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PRINCIPAL INVESTIGATOR:
Gerald T. Nepom MD PhD

CONTRACTING ORGANIZATION:
Benaroya Research Institute
Seattle, WA 98101

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14. ABSTRACT
We previously reported (now published, 2009) that two human GAD65 555-567-responsive T cell receptors (TcRs) expressed in HLA DR0401 transgenic mice exhibit different mechanisms of self-tolerance. 164 TcR transgenic mice evoke tolerance by strong thymic negative selection and peripheral activation-induced cell death (AICD). 4.13 TcR transgenic mice select the TcR transgene near normal thymic cellularity levels and in the periphery result as a combination of IFNγ-secreting Th1 cells and IL-10-secreting Tr1 cells. Through crosses onto IL-10 knockout mice, we now have preliminary evidence that the regulatory cytokine IL-10 is maintaining an unactivated phenotype in 4.13 mice, which is in contrast to the self-antigen-activated phenotype observed in T cells from 164 mice.

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Autoimmunity, diabetes, T cells, tolerance

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Humanized in vivo Model for Autoimmune Diabetes
Gerald T Nepom, MD, PhD; John A Gebe, PhD
Benaroya Research Institute
Seattle, WA 98101-2795
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Research Technical Report—Year 3

INTRODUCTION

This research study entails the use of humanized mice containing type 1 diabetes (T1D)-associated human HLA class II DR4 genes (DR4 mice) and addresses the fate and pathogenecity of high and low avidity autoreactive human T cells expressed in DR4 mice as transgenes. Both human T cells (4.13 and 164) are reactive to the same diabetes autoantigen glutamic acid decarboxylase 65 (GAD65) 555-567 epitope. Human GAD65 555-567 shares sequence homology with mouse GAD65 and GAD67 and thus serves as true self-antigen in this mouse model of autoimmunity.

BODY

Task 2a: As DR4/164β mice are not diabetes prone, nor do they exhibit pancreatic infiltrates indicative of a diabetic process (data not shown), we crossed DR4/164β mice onto our diabetes-prone RIP-B7/DR4 mice and monitored for diabetes by weekly blood glucose monitoring. As RIP-B7/DR4 mice become diabetic at around 30 weeks of age (1), we began monitoring RIP-B7/DR4/164 β mice at around 30 weeks of age. As shown in Figure 1, all mice maintained normal blood glucose over the monitoring period (diabetes is considered at 250 mg/dl blood glucose). Histological examination of the pancreas did not show signs of pre-diabetic islet infiltrate (data not shown).

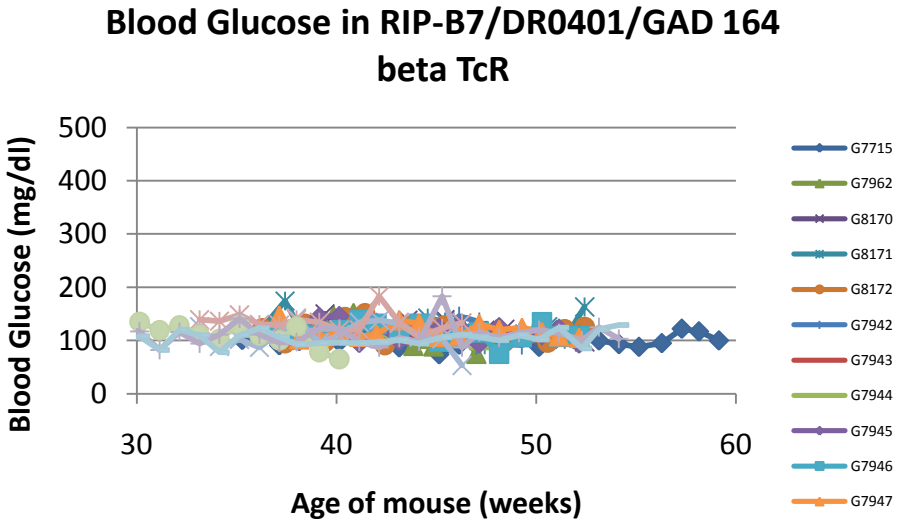

Figure 1. Blood glucose in GAD TcR mice. Blood glucose was monitored weekly by saphenous vein bleeds.

Task 2b: In 2009 we finished a publication comparing and contrasting 164 and 4.13 self-antigen-reactive TcR T cells (2). We explained that CD4+ T lymphocytes in DR4/4.13 TcR transgenic mice (lower functional MHC-TcR avidity) exhibit a combination of IL-10-secreting Tr1 and IFNγ-secreting Th1 cells, while CD4+ T cells in DR4/164 TcR mice (higher functional MHC-TcR avidity) exhibit only IFNγ-secreting Th1 cells (published Figure 5, appendix). Interestingly, CD4+ T cells in DR4/164 mice, but not DR4/4.13 T cells, have an in vivo activated phenotype and on a Rag2-/- background (expressing only a single TcR (published Figure 3, appendix). As DR4/164/Rag2-/- mice exhibit a CD4+ T cell islet infiltrate that correlated with a loss of islet insulin and pancreatic function (3) and IL-10 has an immune regulatory role (4), this observation led us to speculate that T cell-generated IL-10 in DR4/4.13 mice could be preventing its in vivo activation in stark contrast to the activated phenotype seen in non-IL-10-secreting DR4/164 T cells. In this third year, we have finished backcrossing DR4/4.13 TcR mice onto IL-10-/- mice and are testing the hypothesis that IL-10 plays a role in in vivo T cell tolerance of DR4/4.13 GAD65-specific autoreactive T cells. We now report from our initial experiments that CD4+ T cells in these mice directly exhibit ex vivo an upregulation of CD44 and a decrease in CD62L (Figure 2).

