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Low affinity TCRs support regulatory T cell function in autoimmunity

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Abstract

Regulatory T cells utilize a distinct TCR repertoire and are more self-reactive compared to conventional T cells. However, the extent to which TCR affinity regulates the function of self-reactive Tregs is largely unknown. In this study, we utilized a two-TCR model to assess the role of TCR affinity in Treg function during autoimmunity. We observed that both high and low affinity Tregs were recruited to the pancreas and contributed to protection from autoimmune diabetes. Interestingly, high affinity cells preferentially upregulated TCR-dependent Treg functional mediators IL-10, TIGIT, GITR and CTLA4, while low affinity cells displayed increased transcripts for *Areg* and *Ebi3*, suggesting distinct functional profiles. The results of this study suggest mechanistically distinct and potentially non-redundant roles for high and low affinity Tregs in controlling autoimmunity.

Keywords

T cell receptor; regulatory T cell; type 1 diabetes; autoimmunity

Introduction

Foxp3$^+$ regulatory T cells (Tregs) are critical for maintaining immune homeostasis and preventing the development of tissue-specific autoimmunity. Their functional relevance in type 1 diabetes (T1D) can be observed in IPEX patients, who have mutations in *FOXP3,* and develop T1D at a high frequency (1). Likewise, deletion of Foxp3$^+$ T cells in TCR transgenic or retrogenic (Rg) mice specific for beta-cell antigens leads to accelerated diabetes (2, 3). Consequently, Treg-centric immunotherapies have been vigorously pursued for prevention or treatment of T1D. However, it remains unclear whether boosting overall

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Treg numbers will be sufficient, or whether therapeutic approaches will need to focus on a subpopulation of functional Tregs. It is largely accepted that Tregs develop in response to stronger TCR signals, and are presumed to exhibit an overall higher degree of self-reactivity compared to conventional T cells (4–6). Moreover, recent work has shown that continuous TCR signaling is necessary for optimal Treg function (7, 8). Although it is tempting to assume that high affinity T cells are generally more functional, emerging literature suggests an equal and important role for low affinity effector T cells (Teffs) in responses against pathogens, in autoimmunity, and in tumor surveillance (9–11). However, studies addressing the role of low affinity Tregs in immune homeostasis have not been performed; thus, it remains unclear whether TCR affinity is correlated with Treg recruitment, accumulation, and function in autoimmunity.

In our previous analysis of mice expressing eight TCRs with variable affinity for the immunodominant insulin epitope B:9–23, deletion of Tregs in mice expressing higher affinity TCRs resulted in accelerated autoimmune diabetes, whereas in mice harboring lower affinity TCRs the rate of disease was unaffected by Treg-depletion (3). We therefore hypothesized that low affinity Tregs might not be functional in autoimmune diabetes. However, since in single TCR Rg mice both Teffs and Tregs possessed the same TCR, it remains unclear whether higher affinity Tregs were more functional or whether low affinity Teffs were resistant to suppression. In order to directly compare high and low affinity Tregs *in vivo*, here we utilized a mixed TCR Rg bone marrow chimera model. In this competitive setting, we were able to assess the relative accumulation and capacity of high and low affinity Tregs to control the same population of effector T cells.

Materials and Methods

Mice

NOD/ShiLtJ (NOD), NOD.B6-*Ptprc*^{b} (NOD.CD45.2), NOD.CB17-*Prkdc*^{scid}/J (NOD.*scid*), NOD.129P2(C)-*Tcra*^{tm1Mjo}/DoiJ (NOD.*TCRa*$^{-/-}$) and NOD.Cg-*Foxp3*^{sf}/DoiJ (NOD.*scurfy*) mice were obtained directly from the Jackson Laboratories and maintained at our facility. NOD.*scurfy* mice were crossed with NOD.*scid* at our facility. All mice were housed in specific-pathogen-free conditions. The studies were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Generation of two-TCR Rg mice

Two-TCR Rg mice were generated as previously described (12). Briefly, bone marrow (BM) was harvested from NOD.*scid* and NOD.*scid.scurfy* mice, transduced with retroviral TCR vectors expressing either a GFP or Ametrine fluorescent reporter, and transferred into recipient NOD.*Tcra*$^{-/-}$ mice (Supplemental Fig. 1G, 1I). Mice were either monitored for diabetes development or analyzed 5–6.5 weeks post-bone marrow transfer, at which point the T cell reconstitution was assessed (Supplemental Fig. 1H, 1J). For some experiments, NOD.CD45.2 bone marrow was added at 10% of the total cell number prior to injection.

Assessment of Diabetes

Diabetes incidence was monitored weekly with Diastix (Bayer, Elkhart, IN), and confirmed with Breeze2 glucometer (Bayer, Elkhart, IN). Mice were considered diabetic if their blood glucose was >400 mg/dl.

