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References

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Structure-Based Selection of Small Molecules To Alter Allele-Specific MHC Class II Antigen Presentation

Aaron W. Michels,* David A. Ostrov,† Li Zhang,* Maki Nakayama,* Masanori Fuse,* Kristen McDaniel,* Bart O. Roep,‡ Peter A. Gottlieb,* Mark A. Atkinson,† and George S. Eisenbarth*

Class II major histocompatibility molecules are the primary susceptibility locus for many autoimmune disorders, including type 1 diabetes. Human DQ8 and I-A$^\beta^7$, in the NOD mouse model of spontaneous autoimmune diabetes, confers diabetes risk by modulating presentation of specific islet peptides in the thymus and periphery. We used an in silico molecular docking program to screen a large “druglike” chemical library to define small molecules capable of occupying specific structural pockets along the I-A$^\beta^7$ binding groove, with the objective of influencing presentation to T cells of the autoantigenic insulin B chain peptide consisting of amino acids 9–23. In this study we show, using both murine and human cells, that small molecules can enhance or inhibit specific TCR signaling in the presence of cognate target peptides, based upon the structural pocket targeted. The influence of compounds on the TCR response was pocket dependent, with pocket 1 and 6 compounds inhibiting responses and molecules directed at pocket 9 enhancing responses to peptide. At nanomolar concentrations, the inhibitory molecules block the insulin B chain peptide consisting of amino acids 9–23, endogenous insulin, and islet-stimulated T cell responses. Glyphosine, a pocket 9 compound, enhances insulin peptide presentation to T cells at concentrations as low as 10 nM, upregulates IL-10 secretion, and prevents diabetes in NOD mice. These studies present a novel method for identifying small molecules capable of both stimulating and inhibiting T cell responses, with potentially therapeutic applications. The Journal of Immunology, 2011, 187: 5921–5930.

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Materials and Methods

Molecular modeling and docking

We used crystal structures of I-A$^\beta^7$ complexed to the GAD65 peptide, Protein Data Bank code 1ES0, and HLA-DQ8 complexed to insulin B:9–23, Protein Data Bank code 1JK8, as the basis for molecular docking (12). An atomic model of I-A$^\beta^7$ complexed to insulin B:9–23 was generated by superimposing the Ag binding cleft of HLA-DQ8 on I-A$^\beta^7$ and applying the same rotation and translation to coordinates for the B:9–23 peptide. To prepare the site for docking, all water molecules were removed and protonation of I-A$^\beta^7$ residues was done with SYBYL (Tripos) (13). The molecular surface of the structure was explored using sets of spheres to describe potential binding pockets. The sites selected for molecular docking were defined using the SPHGEN program (generates a grid of points that reflect the shape of the selected site) and filtered through CLUSTER. The CLUSTER program groups the selected spheres to define the points that are used by DOCK v5.1.0 (14) to match potential ligand atoms with spheres. Intermolecular AMBER energy scoring (van der Waals + columbic), contact scoring, and bump filtering were implemented.

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The online version of this article contains supplemental material.

Abbreviations used in this article: B:9–23, insulin B chain peptide consisting of amino acids 9–23; NCI/DTP repository, National Cancer Institute Developmental Therapies Program.

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in the DOCK program algorithm. Atomic coordinates for ~140,000 small molecules in the National Cancer Institute Developmental Therapeutics Program (NCT/DTT repository) were positioned in the selected structural pocket in 1000 different orientations and scored based on predicted polar (H bond) and nonpolar (van der Waals) interactions. The best orientation and scores (contact and electrostatic) were calculated. PYMOL was used to generate molecular graphic images.

Generation of insulin B:9–23–specific T cell hybridomas

Creation of BDC 12-4.1 and BDC 12-4.4 T cell hybridomas was previously reported (9). The BDC 8-1.1 α chain line was produced from splenocytes of a retrogenic mouse with a single 8-1.1 TCR following the same procedure. The 4–8 TCR stimulation curve came from the pancreatic islets of an α-chain–only retrogenic mouse (using the α-chain of BDC 12-4.1).

