This information is current as of June 7, 2017.

Maria Bettini, Lori Blanchfield, Ashley Castellaw, Qianxia Zhang, Maki Nakayama, Matthew P. Smeltzer, Hui Zhang, Kristin A. Hogquist, Brian D. Evavold and Dario A. A. Vignali

*J Immunol* 2014; 193:571-579; Prepublished online 18 June 2014;
doi: 10.4049/jimmunol.1400043
http://www.jimmunol.org/content/193/2/571

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/06/18/jimmunol.1400043.DCSupplemental

**References**
This article cites 39 articles, 18 of which you can access for free at:
http://www.jimmunol.org/content/193/2/571.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Autoreactive T cells infiltrating the target organ can possess a broad TCR affinity range. However, the extent to which such biophysical parameters contribute to T cell pathogenic potential remains unclear. In this study, we selected eight InsB\textsubscript{9–23}–specific TCRs cloned from CD4\textsuperscript{+} islet-infiltrating T cells that possessed a relatively broad range of TCR affinity to generate NOD TCR retrogenic mice. These TCRs exhibited a range of two-dimensional affinities (\textsim{}10\textsuperscript{−4}–10\textsuperscript{−3} \textmu{}m\textsuperscript{2}) that correlated with functional readouts and responsiveness to activation in vivo. Surprisingly, both higher and lower affinity TCRs could mediate potent insulitis and autoimmune diabetes, suggesting that TCR affinity does not exclusively dictate or correlate with diabetogenic potential. Both central and peripheral tolerance mechanisms selectively impinge on the diabetogenic potential of high-affinity TCRs, mitigating their pathogenicity. Thus, TCR affinity and multiple tolerance mechanisms converge to shape and broaden the diabetogenic T cell repertoire, potentially complicating efforts to induce broad, long-term tolerance. The Journal of Immunology, 2014, 193: 571–579.
which has been the limiting factor in assessing affinity for multiple TCRs. The 2D micropipette adhesion frequency assay eliminates the TCR purification step because T cells are used for affinity determination of multiple TCRs specific to the same antigenic peptide epitope with sensitivity to a single TCR:pMHC bond. This technique has been highly predictive of the functional outcome of TCR ligation in CD4+ and CD8+ T cells (12–15).

We focused our analysis on the dominant epitope of insulin, a target Ag in autoimmune diabetes in NOD mice and in type 1 diabetes in humans, and obtained a panel of eight InsB9–23–specific MHC II–restricted TCRs, most of which were cloned from T cells infiltrating the pancreatic islets. We performed biological and biological measurements of relative TCR affinities, and then assessed these TCRs for their in vivo function and pathogenicity in autoimmune diabetes to determine the parameters that shape TCR diabetogenic potential.

Materials and Methods

2D TCR affinity measurements

The details of the micropipette adhesion frequency assay are described in detail elsewhere (13, 16). In brief, a pMHC-coated RBC and T cell were placed on opposing micropipettes and mechanically brought into contact for a controlled contact area (Ac) and time (t). The T cell was retracted at the end of the contact period, and the presence of adhesion (indicating TCR–pMHC ligation) was observed microscopically by elongation of the RBC membrane. This contact–retraction cycle was performed 50 times per T cell–RBC pair to calculate an adhesion frequency (Pc). For each experiment, a mean Pc was calculated based on T cells that bound specifically to Ag. The population-averaged 2D affinity (\( \text{AcK}_a \)) using the mean Pa at equilibrium (where \( t \to \infty \)) was calculated using the following equation: 
\[
\text{AcK}_a = \text{ln}(1 - \text{Pa})/\text{Pa} \times \text{m} \times \text{n}
\]
where m and n reflect the receptor (TCR) and ligand (pMHC) densities, respectively. Insulin peptide/MHC monomers used in the micropipette assay were previously published (17) and provided by the National Institutes of Health tetramer core.

Mice

NOD.scid and NOD/ShiLtJ mice were obtained from The Jackson Laboratory. All mice were bred and housed at the St. Jude Animal Resources Center (Memphis, TN) in a Helicobacter-free specific pathogen–free facility following state, national, and institutional mandates. NOD.129S2 (8-10– mouse) and NOD.129S2 (8-10–/– mouse), originally obtained from The Jackson Laboratory, NOD.Foxp3DTR mice, were isolated from Juvenile Diabetes Research Foundation repository (18), and C57BL/6-Tg(Ins2–IAPP(cre)82Kohg/J in our facility. NOD.scid mice (99.3% NOD by single-genotype analysis) were provided by the National Institutes of Health tetramer core.