Isolation of Pancreatic Islets

Pancreata were digested with collagenase IV (Worthington, Lakewood, NJ), and single islets were isolated for further analysis as previously described (3).

Flow Cytometry and Antibodies

Flow cytometry analyses were performed on LSRFortessa II (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.). Monoclonal antibodies against the following molecules were used: Foxp3 (FJK-16s), Vβ12 (MR11-1), and TIGIT (GIGD7) from eBioscience; CD5 (53-7.3), Ki67 (B56), and Vβ11 (RR3-15) from BD Biosciences; CD3 (145-2C11), CD4 (GK1.5), CD25 (PC61), CTLA-4 (UC10-4B9), CD8 (53-6.7), GITR (YGITR 765), Vβ2 (B20.6), and IL-10 (JES5-16E3) from Biolegend.

RNAseq

Tregs were sorted from pancreatic islets and spleens of *wt/wt* two-TCR Rg mice based on Ametrine or GFP TCR fluorescent reporter and CD4+CD3+GITR+CD25+ gating strategy (Supplemental Fig. 2G). Samples were sorted with an average purity of 92.6% Foxp3+ for 4–8, and 92.5% Foxp3+ for 12-4.4m1. cDNA was synthesized using the SMARTer Ultra Low Input RNA Kit (Clonetech). Library preparation was performed with the Illumina Nextera XT kit before paired-end RNA-sequencing using the Illumina NextSeq500 platform for 150 cycles (NextSeq500 Mid Output Kit). Sequencing reads were aligned to the mm10 genome using TopHat Alignment Trapnell, et al. (13) and gene expression was quantified by FPKM. Cufflinks Assembly & DE (14) were used to compute differential expression ($q<0.05$) between groups, with Benjamini-Hochberg correction for multiple testing. Heatmaps and principle component analysis (PCA) were generated in R (version 3.2.3) using pheatmap from gplots package (version 2.17.0) with viridis (version 0.4.0), and ggbiplots (15). **Data Resources**: The accession number for the raw data reported in this paper is GSE106467 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106467).

Statistical Analysis

Diabetes incidence was subjected to Log-rank Mantel-Cox test. Group comparisons were performed using two-tailed Mann-Whitney nonparametric test in Figures 2 and 3, and Wilcoxon matched-pairs test in Figure 4. The mean ± SEM is shown, ns = not significant, *$p<0.05$, **$p<0.005$, ***$p<0.0005$. Statistical analyses were performed using Prism (La Jolla, CA).

Results and Discussion

High and low affinity Tregs cooperate to control autoimmune diabetes

Although some studies suggest that Treg development can be supported by TCRs with a wide range of affinities for self-antigens (16), there appears to be a positive correlation between TCR affinity and Treg development (4, 6). In order to determine whether TCR affinity for self governs Treg function in autoimmunity, we generated two-TCR Rg mice with mixed bone marrow from NOD.*scid* (‘*wt*’) and NOD.*scid.scurfy* (‘*scurfy*’) mice. In this system, we transduced either *wt* or *scurfy* bone marrow with low or high affinity TCR, and mixed the two bone marrows at an equal ratio prior to injection into the TCRα$^{-/-}$ recipients (Fig. 1A). *Scurfy* mice carry a missense mutation in the *Foxp3* gene, resulting in the complete absence of functional Tregs (17). Therefore, this mixed bone marrow chimera allowed us to limit Treg development to the TCR that was expressed on the NOD.*scid* background, while the Teff population was derived from both NOD.s*cid* and NOD.*scid.scurfy* bone marrows (Fig. 1B and Supplemental Fig. 1A).

We chose to study two pairs of high and low affinity TCRs, which were selected based on their ability to support efficient Treg development. The TCRs were stratified into “high” and “low” affinity based on their biophysical 2D affinities, functional responses to the wild type insulin (InsB:9–23) and agonist InsB:9–23(R22E) peptides, as well as insulin tetramer staining (Supplemental Fig. 1B–E) (3).

We first generated two-TCR Rg mice expressing a combination of the high and the low InsB:9–23 reactive TCRs: 4–8 and 12-4.4m1 (Supplemental Fig.1G, H). The two TCRs were chosen because when expressed individually, they yield similar frequencies of islet-infiltrating Foxp3^{+} Tregs (12% and 9%, respectively), and have similar composition of helios^{hi} or thymically derived Tregs (Supplemental Fig. 1F). Importantly, the two TCRs lead to significantly different disease patterns (3). In single-TCR Rg mice, the 4–8 TCR results in spontaneous diabetes development in about 60% of mice. By contrast, low affinity 12-4.4m1 TCR mice are free from diabetes, despite T cell infiltration of the pancreas (3). The lack of disease in 12-4.4m1 TCR mice could not be solely explained by the presence of Tregs, as Treg ablation in Foxp3^{DTR} or in *scurfy* 12-4.4m1 TCR mice did not lead to diabetes development (data not shown). On the other hand, Treg depletion in 4–8 TCR mice resulted in significant acceleration of diabetes (3). Therefore, we hypothesized that unlike the high affinity 4–8 Tregs, the 12-4.4m1 low affinity Tregs lack sufficient levels of TCR signaling to regulate pathogenic T cells, such as 4–8 Teffs.