TCR stimulation LacZ assay

Ag-specific hybridoma stimulation was estimated by measuring LacZ activity. Small molecules for screening were dissolved in DMSO and diluted in PBS for a final concentration of 0.1% DMSO in each well. Each small molecule was screened at a concentration of 100 μM. After small-molecule screening, glycosine and derivatives were purchased from Sigma-Aldrich and dissolved in PBS, with the pH adjusted to 7.4 for use in the stimulation and ELISPOT assays. Peptides (Genemed Synthesis) were used to cleave the peptide and linker and 10 μM biotinylated peptides (B:9–23, HEL11–25, and En52–68 from Genemed Synthesis) in binding buffer (20 mM HEPES and 150 mM NaCl, pH 7.4), to a total volume of 30 μl overnight at room temperature. Biotinylated peptide–I-Aβ complexes were purified from free peptide using gel filtration Bio-Spin columns (Bio-Rad). A 96-well-high binding plate was coated with 0 μg/ml of an I-Aβ mAb (BD 554926) in 100 mM Na2CO3, pH 9.6, overnight at 4˚C. Plates were washed with PBS and PBS containing Tween-20; then samples were transferred to the Ab-coated plate and incubated for 4 h at room temperature. Plates were developed with streptavidin-europium and read on a fluorimeter.

Generation of hybridomas expressing a TCR

The 4–8 and human TCR constructs, α- and β-chains linked by the PTV1.2A sequence, were cloned into murine stem cell virus-based retroviral vectors carrying GFP (pMIGII) (15). Phoenix cells were cotransfected with the pMIGII plasmids and with the pCL-Eco packaging vector, containing the PTV1.2A sequence, were cloned into murine stem cell virus-based retroviruses encoding TCR sequences. The 5KC hybridomas were sorted by GFP expression, and TCR H57-597; BD Biosciences).

**FIGURE 1.** Small-molecule inhibitors used in B:9–23 activation of T cells. A. Diagram of the structural pockets along the I-Aβ2 binding groove, with the proposed binding sites for the compound structures based upon in silico molecular modeling. B–F. Inhibition curves of compounds that block T cell responses to the B:9–23 peptide. Three TCRs are depicted; 8-1.1α1 (●) and BDC 12-4.1 (○) recognize insulin B:9–23 peptide, whereas BDC 2.5 (▲) responds to a chromogranin mimotope. Percentage of inhibition was calculated from the stimulation of peptide alone for each TCR. Data points represent triplicate wells for each concentration of small molecule with peptide. Inhibition curves are representative of at least three independent experiments.

**II-2 ELISA**

TCR-expressing 5KC hybridomas (2 × 106 cells) were incubated in the presence or absence of 200 μg/ml Ags (insulin B:9–23 or tetanus toxin), along with spleen cells from NOD mice (2 × 105 cells per well) or DQ8 humanized mice overnight. IL-2 secretion in supernatants was measured by an ELISA assay according to the manufacturer’s instructions (R&D Systems). Stimulation of the DO11.10 5KC hybridoma was performed in a similar fashion, with 105 cells in the presence of 1 mg/ml chicken OVA (Sigma-Aldrich A5503) and 2 × 105 BALB/c splenocytes.

**Binding analysis**

Binding assays performed as previously described exempt use of a biotin-streptavidin-europium detection method (3). A total of 1.0 μg purified I-Aβ2/B:12–23 protein complex was incubated with 0.1 μM thrombin (Novagen) to cleave the peptide and linker and 10 μM biotinylated peptides (B:9–23, HEL11–25, and En52–68) in binding buffer (20 mM HEPES and 150 mM NaCl, pH 7.4), to a total volume of 30 μl overnight at room temperature. Biotinylated peptide–I-Aβ complexes were purified from free peptide using gel filtration Bio-Spin columns (Bio-Rad).

**Marine ELISPOT assay**

IL-10 and IFN-γ ELISPOT kits (BD Biosciences) were used for splenocyte analysis. Glycosine was used at a final concentration of 500 nM for in vitro culture with splenocytes (7 × 106 cells per well) and peptide (100 μg/ml). Plates were developed using the AEC substrate system (BD Biosciences), and spots were enumerated using the ImmunoSpot reader and software version 3.1 (Cellular Technology).

