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14. ABSTRACT
The CD4+ T cell response is critical for cellular autoimmunity in human T1D, but incomplete understanding of issues of specific cell frequency, avidity, function, and correlation with disease status presents major obstacles to improved therapies. This research study entails using humanized mice manifesting type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of high and low avidity T cells reactive to the putative autoantigen glutamic acid decarboxylase 65 (GAD65). By modeling the dominant human anti-GAD65 response in HLA- and TCR-transgenic mice, we proposed to determine whether pathogenic and/or regulatory responses correspond to high or low avidity profiles at different points during disease course. These ongoing studies indicate that the tolerance mechanisms used to prevent self-antigen GAD65 reactive T cells from eliciting autoimmunity in humanized DR4 HLA mice are diverse and that no single mechanism is exclusively used to maintain immune tolerance and prevent diabetes.

15. SUBJECT TERMS
Autoimmunity; type 1 diabetes; humanized mouse model; T cell; GAD65

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INTRODUCTION

This research study uses humanized mice containing type 1 diabetes (T1D)-associated human HLA molecules to address the fate and pathogenicity of T cells reactive to the autoantigen glutamic acid decarboxylase 65 (GAD65).

BODY

The focus in the second year of this grant has been on **Task 1** (Statement of Work, 2006, appended): To evaluate diabetes disease progression in the DR4/TCR models in order to test the hypothesis that high avidity autoreactive $CD4^+$ T cells escape from selection and persist in the periphery as dominant clonotypes; and to evaluate the fate and pathogenicity of high and low avidity autoreactive T cells representative of the human T1D repertoire.

In a follow up to last year's report on 164 and 4.13 TcR transgenic mice responsive to naturally processed GAD65 555-567, we now report (enclosed manuscript submitted) that these two T cells, both of which use Va12.1/Vb5.1 structural sequences and differ only in their CDR3 regions, display stark differences in both central and peripheral tolerance mechanisms to maintain peripheral self-tolerance when expressed as TcR transgenes. These differences make them an ideal model for our overall objectives to understand and modify the differing autoreactive T cells in humans.

In addition to the strong negative thymic selection (central tolerance) displayed in 164 mice, peripheral tolerance is also achieved through activation-induced cell death (AICD). Peripheral self-antigen reactive 164 T cells are activated in the periphery in spleen and lymph nodes and are also Annexin V, active Caspase 3, and CD95 positive (see enclosed manuscript, Figure 3). The expression of these molecules specific to 164 T cells, but not 4.13 T cells, is indicative of peripheral tolerance through AICD as a method to maintain tolerance to self antigen[1, 2]. This is likely the primary mechanism for lack of disease progression in these cases, and is sufficient to block disease induction by agonists or Treg deletion (**Task 1b, Task 1e, and Task 1f**).

In contrast, T cells from 4.13 mice are not activated in vivo but, like 164 T cells, do secrete IFN-γ upon in vitro stimulation (see enclosed manuscript, Figure 5). Interestingly, unlike 164 T cells, 4.13 CD4 T cells upon stimulation secrete IL-10 independently of IFN-γ (see enclosed manuscript, Figure 6). The differentiation into IL-10-producing cells (presumably Tr1 type) is a peripheral differentiating event, as proliferating mature T cells from the thymus do not secrete the cytokine (see enclosed manuscript, Figure 7). As IL-

10 has been shown to regulate immune responses[3, 4], these mice have been crossed onto IL-10-deficient mice to test the hypothesis that: 1. IL-10 is preventing these particular GAD65 self-antigen-responsive 4.13 cells from activation that is in contrast to 164 T cells; and 2. the lack of in vivo activation of 4.13 T cells by the IL-10-secreting cells is preventing their migration into the pancreatic islets, as was observed in 164 mice (**Task 1e**). Spontaneous islet infiltration has also not been observed in 4.13 mice on a $Rag2^{o/o}$ background (**Task 1f**).

We recently reported that 164 mice on a $Rag2^{o/o}$ background exhibited a T cell pancreatic islet infiltrate, which was associated with loss of islet insulin in infiltrated islets. However, the disease process in these mice did not continue to progress to frank diabetes. This intriguing finding leads to three alternatives:

1. The B cell compartment may regulate diabetes induction in our model[5]. HLA transgenic diabetic RIP-B7/DR4 mice do have a B cell islet infiltrate[6]. 164 mice have been crossed onto TcR $Ca^{o/o}$ mice to maintain monoclonality of the T cell repertoire and reintroduce the B cell compartment, and they are currently being monitored for islet infiltrate and diabetes.

2. In both the NOD mouse and human disease, other genetic components contribute to disease[7]. To study IL-2 and its association with Treg function, we are currently crossing 164 mice to B6 congenic strains containing both insulin-dependent diabetes (idd)3 (IL-2-containing loci) and idd5-susceptible loci.

3. Recent work has shown the importance of RORγt-mediated Th17 cells in both experimental autoimmune encypholomyelitis[8] and collagen-induced arthritis[9] models of autoimmunity. Through a collaboration, we have obtained CD4 promotor-driven RORγt mice and are crossing them to 164 mice to skew the cytokine profile from a Th1 (IFN-γ secreting) to a Th17 profile.

KEY RESEARCH ACCOMPLISHMENTS

- Tolerance to autoreactive Th1 164 T cells is maintained through a strong thymic negative selection (central tolerance) and also in the periphery by activation-induced cell death (peripheral tolerance).

- Central tolerance in 4.13 mice is also achieved by negative selection, albeit to a lesser extent, likely a reflection of its lower avidity. In contrast to peripherally activated 164 T cells, peripheral 4.13 T cells appear to achieve tolerance through the generation of IL-10-secreting Tr1 cells. 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$^{o/o}$ background, 4.13 mice on a Rag2$^{o/o}$ background do not exhibit islet infiltrates.

