenic mice on HLA DR4 Rag2 sufficient background did not show evidence for loss in pancreatic function, GAD65 TcR transgenic mice on a Rag2$^{o/o}$ background (DR4/164/Rag2$^{o/o}$) beginning at about 25 weeks of age showed signs of impaired islet function, as demonstrated by a CD4$^+$ T-cell islet-specific infiltrate into the pancreas that was correlated with a loss in islet insulin staining and an abnormal response to an intraperitoneal glucose tolerance test. Thus, the GAD65 (555–567) specificity is sufficient for initiation of insulitis, resulting in metabolic compromise characteristic of pre-diabetes. Since this phenotype occurs on the relatively autoimmune resistant C57BL/6 background, it suggests that T-cell autoreactivity to GAD65 is sufficient for early immune activation associated with early autoimmunity to pancreatic islets, but that additional autoimmunity predisposition is likely to be necessary for full disease penetrance. This phenotype is distinct from that described by Tarbell et al. [26] and Kim et al. [25], in which GAD65 reactive I-Ag7-restricted TcR transgenic NOD mice appeared to the protected from diabetes and correlated with IL-10 and IFN-γ being secreted by CD4$^+$ T cells. A determination of whether the protection in those GAD TcR models was mediated by T-cell produced IL-10 was not addressed. Other islet responsive (including GAD) IL-10 producing T cells have been shown to protect from diabetes in transfer models [27,28,41] with the later study showing abrogating effects by blocking IL-10 signaling. In contrast, the insulitic behavior of 164 GAD T cells in DR4 mice shown here do not produce IL-10.

The FOXP3 expression profiles differed in DR4/164 TcR Rag2 sufficient and Rag2$^{-/-}$ mice. In the Rag2-sufficient animals, a substantial population of transgene-negative FOXP3$^+$ T cells was present in the periphery, and in spleen approximately 3 times the frequency found in the non-TcR transgenic DR4 mice. It is possible that these cells arise as a compensatory mechanism suppressing the subpopulation of transgene-positive cells; in any event, the presence of these FOXP3$^+$ cells correlated with a lack of insulitis or hyperglycemia. In the Rag2 deficient mice, we were therefore surprised to find a small percentage of clonotypic CD4$^+$/FoxP3$^+$ cells (Fig. 4). Studies are underway to evaluate the functional suppressive capacity of this small population in animals with insulitis, but lacking overt diabetes.

The insulitis phenotype of GAD TcR and HLA transgenic mice supports the hypothesis that T-cell reactivity to GAD restricted by a diabetes-associated human MHC molecule plays a role in diabetes or pre-diabetes pathogenesis. These mice had impaired glucose tolerance but did not become hyperglycemic; potential reasons could be that other T-cell specificities (or B cells) are required for disease progression, or alternatively that regulatory mechanisms in the context of the C56Bl/6 genome are sufficient to reduce penetrance. A requirement for B cells in non-TcR transgenic NOD diabetes has been established [42,43]. Mice are presently being crossed onto TcR Cα$^{o/o}$ mice to determine if the presence of B cells in the presence of clonotypic 164 T cells will lead to hyperglycemia. The results from this initial study indicate that T_H1 GAD specific T cells can spontaneously migrate specifically to pancreatic islets in DR4 humanized mice on a relatively non-autoimmune background and are capable of mediating a loss in β-cell function.

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Appendix I. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jaut.2007.08.001.

References

[1] Wen L, Wong FS, Tang J, Chen NY, Altieri M, David C, et al. In vivo evidence for the contribution of human histocompatibility leukocyte antigen (HLA)-DQ molecules to the development of diabetes. J Exp Med 2000;191:97–104.

[2] Gebe JA, Unrath KA, Falk BA, Ito K, Wen L, Daniels TL, et al. Age-dependent loss of tolerance to an immunodominant epitope of glutamic acid decarboxylase in diabetic-prone RIP-B7/DR4 mice. Clin Immunol 2006;121:294–304.

[3] Wen L, Chen NY, Tang J, Sherwin R, Wong FS. The regulatory role of DR4 in a spontaneous diabetes DQ8 transgenic model. J Clin Invest 2001;107:871–80.

[4] Gebe JA, Falk BA, Unrath KA, Nepom GT. Autoreactive T cells in a partially humanized murine model of T1D. Ann. NY Acad Sci 2007;1103: 69–76.

[5] Abraham RS, Wilson SB, de Souza Jr NF, Strominger JL, Munn SR, David CS. NOD background genes influence T-cell responses to GAD 65 in HLA-DQ8 transgenic mice. Hum Immunol 1999;60:583–90.

[6] Congia M, Patel S, Cope AP, De Virgiliis S, Sonderstrup G. T-cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin. Proc Natl Acad Sci U S A 1998;95:3833–8.

[7] Patel SD, Cope AP, Congia M, Chen TT, Kim E, Fugger L, et al. Identification of immunodominant T-cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR(alpha1*0101,beta1*0401) transgenic mice. Proc Natl Acad Sci USA 1997;94:8082–7.

[8] Wicker LS, Chen SL, Nepom GT, Elliott JF, Freed DC, Bansal A, et al. Naturally processed T-cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes- associated human MHC class II allele, DRB1*0401. J Clin Invest 1996;98:2597–603.

[9] Kim J, Richter W, Aanstoot HJ, Shi Y, Fu Q, Rajotte R, et al. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. Diabetes 1993;42:1799–808.

[10] Danke NA, Yang J, Greenbaum C, Kwok WW. Comparative study of GAD65-specific $CD4^+$ T cells in healthy and type 1 diabetic subjects. J Autoimmun 2005;25:303–11.

[11] Reijonen H, Mallone R, Heninger AK, Laughlin EM, Kochik SA, Falk B, et al. GAD65-specific $CD4^+$ T-cells with high antigen avidity are prevalent in peripheral blood of patients with type 1 diabetes. Diabetes 2004;53:1987–94.

[12] Reijonen H, Novak EJ, Kochik S, Heninger A, Liu A, Kwok W, et al. Detection of GAD65 specific T-cells by MHC class II multimers in type 1 diabetes patients and at-risk subjects. Diabetes 2002;51:1375–82.

[13] Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. Diabetes 1997;46:1701–10.

[14] Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes 1996;45:926–33.

[15] 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;46:1975–82.

[16] 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;7:851–8.

[17] Tisch R, Yang XD, Liblau RS, McDevitt HO. Administering glutamic acid decarboxylase to NOD mice prevents diabetes. J Autoimmun 1994;7: 845–50.

[18] Denes B, Yu J, Fodor N, Takatsy Z, Fodor I, Langridge WH. Suppression of hyperglycemia in NOD mice after inoculation with recombinant vaccinia viruses. Mol Biotechnol 2006;34:317–27.

[19] Bridgett M, Cetkovic-Cvrlje M, O'Rourke R, Shi Y, Narayanswami S, Lambert J, et al. Differential protection in two transgenic lines of NOD/Lt mice hyperexpressing the autoantigen GAD65 in pancreatic beta-cells. Diabetes 1998;47:1848–56.

[20] Yoon JW, Yoon CS, Lim HW, Huang QQ, Kang Y, Pyun KH, et al. Control of autoimmune diabetes in NOD mice by GAD expression or suppression in beta cells. Science 1999;284:1183–7.

[21] 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;95:10055–60.

[22] Zekzer D, Wong FS, Ayalon O, Millet I, Altieri M, Shintani S, et al. GAD-reactive CD4+ Th1 cells induce diabetes in NOD/SCID mice. J Clin Invest 1998;101:68–73.

[23] Judkowski V, Pinilla C, Schroder K, Tucker L, Sarvetnick N, Wilson DB. Identification of MHC class II-restricted peptide ligands, including a glutamic acid decarboxylase 65 sequence, that stimulate diabetogenic T cells from transgenic BDC2.5 nonobese diabetic mice. J Immunol 2001; 166:908–17.

[24] Wilson DB. GAD-about BDC2.5: peptides that stimulate BDC2.5 T cells and inhibit IDDM. J Autoimmun 2003;20:199–201.

[25] Kim SK, Tarbell KV, Sanna M, Vadeboncoeur M, Warganich T, Lee M, et al. Prevention of type I diabetes transfer by glutamic acid decarboxylase 65 peptide 206-220-specific T cells. Proc Natl Acad Sci U S A 2004;101:14204–9.

[26] Tarbell KV, Lee M, Ranheim E, Chao CC, Sanna M, Kim SK, et al. CD4(+) T cells from glutamic acid decarboxylase (GAD)65-specific T-cell receptor transgenic mice are not diabetogenic and can delay diabetes transfer. J Exp Med 2002;196:481–92.

[27] You S, Chen C, Lee WH, Brusko T, Atkinson M, Liu CP. Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. J Immunol 2004;173:6777–85.

[28] Maron R, Melican NS, Weiner HL. Regulatory Th2-type T-cell lines against insulin and GAD peptides derived from orally- and nasally-treated NOD mice suppress diabetes. J Autoimmun 1999;12:251–8.

[29] Arnold PY, Burton AR, Vignali DA. Diabetes incidence is unaltered in glutamate decarboxylase 65-specific TCR retrogenic nonobese diabetic mice: generation by retroviral-mediated stem cell gene transfer. J Immunol 2004;173:3103–11.

[30] Ito K, Bian HJ, Molina M, Han J, Magram J, Saar E, et al. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. J Exp Med 1996;183: 2635–44.

[31] Kouskoff V, Signorelli K, Benoist C, Mathis D. Cassette vectors directing expression of T-cell receptor genes in transgenic mice. J Immunol Methods 1995;180:273–80.