**Animals**

Mice were bred and housed under specific pathogen-free conditions at the University of Colorado Denver Center for Comparative Medicine with an approved protocol from the University of Colorado Denver Animal Care and Use Committee. Spleens for TCR stimulation LacZ assays and
ELISPOT assays were obtained from the Barbara Davis Center-derived NOD mouse colony. DQ8 splenocytes were obtained from DQ8/human CD4/I-Ag7null/Rag KO NOD mice from The Jackson Laboratory. BALB/c, C57BL/6, and C57BL/6 congenic for I-A\textsuperscript{\alpha} mice were also obtained from The Jackson Laboratory for ELISPOT assays, as were NOD mice for the diabetes prevention study.

**Assessment of diabetes**

Glucose was measured weekly with the ReliOn blood glucose monitoring system (SolarTek), and mice were considered diabetic after two consecutive blood glucose values of \( > 250 \) mg/dl.

**Glyphosine administration**

Glyphosine was purchased from Sigma-Aldrich (catalog no. 15149), dissolved in PBS, and injected into the peritoneal cavity. A dose of 80 mg/kg/d was administered in a final volume of 200 \( \mu \)l on 5 d consecutively. Control mice received 200 \( \mu \)l PBS.

**HLA genotyping**

Informed consent was obtained after the nature and possible consequences were explained to individuals providing serum samples for HLA genotyping and cytokine ELISPOT assay with an approved protocol from the Colorado Institutional Review Board. DRB1 and DQB1 genotyping was performed on patient samples, using linear arrays of immobilized sequence-specific oligonucleotides, similar to previously described methodology (17).

**Human indirect ELISPOT assay**

Indirect ELISPOT analyses were conducted as previously described (18), using the human INF-\( \gamma \) and IL-10 ELISPOT kits (U-CyTech Biosciences, Utrecht, The Netherlands). Briefly, freshly isolated PBMCs (1 \( \times \) \( 10^6 \)) were cultured in 250 \( \mu \)l RPMI 1640 containing 10% heat-inactivated human AB serum (PAA Laboratories, Dartmouth, MA) and 10 \( \mu \)M peptide. The cells were fed an additional 250 \( \mu \)l medium after 24 h, and harvested 24 h later. After washing, the cells were resuspended in 300 \( \mu \)l RPMI 1640.

**FIGURE 2.** Small-molecule inhibitors block presentation of endogenously processed insulin by NOD splenocytes. Inhibition curves for p1 molecules (A) and p6 molecules (B). Dotted line represents IL-2 production by the 4–8 TCR to insulin in the absence of a small molecule. C, IL-2 production from a T cell hybridoma cultured with whole islets used as APCs. The p6:4 molecule inhibits IL-2 production when islets isolated from an adult NOD mouse without diabetes are used for Ag presentation to the 4–8 TCR. *\( p = 0.004 \), **\( p = 0.002 \), ***\( p < 0.001 \). D, p6:4 inhibits B:9–23 peptide binding to I-A\textsuperscript{\alpha}, as determined using a soluble binding assay with purified I-A\textsuperscript{\alpha} protein and biotinylated insulin B:9–23 peptide. Negative control is uncut I-A\textsuperscript{\alpha} (thrombin was not used to cleave the flexible linker and linked peptide), with biotinylated B:9–23 peptide added to the assay. *\( p = 0.023 \), **\( p < 0.001 \). E, p6:4 inhibits a human DQ8-restricted TCR to insulin B:9–23. *\( p = 0.004 \), **\( p < 0.001 \). TT, tetanus toxin (negative control peptide).
medium and transferred as three 100-μl aliquots to 96-well clear polystyrene culture plates coated with the appropriate cytokine capture mAb and subsequently treated with 1 × blocking solution (U-CyTech). At 17 h later, the wells were removed by decanting, and the wells were washed (2× with PBS and 5× with PBS containing 0.05% Tween-20). Spots were then formed by sequential incubations with the biotinylated second site anti–IFN-γ or anti–IL-10, gold-labeled goat anti-biotin, and a precipitating silver substrate. Spots were enumerated with a Bioreader 4000 Pro X (BIOSYS, Karben, Germany). No Ag wells contained DMSO, as peptides are dissolved in DMSO for the assays. Positive control samples from incubations with tetanus toxin were included in each assay.

