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Reliable Diagnosis of Murine Type 1 Diabetes Using A Panel of Autoantigens and "Antigen Surrogates" Mounted Onto A Liquid Array

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Abstract: Autoantibodies raised against β cell antigens are the most reliable preclinical biomarkers for predicting the imminent onset of type 1 diabetes mellitus (T1DM). The most current detection platforms are technically challenging or are run on clinically esoteric equipment. Here, we present a straightforward approach to detect autoantibody biomarkers that employs highly PEGylated microspheres onto which are mounted various capture agents that include affinity-tagged antigens or small molecule "antigen surrogates." After incubation with small quantities of serum, the bound autoantibodies can be measured using a standard flow cytometer. By multiplexing this assay, we show that a panel of antigen and antigen surrogates reliably predicts hyperglycemia in a mouse model of diabetes without false positives.

Introduction

There is great interest in developing a simple diagnostic blood test for autoantibody biomarkers that are expressed during the preclinical stages of type 1 diabetes mellitus (T1DM). This is clearly feasible since it has already been shown that the presence of autoantibodies against two or more of the major autoantigens involved in T1DM (insulin, GAD65, IA-2A, ZnT8) accurately reflects risk of developing the disease.[1] Unfortunately, monitoring these autoantigens in a clinical setting is not straightforward. ELISA assays, for example, perform quite poorly with these antigens, presumably because immobilization of the autoantigen on a surface disrupts binding of some autoantibodies to key epitopes.[2] Therefore, most published studies have relied on radioimmunoassays (RIAs), in which radioactive autoantigens can be employed free in solution.[1, 3, 4] RIAs are inconvenient, notoriously difficult to standardize and the reproducibility is poor.[5] Recently, electrochemiluminescent assays have been reported that are an improvement over the RIA approach.[6, 7] Other, more exotic, binding platforms have also been investigated.[8] Nonetheless, there remains a need for a simple, multiplexed blood test that can be carried out with equipment available in a standard clinical laboratory.

In this paper, we bring together three innovations to achieve this goal in the non-obese diabetic (NOD) mouse model system. First, we demonstrate that at least one of the known autoantigens, GAD65, can be employed as an immobilized antibody capture agent in the form of a fusion protein where the fusion partner, not GAD65, adheres to the bead. Second, we make use of synthetic small molecules that are good ligands for diabetes-linked autoantibodies, acting as surrogates of native epitopes in diabetes-linked autoantigens. These molecules bind to autoantibodies quite well even when displayed on a bead surface. Finally, we mount these small molecules and fusion protein probes on a Luminex-like multiplexed, color-coded liquid array of beads that display an extraordinarily low level of non-specific antibody binding, thus allowing accurate detection of even low level autoantibodies. We show that this multiplexed assay, which

is easy to run and requires only a common flow cytometer, allows diagnosis of murine T1DM with excellent sensitivity and specificity.

Results

As mentioned above, several known proteins recognized by T1DM-linked autoantibodies perform poorly as diagnostic agents when immobilized on a surface. Therefore, with the intent of developing a multiplexed assay for T1DM, we searched for synthetic "antigen surrogates"[9] that would bind to T1DM-linked autoantibodies, though not necessarily within the antigen-binding site, even when displayed on a bead surface. This is done by screening combinatorial libraries of diverse peptidomimetic oligomers for compounds that bind to IgG antibodies present in the serum of animals or patients with a particular disease of interest, but not in appropriate controls. We have demonstrated this approach to be effective for the identification of synthetic antigen surrogates in a number of disease states.[9-13]

The one bead one compound (OBOC) library shown in Fig. 2A was constructed using the split and pool strategy[14] and the "sub-monomer" synthesis method[15, 16,17] (oligomers of N-alkylated glycine). Each bead displays many copies of a single molecule. A detailed depiction of this complex library is included in Fig. S1. The theoretical diversity of the library is 127,000 compounds.