[32] Kruisbeek AM. Isolation and fractionation of mononuclear cell populations. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current Protocols in Immunology. John Wiley & Sons; 2000. p. 3.1.1–5.

[33] Nepom GT, Lippolis JD, White FM, Masewicz S, Marto JA, Herman A, et al. Identification and modulation of a naturally processed T-cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). Proc Natl Acad Sci U S A 2001; 98:1763–8.

[34] Gebe JA, Falk BA, Rock KA, Kochik SA, Heninger AK, Reijonen H, et al. Low-avidity recognition by $CD4^+$ T cells directed to self-antigens. Eur J Immunol 2003;33:1409–17.

[35] Arden B, Clark SP, Kabelitz D, Mak TW. Human T-cell receptor variable gene segment families. Immunogenetics 1995;42:455–500.

[36] Quaratino S, Badami E, Pang YY, Bartok I, Dyson J, Kioussis D, et al. Degenerate self-reactive human T-cell receptor causes spontaneous autoimmune disease in mice. Nat Med 2004;10:920–6.

[37] Ranheim EA, Tarbell KV, Krogsgaard M, Mallet-Designe V, Teyton L, McDevitt HO, et al. Selection of aberrant class II restricted $CD8^+$ T cells in NOD mice expressing a glutamic acid decarboxylase (GAD)65-specific T-cell receptor transgene. Autoimmunity 2004;37:555–67.

[38] Viret C, Janeway Jr CA. Self-specific MHC class II-restricted CD4-CD8- T cells that escape deletion and lack regulatory activity. J Immunol 2003;170:201–9.

[39] Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol 2001;2: 301–6.

[40] Walker LS, Chodos A, Eggena M, Dooms H, Abbas AK. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. J Exp Med 2003;198:249–58.

[41] Han HS, Jun HS, Utsugi T, Yoon JW. A new type of CD4+ suppressor T-cell completely prevents spontaneous autoimmune diabetes and recurrent diabetes in syngeneic islet-transplanted NOD mice. J Autoimmun 1996; 9:331–9.

[42] Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. Diabetes 1997;46:941–6.

[43] Serreze DV, Chapman HD, Varnum DS, Hanson MS, Reifsnyder PC, Richard SD, et al. B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. J Exp Med 1996;184:2049–53.

Restricted Autoantigen Recognition Associated with Deletional and Adaptive Regulatory Mechanisms[1]

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Autoimmune diabetes (T1D) is characterized by $CD4^+$ T cell reactivity to a variety of islet-associated Ags. At-risk individuals, genetically predisposed to T1D, often have similar T cell reactivity, but nevertheless fail to progress to clinically overt disease. To study the immune tolerance and regulatory environment permissive for such autoreactive T cells, we expressed TCR transgenes derived from two autoreactive human T cells, 4.13 and 164, in HLA-DR4 transgenic mice on a C57BL/6-derived "diabetes-resistant" background. Both TCR are responsive to an immunodominant epitope of glutamic acid decarboxylase $65_{555–567}$, which is identical in sequence between humans and mice, is restricted by HLA-DR4, and is a naturally processed self Ag associated with T1D. Although both TCR use the identical Vα and Vβ genes, differing only in CDR3, we found stark differences in the mechanisms utilized in vivo in the maintenance of immune tolerance. A combination of thymic deletion (negative selection), TCR down-regulation, and peripheral activation-induced cell death dominated the phenotype of 164 T cells, which nevertheless still maintain their Ag responsiveness in the periphery. In contrast, 4.13 T cells are much less influenced by central and deletional tolerance mechanisms, and instead display a peripheral immune deviation including differentiation into IL-10-secreting Tr1 cells. These findings indicate a distinct set of regulatory alternatives for autoreactive T cells, even within a single highly restricted HLA-peptide-TCR recognition profile. ***The Journal of Immunology*****, 2009, 183: 59–65.**

Central and peripheral mechanisms maintaining T cell tolerance to self Ags are variable in degree of completeness, and autoreactive T cells populate the peripheral immune system. Central tolerance in the thymus is largely governed through the interaction of the TCR with self-peptide-MHC complexes, in which high-avidity T cells are eliminated through apoptosis (1–3) or potentially differentiated into $CD4^+CD25^+$Foxp3-expressing regulatory T cells (Treg)[3] (4, 5). Strategies by which autoreactive T cells may escape central tolerance to self Ags include down-modulation of receptor or costimulatory molecules (6) and skewing of CD4/CD8 coreceptor expression (7, 8). These mechanisms are incomplete, however, such that self reactivity by some peripheral T cells is an intrinsic property of normal immunity, perhaps required to enable the immune repertoire to respond to the diverse nature of foreign Ags (9).

Once in the periphery, several additional mechanisms operate as checkpoints to limit T cell activation to self Ags, including functional inactivation or anergy of the T cell (10, 11), activation-induced T cell deletion (12–14), generation of suppressive cytokine-secreting T cells (Tr1 and Th3) (15, 16), and differentiation of uncommitted T cells into Foxp3-expressing regulatory T cells (17, 18).

While several TCR transgenic mice have been developed to study tolerance to self Ags, the vast majority of studies use either alloreactive T cells or a foreign Ag-reactive T cell expressed as a TCR transgene along with the foreign Ag as a second transgene (4, 19, 20). In human type 1 diabetes (T1D), HLA-DR4 subjects commonly carry peripheral T cells reactive to a variety of islet-associated self Ags, including the immunodominant glutamic acid decarboxylase (GAD)$65_{555–567}$ peptide, a naturally processed epitope of glutamic acid decarboxylase (21–24). Interestingly, recognition of this epitope displays a biased TCR repertoire, with prevalent use of Vβ5.1/Vα12.1, although CDR3 regions are variable (22). To study tolerance mechanisms associated with this dominant autoreactive specificity, we introduced transgenic TCR from two human $CD4^+$ T cells specific for $GAD65_{555–567}$, which differ only in their CDR3 regions, intercrossed into HLA-DR4 transgenic mice. Despite the close structural features of these two autoreactive TCR, stark differences in both central and peripheral tolerance mechanisms were elicited.

Materials and Methods

Mice

DR0401-IE mice (DR4) were obtained from Taconic. These C57BL/6 I-$Ab^{o/o}$ mice express a human-mouse chimeric class II molecule in which the TCR-interacting and peptide-binding domains of mouse I-E (domains α_1 and β_1, exon 2 in both genes) have been replaced with the α_1 and β_1 domains from DRA1*0101 and DRB1*0401, respectively. Retention of the murine α_2 and β_2 domains allows for the cognate murine CD4-murine MHC interaction (25).

TCR sequences for generation of the two T cell transgenic mice were obtained from human $CD4^+$ T cell clones 164 (26)and 4.13 (22). Both human T cells are responsive to the same self Ag $GAD65_{555–567}$ and both use human Vα12.1/Vβ5.1 T cell receptors. The 164 T cell was cloned from peripheral blood from an HLA DRA1*0101/B1*0401 diabetes at-risk individual as previously described (26).

Clone 4.13 was cloned from the peripheral blood of an HLA DRA1*0101/B1*0401 diabetic individual (22). Human-mouse chimeric TCR transgenes were constructed by subcloning PCR amplified regions encoding rearranged VαJα and VβDβJβ domains from the human clones into pTαcass and pTβcass TCR transgenic vectors, respectively (27). TCR transgenic vectors pTαcass and pTβcass contain the natural mouse TCR α

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[3] Abbreviations used in this paper: Treg, regulatory T cell; GAD, glutamic acid decarboxylase; T1D, type 1 diabetes.

and β promoter/enhancer elements and mouse Cα and Cβ constant regions, respectively. DNA injection into C57BL/6/I-Ab$^{o/o}$ (164 TCR) or F1-B6/C3H (4.13 TCR) mouse embryos was performed at the University of Washington (Seattle, WA) in the Comparative Medicine Animal Facility. Founder mice containing the human TCR transgenes were then crossed onto DR0401-IE mice to generate DR4/164 and DR4/4.13 mice. Additional crosses were made onto Rag2$^{o/o}$ mice. A founder mouse was also identified that contained only the 164 TCR β transgene. This was also crossed onto DR0401-IE mice. The 4.13 TCR transgenic mice generated in F1-B6/C3H were crossed for nine generations into DR0401-IE mice. All animal work was approved by the Benaroya Research Institute Animal Care and Use Committee and animals were housed in the Benaroya Research Institute American Association of Laboratory Animal Care-accredited animal facility.

Tissue processing and flow cytometry

Thymus, spleen, and lymph node tissues were processed into single-cell suspensions by gently pressing through 0.40-μm cell strainers (BD Falcon; ref. no. 352340) using the rubber end of a 1-ml tuberculin syringe in DMEM-10 media (Invitrogen; catalog no. 11965-092) supplemented with 10% FBS (HyClone), 100 μg/ml penicillin, 100 U/ml streptomycin, 50 μM 2-ME, 2 mM glutamine, and 1 mM sodium pyruvate (Invitrogen). Cell suspensions were centrifuged at 200 $\times$ *g* for 10 min, aspirated, and either 1) resuspended in DMEM-10 media (lymph node and thymus) or 2) RBC were lysed (for spleens) using 1 ml of ACK lysis buffer (28) for 5 min at 37°C at which time 30 ml of media was added and cells spun down (200 $\times$ *g*), aspirated, and resuspended in DMEM-10 media. The following chromophore-labeled Abs were used in flow cytometric analysis: anti-mouse CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD62L (Mel-14), anti-human active caspase-3 (polyclonal, catalog no. 557091), CD44 (IM7), Fc block (2.4G2), and PE-labeled annexin V (all from BD Pharmingen), anti-human Vβ5.1-PE (clone IMMU 157; Immunotech/Coulter), and Vα12.1-FITC (clone 6D6; Endogen). FACS samples in media were prestained with Fc block for 10 min on ice and then stained with specific Abs on ice for 45 min, washed once, and resuspended in FACS stain buffer (PBS containing 1% FBS, 0.1% Na azide) before being run on a FACSCalibur or LSR II flow cytometer (BD Biosciences). Intracellular staining of cells for Foxp3 was performed using eBioscience kit (FJK.16a Ab) according to the manufacturer's instructions. Intracellular staining for active caspase-3, mouse anti-IFN-γ (XMG1.2; eBioscience), and anti-IL-10 (clone JES5-16E3; eBioscience) was performed using eBioscience intracellular staining kit (catalog no. 88-8823-88; eBioscience).