**Statistical analysis**

IL-2 concentrations for T cell stimulation assays, europium counts for protein binding, stimulation indices for glyphosine, and mean spot numbers from ELISPOT assays were analyzed with an unpaired Student t test. Survival curves for the diabetes prevention study were analyzed with the log-rank test. Wilcoxon matched pairs test compared means between groups in the human cytokine ELISPOT assays. Linear regression analysis was done to compare IL-10 ELISPOT assays with B:9–23 glyphosine for type 1 diabetic individuals and controls. Statistical tests used GraphPad Prism 4.0 software.

**Results**

**Molecular docking and virtual screening of a small-molecule library**

To identify candidate molecules predicted to bind specific structural pockets of the I-A^d^ Ag binding groove, we used a supercomputer to screen ~140,000 small molecules from the NCI/DTP repository of druglike compounds. Existing crystal structures available for modeling include I-A^d^ with a bound GAD65 peptide (19), as well as B:9–23 bound to DQ8 (20). To model the binding of B:9–23 to I-A^d^, we superimposed the Ag binding clefts of DQ8 and I-A^d^, determined sites of critical contacts between peptide and MHC, and displayed the atomic coordinates of B:9–23 peptide from the solved crystal structure with coordinates of I-A^d^ for the solved crystal structure. The identified orientation and conformation of the insulin B:9–23 peptide was complementary with the Ag binding cleft for the crystal structure of I-A^d^ (Supplemental Fig. 1). All NCI organic compounds were docked in 1000 orientations, using the DOCK program algorithm (14), and scored based on the sum of attractive and repulsive polar and nonpolar interactions to compute free energy (ΔG) of binding. Supplemental Table I lists the top 40 scoring compounds for each pocket along with the corresponding NCI identifier.

**In vitro screening of lead candidate small molecules**

The top 40 scoring compounds for each pocket were screened for their ability to alter anti–B:9–23 T cell responses for different T cell hybridomas (BDC 8-1.1α1, BDC 12-4.1, and BDC 12-4.4) (Supplemental Fig. 2). The in vitro T cell stimulation assays used hybridomas engineered to produce β-galactosidase following TCR Ag activation (NF of activated T cell promoter) (21). Upon

**FIGURE 3.** Glyphosine specifically enhances the T cell response to insulin B:9–23 presented by I-A^d^. A, Glyphosine enhanced stimulation of the T cell hybridoma 8-1.1α1 only when insulin B:9–23 peptide was present. Glyphosine concentrations are indicated, and the reaction mixture contained B:9–23 peptide unless otherwise noted. The glyphosine concentration used in subsequent experiments was 500 nM. TT, tetanus toxin (negative control peptide). *p = 0.011, **p = 0.049 (unpaired Student t test). B, The glyphosine response in the presence of B:9–23 is blocked by a mAb to I-A^d^ and unchanged with an isotype control Ab. *p < 0.001. C and D, Glyphosine does not alter TCR reactivity to a chromogranin peptide presented by I-A^d^ to the BDC 2.5 T cell hybridoma or to OVA presented by I-A^d^ to the DO11.10 T cell hybridoma. Results are given as a stimulation index (SI) from triplicate cultures ± SEM. Data are representative of at least three independent experiments.
stimulation, LacZ production correlates with IL-2 production. From the top-scoring 40 compounds predicted to occupy each p1 and p6, a subset resulted in inhibition of B:9–23 TCR activation: 2 of 40 for p1 and 3 of 40 for p6 (Fig. 1A). In contrast, multiple compounds predicted to bind p9 (20 of 40) resulted in an enhancement of hybridoma responsiveness ($p < 0.001$, p9 compounds versus pocket 1 and 6, using a rank sum test). Pocket 4 small molecules did not dramatically alter T cell responses to the B:9–23 peptide.