Cloning of P2 TCR from pancreatic islets

CD4+ T cells were isolated from the islets of WT NOD mice (10 wk of age), expanded in vitro with PMA and ionomycin, and then sorted on the basis of insulin tetramer binding and fused with a fusion partner expressing an IL-2 GFP reporter, facilitating screening of the clones for Ag sensitivity. P2 TCR was cloned from the hybrid clone that screened positive for expansion or IL-2 secretion in response to wild-type (WT) InsB9–23-specific TCR reagents and retroviral-mediated stem cell gene transfer

All the TCRs used for this study were chosen based on their ability to mediate T cell expansion or IL-2 secretion in response to wild-type (WT) InsB9–23 peptide. Most of the TCRs used in this study were derived from islet-infiltrating T cells (Supplemental Table I). Generation of TCR transgenic constructs and TCR retrogenic mouse generation have been previously published (19–23). Briefly, female NOD.scid mice were injected i.p. with 150 mg/kg 5-fluorouracil (American Pharmaceutical Partners, Schaumburg, IL), bone marrow was harvested 72 h later and cultured for 24 h in complete DMEM supplemented with 20% FBS, 20 ng/ml IL-3, 50 ng/ml human (h)IL-6, and 50 ng/ml mouse stem cell factor (R&D Systems, Minneapolis, MN). Bone marrow cells were spin transduced with retroviral supernatant. 6 µg/ml polybrene, and freshly added cytokines for 1 h at 37°C at 2500 rpm, at 24 and 48 h; fresh media were added at 72 h. After 96 h, bone marrow cells were injected at ~2 × 10^6 cells per recipient approximately one donor/recipient). Mice were then bled for TCR reconstitution 8 wk posttransplant for diabetes analysis, and analyzed 8 wk posttransplant for all other experiments. The 12-4.4m1 sequence used in this study was an artificially modified version of 12-4.4 (see Supplemental Table I).

Ilet isolation

Pancreata were perfused by injecting 3 ml collagenase 4 (Worthington, Lakewood, NJ) (400 U/ml in HBSS and 10% FBS), harvested, and placed in 3–5 ml collagenase 4. The pancreata were then incubated at 37°C for 25 min, after which they were washed three times with 7 ml 5% FBS/HBBSS and resuspended in 10 ml 5% FBS/HBBSS. Islets were handpicked and incubated at 37°C for 15 min in 1 ml dissociation buffer (Invitrogen, Carlsbad, CA) and then further dissociated by vortexing and pipetting. Cells were then washed in 10 ml 5% FBS/HBBSS, counted, and analyzed by flow cytometry.

Assessment of insulitis and diabetes

Pancreata of mice were harvested at 8–9 wk post–bone marrow transfer. Paraffin-embedded 4–µm-thick section were cut at 150 µm apart and stained with H&E at the St. Jude Histology Core Facility. Islets (50–90 per mouse) were scored in a blinded manner using the method outlined in Current Protocols in Immunology (24). Diabetes incidence was monitored weekly by testing for the presence of glucose in the urine by Diastix (Bayer, Elkhart, IN). Mice testing positive by Diastix were tested with a Breeze2 glucometer (Bayer, Elkhart, IN) for elevated blood glucose levels and were considered diabetic if their blood glucose was >400 mg/dl.

T cell line development and in vitro stimulation

The TCR 4G4 CD4+ T cell hybridoma line was transfected with TCRs and sorted on an identical narrow range of TCR expression. A total of 50,000 TCR transfectants were stimulated with 200,000 NOD splenocytes and InsB9–23. Phospho-Erk expression was analyzed after 1% formaldehyde fixation and methanol permeabilization. IL-2 secretion was analyzed after 24 h via IL-2 ELISA.

To generate T cell lines, splenocytes from NOD.scid or NOD.scid, Nur77GFP TCR retrogenic mice were sorted on CD4+Antimete’ cells and expanded in vitro with 1 µg/ml ionomycin, 10 ng/ml PMA (Sigma-Aldrich), and 1000 units/ml hIL-2 for 2 d. Media were changed every 2 d and replenished with 1000 units/ml hIL-2. After 10– to 14-d expansion, cells were used for 2D measurements, or in stimulation assays. A total of 25,000 NOD.scid,Nur77GFP T cells were stimulated in a 96-well flat-bottom plate with plate-bound OVA, and in a 96-well U-bottom plate with 200,000 NOD splenocytes as a source of APCs that were preincubated with 10 µM InsB9–23, 2.5 U/ml Humulin, or 200,000 islet cells from NOD female mice 16 h prior to stimulation. After 24-h stimulation, cells were stained for CD4 and analyzed by flow cytometry for expression of GFP.

