Correction

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Correction for “Revealing the specificity of regulatory T cells in murine autoimmune diabetes,” by Allyson Spence, Whitney Purtha, Janice Tam, Shen Dong, Youmin Kim, Chia-Hsin Ju, Teague Sterling, Maki Nakayama, William H. Robinson, Jeffrey A. Bluestone, Mark S. Anderson, and Qizhi Tang, which was first published April 30, 2018; 10.1073/pnas.1715590115 (Proc Natl Acad Sci USA 115:5265–5270).

The authors note that Fig. 4 appeared incorrectly. The corrected figure and its legend appear below.

Fig. 4. Insulin-specific Tregs in NOD mice. (A) Representative contour plots of pooled insulin B:9–23 tetramers p8G and p8E staining versus control HEL tetramer staining in islet Tconv cells (Upper) and Tregs (Lower). Cells were gated as live CD45+ Thy1.1+ CD8− and Foxp3+ (Tregs) or Foxp3− (Tconv cells). Results are representative of at least eight mice analyzed in three independent experiments. (B) Quantification of insulin tetramer staining in islet Tconv cells and Tregs. Each pair of connected circles represents one mouse, and the mice (n = 24) are grouped according to age. Results shown are a summary of six independent experiments. Statistical significance was determined using a paired Student’s t test, and P values are listed at the top of the groups. (C) Total number of insulin tetramer-positive Tregs and Tconv cells from islets. Results shown are a summary of three independent experiments. Statistical significance was determined using a paired Student’s t test; *P < 0.05. (D) Nur77GFP MFI of insulin tetramer-positive and -negative Tregs in islets. Results shown are a summary of three independent experiments. Statistical significance was determined using a paired Student’s t test; **P < 0.001.

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Revealing the specificity of regulatory T cells in murine autoimmune diabetes

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Regulatory T cells (Tregs) control organ-specific autoimmunity in a tissue antigen-specific manner, yet little is known about their specificity in a natural repertoire. In this study, we used the nonobese diabetic (NOD) mouse model of autoimmune diabetes to investigate the antigen specificity of Tregs present in the inflamed tissue, the islets of Langerhans. Compared with Tregs present in spleen and lymph node, Tregs in the islets showed evidence of antigen stimulation that correlated with higher proliferation and expression of activation markers CD103, ICOS, and TIGIT. T cell receptor (TCR) repertoire profiling demonstrated that islet Treg clonotypes are expanded in the islets, suggesting localized antigen-driven expansion in inflamed islets. To determine their specificity, we captured TCRβ pairs from islet Tregs using single-cell TCR sequencing and found direct evidence that some of these TCRs were specific for islet-derived antigens including insulin B9–23 and proinsulin. Consistently, insulin B9–23 tetramers readily detected insulin-specific Tregs in the islets of NOD mice. Lastly, islet Tregs from prediabetic NOD mice were effective at preventing diabetes in Treg-deficient NOD.CD28−/− recipients. These results provide a glimpse into the specificities of Tregs in a natural repertoire that are crucial for opposing the progression of autoimmune diabetes.

Significance

Tissue antigen-specific Tregs are critical for the prevention of organ-specific autoimmune diseases, yet very little is known about their specificity in a natural repertoire. Here, we show an accumulation of antigen-experienced Tregs in the inflamed islets using a mouse model of autoimmune diabetes. MHC tetramer staining and single-cell T cell receptor (TCR) analyses of islet Tregs reveal their specificity for insulin and other islet-derived antigens. Tregs from inflamed islets prevent diabetes when transferred to diabetes-susceptible recipients. The identification of functional islet antigen-specific Tregs with distinct TCR usage paves the way for future investigations of their development and homeostasis to better understand pathogenesis and treatment of autoimmune diabetes.

Antigen specificities of these diabetogenic T cells include insulin, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), glutamic acid decarboxylase (GAD), zinc transporter 8 (ZnT8), insulinoma antigen 2 (IA-2), phogrin (IA-2δ), chymogranin, and islet amyloid polypeptide (IAPP) (25, 26). These specificities likely arise from inefficient deletion of autoreactive T cells during thymic development and creation of neoantigen in the islets from posttranslational modifications such as hybrid insulin peptide (HIP) formation (27–30).