Proliferation assays

In lymph node or purified CD4$^+$ T cell proliferation assays 1 $\times$ 10^5 lymph node cells were cultured with 2 $\times$ 10^5 (3000 rad) Cs-gamma-irradiated splenocytes (final volume 150 μl). Supernatants for cytokine analysis were taken (50 μl) at 48 h, and 1 μCi/well [^{3}H]thymidine was added at 72 h. Thymidine incorporation was assayed at 96 h using liquid scintillation counting analyzed on a Microbeta TriLux 1450 scintillation counter (Wallac-PerkinElmer Life Sciences). Splenocyte responses were measured in the same manner using 5 $\times$ 10^5 splenocytes per well. CD4 and CD8 single-positive cells were obtained using Mitenyi Biotec beads with purity of 90% or greater or by Ab labeling with CD4 and CD8 and sorting by flow cytometry.

Cytokine analysis

Cytokines IL-2, IL-4, IL-5, TNF-α, and IFN-γ were assayed using a mouse Th1/Th2 cytokine CBA kit (BD Biosciences; catalog no. 551287). IL-10 was assayed using a BD OptEIA mouse IL-10 ELISA set and mouse TGF-β1 was measured using a human/mouse TGF-β1 ELISA Ready-SET-Go! kit (BD Biosciences; catalog nos. 555252 and 88-7344, respectively). Supernatants from triplicate proliferation wells (50 μl/well) were combined for cytokine analysis, with 50 μl used for CBA analysis and 50 μl for IL-10 ELISA.

Results

Thymic selection of autoreactive T cells

Utilizing TCR from two structurally related DRB1*0401 (DR4) restricted human CD4$^+$ T cell clones reactive to the autoantigen GAD65, human TCR transgenic mice were generated to investigate differential modes of T cell tolerance to the naturally processed GAD65$_{555–567}$ autoantigen. The human CD4$^+$ T cell clones 164 and 4.13 (obtained from two different subjects) are structurally related in that they both use TCR with human Vα12.1 (hVα12.1) and Vβ5.1 (hVβ5.1) gene sequences, which differ only in their CDR3 regions (Table I). Both of the human T cells recognize GAD65$_{555–567}$ (22, 26), a region within the naturally processed and presented GAD65$_{552–572}$ epitope (21, 23). The sequence of the DR4-binding minimal stimulating epitope GAD65$_{555–567}$ is identical for GAD65 and GAD67 in both human and mouse and thus serves as a naturally processed self Ag T cell epitope in both species (29). Both 4.13/Rag2$^{+/+}$ and 164/Rag2$^{+/+}$ mice display reduced thymus cellularity (Fig. 1*A*), with 164 mice exhibiting a profound reduction in CD4$^+$CD8$^+$ double-positive cells (Fig. 1*B*). The reduction in cellularity and a decrease in double-positive cells is indicative of negative selection (20, 30, 31). While positively

Table I. *Comparison of 164 and 4.13 TCR*[a]

Clone		CDR3 Region sequence													
						(--)									
	TcRAV					Glu	Gly			Ala	Asn				
164	5.1	A	L	S	E	E	G	G	G	A	N	S	K	L	T
4.13	5.1	A	L	S	E	N	R	G	G	T	A	S	K	L	T
						Asn	Arg			Thr	Ala				
							(+)								(+)
	TcRBV					Ala				Ala	Asn		Pro	Leu	His
164	12.1	A	S	S	L	A	G	G		A	N	S	P	L	H
4.13	12.1	A	S	S	L	V	G	G		P	S	S	E	A	F
						Val				Pro	Ser		Glu	Ala	Phe
													(--)		

[a] Gray highlighted areas denote differences between 164 and 4.13 TCR CD3 sequences; boldface, nonpolar-to-nonpolar amino acid changes; underlined residues, polar-to-polar changes; and parentheses, charge changes.

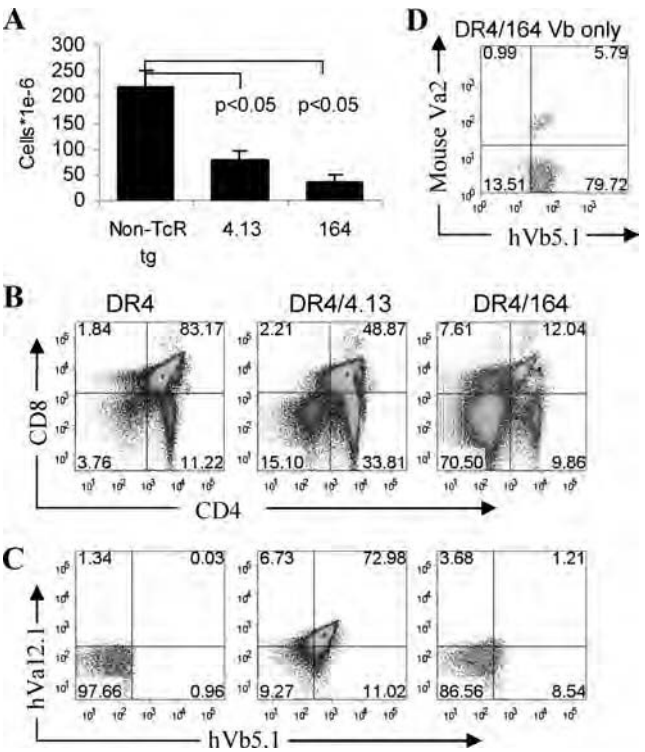

FIGURE 1. Thymic lymphocyte profiles in 164 and 4.13 human TCR transgenic HLA-DR4 mice. Thymus cellularity of 8- to 10-wk-old mice (n = 3) (*A*), CD4/CD8 profiles (*B*), and TCR hVb5.1 vs hVa12.1 expression on CD4$^+$CD8$^-$ gated cells (*C*). Human Vb5.1 expression on CD4$^+$CD8$^-$ thymocytes from 164 Vb only TCR mice (*D*).

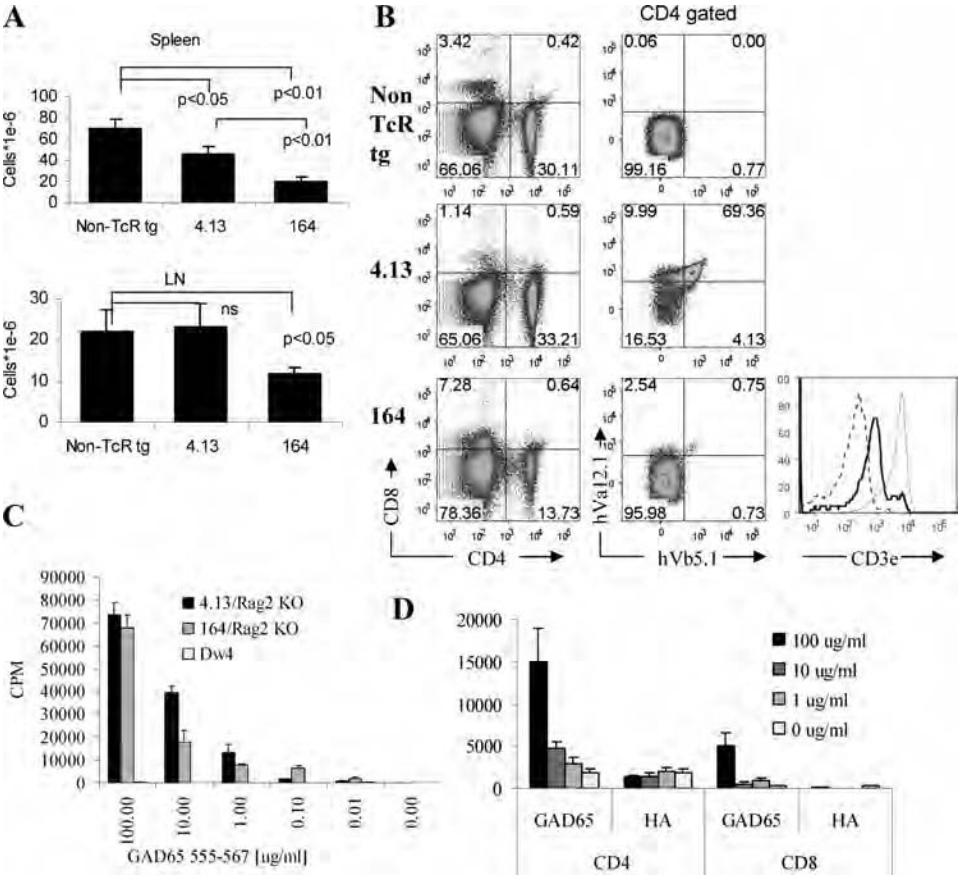

FIGURE 2. Peripheral lymphocyte profiles in 8- to 10-wk-old 164 and 4.13 TCR transgenic DR4 mice. Peripheral spleen and lymph nodes (inguinal and para-aortic combined) cellularity ($n = 3$) (*A*). CD4 vs CD8 profile and TCR human Va12.1 and Vb5.1 expression on $CD4^+CD8^-$-gated cells from spleen (*B*). Histogram in *B* shows CD3e expression on $CD4^+CD8^-$-gated cells from 164 mice (black line), 4.13 mice (gray line), and isotype control (dotted line). Splenocyte Ag dose response in 164 (gray) and 4.13 (black) TCR transgenic DR4 mice on a $Rag2^{o/o}$ background (*C*). For $CD4^+CD8^-$ and $CD4^-CD8^+$ T cell stimulation (*D*) cells were sorted by flow and tested for proliferation in response to $GAD65_{555–567}$ or control HA Ag. All experiments were repeated at least three times with similar results.