In the 4–8 and 12-4.4m1 NOD.*scid* mixed bone marrow chimeras, where both TCRs gave rise to Tregs, mice were partially protected from diabetes, with only 40% developing disease by 20 weeks post bone marrow transfer (Fig. 1C, black line). However, when both TCRs were expressed on the NOD.*scid.scurfy* background, we observed an accelerated disease course with 100% penetrance (Fig. 1C, green line). Surprisingly, the absence of either low or high affinity Tregs resulted in similar acceleration of disease, with about 70% of mice developing diabetes (Fig. 1C, blue and red lines), indicating that both Treg populations were critical for protection and acted in a cooperative manner.

In order to confirm our observations with a different set of InsB:9–23 specific TCRs, we used a combination of the high affinity TCR 1–10 and low affinity 8-1.1, both of which displayed similar frequencies of Tregs when expressed individually (9% and 8%, respectively), and were both pathogenic (Supplemetal Fig. 1I, J) (3). As with the first pair of TCRs, we observed similar levels of helios expression, suggesting equal distribution of thymically and peripherally derived cells within the two Treg populations (Supplemental Fig. 1F). Importantly, as observed with the first set of TCRs, disease onset was accelerated when both or either of the two Treg populations were absent (Fig. 1D). Taken together, these data indicate that both high and low affinity Tregs can contribute to regulation of autoimmunity.

Treg frequencies are regulated by TCR intrinsic mechanisms

Since elimination of either Treg population accelerated diabetes development comparable to the *scurfy/scurfy* group with no functional Tregs, we considered the possibility that the absence of one Treg population might have a negative effect on the survival and homeostasis of the remaining Tregs in the increasingly inflammatory environment. Therefore, we assessed the frequencies of Foxp3$^+$ Tregs in the spleens and pancreatic islets of 4–8/12-4.4m1 two-TCR chimeras. Indeed, we observed an overall decrease in Treg frequencies in *wt/scurfy* chimeras; however, in general, the decrease in Treg frequencies and numbers did not exceed 50% loss (Fig. 2A, Supplemental Fig. 2A). Intra-TCR analysis confirmed that the absence of either Treg population had minimal effect on the frequencies or numbers of the other population (Fig. 2B, Supplemental Fig. 2B). This suggests that Treg frequencies are likely determined by the strength of TCR signaling during thymic development and tonic TCR signaling in the periphery, and not affected by the size or composition of the regulatory T cell compartment. Overall, these data indicate that a net Treg to Teff ratio, rather than Treg TCR affinity, is more important for controlling the pathogenic Teff population in autoimmunity.

Tregs have been known to take on specialized functional characteristics appropriate for suppression of specific T helper subsets (18, 19). We considered that low and high affinity Tregs might be specialized for controlling either high or low affinity effector populations. Therefore, we asked whether deletion of high or low affinity Tregs resulted in preferential expansion of either 4–8 or 12-4.4m1 effectors. Contrary to our expectations, we observed a relative increase in low affinity 12-4.4m1 Teffs when either Treg population was removed, indicating perhaps that low affinity Teffs were generally more susceptible to regulation by either Treg population (Fig. 2C).

Low affinity Tregs are competitive in a polyclonal environment

While both high and low affinity Tregs infiltrated the pancreas, high affinity TCRs supported an overall larger frequency of Tregs in periphery (Fig. 3A). Moreover, when we analyzed the relative contribution of high and low affinity Tregs to the whole Treg population by first gating on Foxp3+ T cells and then separating high and low affinity cells based on Vβ expression, the relative proportion of high affinity 4–8 Tregs was increased at the site of inflammation (Fig. 3B). In order to examine whether the increase of high affinity Tregs is reflected in their increased proliferation we compared expression of Ki67, a marker of cell

cycle, between the two populations. Only the high affinity Tregs exhibited signs of activation and proliferation in the draining pancreatic lymph nodes (PLN), the site of initial antigen exposure, based on the increase of $Ki67^+$ cells (Fig. 3C). Once in the islets, however, both Treg populations reached similar high levels of proliferation. This observation suggested that competition for antigen between the two Treg populations was limited to the site of initial antigen exposure – draining pancreatic LN.