Flow cytometric analysis

Analysis was performed on a Fortessa flow cytometer (BD Pharmingen, San Diego, CA). All Abs were from BioLegend, except anti-Ki67, which was obtained from BD Pharmingen, anti-pErk 1/2 from Cell Signaling, and anti-rabbit 647 polyclonal from Life Technologies. Foxp3 and Ki67 staining was done with a Foxp3 buffer staining kit obtained from eBioscience. Flow analysis was performed using FloJo software. For peripheral organs, analysis was done by gating on the lymphocyte gate based on forward scatter and side scatter, followed by gating on Antimete’ TCR/CD4+ cells; thymic analysis was done after initially gating on lymphocytes, followed by gating on Antimete’ cells, and then on CD4+CD8+ T cells.

Statistical analysis

TCR groups were ranked based on the 2D affinity and pErk point estimates. Each measure was given equal weight in a combined ranking calculated based on the sum of the individual rankings. Diabetes incidence curves were compared using the log-rank test. All other group comparisons were done using the Kruskal–Wallis nonparametric test. A Dunn posttest analysis was performed on all pairs for 2D affinity, or on selected pairs, comparing InsB9–23–specific TCRs to the control 14H4 TCR, or higher affinity TCRs (P2, 1-10, 4-8, 3-4) to lower affinity TCRs (12-4.4, 12-4.4m1, 8-1.1), as noted in the figure legends. All correlation analyses, except Spearman correlation in Supplemental Table II, were based on Pearson correlation...
coefficient. For Spearman correlation analysis, mean values of each variable were taken for each TCR. Spearman correlation coefficient was chosen because several of the variables included failed the Shapiro–Wilk test for normality. A significant p value indicates that the correlation coefficient is statistically significantly different from zero. All statistical tests were two tailed, and p < 0.05 was considered statistically significant (***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05). Statistical analyses were performed using Prism and SAS Version 9.2 (Cary, NC).

Results

Insulin-reactive TCRs possess a broad range of biophysical and functional affinities

TCR affinity can be assessed by determining the biophysical parameters of ligand:receptor interaction, as well as the relative functional biological outcomes of TCR ligation. We assembled a panel of eight InsB9–23-specific TCRs, which were predominantly isolated from islet-resident T cells, thereby ensuring physiological relevance (Supplemental Table I). Furthermore, all the TCRs selected can mediate T cell expansion or IL-2 secretion in response to the WT InsB9–23 peptide. We used two expression systems to assess their biophysical, functional, and diabetogenic potential. First, a TCR-negative hybridoma was transduced with retrovirus encoding each of the insulin-specific TCRs or an irrelevant control (14H4 HEL11–25–restricted TCR) plus a fluorescent protein (Supplemental Table I). Transductants were sorted to ensure comparable TCR cell surface expression. Second, we generated TCR retrogenic mice by transducing NOD.scid bone marrow with TCR-encoding retrovirus that was subsequently used to reconstitute irradiated NOD.scid mice (20, 21, 23, 25).

The dominant diabetogenic CD4+ insulin epitope InsB9–23, like many other CD4+ T cell epitopes, can bind into the MHC groove in several different registers (26–28). We assessed four recombinant MHC:peptide monomers, which had been modified using different approaches to stabilize their peptide register, for their capacity to stimulate T cell transductants. Because the WT InsB9–23 binds in a register that is recognized by most insulin-specific TCRs with fairly low affinity, the presumptive MHC anchor residues were modified to increase the peptide:MHC complex stability of this register (17, 28). The InsB9–23(8G):H-2Ag7 monomer was selected for subsequent analysis based on the comparable responses observed with InsB9–23-pulsed APC-stimulated TCR transductants (Supplemental Fig. 1C).