Despite progress in mapping the specificity of diabetogenic T cells, specificities of Tregs that control the progression of diabetes have not been determined. The challenge in determining Treg specificity is partly due to the rarity of islet antigen-specific Tregs in a natural highly diverse Treg repertoire. This is compounded by the difficulty in measuring Treg function in diabetes protection in vivo. Several published Treg repertoire studies used mice with a transgenic TCRβ chain to experimentally restrict T cell repertoire for easier identification of Treg antigen specificity. This practical approach is effective in tracking Treg clonotypes enriched in the colons and in prostate tumors (31–33). However, a normal T cell repertoire of a mouse uses more than 5 × 107 distinct TCRβ chains (34). Thus, the T cell repertoire in a mouse with fixed TCRβ chain is severely skewed, and Treg clonotypes found in these mice may not reflect those in a normal immune repertoire.

In this study, we set out to map the natural TCR repertoire and specificities of Tregs that protect against diabetes in NOD
mice. Our investigation is based on the premise that diabetes develops with delayed kinetics and reduced penetrance in NOD mice compared with Treg-deficient NOD.CD28−/− mice. This suggests a presence of functional Tregs in NOD mice. We hypothesize that these Tregs are islet antigen specific and can be identified by their responsiveness to islet antigens.

Results

TCR Stimulation of Tregs in Inflamed Islets. To investigate TCR activation of Tregs in NOD mice, we backcrossed a strain of Nur77GFP reporter mice (15) more than 10 generations onto the NOD background. The NOD.Nur77GFP mice developed diabetes at comparable kinetics and overall incidence to standard NOD mice (SI Appendix, Fig. S1A) and exhibited normal cellularity and proportions of T cells in peripheral lymphoid organs (SI Appendix, Fig. S1B). We then further crossed the NOD.Nur77GFP mice with NOD.Foxp3RFP mice (35, 36) to aid the isolation and analysis of Tregs. Tregs from the NOD.Nur77GFP, Foxp3RFP mice showed constitutively high GFP expression indicative of antigen stimulation of the TCR as previously reported (14, 15). To further define the correlation between GFP expression and the strength and immediacy of TCR stimulation, we analyzed lymph node (LN) cells from NOD.Nur77GFP.Foxp3RFP mice that had received varying doses of TCR stimulation in vitro. CD4+Foxp3+ Tconv cells showed dose-dependent increases in the percentages of Nur77GFP+ cells and in GFP mean fluorescence intensity (MFI) (SI Appendix, Fig. S1 C and D). Tregs were nearly all GFP positive directly ex vivo before stimulation. Increasing TCR stimulation also led to a dose-dependent increase in GFP MFI in Tregs (SI Appendix, Fig. S1 C and D). To determine the persistence of GFP expression after the cessation of TCR stimulation, we stimulated purified Tconv cells and Tregs with plate-bound anti-CD3 and anti-CD28 in vitro for 24 h, removed the cells from stimulation, and monitored GFP levels over time. GFP MFI in both Tconv cells and Tregs decayed with a half-life of ~3 d (SI Appendix, Fig. S1E). Unstimulated Tregs showed a rate of Nur77GFP decay similar to that seen in vitro stimulated Tregs and Tconv cells. Together, these data demonstrate that the intensity of GFP in T cells of Nur77GFP reporter mice integrates both the strength of the TCR signal received and the recency of antigen exposure.

We then used the NOD.Nur77GFP.Foxp3RFP mice to identify Tregs with more recent islet antigen exposure. We compared GFP expression in Tregs from pancreatic LN (pancLN) and inflamed islets to those from spleen. Tregs from islets had the highest expression of GFP compared with those from pancLN and spleen (Fig. 1A). A subset of Tregs in the inflamed islets coexpressed previously reported TCR-dependent activation markers CD103, TIGIT, and ICOS (21) (Fig. 1B and SI Appendix, Fig. S2A and B) and had higher expression of Foxp3 and CTLA4 than CD103− islet Tregs (SI Appendix, Fig. S2C). These CD103+ islet Tregs had the highest Nur77GFP expression compared with CD103− islet Tregs and Tregs from lymphoid organs (Fig. 1B and C). The expression of CD5, another marker of recent TCR stimulation (37, 38), was also higher on islet Tregs, especially on the CD103+ subset (Fig. 1D). Moreover, islet Tregs were more proliferative than those in the pancLN by Ki67 staining (Fig. 1E), and islet CD103+ Tregs were the most proliferative subset by both Ki67 staining and in vivo BrdU labeling (Fig. 1E and F). These results indicate that Tregs in islets, especially those coexpressing...
Islet Tregs Have a Distinct and Restricted TCR Repertoire. The activation and proliferation of Tregs in inflamed islets may result in local enrichment of islet antigen-specific clonotypes and formation of distinct TCR repertoires. We therefore compared islet Treg TCRβ sequences with those used by Tregs from pancLN, spleen, and nondraining inguinal-popliteal-sciatic LN (iLN) using high-throughput TCRβ sequencing analysis. In each organ, we divided Tregs into CD103− and CD103+ subsets, and the CD103− islet Tregs were further divided into Nur77GFP+ high and low fractions (Fig. 24). Between 1,600 and 600,000 Tregs were analyzed from different compartments, and 1,144 to 62,604 unique TCRβ sequences were identified (SI Appendix, Table S1). We used the clonality index—the inverse of normalized Shannon’s entropy—to quantify the clonal dominance in each population, with 0 indicating that each clone appears only once and 1 representing a monoclonal population. While all Treg populations showed polyclonality, with a diversity index of less than 0.4, the CD103− islet Tregs were the least diverse (Fig. 2B). Clonal dominance can also be visualized by the contribution of the top 10 clones to the repertoire they reside in, which was 30% for CD103+ islet Tregs, markedly higher than the CD103− islet Treg subsets (Fig. 2 C and D). CD103− Tregs in spleen, pancLN, and iLN were highly diverse, with the 10 most abundant clones representing less than 2% of the population (Fig. 2D). These results further support local clonal expansion of activated Tregs in the inflamed islets.