- 164 mice have been successfully crossed onto TcR Ca$^{o/o}$ 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 mice.

REPORTABLE OUTCOMES—Manuscripts

Gebe JA, Yue BB, Unrath KA, Falk BA, and Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol,* in press, 2009.

CONCLUSION

In a detailed analysis of tolerance in GAD65 555-567-responsive 164 and 4.13 TcR transgenic mice, we find that tolerance in 164 TcR transgenic mice is maintained at both the central level (thymic deletion) but also peripherally through Caspase 3-mediated AICD. This finding contrasts sharply to 4.13 TcR mice where peripheral tolerance through AICD is not evident. Furthermore, we find the differentiation of 4.13 T cells into IL-10-secreting Tr1 cells is a peripheral differentiating event as evident by a lack of IL-10 cytokine in stimulated mature CD4$^+$ thymocytes. As both T cells use identical Va12.1/Vb5.1 TcR segments, these data indicate that subtle sequence changes in the CDR3 region of TcRs responsive to the same antigen can lead to drastically different mechanisms of tolerance induction.

While autoreactive 164 TcR mice on a Rag2-deficient background exhibit a pancreatic infiltration[10], we have not observed this to be the case for 4.13 mice. It is tempting to speculate that the IL-10-secreting Tr1 nature of the 4.13 T cells may be responsible for preventing both the peripheral activation and islet infiltration, which is observed in 164 mice. Having generated IL-10 knockout mice containing 4.13 TcR, we are now in a position to answer this question. Having also crossed our TcR mice onto RORγt mice, we also can address the influence of the generation of Th17 cells in the islet infiltrative process in seen in 164 mice.

Understanding of these mechanisms that control the autoreactivity of the human 4.13 and 164 TcR will lead to informed choices for selecting and monitoring novel immunotherapy.

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APPENDIX

Gebe JA, Yue BB, Unrath KA, Falk BA, and Nepom GT. Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms. *J Immunol,* in press, 2009.

Humanized in vivo Model for Autoimmune Diabetes
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Statement of Work (May 2006)

Task 1. Evaluate diabetes disease progression in the DR4/TCR models (months 1-18)

a. Cross the DR4/TCR transgenics onto the DR4/RIP-B7 transgenics for an accelerated disease course (months 1-9)

b. Inject TLR agonists and/or anti-CD40 agonists for further disease acceleration (months 1-9)

c. Inject low-dose streptozotocin to initiate beta cell damage, testing the impact of antigen release in situ (months 1-9)

d. Transfer spleen and LN cells from DR4/TCR transgenic donors, activated in vitro, after labeling with CFSE, to track their in vivo course (months 6-18)

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 (months 6-18)

f. In all cases (a-e), 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 tests at regular intervals (months 1-18)

Task 2. Evaluate tetramer profiles and disease progression in the TCR 164b model (months 12-42)

a. Measure insulitis and glycemia in the DR4/l64b transgenics, while monitoring peripheral blood, LN and spleen cells for DR4-GAD tetramer binding profiles (months 12-42)

b. Measure cytokines and TCR alpha chain utilization in the GAD-responsive T cells, sorting for tetramer+ and activation profiles; compare high tetramer binding cells with low avidity cells (months 12-42)

c. Monitor disease progression comparing tetramer binding with the same parameters as (f) above. Do this in the presence of the disease accelerants selected for activity in Task 1 (months 12-42)

d. Determine if CD4+CD25+ GAD-specific regulatory T cells derive from the low or the high avidity end of the tetramer-binding spectrum, by flow sorting using tetramers and FOXP3 markers (months 12-42)

Task 3. Optimize the translational potential of the tetramer and disease profiles (months 18-42)

a. Compare peripheral blood tetramer profiles and T cell phenotypes with simultaneous LN and spleen profiles, at different stages of disease progression (months 18-42)

b. Elute lymphocytes from infiltrated pancreatic islets and evaluate for tetramer binding and FOXP3 staining, at different stages of disease progression (months 18-42)

c. Create a standardized multiparameter analysis protocol combining tetramer staining with the most informative T cell markers (determined in the above tasks) that is suitable for analysis of peripheral blood-derived lymphocytes (months 18-42)

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: 0000 0000.**

Central and peripheral mechanisms maintaining T cell tol erance 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 com plexes, in which high avidity T cells are eliminated through apo ptosis (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 immu nity, 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 func tional inactivation or anergy of the T cell (10, 11), activation induced T cell deletion (12 14), generation of suppressive cyto kine 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 com monly carry peripheral T cells reactive to a variety of islet asso ciated self Ags, including the immunodominant glutamic acid decarboxylase (GAD)$65_{555–567}$ peptide, a naturally processed epitope of glutamic acid decarboxylase (21 24). Interestingly, rec ognition of this epitope displays a biased TCR repertoire, with prevalent use of Vβ5.1/Vα12.1, although CDR3 regions are vari able (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

DR040 IE m ce (DR4) were obta ned from Tacon c These C57BL/6 I Ab$^{o/o}$ m ce express a human mouse ch mer c c ass II mo ecu e n wh ch the TCR nteract ng and pept de b nd ng doma ns of mouse I E (doma ns α_1 and β_1, exon 2 n both genes) have been rep aced w th the α_1 and β_1 doma ns from DRA *0 0 and DRB *040 , respect ve y Retent on of the mur ne α_2 and β_2 doma ns a ows for the cognate mur ne CD4 mur ne MHC nteract on (25)