Since we observed unequal Treg expansion in the draining lymph nodes, potentially driven by local competition for antigen, we needed to determine whether there was a similar competition within the single islet microenvironment that was obscured by pooling the islets for analysis. We considered the possibility that low affinity Tregs preferentially accumulated and expanded in the islets with lower numbers of high affinity cells - an environment with reduced competition for antigen and IL-2. To this end, we analyzed single pancreatic islets, and observed consistent presence of both Treg populations within the same microenvironment (Supplemental Fig. 2C). Of 27 islets analyzed from 7 different mice, both Treg populations were detected in 24 islets (88.9%). The observed co-existence of high and low affinity Tregs in individual islet microenvironments suggested that low affinity Tregs are competitive at the site of inflammation.

Next, we considered the possibility that in a limited two-TCR system low affinity Tregs had an artificial advantage, and would be less competitive in a polyclonal environment. Alternatively, a diverse repertoire could result in reduced competition for antigen, thus expanding the insulin reactive Treg developmental niche, potentially favoring low affinity T cells (5, 20). We therefore co-transferred two-TCR Rg bone marrow with congenically marked NOD.CD45.2 polyclonal bone marrow cells (Supplemental Fig. 2D). By 5 weeks post-transfer, the sub-population of insulin specific Rg T cells accumulated at the site of antigen in the draining LN and pancreatic islets (3.8% in spleen vs 9.2% in PLN and 10.4% in the islets) (Supplemental Fig. 2D). The expansion of the antigenic niche resulted in a preferential increase of low affinity Tregs. Compared to the lymphopenic environment, the presence of polyclonal T cells resulted in a 1.59-fold increase of low affinity Tregs in PLN (from 3.7% to 5.9%) and 1.74-fold increase in pancreatic islets (from 2.6% to 4.6%) (Fig. 3A and Supplemental Fig. 2E). Although, the relative proportion of high affinity 4–8 and low affinity 12-4.4m1 Tregs was largely unchanged by the expansion of the antigenic niche (Supplemental Fig. 2F). These data suggest that in a polyclonal setting Tregs with low TCR affinity can successfully compete for the developmental niche and accumulate in the inflammatory tissue.

High and low affinity insulin-specific Tregs are poised to utilize distinct suppressive functions

In order to elucidate the suppressive mechanisms utilized by high and low affinity Tregs in the tissue site, we assessed the transcriptional landscape of 4–8 (high affinity TCR) and 12-4.4m1 (low affinity TCR) Tregs isolated from spleens and pancreatic islets. Principle component analysis showed tight clustering of the two Treg populations in the spleens, while the islet infiltrating Treg populations were more variable in their genetic profile and significantly distinct from the spleen (Fig. 4A). Further analysis revealed similar expression

of Treg functional genes including *Foxp3*, *Tnfrsf18* (GITR), *Ctla4*, and *Tgfb1* (Fig. 4B) (21). Interestingly, several genes associated with Treg suppressive function had distinct expression in either high or low affinity Tregs. High affinity Tregs preferentially expressed *Il10*, *Gzmb*, *Lag3*, and *Tigit*, all previously described to be important for Treg suppression of Th1 responses, including autoimmune diabetes (22–25). On the other hand, low affinity Tregs exhibited significantly higher levels of *Areg* and *Ebi3* (a subunit of heterodimeric IL-35) transcripts, which are known to be important for tissue repair, Treg survival, and suppression of autoimmune responses (26–29). To confirm the RNAseq results, we assessed protein expression of GITR, CTLA-4, TIGIT, and IL-10. Interestingly, the slight difference observed in *Gitr* and *Ctla4* transcript expression was significantly enhanced at the protein level (Fig. 4C, 4D and Supplemental Fig. 2H). Although both Treg populations upregulated GITR and CTLA-4 upon entry into the pancreas, high affinity Tregs displayed enhanced expression of these functional markers. Consistent with the transcriptional analysis, expression of TIGIT and IL-10 was significantly higher in 4–8 high affinity Tregs (Fig. 4E–G and Supplemental Fig. 2H). While there was some variability in the frequency of IL-10 expressing Tregs, high affinity Tregs generally expressed greater levels of IL-10 based on MFI (Fig. 4F, 4G). Taken together these data suggest that neither Treg population alone is sufficient to control autoimmune diabetes, and high and low affinity Tregs have the potential to utilize distinct non-redundant suppressive mechanisms for combined effective control of tissue-specific autoimmune responses.

Studies performed in polyclonal and single TCR systems expressing Treg-derived TCRs revealed that Tregs preferentially express TCRs with higher affinity for self-antigens, and unperturbed TCR signaling is critical for optimal Treg function (30–32). The intensity of TCR signaling during Treg activation dictates the expression levels of several key genes involved in Treg homeostasis and function, including CD25 and CTLA4 (7, 30). Thus, it has been widely accepted that functional potential of Tregs is directly correlated with their affinity for self. However, there is little direct evidence to show whether TCR affinity has a direct effect on Treg function in autoimmunity. Importantly, we found that the simultaneous presence of high and low affinity Tregs was necessary to delay the onset of diabetes (Fig. 1C, D). This unexpected observation suggests that while tissue specificity and affinity is necessary for optimal Treg infiltration (Fig. 3B) (33), TCR affinity for tissue antigen might be important in activating distinct regulatory programs. Upon entry into the site of autoimmune inflammation, both populations increased the expression of Treg functional mediators, suggesting at least partial, if not equal, contribution of low affinity Tregs to regulation of autoimmunity.