Islet Antigen Specificity of Islet Tregs. Our results thus far suggest that Tregs specific to islet autoantigens are locally activated, clonally expand, and accumulate in the islets. However, distinct TCRs may recognize the same antigen. To further determine the specificity of islet Tregs, we performed single-cell sequencing analysis of paired TCRαβ chains and then further interrogated for their specificity using an in vitro reporter assay (39, 40). FACS-purified ICOS+TIGIT+ islet Tregs, which also express high levels of CD103 and Nur77GFP (SI Appendix, Fig. S2A), were sorted into single wells for barcoding and sequencing. Among the 71 paired TCRαβ sequences identified, six TCRβ were found more than once in a single mouse, indicating clonal expansion, and two other TCRs were found in multiple mice (SI Appendix, Table S2). We thus selected these eight TCRs for further analysis of their reactivity to islet antigens.

We reconstituted the paired TCRs using retroviral transduction in a hybridoma cell line that expresses an NFATGFP reporter construct so that TCR triggering can be measured by GFP expression. A hen egg lysosome (HEL)-specific TCR (PA21.14H4) was used as a negative control, and a TCR for insulin (p3w17) was used as a positive control in the screening. We stimulated all TCR-pulsed islet-derived CD11c+ dendritic cells (DCs) pulsed with lysate from NOD.Rag2−/− islets. Five of eight clones (i.e., 510, 518, 521, 550, and 551) showed measurable reactivity to islet lysates (Fig. 3A). To determine whether these TCRs respond to islet antigen naturally processed and presented by DCs in the inflamed islets, we stimulated the hybridomas with freshly isolated islet-derived CD11c+ DCs. In this assay, the same five clones responded to islet DCs, but not to control DCs from spleen or colon (Fig. 3A and B and SI Appendix, Fig. S3B). To further map antigen specificity of these TCRs, we screened the hybridomas against 19 peptides previously reported to be stimulatory to islet antigen-specific CD4+ T cells in NOD mice (Table S3). None of the TCRs responded to BDC mimbosites, Hlα, or peptides from ZnT8, GAD, IGRP, IA2, or IA2-p (Table S3). Islet-derived insulin is a dominant autoantigen in type 1 diabetes in both NOD mice and human patients (41, 42). Therefore, we tested Treg TCRs against insulin. One TCR, 521, responded to splenic DCs loaded with insulin B9–23 peptide (Fig. 3C and Table 1). Another TCR, 510, responded to immobilized insulin B9–23 p8E and p8G tetramers (29) (Fig. 3C and Table 1). To determine reactivity

**Fig. 2.** Treg TCR repertoires in prediabetic NOD mice. (A) A summary of Treg populations used for TCRβ chain sequencing analysis. CD103− and CD103+ Treg populations were sorted from spleen, pancLN, and iLN. Islet Tregs were additionally sorted on Nur77GFP expression from islets: CD103+ Nurlo, CD103+ Nurhi, and CD103− Nurhi. A total of 32 populations from four mice were analyzed individually (SI Appendix, Table S1). (B) Clonality of each Treg population’s TCR repertoire. Each triangle represents a single mouse, and the columns represent the mean and SD of the group. Statistical significance was determined using a one-way ANOVA followed by Tukey’s multiple comparison test; ***P < 0.001. (C) Frequency of the 10 most abundant clones in different islet Treg populations in a representative mouse. (D) Summary graph of the sum frequencies of the 10 most abundant clones in different Treg populations in each mouse. Each triangle represents a single mouse, and the columns represent the mean and SD of the group. Statistical significance determined as described in B; *P < 0.01.