TCR sequences for generat on of the two T ce transgen c m ce were obta ned from human CD4$^+$ T ce c ones 64 (26)and 4 3 (22) Both human T ce s are respons ve to the same se f Ag $GAD65_{555–567}$ and both use human Vα 2 /Vβ5 T ce receptors The 64 T ce was c oned from per phera b ood from an HLA DRA *0 0 /B *040 d abetes at r sk n d v dua as prev ous y descr bed (26)

C one 4 3 was c oned from the per phera b ood of an HLA DRA *0 0 /B *040 d abet c nd v dua (22) Human mouse ch mer c TCR transgenes were constructed by subcloning PCR amplified regions encod ng rearranged VαJα and VβDβJβ doma ns from the human c ones nto pTαcass and pTβcass TCR transgen c vectors, respect ve y (27) TCR transgen c vectors pTαcass and pTβcass conta n the natura mouse TCR α

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and β promoter/enhancer e ements and mouse Cα and Cβ constant reg ons, respect ve y DNA nject on nto C57BL/6/I Ab$^{o/o}$ (64 TCR) or F B6/C3H (4 3 TCR) mouse embryos was performed at the Un vers ty of Wash ngton (Seatt e, WA) n the Comparat ve Med c ne An ma Fac ty Founder m ce conta n ng the human TCR transgenes were then crossed onto DR040 IE m ce to generate DR4/ 64 and DR4/4 3 m ce Add t ona crosses were made onto Rag2$^{o/o}$ m ce A founder mouse was a so identified that contained only the 164 TCR β transgene Th s was a so crossed onto DR040 IE m ce The 4 3 TCR transgen c m ce generated n F B6/C3H were crossed for n ne generat ons nto DR040 IE m ce A an ma work was approved by the Benaroya Research Inst tute An ma Care and Use Comm ttee and an ma s were housed n the Benaroya Re search Inst tute Amer can Assoc at on of Laboratory An ma Care accred ted an ma fac ty

Tissue processing and flow cytometry

Thymus, sp een, and ymph node t ssues were processed nto s ng e ce suspens ons by gent y press ng through 0 40 μm ce stra ners (BD Fa con; ref no 352340) us ng the rubber end of a m tubercu n syr nge n DMEM 0 med a (Inv trogen; cata og no 965 092) supp emented w th 0% FBS (HyC one), 00 μg/m pen c n, 00 U/m streptomyc n, 50 μM 2 ME, 2 mM g utam ne, and mM sod um pyruvate (Inv trogen) Ce suspens ons were centr fuged at 200 × *g* for 0 m n, asp rated, and e ther) resuspended n DMEM 0 med a (ymph node and thymus) or 2) RBC were ysed (for sp eens) us ng m of ACK ys s buffer (28) for 5 m n at 37°C at wh ch t me 30 m of med a was added and ce s spun down (200 × *g*), asp rated, and resuspended n DMEM 0 med a The fo ow ng chro mophore-labeled Abs were used in flow cytometric analysis: anti-mouse CD4 (c one RM4 5), CD8 (c one 53 6 7), CD25 (c one PC6), CD62L (Me 4), ant human act ve caspase 3 (po yc ona , cata og no 55709), CD44 (IM7), Fc b ock (2 4G2), and PE abe ed annex n V (a from BD Pharm ngen), ant human Vβ5 PE (c one IMMU 57; Immunotech/ Cou ter), and Vα 2 FITC (c one 6D6; Endogen) FACS samp es n me d a were presta ned w th Fc b ock for 0 m n on ce and then sta ned w th specific Abs on ice for 45 min, washed once, and resuspended in FACS sta n buffer (PBS conta n ng % FBS, 0 % Na az de) before be ng un on a FACSCalibur or LSR II flow cytometer (BD Biosciences). Intracellular sta n ng of ce s for Foxp3 was performed us ng eB osc ence k t (FJK 6a Ab) accord ng to the manufacturer's nstruct ons Intrace u ar sta n ng for act ve caspase 3, mouse ant IFN γ (XMG 2; eB osc ence), and ant IL 0 (c one JES5 6E3; eB osc ence) was performed us ng eB osc ence ntrace u ar sta n ng k t (cata og no 88 8823 88; eB osc ence)

AQ: D

Proliferation assays

In lymph node or purified CD4$^+$ T ce pro ferat on assays × 0^5 ymph node ce s were cu tured w th 2 × 0^5 (3000 rad) Cs gamma rrad ated splenocytes (final volume 150 μ) Supernatants for cytok ne ana ys s were taken (50 μ) at 48 h, and μC /we ^{3}H]thym d ne was added at 72 h Thym d ne ncorporat on was assayed at 96 h us ng qu d sc nt at on count ng ana yzed on a M crobeta Tr Lux 450 sc nt at on counter (Wa ac Perk nE mer L fe Sc ences) Sp enocyte responses were measured n the same manner us ng 5 × 0^5 sp enocytes per we CD4 and CD8 s ng e pos t ve ce s were obta ned us ng M teny B otec beads w th pur ty of 90% or greater or by Ab labeling with CD4 and CD8 and sorting by flow cytometry

Cytokine analysis

Cytok nes IL 2, IL 4, IL 5, TNF α, and IFN γ were assayed us ng a mouse Th /Th2 cytok ne CBA k t (BD B osc ences; cata og no 55 287) IL 0 was assayed us ng a BD OptEIA mouse IL 0 ELISA set and mouse TGF β was measured us ng a human/mouse TGF β ELISA Ready SET Go! k t (BD B osc ences; cata og nos 555252 and 88 7344, respect ve y) Supernatants from tr p cate pro ferat on we s (50 μ /we) were com b ned for cytok ne ana ys s, w th 50 μ used for CBA ana ys s and 50 μ for IL 0 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 investi gate differential modes of T cell tolerance to the naturally pro cessed GAD65$_{555–567}$ autoantigen. The human CD4$^+$ T cell clones 164 and 4.13 (obtained from two different subjects) are structurally