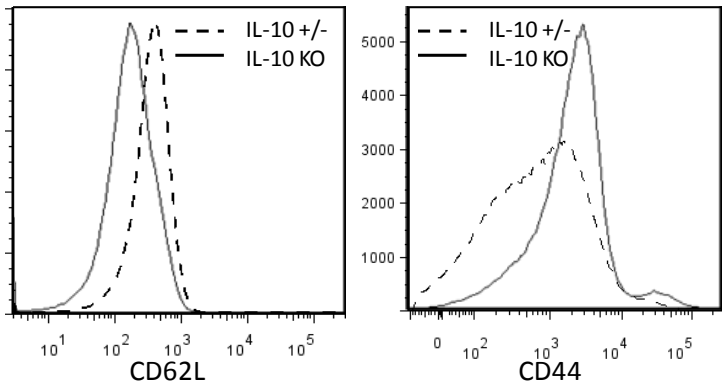

Figure 2. 4.13 transgenic T cells are activated in IL-10 knockout mice. Lymph node $CD4^+/V\alpha12.1^+/V\beta5.1^+$ T cells from 6-week-old DR4/4.13/IL-$10^{-/-}$ and DR4/4.13/IL-$10^{+/-}$ mice were analyzed by flow cytometry for CD62L and CD44 expression.

The increase in CD44 is a marker of antigen-specific activation of naïve or memory T cells (5-7) and down-modulation of CD62L is also a marker of T cell activation (11). The more activated phenotype seen in autoreactive CD4+ T cells from DR4/4.13/IL-10-/- mice is akin to that observed in CD4+ T cells from DR4/164/IL-10+/+ mice. These preliminary data support the hypothesis that IL-10 is mediating a tolerization effect upon autoreactive 4.13 T cells. In our previous published manuscript, we showed that activation-induced cell death (AICD) was a deletional mechanism of tolerance in DR4/164 mice (2). We are currently investigating whether AICD is now exhibited in DR4/4.13/IL-10-/- mice. Interestingly, CD44 also plays a role in maintenance of tolerance through its role in AICD (9).

While the exact mechanism by which IL-10 is preventing activation of 4.13 cells is unknown at this time, we hypothesize that IL-10-producing 4.13 T cells will prevent the T cell-activated phenotype seen in 164 TcR mice when both 4.13 and 164 T cells are present in the same host. These experiments will be done using 4.13/164 bone marrow chimeras. As both 164 and 4.13 TcRs use the same TcR Vα and TcR Vβ gene segments and cannot be tracked independently by TcR specific antibodies, we are generating a 4.13 congenic strain in which DR4/4.13 mice will express the antibody-specific CD45.1 surface antigen. As 164 mice express the CD45.2 antigen, this will allow independent tracking of 4.13 and 164 T cells in mixed bone marrow chimeric

mice. Mixed bone marrow chimeras will allow us to determine the in vivo extent to which autoantigen specific Tr1 (4.13) cells can tolerize Th1 (164) cells.

We have also backcrossed 164 mice onto a TcRCa-/- background in the hope of generating a GAD65 T cell-mediated hyperglycemic diabetes model. As DR4/164/Rag2-/-, but not DR4/164/Rag2+/+, mice exhibited an islet infiltrate, we have backcrossed DR4/164 mice onto TcRCa-/- mice to generate DR4/164/TcRCa-/- mice, which express only the transgenic TcR in the presence of a competent B cell compartment with the hope that the presence of B cells (important in non obese diabetic [NOD] mice [10]) will lead to either hyperglycemic diabetes or a greater islet infiltrate. We have completed generating DR4/164 TcR/TcRCa-/- mice, in which only the GAD65-specific TcR is present along with a full complement of the B cell compartment. As shown in Figure 3, DR4/164 CD4+ T cells in contrast to non-TcR transgenic T cells (non-TcR) are activated when on a TcRCa-/- background, while B cells (CD45R+) are not activated. We are now following a cohort of these mice (now at 20 weeks) for diabetes. Mice will be sacrificed at 30 weeks and assayed for islet infiltration and compared with 164/Rag2-/- mice.