selected 4.13 T cells are heavily skewed toward a single-positive $CD4^+CD8^-$ phenotype reflecting their class II restriction, single-positive thymic T cells in 164 mice are matured into both $CD4^-CD8^+$ and $CD4^+CD8^-$ phenotypes, a profile similar to that observed in other self Ag-responsive TCR transgenic mice under conditions of strong negative selection (8). In addition to the stronger negative selection observed in 164 mice is the down-modulated expression of the TCR on $CD4^+CD8^-$ thymocytes where only ~1% of mature $CD4^+CD8^-$ T cells express both Vα and Vβ transgenes (Fig. 1*C*). This is in stark contrast to the >70% expression of hVα12.1 and hVβ5.1 on $CD4^+CD8^-$ thymocytes from 4.13 mice. As the amino acid sequence in the CDR3 region of 164 TCR is different from 4.13 TCR, it was possible that the low level of hVβ5.1 and hVα12.1 staining on 164 mice could be the result of differential binding of the Ab itself; however, the hVβ5.1 Ab does stain the 164 TCR from 164 β-chain-only TCR transgenic mice (lacking the human TCR Vα12.1 transgene), suggesting that the low level of 164 TCR expression on matured $CD4^+CD8^-$ thymocytes is the result of down modulation of the TCR under thymic selection pressures (Fig. 1*D*). Based on thymic cellularity, CD4 vs CD8 profiles, and TCR expression levels, we conclude that 164 TCR thymocytes, likely due to a higher avidity for peptide-MHC of the 164 TCR relative to the 4.13 TCR, undergo stronger central tolerance and maintain a down-modulated TCR.

Peripheral skewing of autoreactive T cells

$4.13/Rag2^{+/+}$ and $164/Rag2^{+/+}$ mice both show reduced cellularity in the spleen (Fig. 2*A*), but only 164 mice show a reduction in peripheral lymph nodes. The 4.13 T cells in the spleen as in the thymus are heavily skewed toward $CD4^+CD8^-$ lineage, reflecting their class II restriction (Fig. 2*B*). The $164/Rag2^{+/+}$ mice have fewer cells in the spleen, and <1% of $CD4^+CD8^-$ T cells are hVα12.1- and hVβ5.1-positive (Fig. 2*B*). Coinciding with the weak TCR expression in $164/Rag2^{+/+}$ mice is also a low expression of CD3e on $CD4^+CD8^-$ gated cells (Fig. 2*B*, histogram). In contrast to the near absence of $CD4^-CD8^+$ cells in $4.13/Rag2^{+/+}$ mice, $164/Rag2^{+/+}$ mice have nearly one-third of their T cells as $CD8^+CD4^-$ cells, which is also greater than that seen in non-TCR transgenic mice (Fig. 2*B*). The percentages of CD4 cells among all T cells (CD4/(CD4 + CD8)) (average of three mice) are 98 ± 1% in 4.13 mice and 73 ± 2% in 164 mice compared with 90 ± 1% in non-TCR transgenic mice, indicating that 4.13 T cells are strongly selected toward their MHC class II restriction, while T cell selection in $164/Rag2^{+/+}$ mice is skewed toward the CD8 compartment, similar to what is observed in the thymus. The stronger central tolerance in $164/Rag2^{+/+}$ mice is also reflected in the periphery by the greater expression of endogenous mouse mVα and mVβ T cell receptors (supplemental Fig. S1).[4] In assaying for Ag specificity we used splenocytes from $Rag2^{o/o}$ TCR transgenic mice to ensure that all α/β T cells only express the hVα12.1 and hVβ5.1 transgenes. Splenocytes from both $4.13/Rag2^{o/o}$ and $164/Rag2^{o/o}$ mice respond to $GAD65_{555–567}$ in an Ag-specific manner, confirming their specificity for the GAD65 epitope (Fig. 2*C*). Because of the skewing of 164 T cells from $164/Rag2^{+/+}$ mice (also seen in $164/Rag2^{o/o}$ mice) into a $CD8^+CD4^-$ pathway, we sorted $164/Rag2^{o/o}$ T cells into $CD4^+CD8^-$ and $CD4^-CD8^+$ fractions and stimulated these fractions with irradiated splenocytes and peptide. We find that both populations are Ag specific, with the CD8 164 cells having a lower proliferative response (lower functional avidity) (Fig. 2*D*).

[4] The online version of this article contains supplemental material.

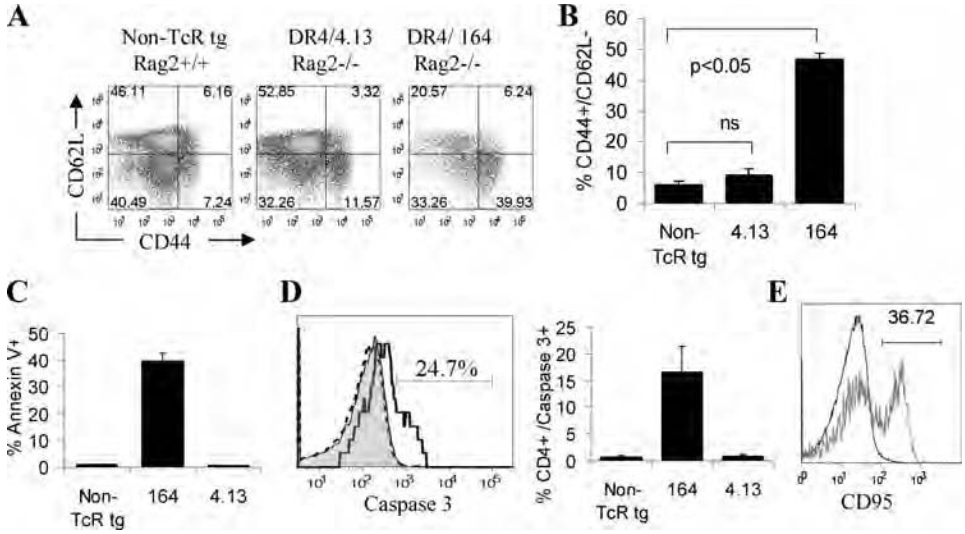

FIGURE 3. Activation and apoptosis in 164 and 4.13 TCR transgenic mice. Spleen cells from 8- to 12-wk-old DR4 mice on a Rag2$^{o/o}$ background were stained with activation markers CD44 and CD62L and gated on CD4$^+$CD8$^-$ cells for analysis (*A* and *B*). Percentage of gated CD4$^+$CD8$^-$ cells that were annexin V$^+$ (*C*) and active caspase-3$^+$ (*D*) are shown. Examples of caspase-3 histograms in (*D*) are non-TCR transgenic (gray filled), DR4/4.13/Rag2$^{o/o}$ (black dashed line), and DR4/164/Rag$^{o/o}$ (black heavy line). Percentages are from three mice in each group. CD4$^+$ spleen T cells from 164 (gray line) mice are also CD95$^+$ compared with non-TCR CD4$^+$ T cells (black line) (*E*).

Peripheral tolerance mediated by apoptosis

As with the low expression of the transgenic TCR on 164/Rag2$^{+/+}$ thymocytes in the thymus (Fig. 2*B*), the TCR expression on 164/Rag2$^{+/+}$ T cells in the periphery is also nearly absent (also true in 164/Rag2$^{o/o}$ mice). This suggested that perhaps the ligand inducing negative selection in the thymus is also activating these cells in the periphery, and thus the extremely low level of TCR expression in the periphery is in part the result of constant activation of 164 cells in the periphery. By surface phenotyping we found that most peripheral 4.13/Rag2$^{o/o}$ CD4$^+$ T cells, like CD4$^+$ cells from non-TCR/Rag2$^{+/+}$ transgenic mice, are of a naive nature expressing high levels of CD62L and intermediate levels of CD44 (CD62L^{high}CD44^{int}) (Fig. 3*A*). In contrast, ~40% of peripheral spleen CD4$^+$ cells from 164/Rag2$^{o/o}$ mice are CD62L^{low}CD44^{high} compared with ~10% in 4.13 and non-TCR transgenic mice, indicating an activated phenotype (Fig. 3, *A* and *B*). A similar activation profile of 164/Rag2$^{o/o}$ CD4$^+$ T cells was observed in other lymph nodes (pancreatic and inguinal, data not shown) and also in Rag2$^{+/+}$ mice (supplemental Fig. S3). Therefore, we tested whether the low numbers of T cells in the peripheral tissues of 164/Rag2$^{o/o}$ mice could be the result of constant peripheral activation and subsequent activation-induced cell death. As shown in Fig. 3*C*, peripheral CD4$^+$ 164/Rag2$^{o/o}$ T cells compared with 4.13/Rag2$^{o/o}$ and non-TCR transgenic cells stain with the apoptotic marker annexin V and additional staining indicated that the CD4$^+$ 164/Rag2$^{o/o}$ T cells are also activated caspase-3$^+$ (Fig. 3*D*). Peripheral CD4$^+$ 4.13/Rag2$^{o/o}$ cells were negative for both annexin V and activated caspase-3 staining. Surface staining on CD4$^+$ 164/Rag2$^{o/o}$ T cells indicated that a significant portion of these cells are also CD95$^+$, suggesting that apoptotic signaling may occur through CD95 (Fig. 3*E*).