Tab e 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	
4.13	5 1	A	L	S	E	N	R	G	G		A	S	K	L	
						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] G ay h ghl ghted a eas denote d ffe ences between 164 and 4 13 TCR CD3 sequences boldface, nonpola -to-nonpola am no ac d changes unde l ned es dues, pola -to-pola changes and pa entheses, cha ge changes

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 iden tical for GAD65 and GAD67 in both human and mouse and thus serves as a naturally processed self Ag T cell epitope in both spe cies (29). Both 4.13/Rag2$^{+/+}$ and 164/Rag2$^{+/+}$ mice display re duced 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

T1

F1

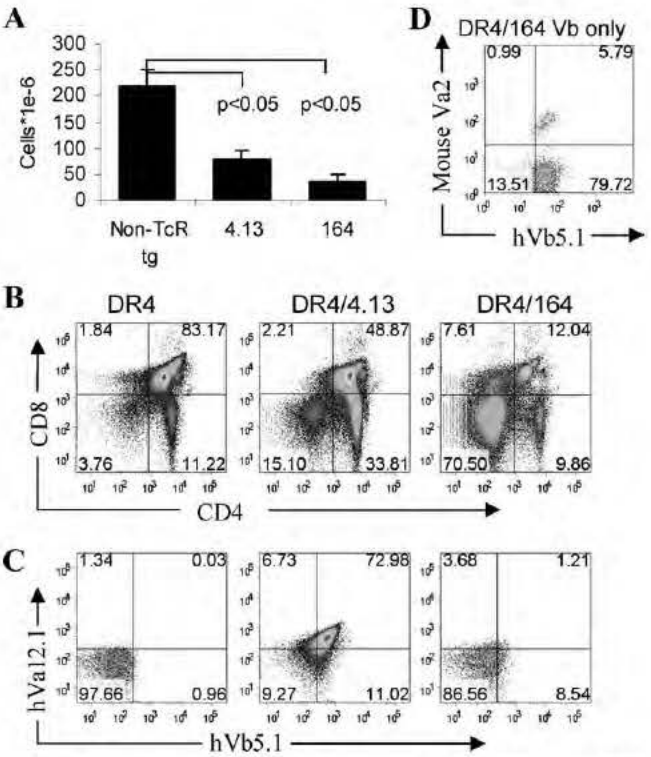

FIGURE 1. Thymic lymphocyte profiles in 164 and 4.13 human TCR transgen c HLA DR4 m ce Thymus ce u ar ty of 8 to 0 wk o d m ce (n = 3) (*A*), CD4/CD8 profiles (*B*), and TCR hVb5 vs hVa 2 expres s on on CD4$^+$CD8$^-$ gated ce s (*C*) Human Vb5 express on on CD4$^+$CD8$^-$ thymocytes from 64 Vb on y TCR m ce (*D*)