DR4/164 TcR mice on a Rag2-/- background (but not on a Rag2+/+) are prone to an autoantigen GAD65-specific CD4+ T cell islet infiltrate, which correlates with a loss of islet insulin and pancreatic function measured by a glucose tolerance test (3). We observed that the absence of an infiltrate in 164/Rag2+/+ mice was also correlates with expression of endogenous murine TcR Vα chains pairing with the transgenic Vβ5.1 chain (diluting out GAD antigen specific T cells) (published Figure 4, appendix) and also with an increase in the percentage of Foxp3+ Treg cells among CD4+ T cells.

Tasks 2c and 2d: Success in these two tasks depended in part on the ability of human HLA DR4 tetramers (TmR) to bind to the two GAD TcR transgenic mouse T cells. While the human clones from which these two GAD TcRs came do bind HLA tetramers (11), unfortunately they do not bind to the same T cell receptor when expressed on mouse T cells (data not shown). We think this may be due in part to the mismatch between mouse CD4 (mouse T cell) and the human β2 domain (on the human TmR). Initially, we thought this might not be a hindrance, as we had shown that human HLA DR4 tetramers can bind to mouse T cells immunized with an antigen (5). As the mouse DR4 MHC is a chimera of peptide-inter-reacting human domains (α1 and 2) and β1 and 2 mouse domains, the correct mouse-mouse CD4-MHC interaction is preserved, and thus the functionality of the human TcR in the mouse is preserved. This was demonstrated in the published manuscript (2).

Tasks 2d: Regulatory cells. A surprising outcome from studying these native self-antigen human TcR transgenic mice was the unexpected near absence of CD4+/CD25+/FoxP3+ regulatory T cells (Treg) in either of these mice (published Figure 4, appendix). It has been demonstrated that Treg cells can be generated when the TcR-responding antigen is transgenically expressed (as a pseudo-self-antigen) (12-14). As the antigen to which 164 and 4.13 respond (GAD) is expressed in the thymus, the lack of selection of peripheral Tregs (mainly thought to occur in the thymus) in these self-reactive TcRs, mice may be more a function of where the antigen is expressed (i.e., type of cells) than whether it is expressed or not. This possibility has already been raised by others (15).

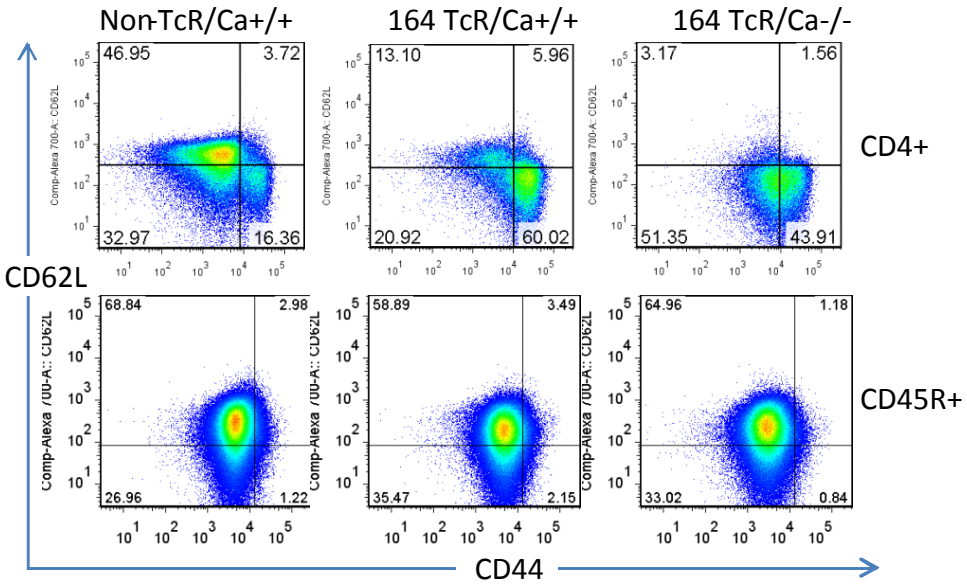

Figure 3. CD44 vs CD62L on CD4+ gated T cells and CD45R+ (B220+) gated B cells from DR4/non-TcR transgenic/Ca+/+, 164 TcR/Ca+/+, and 164 TcR/Ca-/- mice.

KEY RESEARCH ACCOMPLISHMENTS

- Published peer-reviewed manuscript (see reportable outcomes)
- Generation of 4.13 GAD TcR IL-10 knockout mice. IL-10 appears to have a regulatory role in the prevention of activation of self-antigen responsive 4.13 T cells.
- Generation of 164 GAD TcR/TcR $Ca^{-/-}$ mice. We can now test the hypothesis that the B cell compartment of the immune system will lead to a more severe form of insulitis and/or diabetes in 164 TcR mice.
- Generated 164 GAD TcR mice congenic for NOD idd3 and idd5 diabetes-susceptible loci.

REPORTABLE OUTCOMES—MANUSCRIPT

- 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. PMID: 19535636. PMCID: 2440666.