We first determined the biophysical and functional affinities of the InsB9–23-specific TCRs. Biophysical measurements were obtained using the 2D micropipette adhesion frequency assay, a mechanical approach to measure TCR affinity between the T cell and an RBC coated with the stabilized InsB9–23(8G):H-2Ag7 monomer (10, 13, 17). The utilization of this approach in this study has allowed us to assess the affinity of multiple TCRs specific to the same antigenic peptide epitope. Importantly, the eight TCRs exhibited a range of statistically separable biophysical affinities that fell within the polyclonal range of TCR 2D affinities determined previously (Fig. 1A) (Refs. 10, 15; J. Hood and B. Evavold, unpublished data). The affinities calculated for insulin-specific TCRs were relatively high compared with those of other self-reactive TCRs specific to myelin Ags (10−3–10−4 compared with ~10−6 μM); however, they were similar to 2D affinities of other islet Ag-specific TCRs (Refs. 10, 14, 15; J. Hood and B. Evavold, unpublished data). Still, it is unknown whether the eight TCRs span the entire range of affinities for InsB9–23 represented by islet-infiltrating T cells.

We next obtained functional affinities with TCR hybridoma transductants based on pErk activation and IL-2 secretion in response to InsB9–23 peptide (Fig. 1B, Supplemental Fig. 1A). Importantly, a strong correlation was found between the functional affinity and the biophysical 2D affinity determined for all InsB9–23-specific TCRs, except for 8-1.1 that appeared to mediate a surprisingly high pErk activation and IL-2 induction relative to its 2D affinity (Fig. 1C, Supplemental Fig. 1A). Although the 2D affinities were on the upper end of what has been published for CD4+ T cells in the 2D micropipette system, insulin-specific TCR transfectants responded only to high concentrations of WT peptide compared with the control non–self-reactive TCR (Supplemental Fig. 1B). To facilitate subsequent comparison with other parameters, we arranged and color-coded the TCRs, based on the ranking that incorporated the biophysical 2D affinity and functional affinity (Fig. 1D). Taken together, these data show a panel of InsB9–23-specific TCRs that exhibit a broad range of physiologically relevant, correlative functional, and biophysical affinities.

Diabetogenic potential exhibited over a broad range of TCR affinities

We next generated TCR retrogenic NOD.scid mice to determine the insulitic and diabetogenic potential of our panel of InsB9–23-specific TCRs. We had previously shown that TCR specificity dictated T cell–autonomous islet infiltration (21). Consistent with these findings, we observed that all the InsB9–23-specific TCRs, but not the HEL11–25–specific control TCR, were able to mediate T cell islet infiltration (Fig. 2A). Histological assessment of the pancreatic tissue sections confirmed extensive insulitis driven by almost all the TCRs, albeit with differing severity (Fig. 2B, 2C).
Of interest, the two TCRs mediating limited insulitis possessed the highest and one of the lowest TCR affinities (P2 and 12-4.4m1).

We next assessed the ability of the InsB9–23-specific TCRs to induce spontaneous disease development in NOD.scid mice. We found that the majority of the TCRs (six of eight) were able to induce spontaneous diabetes within 20 wk post–bone marrow transfer (Fig. 2D, 2E). Because most TCRs were isolated from infiltrated islets, the data suggest that a heterogeneous population of T cells exhibiting a range of TCR affinities is involved in autoimmune organ destruction. Not surprisingly, P2 and 12-4.4m1, the two TCRs that mediated limited islet infiltration and insulitis, did not induce diabetes development, although both P2 and 12-4.4m1 have the capacity to induce diabetes upon in vitro expansion, activation, and transfer (Supplemental Fig. 2A). The remaining TCRs induced a surprisingly broad range of diabetes onset and incidence, with neither one of these parameters having any obvious correlation with TCR affinity or, by extension, the functional capacity of these TCRs (data not shown).

To assess the impact of TCR affinity on TCR signaling in vivo, we expressed InsB9–23-specific TCRs on a NOD.scid/Nur77GFP background to facilitate quantification of pErk activation via the early immediate downstream gene Nur77 (33). There was a correlation between TCR affinity and GFP expression in thymocytes (Fig. 2D). The overall level of GFP expression in the thymus was also similar to the peripheral nondraining lymph nodes (LNs) and spleen. Of note, the level of GFP expression was substantially increased in the draining pancreatic lymph nodes (PLNs), consistent with increased Ag availability. However, there was minimal correlation between GFP expression in the PLN and 2D TCR affinity (Fig. 2D). Interestingly, upon T cell islet infiltration GFP expression was comparable among all InsB9–23-specific TCRs, suggesting that an inflammatory setting environmental signals compensate for varying TCR affinity. No correlation was noted between CD5 and Nur77GFP expression in the draining LNs (data not shown).