Interestingly, in the recipients of haplodeficient bone marrow, disease kinetics and incidence were similar to *scurfy* bone marrow recipient mice, which were completely devoid of functional Tregs. These results are in contrast to previous studies of polyclonal haplodeficient or insufficient systems where the remaining Tregs are able to expand and compensate for the deficiency (34). Treg frequencies are regulated by both TCR intrinsic factors and the availability of IL-2 (8, 20, 35). Although in a polyclonal system partial deletion of Tregs presumably relieves IL-2 sources that drive Treg expansion to fill the niche, in our two-TCR system Treg frequencies do not change within each TCR population in response to reductions in the overall Treg compartment (Fig. 2B). Therefore, within the

context of fixed antigen availability intra-TCR Treg frequencies seem to be limited by TCR intrinsic parameters and remain stable.

Recently, we began to appreciate the heterogeneity of the Foxp3$^+$ Treg population, which often mirrors effector T cells in their ability to utilize a wide range of tissue specific and context dependent responses (36, 37). Heterogeneity of effector T cell responses is primarily regulated at the level of TCR signaling, which dictates the level of activation, as well as instructs the type and relative proportion of helper lineage development (38, 39). It is likely that the range of Treg phenotype and function is similarly dependent on the level of TCR activation. Moreover, regulatory mechanisms employed by Tregs are differentially dependent on TCR signaling, and some of these are induced by inflammatory cytokines rather than TCR activation. Perhaps not surprisingly one of these genes, *Areg* or amphiregulin (26), was preferentially upregulated in low affinity insulin reactive Tregs (Fig. 4B), suggesting that in the absence of strong TCR signaling Tregs are more likely to utilize non-TCR dependent suppressive functions. While on the other hand, high affinity Tregs preferentially upregulated TCR-dependent regulatory molecules including CTLA-4, TIGIT, and IL-10 (Fig. 4D–G) (7, 30). Given the dramatic disease acceleration in mice devoid of either the high or the low affinity Tregs, it is tempting to postulate that the two Treg populations utilize distinct regulatory mechanisms and both are necessary for regulation of autoimmunity (37). An alternative explanation is that the regulation of autoimmunity is highly dependent on Teff to Treg ratio, and once that ratio is compromised the regulation fails completely. Collectively, our data suggest that functional Tregs span a range of TCR affinities, and high and low affinity populations cooperatively prevent autoimmune pathology. These results might have important implications for the development of Treg-based approaches for the monitoring and treatment of autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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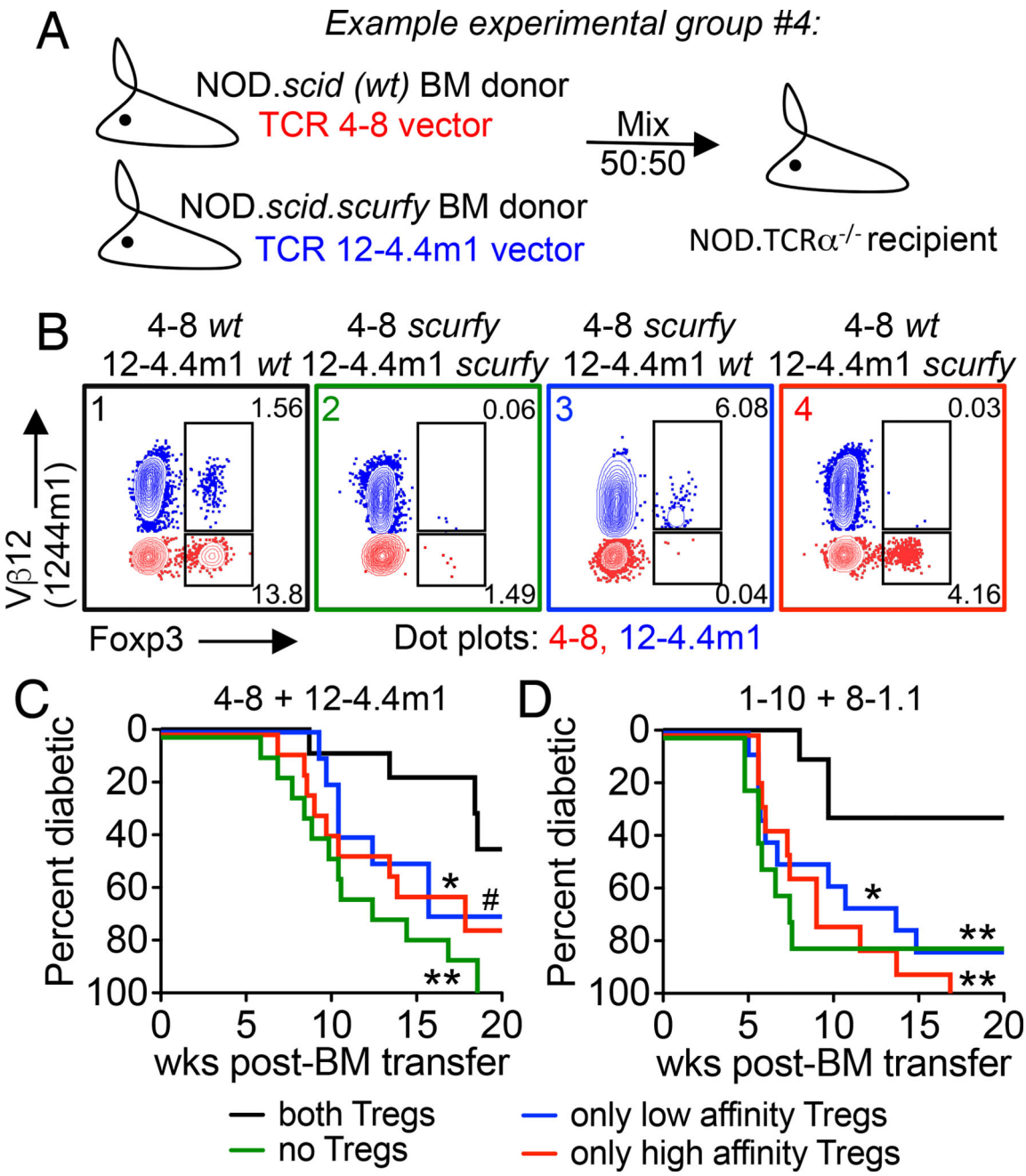