Approximately three copies of the library (≈ 380,000 beads; on average, each compound in the library should be displayed on three different beads) were employed in the screen (Fig 1D). The first step was to remove from the library any beads that display compounds that are ligands for antibodies not associated with T1DM. To do so, the beads were treated with a StartingBlock™, then incubated with a pool of serum samples (total protein = 250 μg mL^{-1}) obtained from SWR/J (Swiss) control mice that never developed hyperglycemia. After washing to remove unbound proteins, the beads were exposed to a biotin-conjugated secondary antibody

followed by a saturating amount of streptavidin-coated magnetic beads to magnetize beads that retained significant amounts of antibodies from serum. These beads were removed from the library using a strong magnet. To even more effectively cleanse the library of unwanted antibody ligands, the population of beads that were not magnetized strongly in the first round were incubated with another pool of control serum samples, this time obtained from C57BL/6 mice that had been immunized with a peptide comprising residues 35–55 amino acid of myelin oligodendrocyte glycoprotein (MOG(35–55)), a common mouse model for multiple sclerosis.[18] After washing and hybridizing a biotin-conjugated secondary antibody to the beads, magnetic streptavidin particles were introduced and beads that bound significant quantities of antibody were removed using a strong magnet. Approximately 2,500 beads were removed from the population during this pre-screening phase.

Having cleansed the library of uninteresting antibody ligands to the best of our ability, the remaining library beads were exposed to pooled NOD mouse serum diluted to 100 μg mL^{-1} total protein in StartingBlock™ and hits were removed using the magnetic screening protocol. Based on experience from previous screens, we were suspicious that many of the beads harvested at this step are "false positives". The magnetic pull-out method described above is far from perfect. Control experiments using known peptide-antibody complexes have shown that some bona fide antibody ligands are left behind and many false positives are collected on the magnet (Mendes, et al., in preparation). Therefore, the approximately 2500 "hits" from this screening procedure were subjected to another round of pre-screening with control sera (Fig. 1E). In this case, beads displaying antibody-binding compounds were visualized by staining the beads with secondary antibody conjugated to fluorescent red quantum dots (Fig. 1E). The highly fluorescent beads were removed manually using a micropipette using visual inspection under a low power fluorescence microscope. This is far more reliable than the magnetic capture protocol, but much more tedious. Thus it is feasible to do on a small population of beads, but more difficult to employ for primary screening. Finally, the remaining beads were again

incubated with NOD mouse serum and stained with quantum dot-labeled secondary antibody. 146 beads displaying a bright red halo were collected. Each bead was placed in a well of a microtiter plate and released into solution by cleavage of the linker that tethers the compounds to the bead. Their structures were then determined by tandem MALDI mass spectrometry.

Of the 146 beads that were isolated as potential hits at this step, only two displayed the same compound. OBOC library screens have a notoriously high false positive rate, but we have shown previously that compounds isolated more than once from a redundant library are almost always bona fide ligands for the target protein.[19] Therefore, we focused exclusively on the redundant hit, compound **1** (Fig. 2A), as a potentially useful diagnostic agent.

Compound **1** and a control compound **2**, which should not bind selectively to T1DM-linked autoantibodies, were re-synthesized and purified by HPLC. To assess the ability of **1** to distinguish NOD mouse autoantibodies from other antibody populations, the molecules were linked covalently to the exterior of 10 μm TentaGel beads encoded in their protein-inaccessible interior with a characteristic ratio of Pacific Blue and Pacific Orange[20]. The encoded beads displaying **1** or **2** were combined and added to serially diluted serum from a NOD or Swiss mouse from the discovery serum set. After a short incubation, the beads were washed, mixed with an Alexa Fluor 647-conjugated secondary antibody and analyzed using flow cytometry. Fig 2B shows that **1** retained ≈7-fold more IgG antibody from the NOD mouse sample than from the Swiss mouse sample. Conversely, little antibody retention was observed for control compound **2** relative to **1** (Fig. 2C), suggesting that **1** is a ligand for NOD mouse antibodies with reasonably good selectivity.