**Small molecules inhibit insulin presentation**

Five compounds (Fig. 1A; Supplemental Table II) inhibited the response of multiple anti–B:9–23 TCRs to varying degrees, with IC50 values in the low micromolar range. The inhibition curves for the compounds from stimulation assays in the presence of B:9–23 peptide are shown in Fig. 1B–F. Next, we tested the ability of the small molecules to block endogenous insulin presentation. Incubation of whole insulin with I-A$^d$-containing splenocytes activates 5KC cells (T cell hybridoma lacking endogenous TCRα— and β-chains) transduced with a 4–8 TCR. The small molecules examined were able to block T cell stimulation from endogenously processed insulin, with the small molecules p1:17 (pocket 1: compound 17), p6:4, and p6:18 having IC50 values in low nanomolar concentrations (Fig. 2A, 2B). The p6:4 molecule, tetraazatricyclododecane, was studied in additional detail owing to lower IC50 values and was able to inhibit TCR response to isolated whole islets from an adult non-diabetic NOD mouse (Fig. 2C), which have been shown to contain dendritic cells with insulin peptides, notably B:9–23 (22). To document direct effects of these small molecules on inhibiting peptide binding to I-A$^d$, we expressed an I-A$^d$ protein construct in baculovirus with a linked peptide (23). The flexible linker contained a thrombin cleavage site, allowing for thrombin cleavage of the linker and release of the peptide. In a soluble binding assay, the p6:4 molecule was able to inhibit B:9–23 peptide binding to the empty I-A$^d$ in a dose-dependent manner (Fig. 2D). We further investigated the binding

<table>
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<th>EC50 for B:9–23 Peptide</th>
<th>Generality</th>
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</thead>
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<td>Glyphosine</td>
<td>70.6 nM</td>
<td>Enhances stimulation of anti-B:9–23 hybridomas and not the antichromogranin BDC 2.5 hybridoma</td>
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<tr>
<td>Derivative 1</td>
<td>No stimulation or inhibition</td>
<td>NA</td>
</tr>
<tr>
<td>Derivative 2</td>
<td>194 nM</td>
<td>Enhances stimulation of both anti-B:9–23 hybridomas and BDC 2.5 hybridoma</td>
</tr>
<tr>
<td>Derivative 3</td>
<td>No stimulation or inhibition</td>
<td>NA</td>
</tr>
<tr>
<td>Derivative 4</td>
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<td>NA</td>
</tr>
</tbody>
</table>
inhibition with higher affinity Ags for I-A<sup>d</sup>, HEL11–25, and E<sub>a</sub>52–68. We found p6:4 was able to inhibit binding of these biotinylated peptides to a similar degree as the B:9–23 peptide (data not shown).

To test whether these inhibitory small molecules decrease TCR reactivity selectively to B:9–23 insulin peptides, we studied a chromogranin peptide mimotope presented by I-A<sup>d</sup> to the BDC 2.5 T cell hybridoma (24). Two of the five compounds tested, p1:6 and p6:17, showed no inhibition of chromogranin presentation to the BDC 2.5 hybridoma (Fig. 1B–F), whereas the other three compounds inhibited both B:9–23 and chromogranin presentation to their respective hybridomas. Other compounds predicted to bind pocket 1 and 6 blocked TCR responses, but not all of these molecules were specific for the B:9–23 peptide. Taken together, these data demonstrate that in vitro small molecules are able to inhibit endogenous insulin presentation and displace B:9–23 peptide binding to I-A<sup>d</sup>, and certain small molecules have specificity for inhibiting insulin presentation to T cells.

Significant homology exists between the high-risk class II allele DQ8 of humans (DQA1*0301-DQB1*0302) and I-A<sub>g7</sub> of the mouse (25). Furthermore, the amino acid sequences of the murine insulin 2 B:9–23 is identical to human B:9–23. We performed in silico molecular docking, using the compounds from the NCI/DTP repository for pockets along the binding groove of DQ8. Twenty of the top 40 scoring compounds for both pocket 6 of I-A<sub>g7</sub> and DQ8 are identical, suggesting that small molecules targeted to I-A<sup>d</sup> may similarly bind to DQ8 (for p1, 0 of 40; p4, 5 of 40; and p9, 6 of 40 are identical). We evaluated the p6:4 molecule for the ability to block B:9–23 peptide presentation by DQ8. Using a TCR sequence obtained from a T cell line generated from the peripheral blood of a diabetic individual with the DQ8 molecule, we identified a TCR that is DQ8 restricted and responds to the insulin B:9–23 peptide (26). Using this TCR along with DQ8 splenocytes from a humanized mouse, we show the p6:4 molecule inhibits TCR activation (Fig. 2E), suggesting that small molecules targeted to pocket 6 of I-A<sup>d</sup> also bind in a manner similar to that of the human homolog DQ8.