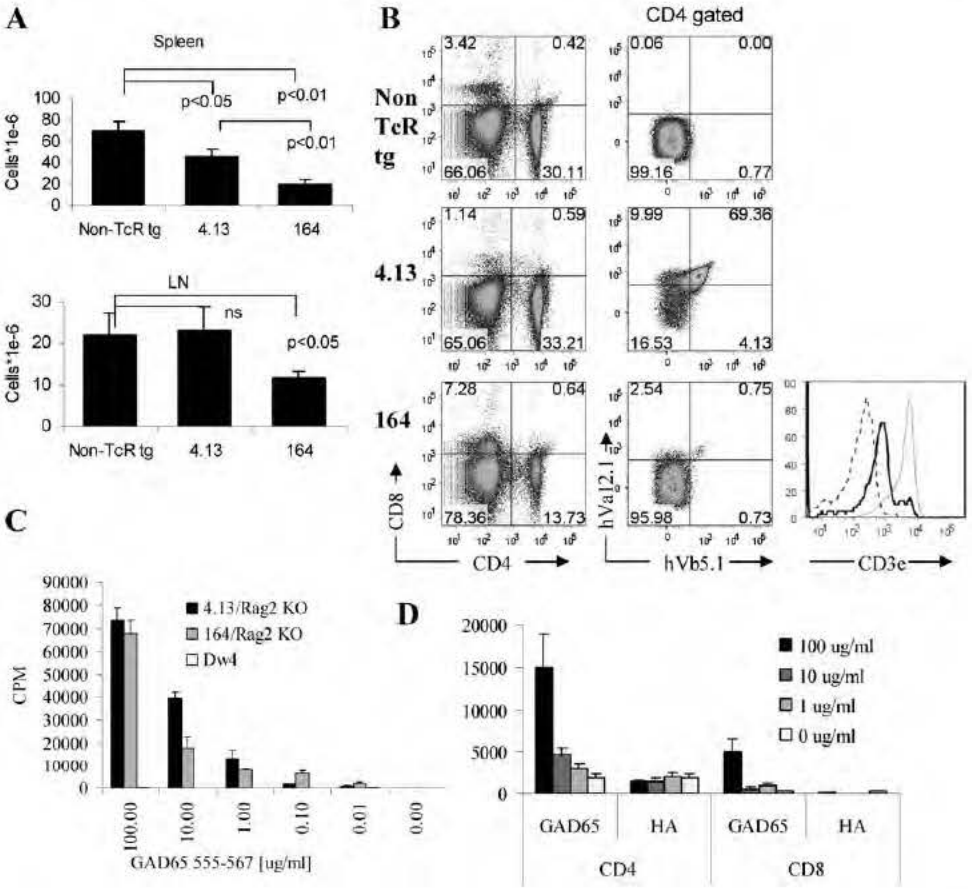

FIGURE 2. Per phera ympho cyte profiles in 8- to 10-wk-old 164 and 4 3 TCR transgen c DR4 m ce Per phera sp een and ymph nodes (ngu na and para aort c comb ned) ce u ar ty (n = 3) (A) CD4 vs CD8 profile and TCR human Va12.1 and Vb5 express on on $CD4^+CD8^-$ gated ce s from sp een (B) H sto gram n B shows CD3e express on on $CD4^+CD8^-$ gated ce s from 64 m ce (b ack ne), 4 3 m ce (gray ne), and sotype contro (dotted ne) Sp enocyte Ag dose response n 64 (gray) and 4 3 (b ack) TCR transgen c DR4 m ce on a $Rag2^{o/o}$ background (C) For $CD4^+CD8^-$ and $CD4^-CD8^+$ T ce st mu at on (D) cells were sorted by flow and tested for pro ferat on n response to $GAD65_{555–567}$ or contro Ag A ex per ments were repeated at east three t mes w th s m ar resu ts

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 stron ger negative selection observed in 164 mice is the down modu lated expression of the TCR on $CD4^+CD8^-$ thymocytes where only ~1% of mature $CD4^+CD8^-$ T cells express both Vα and Vβ transgenes (Fig. 1C). This is in stark contrast to the >70% expres sion 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. 1D). 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 cellular F2 ity in the spleen (Fig. 2A), 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. 2B). 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. 2B). Coinciding with the weak TCR expression in $164/Rag2^{+/+}$ mice is also a low expres sion of CD3e on $CD4^+CD8^-$ gated cells (Fig. 2B, 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. 2B). 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 stron ger 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 AQ: E 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. 2C). Be cause 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 pep tide. We find that both populations are Ag specific, with the CD8 164 cells having a lower proliferative response (lower functional avidity) (Fig. 2D).

Peripheral tolerance mediated by apoptosis

As with the low expression of the transgenic TCR on $164/Rag2^{+/+}$ thymocytes in the thymus (Fig. 2B), the TCR expression on

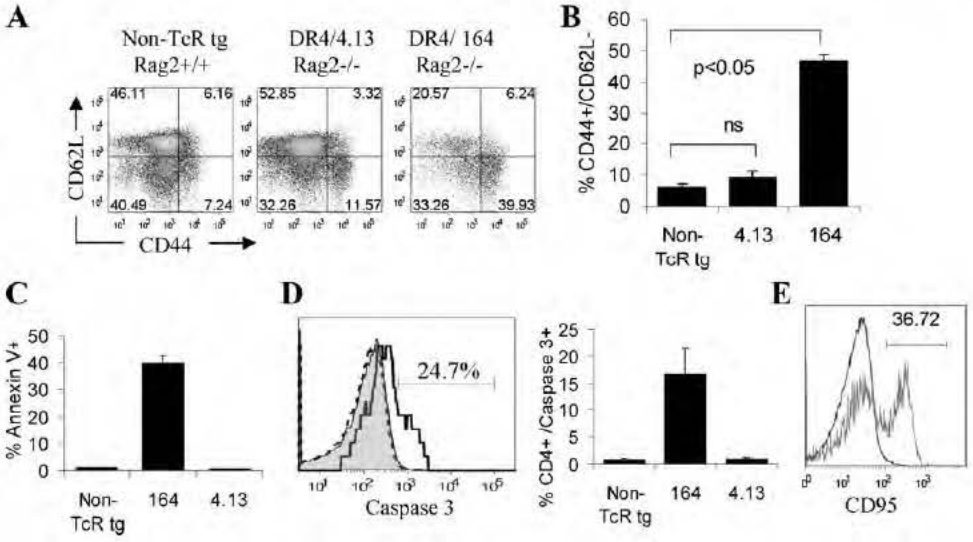

FIGURE 3. Act vat on and apo ptos s n 64 and 4 3 TCR trans gen c m ce Sp een ce s from 8 to 2 wk o d DR4 m ce on a Rag2$^{o/o}$ background were sta ned w th act va t on markers CD44 and CD62L and gated on CD4$^+$CD8$^-$ ce s for ana y s s (*A* and *B*) Percentage of gated CD4$^+$CD8$^-$ ce s that were annex n V$^+$ (*C*) and act ve caspase 3$^+$ (*D*) are shown Examp es of caspase 3 h sto grams n (*D*) are non TCR transgen c (gray filled), DR4/4.13/Rag2$^{o/o}$ (b ack dashed ne), and DR4/ 64/ Rag$^{o/o}$ (b ack heavy ne) Percent ages are from three m ce n each group CD4$^+$ sp een T ce s from 64 (gray ne) m ce are a so CD95$^+$ com pared w th non TCR CD4$^+$ T ce s (b ack ne) (*E*)

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 activa tion 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 na ture expressing high levels of CD62L and intermediate levels of CD44 (CD62L^{high}CD44^{int}) (Fig. 3*A*). In contrast, ~40% of pe F3 ripheral 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). There fore, we tested whether the low numbers of T cells in the periph eral 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 com pared 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*).