CONCLUSION

Our studies over the past two years have shown that clonotypically unique autoreactive T cells specific to the same antigen can have pathogenic effects as well as regulatory effects. This outcome was observed in two DR4-humanized mice expressing human GAD65 555-567 epitope-specific autoreactive T cells. High avidity GAD65-specific 164 T cells are of an IFNγ-secreting Th1 phenotype, in vivo activated, and are pathogenically capable of infiltrating islets. In contrast, 4.13 T cells specific for the same GAD65 epitope exhibit a more regulatory position as indicated by an IL-10-secreting Tr1 phenotype. Our preliminary data supported this regulatory potential, as CD4+ T cells from DR4/4.13/IL-10-/- mice displayed a more activated phenotype similar to that observed in 164 mice. 4.13 and 164 TcRs are two structurally similar autoreactive T cells responsive to the same autoantigen, use the same Vα and Vβ structural elements, and differ only in the structurally small CDR3 region. These results have implications in new therapeutic approaches aimed at antigen-specific targeting of autoreactive polyclonal T cell populations. Such an endeavor may inadvertently target protective antigen responses at the same time as eliminating pathogenic ones.

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APPENDIX

Gebe, J. A., B. B. Yue, K. A. Unrath, B. A. Falk, and G. T. **Nepom**. 2009. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J. Immunol.* 183: 59-65. PMID: 19535636. PMCID in process.

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

John A. Gebe,[2]* Betty B. Yue,* Kelly A. Unrath,† Ben A. Falk,* and Gerald T. Nepom*‡

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

*Benaroya Research Institute, Seattle WA 98101; †Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331; and ‡Department of Immunology, University of Washington School of Medicine, Seattle WA 98195

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[2] Address correspondence and reprint requests to Dr. John A. Gebe, Department of Diabetes, Benaroya Research Institute, 1201 9th Avenue, Seattle, WA 98101. E-mail address: jgebe@benaroyaresearch.org

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

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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.

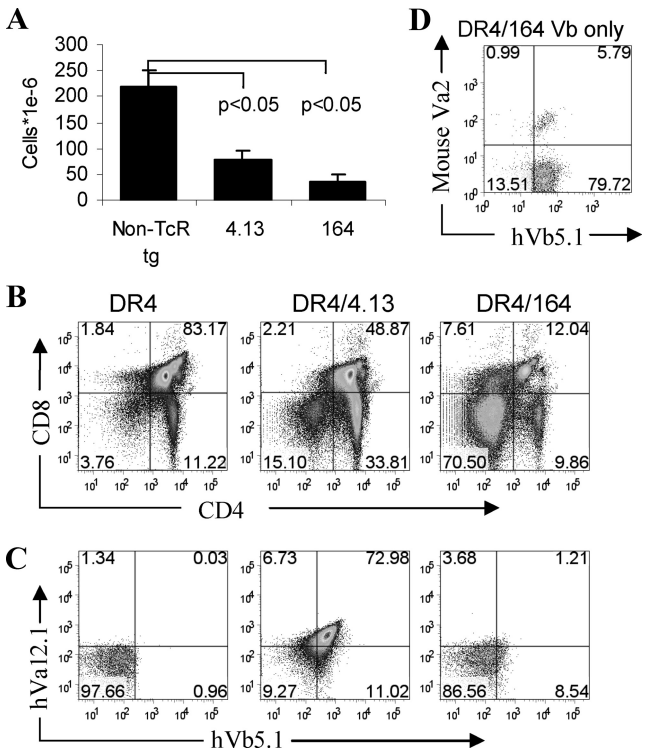

FIGURE 1. Thymic lymphocyte profiles in 164 and 4.13 human TCR transgenic HLA-DR4 mice. Thymus cellularity of 8- to 10-wk-old mice (n = 3) (*A*), CD4/CD8 profiles (*B*), and TCR hVb5.1 vs hVa12.1 expression on CD4$^+$CD8$^-$ gated cells (*C*). Human Vb5.1 expression on CD4$^+$CD8$^-$ thymocytes from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