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Insulin Specificity Among Islet Tregs. Our results suggest that insulin specificity may be common among islet-infiltrating Tregs. To further test this notion, we analyzed Tregs and Tconv cells in islets and pancLN of NOD mice (8 to 28 wk old) using insulin I-A^d tetramer containing modified insulin B:9–23 peptides p8E and p8G (29). Insulin B:9–23-specific cells were readily detectable among islet Treg and Tconv cells but were not consistently found in the pancLN (SI Appendix, Fig. S4). Insulin B:9–23-specific islet Tregs were often present at higher frequencies than insulin B:9–23-specific Tconv cells in prediabetic mice (Fig. 4B). Overall, the total numbers of insulin-specific Tconv cells per islet were slightly higher than the total number of insulin-specific Tregs (Fig. 4C). Moreover, insulin tetramer-positive Tregs had higher Nur77^GFP expression than insulin tetramer-negative Tregs, indicating stronger TCR activation (Fig. 4D). Thus, the insulin tetramer stain result is consistent with single-cell TCR sequencing analysis and shows that a demonstrable proportion of islet Tregs is insulin specific.

Function of Islet Tregs. Islet antigen-specific Tregs are more effective at protecting against diabetes (17, 18). Given their islet antigen specificity and propensity to be activated in the islets, we hypothesized that islet Tregs in the NOD mice would be effective at suppressing diabetes. We assessed the in vivo capacity of islet Tregs to prevent diabetes in an adoptive transfer model using NOD.CD28^−/− mice as recipients. These mice develop diabetes at a younger age and at higher penetrance due to Treg defects

to other insulin epitopes, we stimulated the hybridomas with splenic DCs pulsed with metabolically inactive human insulin, B25D-insulin (43). The use of B25D-insulin avoided the hormonal activation of the hybridomas that altered the assay background. TCR 551 responded to B25D-insulin (Fig. 3C and Table 1). Additionally, we found that TCR 518 responded to a previously described proinsulin peptide, insulin B:24–36 (Fig. 3C and Table 1) (44). The remaining islet antigen-specific TCR, 550, did not respond to any of the above insulin stimulations (Fig. 3C, Table 1, and SI Appendix, Table S3). In summary, five of eight islet Treg-derived TCRs were stimulated by islet antigens, and four of the five were specific for insulin (Table 1).

Fig. 3. Islet antigen reactivity of activated islet Tregs. (A) Hybridomas expressing NFAT^GFP reporter and TCRs derived from activated islet Tregs were stimulated for 20 to 24 h with splenic DCs or splenic DCs with islet lysates. GFP expression was determined using flow cytometry, and contour plots for all of the hybridomas analyzed are shown. (B) As described in A, except the hybridomas were stimulated with islet DCs. (C) As described in A, except the hybridomas were stimulated with splenic DCs with insulin B:9–23 peptide, plate-bound insulin tetramers p8E and p8G, human B25D-insulin, or proinsulin B:24–36 peptide. Filled histograms are the HEL-specific hybridoma; open histograms are the TCR hybridoma listed at the top of the column. In A–C, representative flow plots for at least two independent experiments are shown.

Table 1. Summary of islet Treg reactivity

<table>
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<tr>
<th>Sequence ID</th>
<th>Insulin*</th>
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<th>Islet antigen specific?</th>
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<td>+</td>
<td>+</td>
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<td>Insulin B:9–23</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>Yes</td>
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<td>–</td>
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<tr>
<td>521</td>
<td>+</td>
<td>±</td>
<td>±</td>
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<td>+</td>
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<td>–</td>
<td>–</td>
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<td>Unknown</td>
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</table>

*Definition of insulin reactivity includes specificity to mature insulin, proinsulin, and preproinsulin.
More than 80% of NOD.CD28−/− mice become diabetic between 8 and 12 wk of age, which can be largely prevented by adoptive transfer of 50,000 islet antigen-specific Tregs from BDC-2.5 TCR transgenic mice (Fig. 5A). The same dose of Tregs isolated from spleen, pancLN, or nonpancreas draining LNs (mesenteric LN and inguinal LN) of prediabetic NOD mice were unable to prevent diabetes (Fig. 5B). Transfer of 50,000 islet Tregs prevented diabetes as efficiently as BDC-2.5 Tregs (Fig. 5C). Taken together, this study demonstrates that inflamed islets contain an enrichment of islet antigen-specific Tregs that exhibit potent antidiabetes activity.