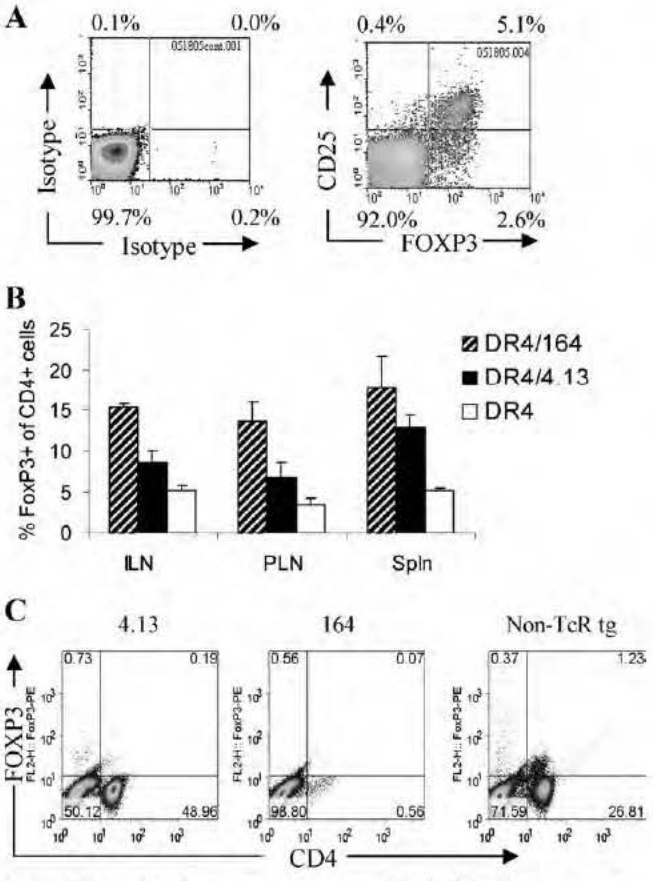

FIGURE 4. Foxp3 express on on sp een CD4$^+$CD8$^-$ T ce s from non TCR transgen c, 64, and 4 3 GAD TCR transgen c m ce E ght to 2 wk o d mouse sp een ce s from TCR and non TCR transgen c m ce were surface sta ned w th CD4, CD25, and then ntrace u ar y for Foxp3 Ex amp e of sta n ng s shown n (*A*) on non TCR transgen c DR4 sp enocytes Foxp3 express on n Rag2$^{+/+}$ m ce as a percentage of CD4$^+$ ce s s shown n (*B*) (average of three m ce) Foxp3 express on on CD4$^+$ T ce s from 64 and 4 3 TCR transgen c m ce on a Rag2$^{o/o}$ background s shown n *C*

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

CD4$^+$CD25$^+$ cells that express Foxp3 participate in immune reg ulation, and the selection of these Treg can be mediated in foreign

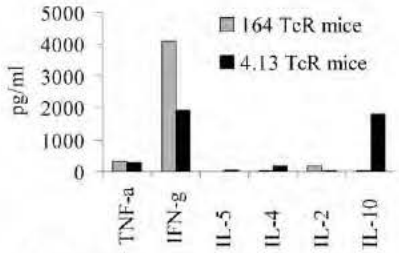

FIGURE 5. Cytokine profile of 164 and 4.13 T cells to GAD65$_{552–572}$ Sp enocytes from DR4/ 64/Rag2$^{o/o}$ and DR4/4 3/Rag2$^{o/o}$ m ce were st m u ated w th 00 μg/m GAD65 or contro pept de for 96 h Supe natants were co ected at 48 h and TNF α, IFN γ, IL 2, IL 4, and IL 5 were mea sured us ng a mouse Th /Th2 k t, and IL 0 was measured by ELISA Exper ment was done three t mes w th s m ar resu ts

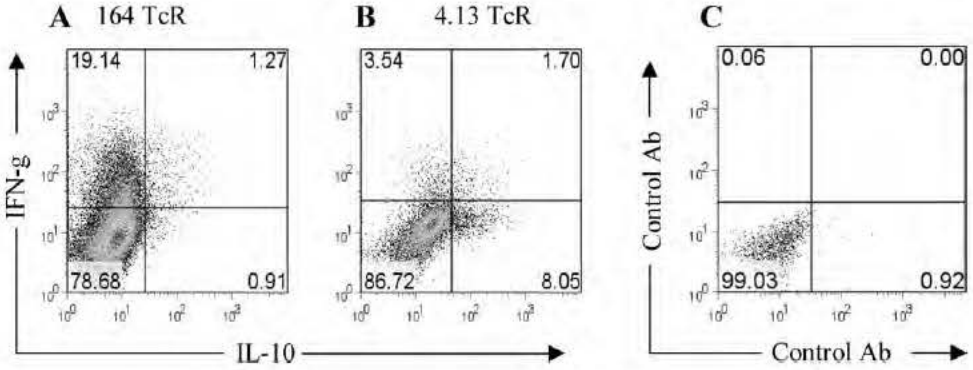

FIGURE 6. Interna cytok ne sta n ng for IL 0 and IFN γ ce s from 64 (*A*) and 4 3 (*B*) m ce were ntrace u ar y sta ned w th IL 0 and IFN γ d rect y conjugated Abs Sp een ce s from Rag2$^{o/o}$ 64 and 4 3 m ce were st mu ated for 4 days w th GAD65$_{552–572}$ and then cu tured w th PMA/ ono myc n for 4 h w th brefe d n A dur ng the ast 2 h Exper ment was done three t mes w th s m ar resu ts

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 cyto kine measurements revealed that 4.13 T cells do not secrete TGF β1 (supplemental Fig. S2). Because IL 10 can be immuno regulatory, 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 DR4/164/Rag2$^{o/o}$ and DR4/4.13/Rag2$^{o/o}$ 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).