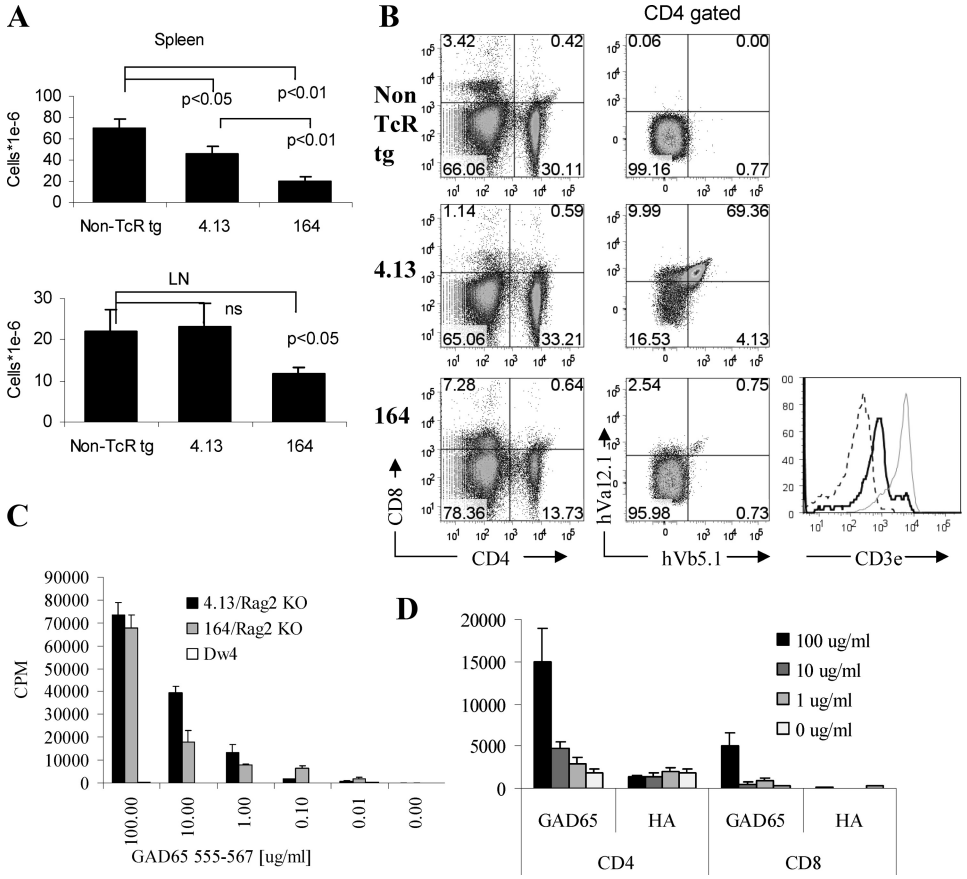

FIGURE 2. Peripheral lymphocyte profiles in 8- to 10-wk-old 164 and 4.13 TCR transgenic DR4 mice. Peripheral spleen and lymph nodes (inguinal and para-aortic combined) cellularity ($n = 3$) (*A*). CD4 vs CD8 profile and TCR human Va12.1 and Vb5.1 expression on $CD4^+CD8^-$-gated cells from spleen (*B*). 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.

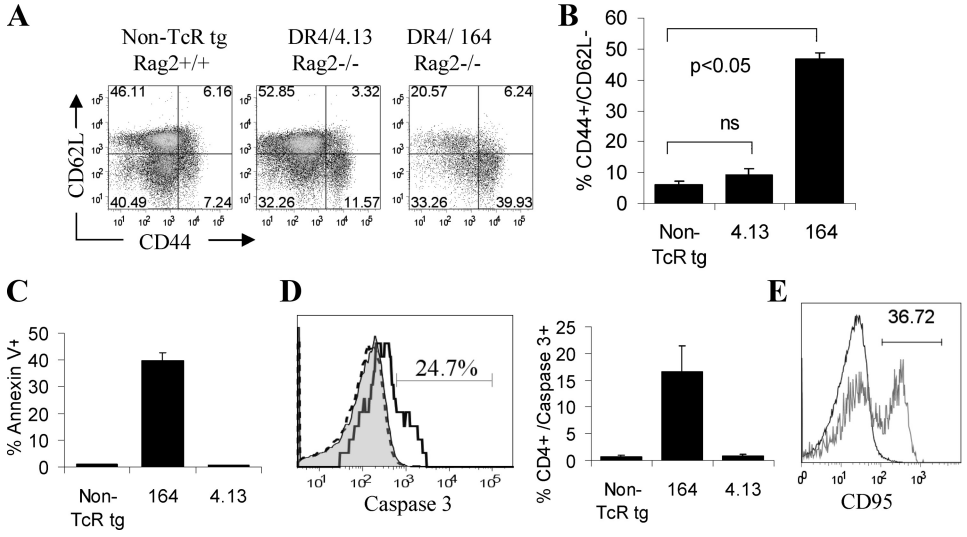

FIGURE 3. Activation and apoptosis in 164 and 4.13 TCR transgenic mice. Spleen cells from 8- to 12-wk-old DR4 mice on a Rag2$^{o/o}$ background were stained with activation markers CD44 and CD62L and gated on CD4$^+$CD8$^-$ cells for analysis (*A* and *B*). Percentage of gated CD4$^+$CD8$^-$ cells that 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*).