Because we have amassed a large dataset comparing the in vivo function of eight InsB9–23-specific TCRs, we performed a simultaneous correlative analysis of multiple parameters associated with TCR affinity obtained from our study (Supplemental Table II). The Spearman rank correlation coefficients were used to rank the strength of correlation of the TCR functional parameters, including TCR affinity and disease development. Effective 2D TCR affinity correlated strongly with the strength of TCR signaling in the thymus, as assessed by the level of Nur77GFP expression ($r = 0.7381$) and downregulation of TCR expression (negative correlation with the level of TCR expression in the
The strongest correlation was observed between CD5 expression in the islets and PLN (*r = 1). CD5 expression in the thymus was also correlated with both islets and PLN (*r = 0.6667), which suggests that CD5 levels are stabilized in the periphery and do not change after prolonged stimulation in the islets. Interestingly, CD5 expression correlated with the frequencies of CD4+ T cells in the spleen and PBMCs (*r = 0.7619, *r = 0.8095), which suggests that T cell accumulation in the peripheral organs is positively associated with the strength of TCR signaling. However, diabetes incidence did not show a strong correlation with any parameter associated with TCR affinity, and correlated only with the level of insulitis, and the frequency and number of infiltrating CD4+ T cells (*r = 0.8264; *r = 0.8024; *r = 0.7306, respectively).

Tolerance mechanisms preferentially affect higher affinity diabetogenic TCRs

Even though TCR affinity for the most part correlated with in vivo and in vitro TCR signaling in response to Ag (Figs. 1C, 3F), we observed only minimal correlation between TCR affinity and disease incidence or onset: specifically, lack of any diabetes onset for the highest and one of the lowest affinity TCRs (P2 and 12-4.4m1), and delayed onset for the lower affinity TCRs (8-1.1 and 12-4.1) (Supplemental Fig. 2B, 2C). We postulated that downstream signaling after initial TCR stimulation might lead to activation of negative regulatory feedback mechanisms, including variable expression of CD5, which normalizes the level of TCR signaling during chronic disease progression among high- and low-affinity TCRs. This result would be consistent with normalization of Nur77GFP expression in T cells that have been recruited to the pancreatic islets (Fig. 3D). We therefore assessed the in vitro response of T cell lines derived from Nur77GFP mice to WT peptide and also to naturally processed insulin under controlled stimulation conditions. Nur77GFP expression after 24 h in culture with Ags and APCs was analyzed by flow cytometry (Fig. 4A). A significant correlation was found between pErk activation in transfectants and GFP expression in the T cell lines in response to WT peptide (Fig. 4B, 4C), although the discordance between these values suggested modification of TCR signaling in T cells. Of interest, the T cell response to whole protein best correlated with in vivo GFP expression in the draining LNs over CD5 or 2D affinity (Fig. 4B, 4D), which supported our hypothesis that activation of feedback negative regulatory mechanisms modulates TCR signaling in vivo and in vitro. Moreover, the Spearman correlation analysis showed that 2D TCR affinity and Nur77GFP correlated with response to protein stimulation when it was normalized to anti-CD3 stimulation (Supplemental Table II, Nur77GFP, insulin; *r = 0.7857), which suggests that InsB9–23(8G):H2-Ag7 monomer used for measurements of 2D affinity closely mimics the naturally processed epitope, and supports our hypothesis that permanent reg-
ulatory changes occur downstream of TCR ligation, modulating signaling in the higher affinity TCRs (Supplemental Table II). We therefore assessed the role of canonical central and peripheral tolerance mechanisms in regulating diabetogenic potential.

First, NOD.\textit{Ins2}^{−/−} mice develop accelerated and exacerbated autoimmune diabetes (34, 35). Deletion of the \textit{Ins2} gene, which is expressed in both the thymus and pancreas, unlike the \textit{Ins1} gene, which is expressed only in the pancreas, results in increased susceptibility to diabetes largely owing to inefficient deletion of insulin-reactive clones during thymic selection (35). We expressed the panel of \textit{InsB}^{9−23}-specific TCRs in NOD.\textit{scid}.\textit{Ins2}^{−/−} mice. Strikingly, there was a clear impact of \textit{Ins2}-mediated central tolerance on T cells expressing predominantly high- but not low-affinity TCRs (Fig. 5).