**Glyphosine, a pocket 9 compound, enhances T cell responses to insulin B:9–23**

We hypothesized that small molecules binding to the I-A<sup>d</sup> binding groove would inhibit Ag presentation and subsequent T cell stimulation; however, compounds predicted to bind pocket 9 proved to be stimulatory. Twenty of the 40 top scoring compounds enhanced T cell stimulation of at least one of our three anti-B:9–23 hybridomas in the presence of insulin B:9–23 peptide. One compound, p9:12 (pocket 9: compound 12) glyphosine, enhanced TCR stimulation to B:9–23 when tested with all three of our anti-B:9–23 hybridomas, prompting further evaluation of this compound. To assess specificity of the hybridoma response to glyphosine, the same assays evaluating
Glyphosine treatment delays diabetes onset in NOD mice

IL-10 has anti-inflammatory properties reported to downregulate Th1 cytokines, such as IFN-γ (27), and is associated with T1 regulatory T cells (28, 29). Glyphosine could be an immunomodulatory agent for the treatment and prevention of type 1 diabetes. We evaluated whether in vivo administration would delay the development of diabetes (Fig. 4E). NOD mice received glyphosine starting at 4 wk of age, with treatment concluded at 21 wk of age. Prevention of diabetes occurred as long as therapy was administered (p < 0.001); however, cessation of glyphosine treatment resulted in diabetes development at a rate similar to that in control mice between 10 and 30 wk of age.

Glyphosine enhances IL-10 ELISPOT assays in HLA-DQ8 type 1 diabetic individuals

With the remarkable homology between murine I-A^β^ and human DQ8, we evaluated the glyphosine IL-10 response in human cells, using an indirect ELISPOT assay (30) from DQ8 individuals with and without type 1 diabetes; clinical characteristics and DR genotyping are depicted in Table II. PBMCs were isolated from DQ8 individuals and cultured with peptides (including insulin B:9–23) and glyphosine. The PBMCs were washed, resuspended in media, and then IL-10 and IFN-γ spots were developed and enumerated. In type 1 diabetic individuals, IL-10 ELISPOTs were increased with glyphosine and B:9–23 peptide, compared with B:9–23 peptide alone or glyphosine alone (Fig. 5A). IFN-γ ELISPOTs were unchanged with the addition of glyphosine and B:9–23 peptide (Fig. 5B). DQ8 controls without diabetes did not show any difference in ELISPOT number for IL-10 (Fig. 5C) and IFN-γ (Fig. 5D) with the addition of glyphosine. Glyphosine did not alter the IL-10 or IFN-γ response to tetanus toxin in type 1 patients with diabetes or controls. In summary, a correlation exists between the number of IL-10 spots with B:9–23 peptide and glyphosine, compared with B:9–23 peptide alone in the diabetic group (Fig. 6A); however, no correlation exists in the DQ8 controls without diabetes (Fig. 6B).

Table II. Clinical characteristics and HLA genotype in patients with type 1 diabetes and nondiabetic control subjects

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<th>Sex</th>
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*DQ8 is part of the DR4 haplotype.

TID, type 1 diabetes; x, unable to obtain complete DR genotype.
Discussion

Our studies indicate that small organic molecules identified by an in silico docking algorithm targeted to pockets in the binding groove of the NOD MHC class II molecule, I-A^7, can alter presentation of the insulin peptide B:9–23. Furthermore, these molecules have similar effects with human components of the anti-insulin trimolecular complex. Pocket 9 compounds resulted in enhanced TCR response, whereas compounds predicted to occupy p1 and p6 inhibited TCR activation.

Small molecules have been used in the context of MHC class II Ag presentation. Molecules exist that act as a catalyst and facilitate peptide loading onto human DR (31, 32), and a recent study by Call and colleagues (33) identified a small molecule with in vivo activity that accelerated the rate of peptide loading onto DR.  