Figure 1.

Both high and low affinity Tregs contribute to protection during autoimmunity. (**A**) An example of a two-TCR retrogenic chimera experimental group where Foxp3+ Treg development is limited to 4–8 TCR expressing T cells. (**B**) Representative flow plots of Foxp3+ Tregs from the spleen of 4–8/12-4.4m1 two-TCR Rg BM chimeras. Analysis is gated on CD4+CD3+ cells, Vβ12+ (blue) are 12-4.4m1, and Vβ12– (red) are 4–8 T cells. Mice were analyzed 5.3 weeks post-bone marrow transfer. (**C** and **D**) Diabetes incidence for two-TCR Rg chimeras expressing either 4–8 and 12-4.4m1 (**C**) or 1–10 and 8-1.1 TCRs (**D**).

Mice were monitored for spontaneous diabetes development for 20 weeks (n=10–13 mice per group, #p=0.057). Data are pooled from six (**C**) and nine (**D**) independent experiments.

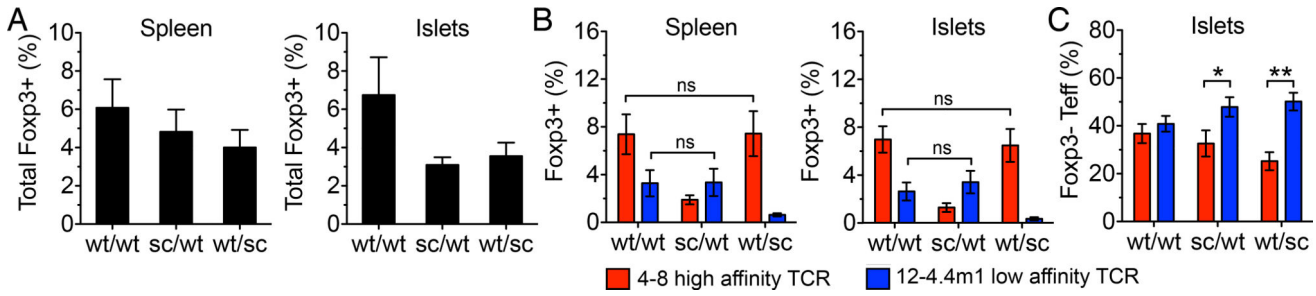

Figure 2.

Treg frequencies are regulated by TCR intrinsic mechanisms and are not affected by changing the size of the regulatory compartment. (**A**) Total Foxp3+ Treg frequencies in the spleens and islets of 4–8/12-4.4m1 two-TCR Rg BM chimeras. Analysis is gated on CD4+CD3+ (n=13–20 mice per group). (**B**) Frequencies of 4–8 or 12-4.4m1 Foxp3+ T cells in the spleens and islets of two-TCR Rg BM chimeras. Analysis is gated on CD4+CD3+Vβ2+ or Vβ12+ (n=13–20 mice per group). (**C**) Relative frequencies of 4–8 or 12-4.4m1 Foxp3– Teff cells in the islets of two-TCR Rg BM chimeras. Analysis is gated on CD4+CD3+Foxp3–Vβ2+ or Vβ12+ (n=13–20 mice per group). Mice were analyzed 5–6.5 weeks post-bone marrow transfer. Data are pooled from six independent experiments.