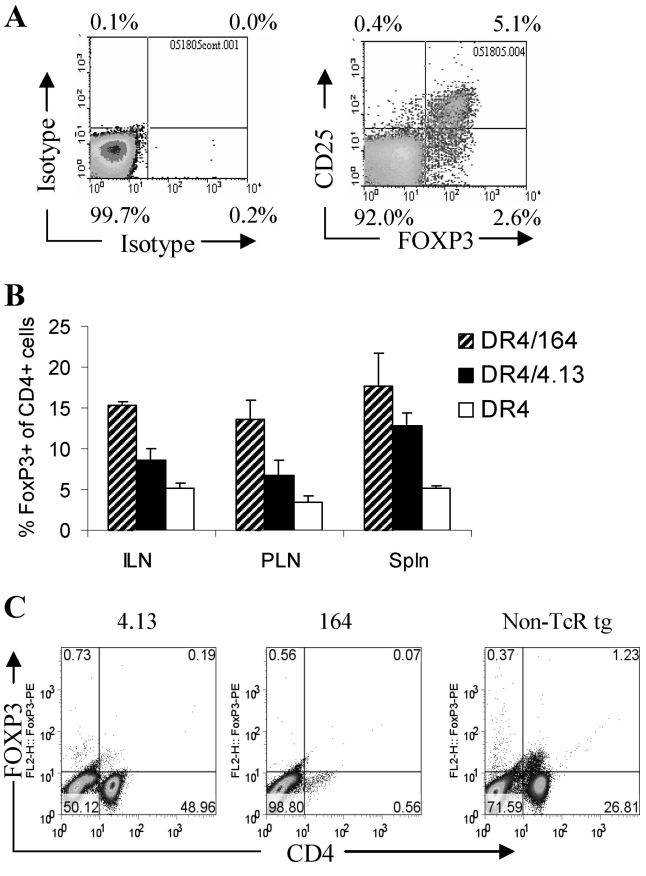

FIGURE 4. Foxp3 expression on spleen CD4$^+$CD8$^-$ T cells from non-TCR transgenic, 164, and 4.13 GAD TCR transgenic mice. Eight- to 12-wk-old mouse spleen cells from TCR and non-TCR transgenic mice were surface stained with CD4, CD25, and then intracellularly for Foxp3. Example of staining is shown 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*.

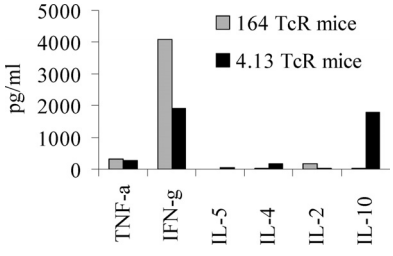

FIGURE 5. Cytokine profile of 164 and 4.13 T cells to GAD65$_{552–572}$. Splenocytes from DR4/164/Rag2$^{o/o}$ and DR4/4.13/Rag2$^{o/o}$ mice were stimulated with 100 μg/ml GAD65 or control peptide for 96 h. Supernatants were collected at 48 h and TNF-α, IFN-γ, IL-2, IL-4, and IL-5 were measured using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

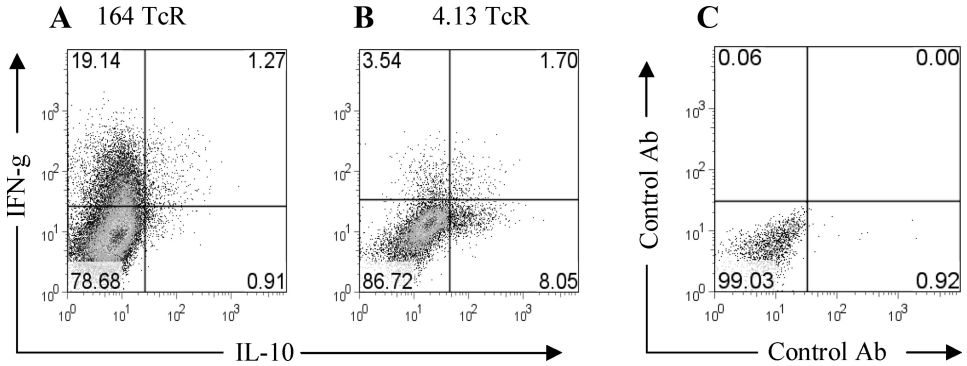

FIGURE 6. Internal cytokine staining for IL-10 and IFN-γ cells from 164 (*A*) and 4.13 (*B*) mice were intracellularly stained with IL-10 and IFN-γ directly conjugated Abs. Spleen cells from Rag2$^{o/o}$ 164 and 4.13 mice were stimulated for 4 days with GAD65$_{552–572}$ and then cultured with PMA/ionomycin 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).

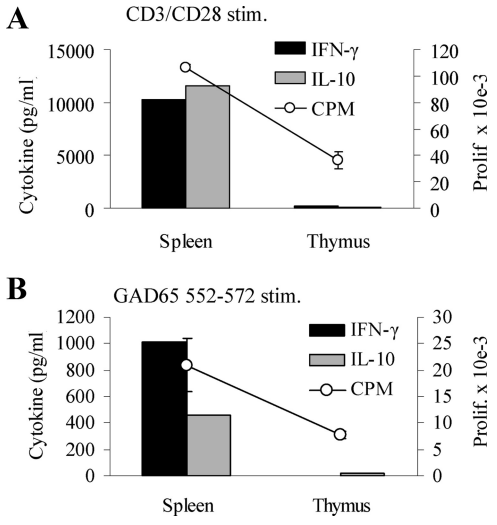

FIGURE 7. IFN-γ and IL-10 production from stimulated sorted CD4$^+$CD8$^-$ cells from DR4/4.13/Rag2$^{+/+}$ mice. CD4$^+$CD8$^-$ cells from spleen, lymph node, and thymus were FACS sorted from tissues taken from 8- to 12-wk-old mice and stimulated with irradiated APC and either CD3/CD28 Ab (*A*) or GAD65$_{552–572}$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