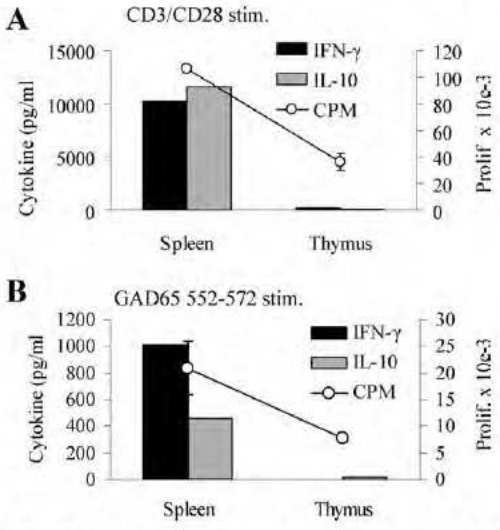

FIGURE 7. IFN γ and IL 0 product on from st mu ated sorted CD4$^+$CD8$^-$ ce s from DR4/4 3/Rag2$^{+/+}$ m ce CD4$^+$CD8$^-$ ce s from sp een, ymph node, and thymus were FACS so ted from t ssues taken from 8 to 2 wk o d m ce and st mu ated w th rrad ated APC and e ther CD3/CD28 Ab (*A*) or GAD65$_{552–572}$ (*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 mod ulation 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 re ceptors 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 auto immune 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 re-activity. 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 ap pears 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 over all 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 path way. The end result of all these simultaneous high avidity toler ance 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 recep tor 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
AQ: F 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
AQ: G periphery, which contrasts with the activated phenotype in periph eral 164 mice. This hypothesis is currently being testing by cross ing 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 un known 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. How ever, 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
AQ: H 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). Con sidering 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 dis ease, and in normal HLA matched individuals (48 52). Neverthe less, 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. In deed, 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 immunomodu latory mechanisms. That such distinction occurs even with closely related autoreactive TCR underscores the importance of under standing 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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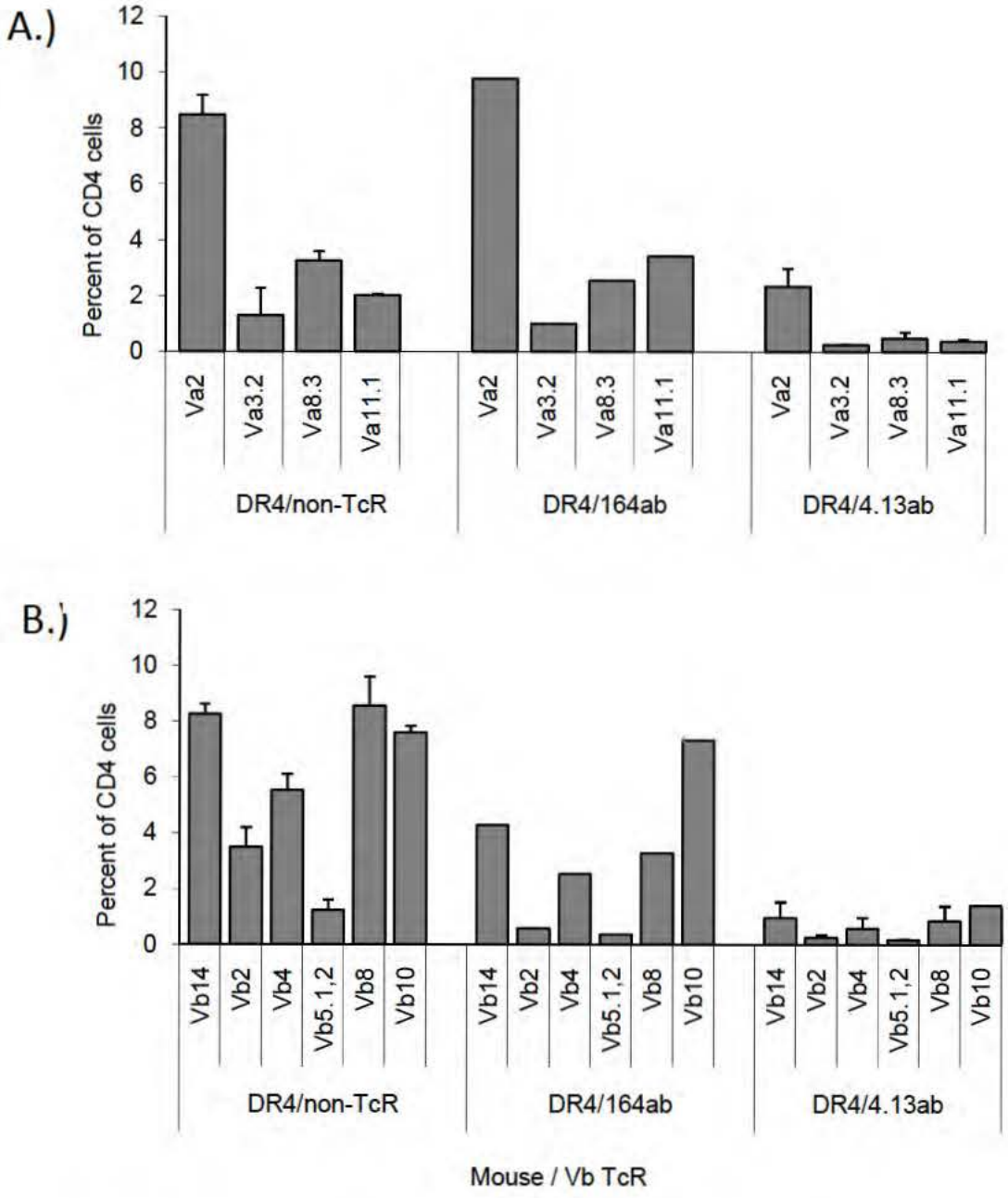

S1. TcR Valpha (A) and Vbeta (B) expression on CD4 gated T cells in DR4 non-TcR tg, DR4/164ab, and DR4/4.13ab mice. Data are from mice between the ages of 8-12 weeks