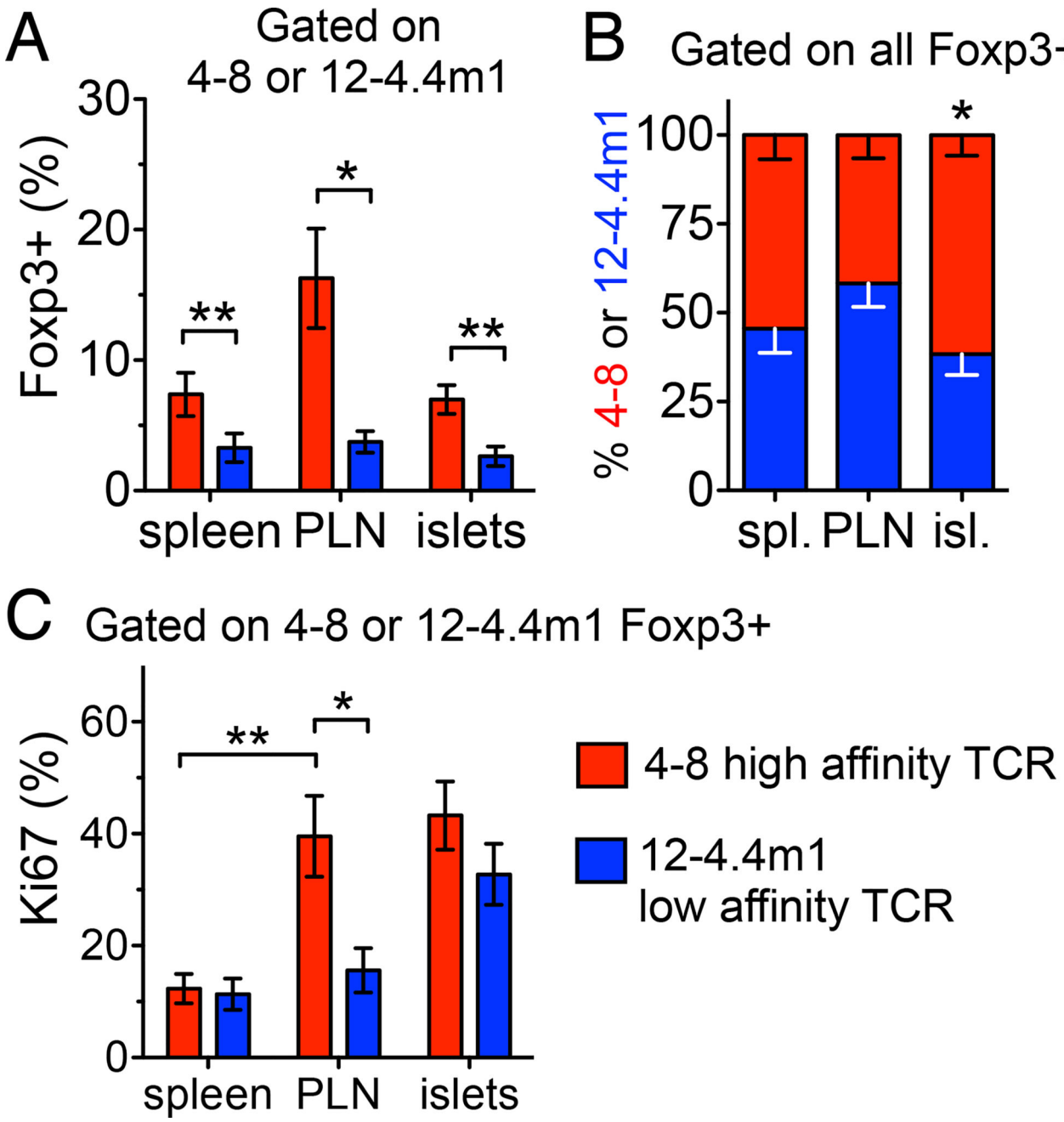

Figure 3.

Increased activation in PLN and preferentially accumulation in the islets by high affinity Tregs. (**A**) Intra-TCR frequencies of 4–8 and 12-4.4m1 Foxp3+ Tregs in wt/wt two-TCR Rg BM chimeras. CD4+CD3+ T cells are initially separated based on Vβ2+ or Vβ12+ expression, followed by analysis of Foxp3+ frequencies within each TCR population (n=17–20 mice per group). (**B**) Relative frequencies of 4–8 and 12-4.4m1 cells within the whole Foxp3+ Treg population in wt/wt two-TCR Rg BM chimeras. Analysis is first gated on all CD4+CD3+Foxp3+ cells, followed by the analysis of relative frequency of Vβ2+ or Vβ12+ cells within all Foxp3+ T cells (n=17–20 mice per group). (**C**) Percent Ki67+ Tregs in wt/wt

two-TCR Rg BM chimeras. (n=17–20 mice per group). Mice were analyzed 5–6.5 weeks post-bone marrow transfer. Data are pooled from six independent experiments.