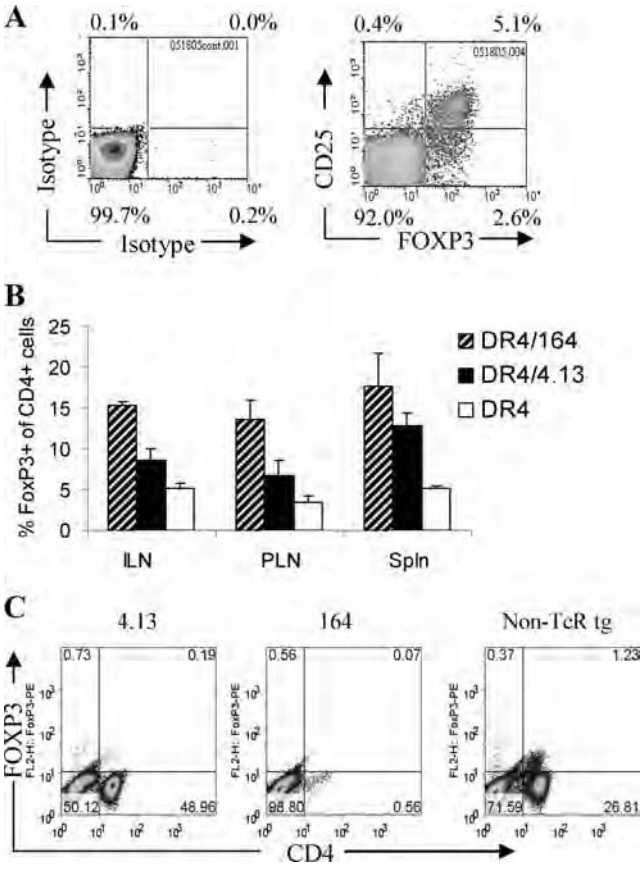

FIGURE 4. Foxp3 expression on spleen CD4$^+$CD8$^-$ T cells from non-TCR transgenic, 164, and 4.13 GAD TCR transgenic mice. Eight- to 12-wk-old mouse spleen cells from TCR and non-TCR transgenic mice were surface stained with CD4, CD25, and then intracellularly for Foxp3. Example of staining is shown in (*A*) on non-TCR transgenic DR4 splenocytes. Foxp3 expression in Rag2$^{+/+}$ mice as a percentage of CD4$^+$ cells is shown in (*B*) (average of three mice). Foxp3 expression on CD4$^+$ T cells from 164 and 4.13 TCR transgenic mice on a Rag2$^{o/o}$ background is shown in *C*.

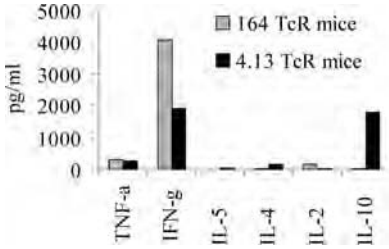

FIGURE 5. Cytokine profile of 164 and 4.13 T cells to GAD65$_{552–572}$. Splenocytes from DR4/164/Rag2$^{o/o}$ and DR4/4.13/Rag2$^{o/o}$ mice were stimulated with 100 μg/ml GAD65 or control peptide for 96 h. Supernatants were collected at 48 h and TNF-α, IFN-γ, IL-2, IL-4, and IL-5 were measured using a mouse Th1/Th2 kit, and IL-10 was measured by ELISA. Experiment was done three times with similar results.

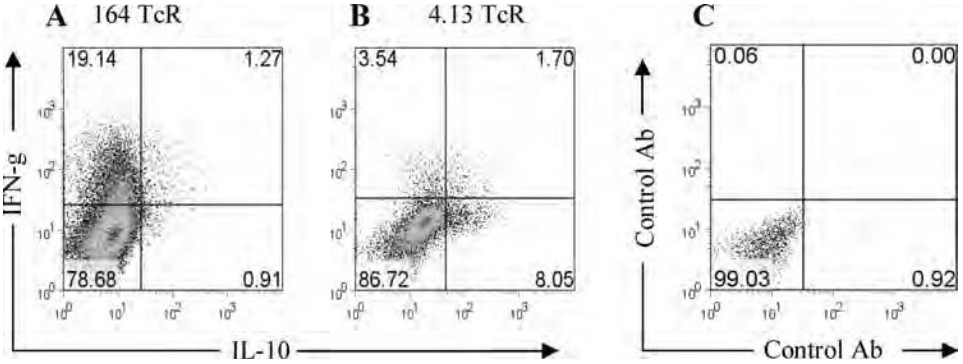

FIGURE 6. Internal cytokine staining for IL-10 and IFN-γ cells from 164 (*A*) and 4.13 (*B*) mice were intracellularly stained with IL-10 and IFN-γ directly conjugated Abs. Spleen cells from Rag2$^{o/o}$ 164 and 4.13 mice were stimulated for 4 days with GAD65$_{552–572}$ and then cultured with PMA/ionomycin for 4 h with brefeldin A during the last 2 h. Experiment was done three times with similar results.

Both 164 and 4.13 mice show an enhanced selection of peripheral Foxp3$^+$ cells

CD4$^+$CD25$^+$ cells that express Foxp3 participate in immune regulation, and the selection of these Treg can be mediated in foreign Ag-specific TCR transgenic mice by expression of the stimulatory Ag as a neo-self peptide driven by tissue-specific promoters (4, 32). It has also been shown that increasing avidity of the TCR for the peptide-MHC correlates with a propensity to develop along the thymic-derived Foxp3 Treg pathway (4). In our setting involving endogenous self Ag recognition, we find that peripheral CD4$^+$ T cells from both autoreactive 4.13/Rag2$^{+/+}$ and 164/Rag2$^{+/+}$ TCR transgenic mice express increased numbers of Foxp3 cells, and that the percentage of CD4$^+$ cells that express Foxp3 is highest in 164 mice compared with 4.13 mice, and both are greater than that seen in non-TCR transgenic mice (Fig. 4*B*). However, upon crossing TCR transgenic mice onto a Rag2-deficient background, peripheral Foxp3$^+$ cells were near undetectable levels in either 164 or 4.13 mice (Fig. 4*C*), consistent with the induction of Treg populations in the nontransgenic fraction of endogenous T cells.

Peripheral 4.13 CD4$^+$ T cells exhibit Th1 and Tr1 profiles

Cytokine analysis on in vitro-stimulated cells from both Rag2$^{o/o}$ TCR transgenic mice responding to GAD65$_{555–567}$ stimulation is shown in Fig. 5. Peripheral 164 T cells are of a Th1 phenotype expressing IFN-γ and little or no IL-4, IL-5, IL-10, or TNF-α, while CD4$^+$ 4.13 T cells secrete IFN-γ and IL-10 and little or no IL-4, IL-5, or TNF-α. The same pattern was observed in Rag2$^{+/+}$ mice (data not shown). Because of the unexpected finding of both IFN-γ and IL-10 from GAD65$_{555–567}$ stimulation, we performed intracellular staining for IFN-γ and IL-10 to determine whether both of these cytokines are derived from the same cell. As shown in Fig. 6, we found that T cells from 4.13/Rag2$^{o/o}$ mice generate IFN-γ independently of IL-10 and therefore peripheral 4.13 CD4$^+$ T cells are of a mix of Th1 and Tr1 cells types, while 164 T cells are of a Th1 phenotype generating only IFN-γ. Additional cytokine measurements revealed that 4.13 T cells do not secrete TGF-β1 (supplemental Fig. S2). Because IL-10 can be immunoregulatory, we addressed whether the commitment of 4.13 T cells to a Tr1 phenotype is a central or peripheral tolerizing event. CD4$^+$ T cells from thymus and spleens of and DR4/4.13/Rag2$^{+/+}$ mice were FACS sorted and stimulated with irradiated APC, and then assayed for IL-10 and IFN-γ production. The 4.13 CD4$^+$ T cells from spleen generated IL-10 and IFN-γ in response to either CD3/CD28 or GAD65$_{552–572}$ stimulation, while thymus-derived CD4$^+$CD8$^-$ 4.13 T cells secreted neither cytokine (Fig. 7).

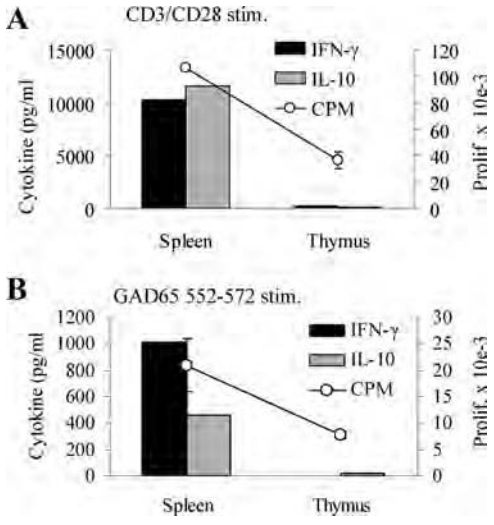

FIGURE 7. IFN-γ and IL-10 production from stimulated sorted CD4$^+$CD8$^-$ cells from DR4/4.13/Rag2$^{+/+}$ mice. CD4$^+$CD8$^-$ cells from spleen, lymph node, and thymus were FACS sorted from tissues taken from 8- to 12-wk-old mice and stimulated with irradiated APC and either CD3/CD28 Ab (*A*) or GAD65$_{552–572}$ (*B*).