Interestingly, the highest affinity TCR P2 did not show any disease development on the NOD.\textit{scid}..\textit{Ins2}^{−/−} background. However, upon in vitro activation and expansion and subsequent adoptive transfer P2 T cells are capable of inducing diabetes to some extent (Supplemental Fig. 2A), suggesting that additional parameters other than \textit{Ins2}-mediated central tolerance in the thymus regulate its pathogenic potential. Although the impact of \textit{Ins2}^{−/−} background on diabetogenic potential of high-affinity TCRs was dramatic, we did not notice any obvious changes in thymic selection based on frequency or number of SP CD4+ thymocytes, or in the levels of TCR or CD5 expression (data not shown).

Second, we examined the contribution of Foxp3+ regulatory T cell (Treg)–mediated peripheral tolerance to disease outcome in \textit{InsB}^{9−23}-specific TCR retrogenic mice. Treg accumulation was observed in the periphery of all \textit{InsB}^{9−23}-specific TCR retrogenic mice, albeit over a broad range (0.5–12%), whereas a more consistent Treg percentage was observed in the PLNs (1.5–5%; Fig. 6A). Of interest, there appeared to be a trend toward higher Treg percentages, especially in the islets, of mice expressing high-affinity TCRs. On the basis of the Spearman correlation analysis, frequencies of Foxp3+ T cells in the islets were associated with CD5 expression in the thymus and CD4+ T cell frequency and number in the periphery (Supplemental Table II; *$r=0.7143$, *$r=0.7381$, *$r=0.7143$), which points to the positive association of TCR strength of signal and/or T cell numbers and Treg accumulation in the inflammatory setting.

We then assessed the contribution of these Treg populations in moderating autoimmune diabetes by their selective deletion in NOD.\textit{scid}.\textit{Foxp3}^{DTR} TCR retrogenic mice following diphtheria toxin treatment from 5.5 wk post–bone marrow transfer (18). Synonymous with the observations made in the NOD.\textit{scid}.\textit{Ins2}^{−/−} experiments above, a selective contribution of Foxp3+ Tregs was...
observed in mice expressing higher affinity InsB9–23-specific TCRs (1-10, 3-4, and 4-8; Fig. 6B). It is not clear whether the impact of Tregs in high-affinity TCR retrogenic mice was due to the increased number of Tregs or the increased suppressive capacity of those Tregs owing to their higher affinity TCR. The rate of disease acceleration was different among the three TCRs, and slower than was observed for the BDC2.5/NOD.Foxp3DTR mice, which was probably due to the differences in Treg/T effector ratio or function of cells with variable TCR affinities, different antigenic specificities (for BDC2.5), and/or wider Treg TCR repertoire (18). Taken together, these data suggest that central and peripheral tolerance mechanisms selectively impinge on the pathogenicity of autoreactive TCRs, with higher affinity to mitigate their diabetogenic potential to a level comparable to that of TCRs with lower affinity.

Discussion
In this study, we assessed whether the biophysical properties of the TCR, such as affinity, alone can determine the autoimmune pathogenicity of T cells or whether other factors converge to shape disease outcome. Previous efforts to address this important but often contentious issue have been limited by the number of TCRs examined (6–10). We have overcome this barrier using TCR retrogenic technology to generate mice expressing one of eight InsB9–23-specific TCRs with a broad affinity range. This approach has three key advantages: 1) Any genetic background can be used, which remains consistent between groups; 2) multiple TCRs can be simultaneously analyzed; and 3) a panel of TCRs to a single T cell epitope can be analyzed. Surprisingly our study showed that 2D TCR affinity did not predict disease outcome. In fact both high- and low-affinity TCRs, at least within the range assessed, caused significant insulitis and autoimmune diabetes. These data also suggest that relatively small changes in 2D TCR affinity (within a 10-fold range) can result in a very broad range of biological, regulatory and pathogenic outcomes. Given that most of the TCRs used in this study were directly derived from islet-infiltrating T cells, an autoimmune CD4+ T cell population specific for a single self-epitope can be composed of highly pathogenic, nonpathogenic, and regulatory clonal populations.