Supplemental Figure S2

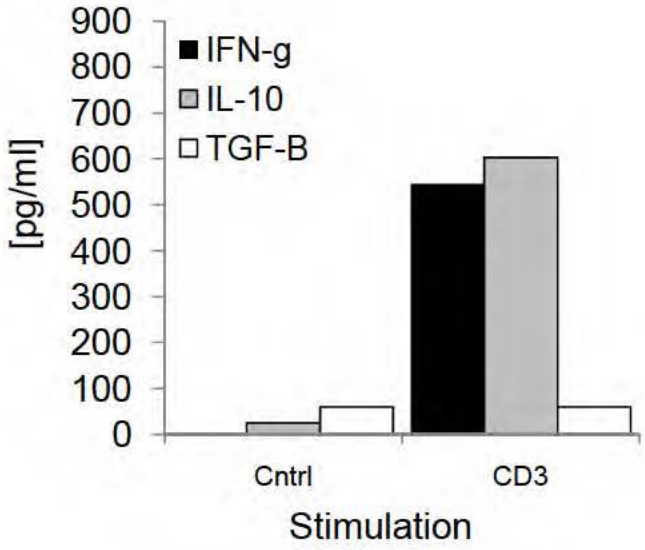

Figure S2. 4.13 TcR transgenic mice secrete IFN-g and IL-10, but not TGF–b1 upon stimulation. Purified CD4+ cells from DR4/4.13 mice were stimulatied with anit-CD3/CD28 at 2.0/0.2 ug/ml. Supernatants were taken at 72 hours and assayed for cytokines. Limit of detection for TGF-b1 was 60 pg/ml

Supplemental Figure S3

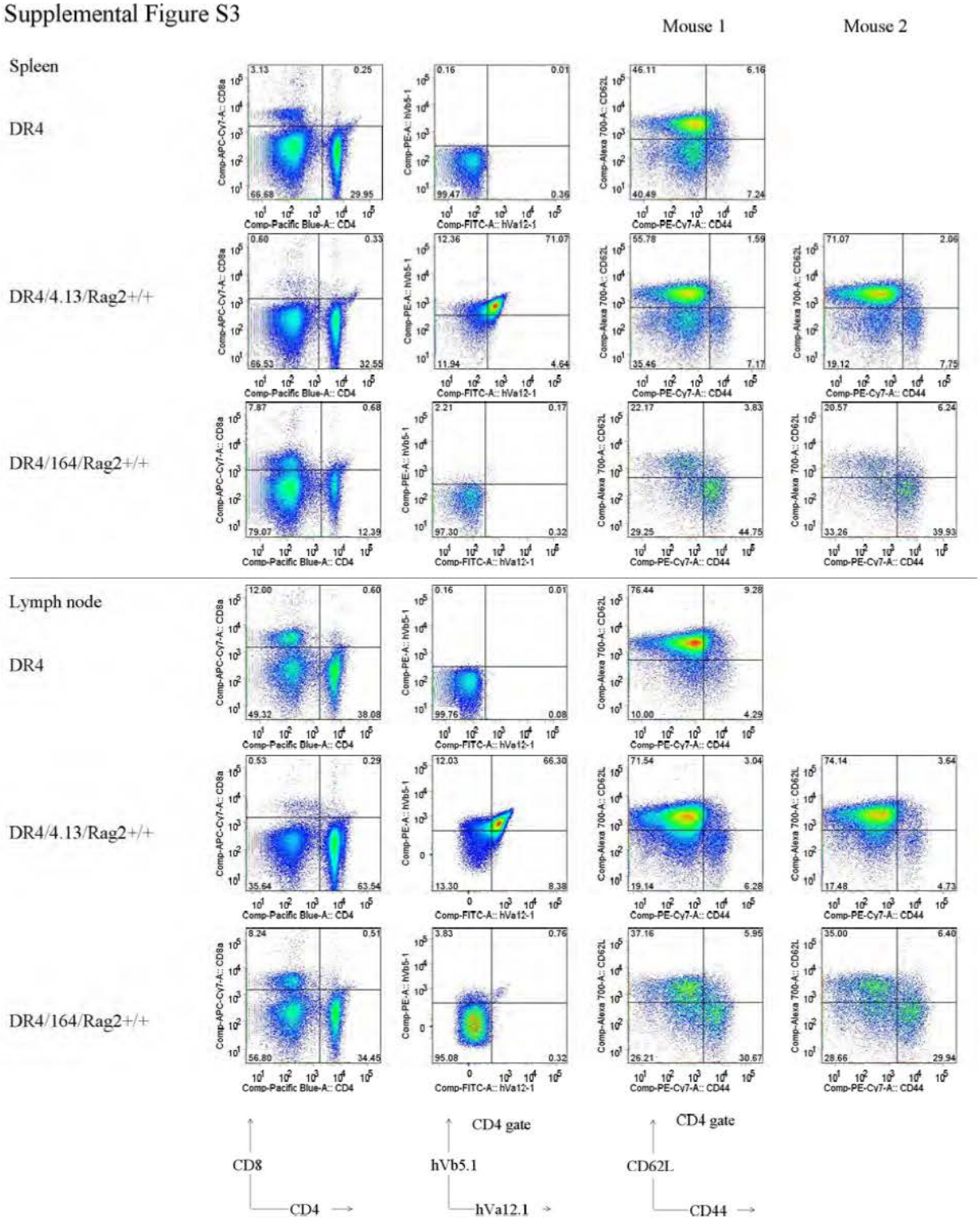

Figure S3. Surface phenotyping of spleen and lymph node cells from non-TcR transgenic 164/Rag2+/+, and 4.13/Rag2+/+ transgenic DR4 mice. Human TcR staining and CD44 vs CD62L Expression were done on CD4+/CD8- gated cells. Mice were 8-12 weeks of age.