Discussion

Limiting pathogenic autoreactivity is of the utmost importance for a successful immune system, and several mechanisms provide functional checkpoints for this control. These mechanisms broadly fit into three categories: those that involve deletion of autoreactive cells, centrally and/or peripherally; those that involve down-modulation of activation molecules or receptors, changing activation thresholds; and those that involve active immune regulation. In this study we evaluated central and peripheral tolerance mechanisms using two TCR transgenic mice containing structurally similar receptors specific for a naturally processed self Ag. These TCR were derived from autoreactive CD4$^+$ T cells present in humans with immunity to GAD65, an important islet Ag associated with autoimmune diabetes. On a C57BL/6 "diabetes-resistant" background transgenic for HLA-DR4, the human class II-restricting element for these TCR, very potent in vivo tolerance mechanisms were observed. The 164 TCR was associated with strong deletional events, both in the thymus and in the periphery, and surviving 164 T cells down-modulated TCR expression and/or switched from CD4 to CD8 phenotype, even as they maintained specific Ag reactivity. In marked contrast, the 4.13 TCR had less sensitivity to negative selection and no CD4-to-CD8 skewing, but instead used a predominant pathway of immunomodulation, skewing toward an IL-10 phenotype.

Both 164 and 4.13 T cells use Vα12.1/Vβ5.1 TCR and differ only in CDR3, a region that conventionally interacts primarily with the peptide in the Ag-binding MHC (33). Based on the higher thymic cellularity in 4.13 mice compared with 164 mice and the

absence of differentiation toward the $CD4^-CD8^+$ pathway, it appears that the 164 TCR is of a higher avidity to peptide-MHC complexes in the thymus. As T cell CD4 avidity interaction with the β_2 domain of the MHC class II has been shown to contribute positively to thymic T cell selection (21, 34), the differentiation of immature $CD4^+CD8^+$ double-positive 164 thymocytes into $CD4^-CD8^+$ mature cells would presumably lower the TCR overall avidity to the MHC complex and enable escape from negative selection. This skewing toward a $CD4^-CD8^+$ expression pathway and away from a $CD4^+CD8^-$ pathway occurred despite the class II restriction of the original human 164 T cell clone. Consistent with this interpretation is our observation that peripheral $CD4^-CD8^+$ 164/$Rag2^{o/o}$ T cells have less functional avidity to $GAD65_{555–567}$ stimulation than do $CD4^+CD8^-$ 164/$Rag2^{o/o}$ T cells. The skewing of class II-restricted self Ag-reactive T cells toward a CD8 lineage has been observed in other TCR transgenic models, also in the context of strong negative selection (7, 8).

In addition to thymic deletion and CD4-to-CD8 skewing, T cells surviving in the 164 TCR mice showed significant down-regulation of the TCR molecule itself. This also is consistent with a strategy invoked for lowering avidity, and correlated in the mice with evidence of a very strong activation-induced cell death pathway. The end result of all these simultaneous high-avidity tolerance checkpoints was the presence in the peripheral circulation of a low number of autoreactive T cells, which nevertheless displayed strong Ag-specific proliferative and Th1 characteristics.

Considering that both 164 and 4.13 TCR use Vα12.1 and Vβ5.1 and are responsive to the same Ag, it was remarkable that 4.13 T cells showed a completely different tolerance induction profile. A more modest central tolerance for 4.13 T cells was reflected in less thymic deletion and normal $CD4^+CD8^-$ maturation, and similarly no evidence for peripheral activation-induced cell death or receptor down-modulation was observed. A likely explanation for the absence of peripheral activation of 4.13 T cells was the peripheral generation of IL-10-producing Tr1 regulatory cells in these mice. IL-10 is a potent regulatory cytokine and has been shown to be important in regulating colitis and autoimmunity in experimental autoimmune encephalomyelitis and collagen-induced arthritis models (35–38). The absence of IL-10 from sorted $CD4^+CD8^-$ T cells from the thymus upon stimulation with either CD3/CD28 or Ag-specific $GAD65_{552–572}$ peptide also indicates that generation of these IL-10-secreting T cells was a peripheral differentiation event. It is interesting to speculate that T cell-generated IL-10 in 4.13 mice could be preventing the activation of 4.13 T cells in the periphery, which contrasts with the activated phenotype in peripheral 164 mice. This hypothesis is currently being testing by crossing DR4/4.13/$Rag2^{o/o}$ mice onto IL-10-deficient mice. While both 164 and 4.13 peripheral T cells are specific for $GAD65_{555–567}$, because many TCR are degenerate in peptide recognition (39), we cannot exclude the possibility that cross-reactivity with other unknown ligands might contribute to the differences in functional profiles.

In the periphery, both 4.13 and 164 mice show an increase in $Foxp3^+$ cells, which is consistent with that seen in quasi-self Ag models (4, 40). The larger increase in the percentage of $Foxp3^+$ cells in 164 mice relative to 4.13 mice correlates with the increase in negative selection (higher avidity TCR) in the thymus. However, upon crossing to Rag2-deficient mice we did not detect peripheral $CD4^+CD25^+$ ($Foxp3^+$) cells from either 164 or 4.13 mice. This is in contrast to HA-specific and OVA-specific TCR transgenic mice on a Rag-deficient background where the Ag is expressed as a neo-self Ag (40–42). In these models up to half of peripheral T cells are $CD25^+$ and have a regulatory function. However, in TCR transgenic mice where the T cell-responsive Ag is endogenously expressed, $CD4^+CD25^+$ ($Foxp3^+$) Treg do not develop on a Rag-deficient background. This includes a myelin basic protein-specific TCR (43) and the BDC2.5 TCR (44). It has been suggested that a high-avidity interaction between T cells and APC in the thymus is required for Treg development (45). Considering the strong negative selection in the thymus of both TCR mice suggesting a high functional avidity of the TCR for MHC-Ag, we were surprised to not find $CD4^+CD25^+Foxp3^+$ cells in the periphery on $Rag2^{o/o}$ mice. A possible explanation for a lack of $Foxp3^+$ Treg in these mice may be that both of these TCR are of high enough avidity that they are beyond the threshold for Foxp3 differentiation (5).

Peripheral tolerance methods of anergy (10, 11), deletion (12–14), or the generation of Tr1(15) and Th3(16) cells are a second line of defense against T cell autoimmunity. Once in the periphery 164 cells displayed a strong activation phenotype in both spleen and lymph nodes resulting in continued down-modulation of their TCR and concomitant activation-induced cell death through an activated caspase-3 pathway. Consistent with this is the expression of CD95 (FAS) on 164 T cells through which signaling has been shown to mediate deletion-induced peripheral tolerance (46, 47). The 4.13 T cells, which populate the periphery to a greater extent, do not undergo this type of peripheral tolerance, most likely due to their apparent lower overall pMHC avidity.

Autoreactive cells, such as those used to derive the 164 and 4.13 TCR in this study, occur frequently in humans with autoimmune disease, in people who are genetically at risk of autoimmune disease, and in normal HLA-matched individuals (48–52). Nevertheless, overt autoimmune disease is relatively rare, reflecting the importance of tolerance checkpoints in normal immune function. Our study, using human autoimmune TCR and human MHC transgenic mice, directly demonstrates multiple mechanisms that, sometimes simultaneously, elicit both central and peripheral tolerance. Indeed, the two structurally similar TCR used, derived from human HLA-DR4 subjects, with specificity for the same Ag and restriction element and differing only in their CDR3 regions, revealed stark differences in deletional, compensatory, and immunomodulatory mechanisms. That such distinction occurs even with closely related autoreactive TCR underscores the importance of understanding the contribution of this variation to disease susceptibility, pathogenic pathways, and response to therapy.