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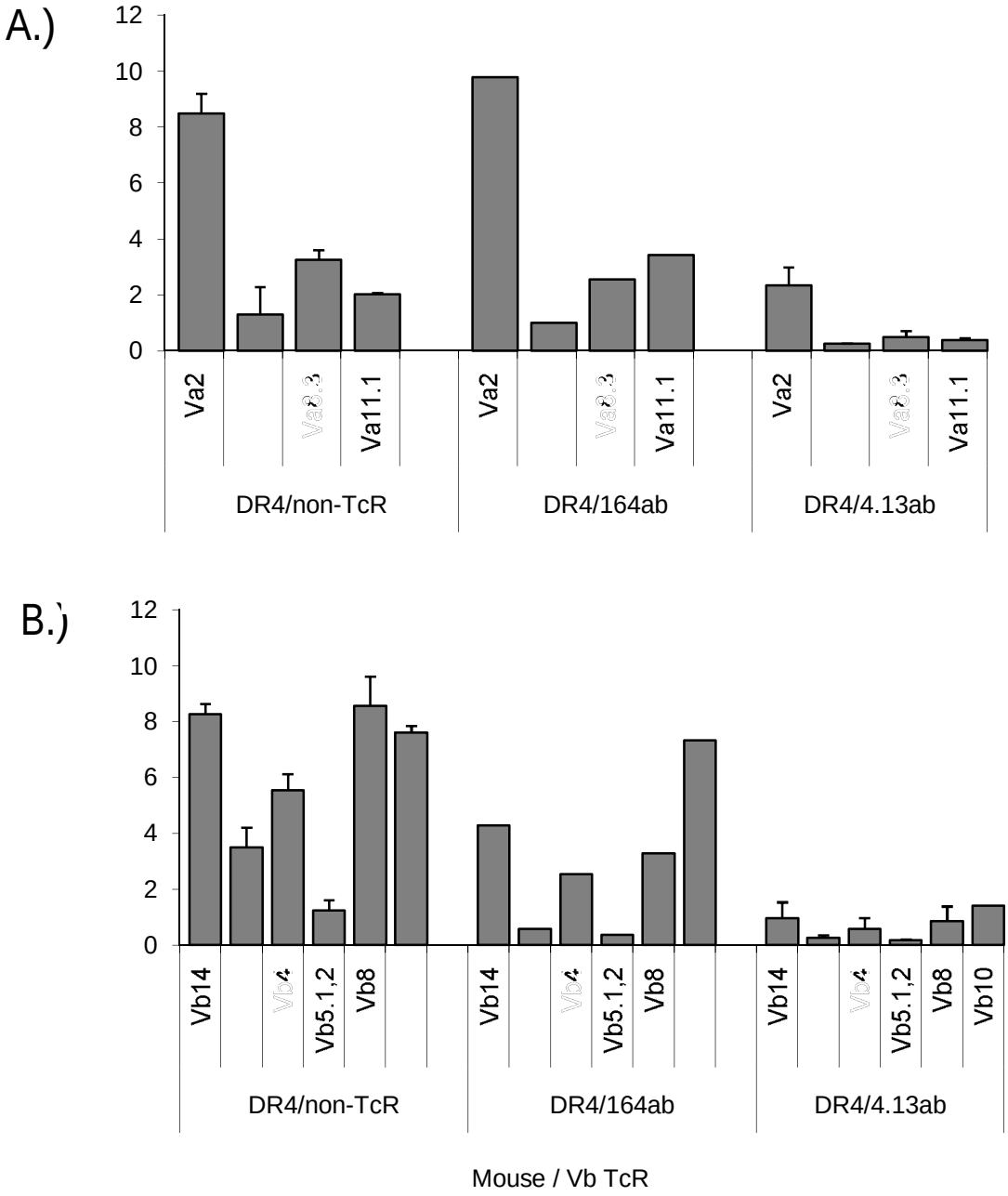

S1. TcR Valpha (A) and Vbeta (B) expression on CD4 gated T cells in DR4 non-TcR tg, DR4/164ab, and DR4/4.13ab mice. Data are from mice between the ages of 8-12 weeks