Discussion

Collectively, results from this study show that islet antigen-specific (particularly insulin-specific) Tregs are enriched in the inflamed islets of NOD mice. These Tregs are functional in curbing the progression of islet destruction. It is worth noting that most NOD mice eventually succumb to diabetes, likely because islet antigen-specific Tregs die due to IL-2 deficiency in inflamed islets and exhaustion of the islet antigen-specific Treg pool from chronic attrition (45).

Insulin is the most abundant protein in beta cells and a key autoantigen for driving the initiation and progression of autoimmune beta cell destruction (42, 46, 47). In this study, we found a prominence of insulin-specific Tregs in inflamed islets. A recent study has reported that human subjects with long-standing antiislet autoimmunity without progression to overt diabetes have increased frequency of insulin-specific Tregs compared to healthy subjects and those with diabetes onset at a young age (48). These results suggest an important role for insulin-specific Tregs in controlling diabetes progression. To our surprise, none of the TCRs responded to any of the 19 peptides previously reported to stimulate islet antigen-specific CD4+ T cells. Previous analyses used CD4+ T cells and likely did not include many Tregs clones. Moreover, the Treg clone analysis in this report is limited and aimed only at identifying dominant clones. Nonetheless, the two insulin epitopes we are able to identify in this study have been previously reported for Tconv cells. Overall, our mapping of Treg specificities in the inflamed islets suggest insulin as a dominant specificity and that insulin-specific Tregs and Tconv cells can recognize the same epitopes.

The prominent and highly localized presence of insulin-specific Tregs in inflamed islets is somewhat unexpected. Insulin is a systemic hormone that can potentially be presented by DCs throughout the body. However, insulin-specific T cells are hard to detect outside islets, and insulin-specific TCRs were not activated by splenic DCs in vitro without addition of islet antigens or insulin peptides. These results suggest that insulin-specific Tregs likely require high abundance of insulin to be activated, which limits their suppressive activity to the islets at the source of insulin production. This observation has important implications for developing islet antigen-specific Treg therapies. Polyclonal Treg therapy is being evaluated in patients with type 1 diabetes in early-phase clinical trials (49, 50). Genetic engineering of Tregs with TCR-specific islet antigens has the potential to increase therapeutic efficacy and minimize systemic immunosuppression. A key issue in engineering TCRs for islet antigen-specific Tregs is to ensure that the targeted
antigens are naturally presented at adequate levels in the islets and draining LN to activate the engineered Tregs. Our results suggest that insulin-specific TCRs are viable candidates for Treg specificity for the protection of beta cells.

Materials and Methods

Bulk TCRβ Sequencing

Total RNA was extracted from FACs-purified Tregs using ARCTURUS PicPure RNA Isolation Kit (Life Technologies) for <100,000 cells or a microRNA extraction kit (QIAEN) for >100,000 cells. TCRβ repertoire analyses were amplified and sequenced using Illumina MiSeq by iRepertoire, Inc. Data analysis was performed using iRepertoire, Inc. web tools (https://www.i repertoire.com). Conalit was calculated using the inverse of normalized Shannon's diversity index.

Single-Cell TCRβ Sequencing

Individual activated islet Tregs were flow sorted into single wells of a 96-well plate, and TCR sequences from single cells were amplified and sequenced using Illumina MiSeq by iRepertoire, Inc. Data analysis was performed using iRepertoire, Inc. web tools (https://www.i repertoire.com).

Hybridoma Assays

Splenic and islet DCs were enriched using a CD11c+ selection kit (STEMCELL Technologies). DCs and hybridomas, 5 x 10^6 per well, were cocultured 20 to 24 h in round-bottom 96-well plates with various forms of islet antigens as described, before flow cytometric analysis of GFP expression.

Intracellular T cell Staining

The APC-conjugated tetramer of I-Aβ bound to insulin B9-23 mimotopes p8E (HLVERLYLVCGEEG) and p8G (HLVERLYLVCGGEG) (29) was purchased from Immudyn (MD). Splenic and islet islets were obtained from the National Institutes of Health Tetramer Core Facility at Emory University and stained for 2 h at 37 °C for panNKT and islet T cell analysis.

Mice

All experiments involving mice have been approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Statistical Analysis

Analyses were performed with GraphPad Prism (GraphPad Software) as detailed in the figure legends. Additional information can be found in SI Appendix, Supplemental Experimental Procedures.

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