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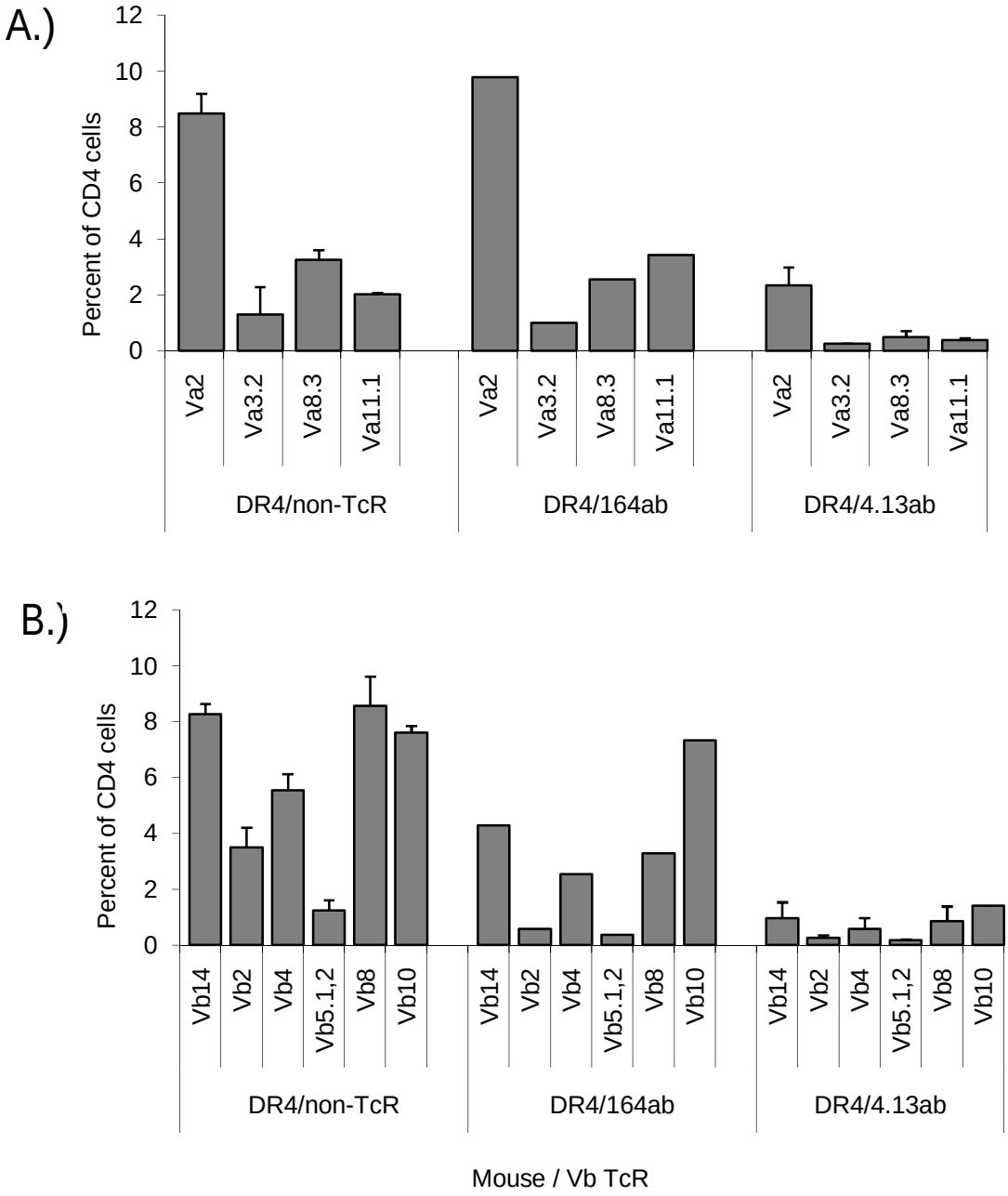
Disclosures

The authors have no financial conflicts of interest.

References

1. Bevan, M. J., K. A. Hogquist, and S. C. Jameson. 1994. Selecting the T cell receptor repertoire. *Science* 264: 796–797.
2. Lo, D., C. R. Reilly, L. C. Burkly, J. DeKoning, T. M. Laufer, and L. H. Glimcher. 1997. Thymic stromal cell specialization and the T-cell receptor repertoire. *Immunol. Res.* 16: 3–14.
3. Jameson, S. C., and M. J. Bevan. 1998. T-cell selection. *Curr. Opin. Immunol.* 10: 214–219.
4. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton. 2001. Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2: 301–306.
5. Fehervari, Z., and S. Sakaguchi. 2004. $CD4^+$ Tregs and immune control. *J. Clin. Invest.* 114: 1209–1217.
6. Barnden, M. J., W. R. Heath, and F. R. Carbone. 1997. Down-modulation of CD8 β-chain in response to an altered peptide ligand enables developing thymocytes to escape negative selection. *Cell. Immunol.* 175: 111–119.
7. Badami, E., L. Maiuri, and S. Quaratino. 2005. High incidence of spontaneous autoimmune thyroiditis in immunocompetent self-reactive human T cell receptor transgenic mice. *J. Autoimmun.* 24: 85–91.

8. Ranheim, E. A., K. V. Tarbell, M. Krogsgaard, V. Mallet-Designe, L. Teyton, H. O. McDevitt, and I. L. Weissman. 2004. Selection of aberrant class II restricted CD8$^+$ T cells in NOD mice expressing a glutamic acid decarboxylase (GAD)65-specific T cell receptor transgene. *Autoimmunity* 37: 555–567.
9. Goodnow, C. C. 1996. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. *Proc. Natl. Acad. Sci. USA* 93: 2264–2271.
10. Schwartz, R. H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21: 305–334.
11. Saibil, S. D., E. K. Deenick, and P. S. Ohashi. 2007. The sound of silence: modulating anergy in T lymphocytes. *Curr. Opin. Immunol.* 19: 658–664.
12. Redmond, W. L., C. H. Wei, H. T. Kreuwel, and L. A. Sherman. 2008. The apoptotic pathway contributing to the deletion of naive CD8 T cells during the induction of peripheral tolerance to a cross-presented self-antigen. *J. Immunol.* 180: 5275–5282.
13. Forster, I., and I. Lieberam. 1996. Peripheral tolerance of CD4 T cells following local activation in adolescent mice. *Eur. J. Immunol.* 26: 3194–3202.
14. Morgan, D. J., H. T. Kreuwel, and L. A. Sherman. 1999. Antigen concentration and precursor frequency determine the rate of CD8$^+$ T cell tolerance to peripherally expressed antigens. *J. Immunol.* 163: 723–727.
15. You, S., C. Chen, W. H. Lee, T. Brusko, M. Atkinson, and C. P. Liu. 2004. Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. *J. Immunol.* 173: 6777–6785.
16. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265: 1237–1240.
17. Curotto de Lafaille, M. A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J. J. Lafaille. 2008. Adaptive Foxp3$^+$ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29: 114–126.
18. Walker, M. R., B. D. Carson, G. T. Nepom, S. F. Ziegler, and J. H. Buckner. 2005. De novo generation of antigen-specific CD4$^+$CD25$^+$ regulatory T cells from human CD4$^+$. *Proc. Natl. Acad. Sci. USA* 102: 4103–4108.
19. Cabarrocas, J., C. Cassan, F. Magnusson, E. Piaggio, L. Mars, J. Derbinski, B. Kyewski, D. A. Gross, B. L. Salomon, K. Khazaie, et al. 2006. Foxp3$^+$CD25$^+$ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc. Natl. Acad. Sci. USA* 103: 8453–8458.
20. Pircher, H., K. Burki, R. Lang, H. Hengartner, and R. M. Zinkernagel. 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature* 342: 559–561.
21. Patel, S. D., A. P. Cope, M. Congia, T. T. Chen, E. Kim, L. Fugger, D. Wherrett, and G. Sonderstrup-McDevitt. 1997. Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR (α1*0101,β1*0401) transgenic mice. *Proc. Natl. Acad. Sci. USA* 94: 8082–8087.
22. Reijonen, H., R. Mallone, A.-K. Heninger, E. M. Laughlin, S. A. Kochik, B. Falk, W. W. Kwok, C. Greenbaum, and G. T. Nepom. 2004. GAD65-specific CD4$^+$ T-cells with high antigen avidity are prevalent in peripheral blood of patients with type 1 diabetes. *Diabetes* 53: 1987–1994.
23. Nepom, G. T., J. D. Lippolis, F. M. White, S. Masewicz, J. A. Marto, A. Herman, C. J. Luckey, B. Falk, J. Shabanowitz, D. F. Hunt, et al. 2001. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65). *Proc. Natl. Acad. Sci. USA* 98: 1763–1768.
24. Masewicz, S. A., N. Meldrum, V. Gersuk, L. Gaur, W. Hagopian, L. Moriarity, and G. T. Nepom. 2001. Complexity of human immune response profiles for CD4$^+$ T cell epitopes from the diabetes autoantigen GAD65. *Autoimmunity* 34: 231–240.
25. Ito, K., H. J. Bian, M. Molina, J. Han, J. Magram, E. Saar, C. Belunis, D. R. Bolin, R. Arceo, R. Campbell, et al. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J. Exp. Med.* 183: 2635–2644.
26. Reijonen, H., E. J. Novak, S. Kochik, A. Heninger, A. Liu, W. Kwok, and G. Nepom. 2002. Detection of GAD65 specific T-cells by MHC class II multimers in type 1 diabetes patients and at-risk subjects. *Diabetes* 51: 1375–1382.
27. Kouskoff, V., K. Signorelli, C. Benoist, and D. Mathis. 1995. Cassette vectors directing expression of T cell receptor genes in transgenic mice. *J. Immunol. Methods* 180: 273–280.
28. Kruisbeek, A. M. 2000. Isolation and fractionation of mononuclear cell populations. In *Current Protocols in Immunology*, suppl. 39th ed. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, eds. Wiley, New York, pp. 3.1.1–3.1.5.
29. Gebe, J. A., B. A. Falk, K. A. Rock, S. A. Kochik, A. K. Heninger, H. Reijonen, W. W. Kwok, and G. T. Nepom. 2003. Low-avidity recognition by CD4$^+$ T cells directed to self-antigens. *Eur. J. Immunol.* 33: 1409–1417.
30. Kisielow, P., H. Bluthmann, U. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4$^+$8$^+$ thymocytes. *Nature* 333: 742–746.
31. Gallegos, A. M., and M. J. Bevan. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* 200: 1039–1049.
32. Kawahata, K., Y. Misaki, M. Yamauchi, S. Tsunekawa, K. Setoguchi, J. Miyazaki, and K. Yamamoto. 2002. Generation of CD4$^+$CD25$^+$ regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression. *J. Immunol.* 168: 4399–4405.
33. Rudolph, M. G., and I. A. Wilson. 2002. The specificity of TCR/pMHC interaction. *Curr. Opin. Immunol.* 14: 52–65.
34. Riberdy, J. M., E. Mostaghel, and C. Doyle. 1998. Disruption of the CD4-major histocompatibility complex class II interaction blocks the development of CD4$^+$ T cells in vivo. *Proc. Natl. Acad. Sci. USA* 95: 4493–4498.
35. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
36. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
37. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161: 3299–3306.
38. Johansson, A. C., A. S. Hansson, K. S. Nandakumar, J. Backlund, and R. Holmdahl. 2001. IL-10-deficient B10.Q mice develop more severe collagen-induced arthritis, but are protected from arthritis induced with anti-type II collagen antibodies. *J. Immunol.* 167: 3505–3512.
39. Mazza, C., and B. Malissen. 2007. What guides MHC-restricted TCR recognition? *Semin. Immunol.* 19: 225–235.
40. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4$^+$CD25$^+$ regulatory T cells in vivo. *J. Exp. Med.* 198: 249–258.
41. Lerman, M. A., J. Larkin, III, C. Cozzo, M. S. Jordan, and A. J. Caton. 2004. CD4$^+$CD25$^+$ regulatory T cell repertoire formation in response to varying expression of a neo-self-antigen. *J. Immunol.* 173: 236–244.
42. Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3: 756–763.
43. Hori, S., M. Haury, A. Coutinho, and J. Demengeot. 2002. Specificity requirements for selection and effector functions of CD25$^+$4$^+$ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA* 99: 8213–8218.
44. Chen, Z., A. E. Herman, M. Matos, D. Mathis, and C. Benoist. 2005. Where CD4$^+$CD25$^+$ Treg cells impinge on autoimmune diabetes. *J. Exp. Med.* 202: 1387–1397.
45. Walker, L. S. 2004. CD4$^+$CD25$^+$ Treg: divide and rule? *Immunology* 111: 129–137.
46. Zhang, H. G., X. Su, D. Liu, W. Liu, P. Yang, Z. Wang, C. K. Edwards, H. Bluethmann, J. D. Mountz, and T. Zhou. 1999. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. *J. Immunol.* 162: 1423–1430.
47. Herndon, J. M., P. M. Stuart, and T. A. Ferguson. 2005. Peripheral deletion of antigen-specific T cells leads to long-term tolerance mediated by CD8$^+$ cytotoxic cells. *J. Immunol.* 174: 4098–4104.
48. Danke, N. A., D. M. Koelle, C. Yee, S. Beheray, and W. W. Kwok. 2004. Autoreactive T cells in healthy individuals. *J. Immunol.* 172: 5967–5972.
49. Oling, V., J. Marttila, J. Ilonen, W. W. Kwok, G. Nepom, M. Knip, O. Simell, and H. Reijonen. 2005. GAD65- and proinsulin-specific CD4$^+$ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects. *J. Autoimmun.* 25: 235–243.
50. Berthelot, L., D. A. Laplaud, S. Pettre, C. Ballet, L. Michel, S. Hillion, C. Braudeau, F. Connan, F. Lefrere, S. Wiertlewski, et al. 2008. Blood CD8$^+$ T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur. J. Immunol.* 38: 1889–1899.
51. Danke, N. A., J. Yang, C. Greenbaum, and W. W. Kwok. 2005. Comparative study of GAD65-specific CD4$^+$ T cells in healthy and type 1 diabetic subjects. *J. Autoimmun.* 25: 303–311.
52. Veldman, C. M., K. L. Gebhard, W. Uter, R. Wassmuth, J. Grotzinger, E. Schultz, and M. Hertl. 2004. T cell recognition of desmoglein 3 peptides in patients with pemphigus vulgaris and healthy individuals. *J. Immunol.* 172: 3883–3892.