Supplemental Figure S2

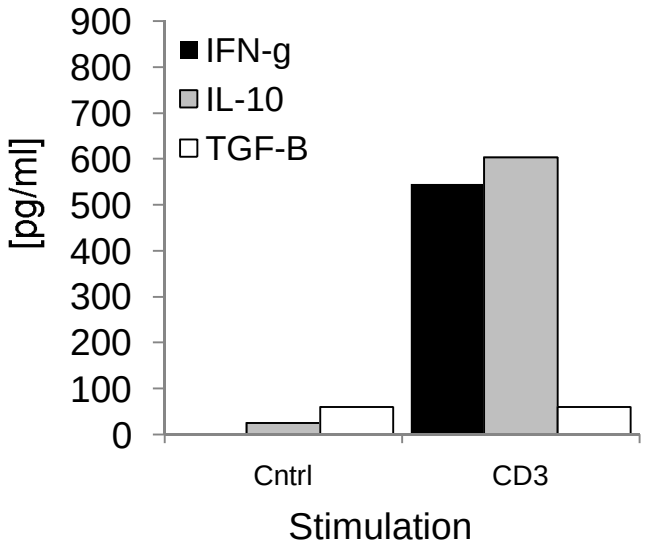

Figure S2. 4.13 TcR transgenic mice secrete IFN-g and IL-10, but not TGF–b1 upon stimulation. Purified CD4+ cells from DR4/4.13 mice were stimulatied with anit-CD3/CD28 at 2.0/0.2 ug/ml. Supernatants were taken at 72 hours and assayed for cytokines. Limit of detection for TGF-b1 was 60 pg/ml

Supplemental Figure S3

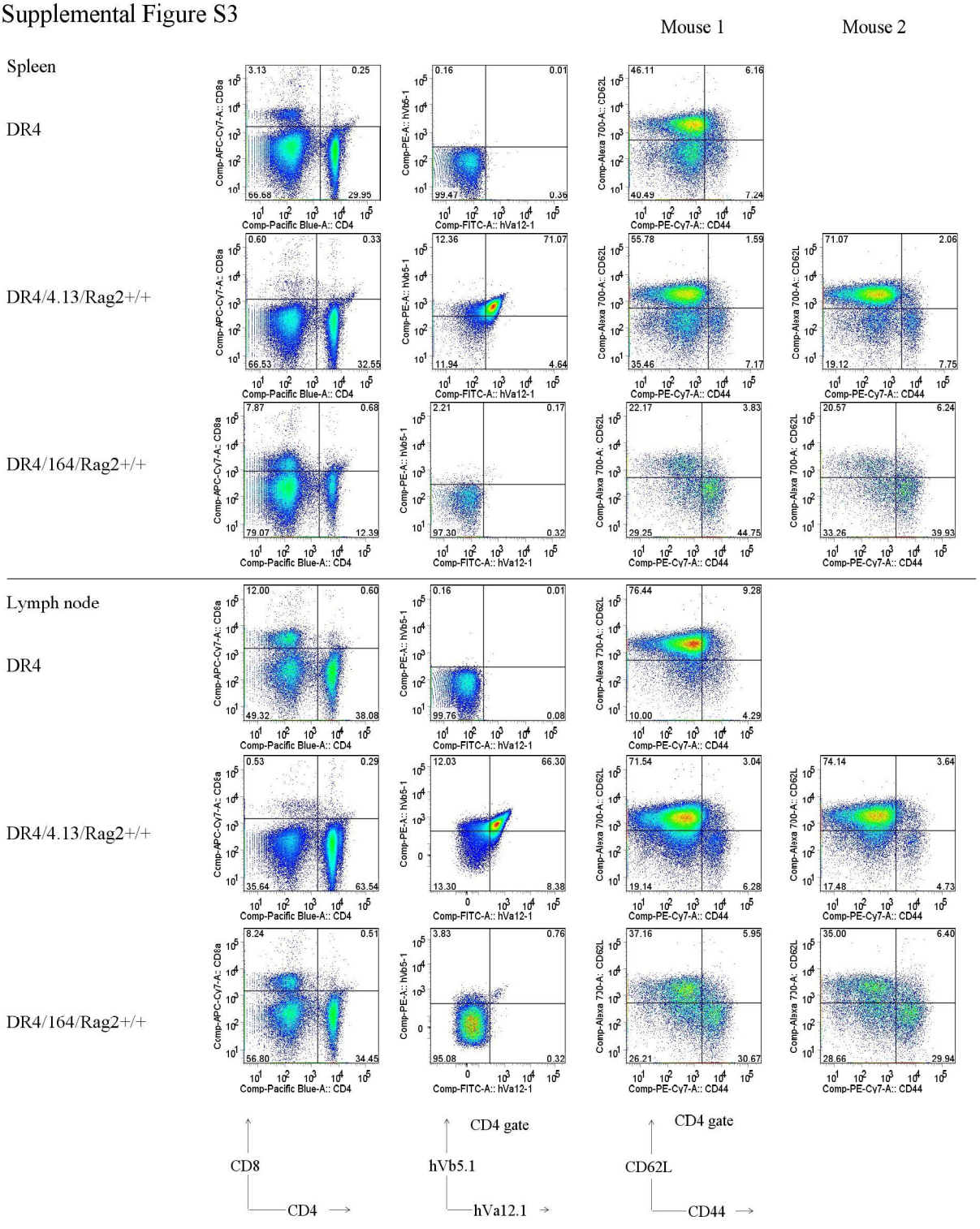

Figure S3. Surface phenotyping of spleen and lymph node cells from non-TcR transgenic 164/Rag2+/+, and 4.13/Rag2+/+ transgenic DR4 mice. Human TcR staining and CD44 vs CD62L Expression were done on CD4+/CD8- gated cells. Mice were 8-12 weeks of age.