FIGURE 5. IL-10 and IFN-γ ELISPOT assays from DQ8 type 1 diabetic individuals and controls. A, IL-10 ELISPOT in type 1 diabetic subjects (n = 14) showing an increase in IL-10 spots with glyphosate and B:9–23 peptide compared with B:9–23 peptide alone. B, IFN-γ ELISPOT showing no change in number when glyphosate is added to culture with or without B:9–23 peptide. IL-10 (C) and IFN-γ ELISPOTs (D) from nondiabetic DQ8 controls (n = 8) showing no change in spot number with glyphosate. All patients produced IFN-γ in response to a tetanus toxin peptide (used as a positive control) that did not change in response to glyphosate. Each data point represents the mean spot number from triplicate wells for an individual. Wilcoxon matched pairs test was used to compare means between groups, and p values are labeled on graphs for ease of comparison.

FIGURE 6. Correlation between IL-10 ELISPOTs with and without glyphosate in the presence of B:9–23 peptide. Mean IL-10 ELISPOTs from DQ8 individuals with type 1 diabetes (n = 14) (A) and nondiabetic DQ8 controls (n = 8) (B). A statistically significant correlation exists between the insulin B:9–23 spot number and spots in the presence of peptide and glyphosate for individuals with type 1 diabetes (p = 0.002, r^2 = 0.56). No correlation is seen with the DQ8 controls (p = 0.30, r^2 = 0.17). T1D, type 1 diabetes.
Although small molecules have been studied in the context of MHC class II Ag presentation, to our knowledge, our study is the first to identify small molecules, using a molecular docking algorithm, that are predicted to interact with the MHC class II peptide binding groove for the purpose of inhibiting or enhancing the T cell response to a specific target peptide.

Only two small molecules predicted to bind pocket 1 and 6 specifically blocked the T cell response to insulin B:9–23. The insulin B:9–23 peptide can bind to I-A\(^\beta\) in multiple different registers (34, 35), and in particular the BDC 12:4.1 hybridoma and other TCRs recognize the B:12–23 peptide in a low-affinity register, termed “register 3.” Given the very low affinity register for B:9–23, and the fact that peptide binding is recognized by multiple autoreactive TCRs, the potential exists that small molecules differentially inhibit TCR responses to specific peptides. This potential may explain why specific small molecules inhibit B:9–23 responses, but not the antichloroguanadin mimotope BDC 2.5 responses, which have a higher affinity for I-A\(^\beta\).

Unlike the pocket 1 and 6 small molecules, the pocket 9 molecule, glyphosine, enhanced the T cell response to the insulin B:9–23 peptide. The low-affinity register of binding for the B:9–23 peptide presumably has the arginine residue of the peptide occupying pocket 9 along the I-A\(^\beta\) binding groove (7). A polymorphism in the gene encoding the β-chain of I-A\(^\beta\) and DQ8 alters the electrostatic nature of pocket 9 owing to the substitution of serine or alanine for aspartic acid at the β57 position (3, 36). This substitution disrupts a salt bridge between β57 aspartic acid and α76 arginine, thus allowing the basic arginine residue to interact with the amino acid side chain of peptides bound in p9 of I-A\(^\beta\) and DQ8 (Supplemental Fig. 1). Glyphosine is capable of being negatively charged at a neutral pH, and we postulate that glyphosine changes the register of peptide binding when occupying pocket 9. It has been shown that the peptide amino acid occupied in pocket 9 of I-A\(^\beta\), either neutral or negatively charged, can affect TCR/peptide-MHC affinity (4). The enhanced IL-10 response we observed in the presence of glyphosine may reflect this altered TCR interaction.

Glyphosine administration to NOD mice increased IL-10 production from splenocytes (Fig. 4B) without changing IFN-γ responses by ELISPOT. With glyphosine, the increased IL-10 spot numbers were more pronounced in the presence of insulin B:9-23 peptide. The low-affinity register of binding for the B:9-23 peptide can bind insulin B:9-23 alpha chains.

In vivo, glyphosine prevention of diabetes was not sustained upon glyphosine withdrawal, and we hypothesize that enhanced IL-10 production following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes. IL-10 has anti-inflammatory properties following glyphosine treatment contributed to protection from diabetes.