S1. TcR Valpha (A) and Vbeta (B) expression on CD4 gated T cells in DR4 non-TcR tg, DR4/164ab, and DR4/4.13ab mice. Data are from mice between the ages of 8-12 weeks

Supplemental Figure S2

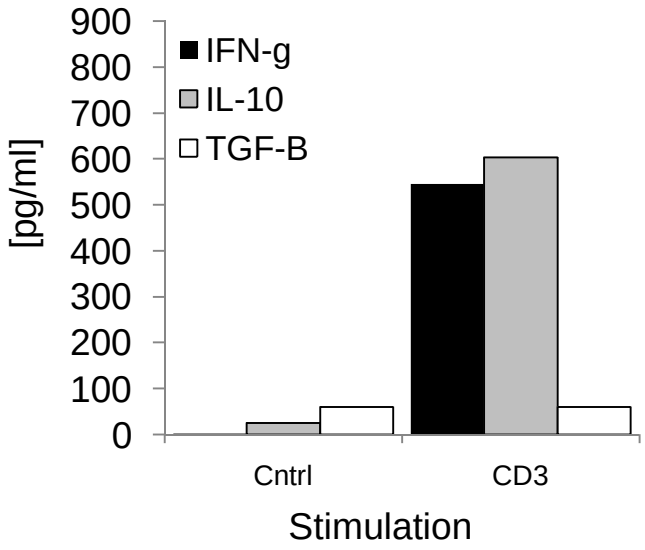


Figure S2. 4.13 TcR transgenic mice secrete IFN-g and IL-10, but not TGF–b1 upon stimulation. Purified CD4+ cells from DR4/4.13 mice were stimulatied with anit-CD3/CD28 at 2.0/0.2 ug/ml. Supernatants were taken at 72 hours and assayed for cytokines. Limit of detection for TGF-b1 was 60 pg/ml

Supplemental Figure S3

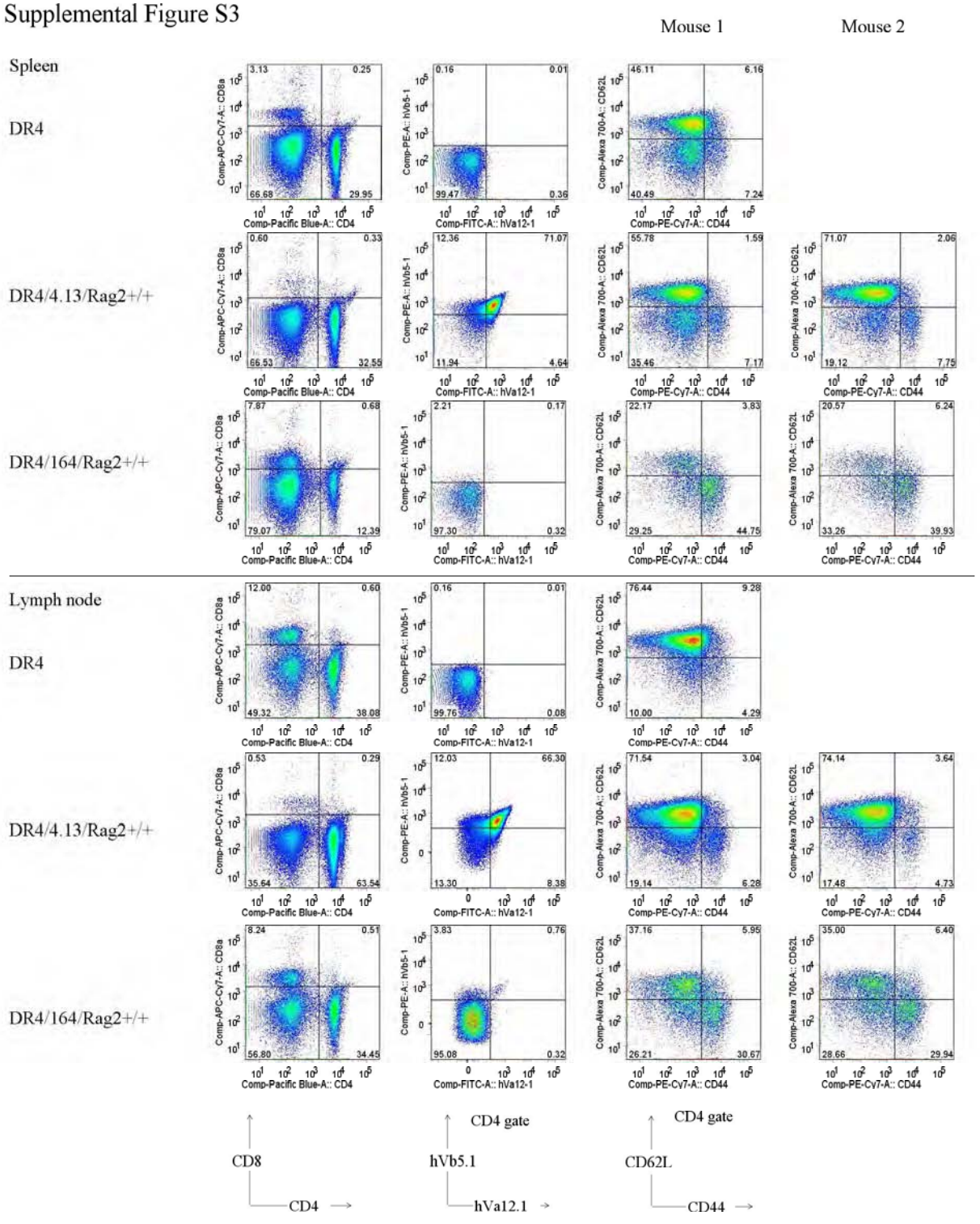


Figure S3. Surface phenotyping of spleen and lymph node cells from non-TcR transgenic 164/Rag2+/+, and 4.13/Rag2+/+ transgenic DR4 mice. Human TcR staining and CD44 vs CD62L Expression were done on CD4+/CD8- gated cells. Mice were 8-12 weeks of age.