The 2D functional affinities we obtained for islet-infiltrating InsB9–23-specific TCRs are relatively high compared with other published autoreactive TCR 2D affinities (10^{-3}–10^{-4} versus 10^{-5} μM), and are similar to nonautoimmune Ag-specific TCRs (10, 13–15). These TCRs may preferentially recognize insulin peptide bound in the nonfavorable register 3 (27), and in vivo the instability of this complex probably has some effect on the dwell time of the TCR, thus allowing escape of InsB9–23-specific T cells from negative selection in the thymus. Indeed, many of these TCRs respond only to relatively high levels of Ag in vitro. Similarly, human InsA1–15-specific T cells cloned from pancreatic islets of type 1 diabetic patients were activated only with high levels of antigenic peptide (36). Thus, it is possible that InsB9–23-specific CD4+ T cell pathogenicity is governed more by the efficiency with which this epitope can be loaded into H-2Aβ in the third register.
and presented to T cells than by TCR affinity per se. It is also possible that peptide loading may be more efficient in the islets and/or PLNs compared with thymus, owing to the differing microenvironments (37). Given that all of the TCRs were isolated based on T cell reactivity to insulin, it is possible that we missed lower affinity TCRs that are present during islet infiltration and may contribute to the disease. Future studies with polyclonal populations analyzed using sensitive 2D TCR affinity measurements will be required to fully address this issue.

Central and peripheral tolerance mechanisms collaborate to limit autoimmune insults. However, the extent to which they shape the clonal diversity of an autoimmune response is poorly understood. Our results and those of a previous study (38) are somewhat different from the data obtained with model Ags in which negative selection has a sharp and well-defined affinity threshold (5). Higher affinity InsB9–23–specific TCRs (especially P2) are affected by the level of Ag expressed in the thymus such that they are rendered nonpathogenic unless activated in vitro, but complete deletion is not observed. Thus, thymically expressed insulin has a partial effect on negative selection in contrast to the well-studied thymic selection events mediated by the male HY Ag. Likewise, a surprisingly large proportion of InsB9–23–specific TCRs can navigate negative selection and induce autoimmune diabetes over a seemingly broad TCR affinity range. However, it was evident that the different regulatory mechanisms that preferentially impinge on the diabetogenic potential of higher affinity TCRs (especially P2) are affected by negative regulatory mechanisms, such as Tregs. Of interest, high-affinity TCRs are more susceptible to which they are affected by negative regulatory mechanisms, as Tregs. Of interest, high-affinity TCRs are more susceptible to Treg-mediated peripheral tolerance mechanisms. With one exception (12-4.4m1), higher affinity TCRs displayed increased frequencies of Foxp3+ T cells accumulating in the pancreatic islets, which is consistent with the idea that Treg development is associated with high-affinity TCRs (40). This finding suggests that the Treg/T effector cell ratio in the islets defines the extent of autoimmune diabetes and may provide the simplest explanation for the impact of Treg deletion on pathogenicity. It is possible that the reduced susceptibility of T cells to Treg-mediated regulation in the later stages of chronic autoimmune disease is due in part to accumulation of lower affinity T cells that are less likely to be converted into regulatory populations and are more refractory to regulation owing to their reduced intrinsic inhibitory mechanisms.

In conclusion, our study shows that the diabetogenic potential of CD4+ T cells is not exclusively governed by TCR affinity and that T cell clones with both high- and low-affinity TCRs can mediate potent pathogenic responses. The mechanistic basis for this lies in the differential effect of central and peripheral tolerance mechanisms that preferentially impinge on the diabetogenic potential of high-affinity TCRs, thereby “leveling the playing field.” This may inadvertently broaden the autoreactive T cell repertoire to a single epitope in which higher and lower affinity T cells may exhibit similar pathogenicity. Whether these criteria shape the human autoimmune response in type 1 diabetes, or any other autoimmune disease, remains to be determined. However, if the observations made in this article represent a general principle, it is conceivable that multiple combinatorial approaches may have to be used to induce effective, broad, long-term tolerance in the clinic.

Acknowledgments

We thank members of the Vignali laboratory for assistance with harvesting bone marrow and helpful discussions; Richard Cross, Stephanie Morgan, Greig Lennon, and Painter Ingle for cell sorting; Scott Brown for assistance with generating T cell fusions; John Altman and Richard Willis, National Institutes of Health Tetramer Core Facility, for providing peptide and MHC monomers; the staff of the Shared Animal Resource Center at St. Jude for the animal husbandry; and the Hartwell Center for Biotechnology and Bioinformatics at St. Jude for primers and sequencing.

Disclosures

The authors have no financial conflicts of interest.

References