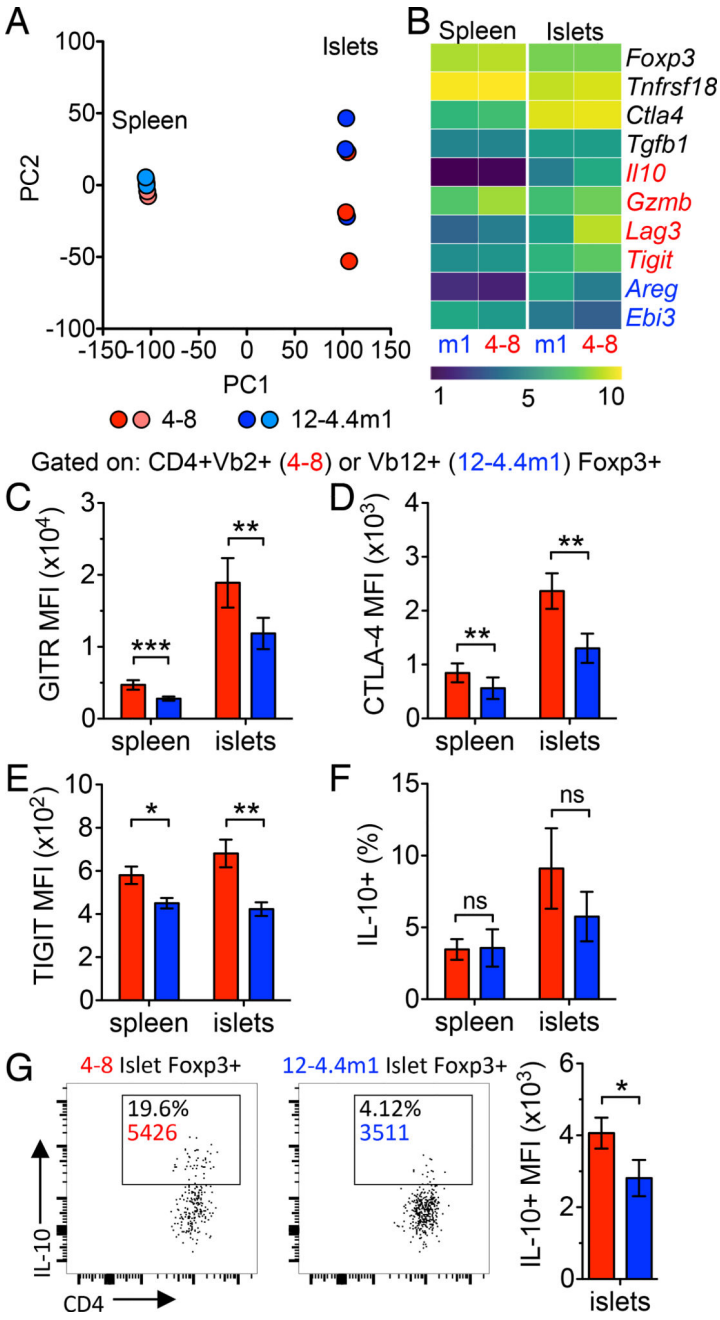

Figure 4.

High and low affinity Tregs are transcriptionally distinct. (**A**) Principle component analysis of 4–8 and 12-4.4m1 Tregs isolated from the spleens and islets of wt/wt two-TCR Rg BM chimeras (n=3). (**B**) rlog transformed heatmap of Treg functional genes (n=3). Genes in black had no statistical difference between 4–8 and 12-4.4m1 Tregs in the islets. Red (increased in 4–8 Tregs) and blue (increased in 12-4.4m1 Tregs) genes had a significant q-value ($q<0.05$) between 4–8 and 12-4.4m1 Tregs in the islets. (**C**) GITR MFI (n=16), (**D**) CTLA-4 MFI (n=7), (**E**) TIGIT MFI (n=10) and (**F**) percent IL-10+ (n=7) of 4–8 and 12-4.4m1 Tregs in wt/wt two-TCR Rg BM chimeras. (**G**) Representative gating of

IL-10+ 4–8 and 12-4.4m1 Tregs in wt/wt two-TCR Rg BM chimeras and quantification of IL-10+ MFI (n=7). Mice were analyzed 5–6.5 weeks post-bone marrow transfer. (**C–D**) Data are pooled from at least 3 independent experiments.