To further ensure that **1** did not bind nonspecifically to antibodies raised during a fulminant immune response, beads containing **1** and **2** were incubated with serum from MOG(35–55)-immunized mice, an experimental autoimmune encephalomyelitis (EAE) model.[18] After incubating the beads with 500 μg mL^{-1} diluted serum, retention of antibodies to the beads was monitored using the cytometer-based assay. Fig. 2C shows that both **1** and **2** bound modest titers of antibodies from the EAE mouse model, but this retention of antibodies was significantly

lower than the titers observed when **1** was incubated with NOD mouse serum. These data further support the idea that compound **1** is a selective ligand for NOD mouse autoantibodies.

The diagnostic utility of **1** was examined through an analysis of a larger number of NOD and control mouse serum samples. Blood was collected from 18 Swiss and 20 NOD mice biweekly for seven months beginning when the mice were five weeks old. Serum was prepared and each sample was diluted to 500 μg mL^{-1} total protein in a blocking buffer. Binding of serum antibodies to **1** was monitored using the flow cytometry assay. The mean of the values measured for all of the Swiss mouse serum samples was calculated and a value of five standard deviations above this was taken as the threshold over which a sample was called positive. Note that levels of autoantibodies rise and fall over time (Figs. S7 and S8), so Table 1 calls a mouse "positive" if the level of antibodies binding to compound **1** exceeded this threshold at any time during the sampling period.

Table 1 summarizes these results, and shows that IgG antibody binding to **1** was able to identify 40% of the NOD mice tested correctly. Above-threshold binding was not observed for any of the control mice.

In a recent study, we identified GAD65 as a humoral autoantigen in the NOD mouse[10] and demonstrated that a GST-GAD65 fusion protein can be immobilized on glutathione-coated TentaGel beads without obstructing epitopes in GAD65 critical for autoantibody binding. We also have reported the isolation of a different antigen surrogate, compound **3** (Fig. 2), that does not compete with **1** or with GAD65 for binding to NOD autoantibodies (Fig. S9).[21] We hypothesized that by using these three molecules together in a multiplexed panel, a highly accurate diagnostic test for murine T1DM might be achieved.

Color-coded TentaGel beads displaying GST-GAD65, compound **1** or compound **3**, as well as beads displaying control compound **2**, were added to serum (500 μg mL^{-1} total protein) collected from NOD mice or control Swiss mice between the ages of 5 and 8 weeks old. At this age, none of the mice had developed hyperglycemia, though all surviving NOD mice in this study eventually developed hyperglycemia (Fig. S10). The beads were then washed and probed with a

fluorescent secondary antibody, washed again, and antibody retention was measured using a flow cytometer. Mice were scored as positive for each autoantibody population if antibody titers for one of the two blood draws surpassed a three-sigma threshold calculated using Swiss control mouse sera[10, 21] (Fig S11). The data in Table 2 show that the use of a panel of capture agents, each of which retains a different antibody population, collectively predicted spontaneous hyperglycemia in all of the NOD mice and none of the Swiss control mice.

TABLE 2

Discussion

Developing a simple blood test for autoantibodies to multiple autoantigens is a major goal for ensuring the early diagnosis and treatment of T1DM in the general population. This has been frustrated by the fact that immobilization of most known autoantigens to a bead or plate surface appears to compromise their ability to bind their cognate autoantibodies, forcing the field to rely largely on RIAs. In this study we set out to attempt to solve this problem in the NOD mouse model system by employing two strategies. The first was to attempt to employ fusion protein derivatives of a known autoantigen (here GAD65) in the hopes that immobilization through the fusion partner would facilitate display of the appropriate epitopes for autoantigen binding. The second was to discover synthetic molecules capable of acting as autoantigen surrogates. Using two such surrogates, compounds **1** (this study) and **3**[10] as well as GST-GAD65, we were able to construct a simple diagnostic assay that has 100% sensitivity and 100% specificity in this modest cohort (40 animals; Table 2). This work suggests that a similar approach may be useful for the development of a blood test for human T1DM. These efforts are underway.

Experimental Section

Supplementary Methods

General. Deuterated (*S*)-(–)-2-bromopropionic acid, *N*-Alloc-1,4-ethylenediamine hydrochlroride, and (*S*)-2-chloro-3-phenylpropanoic acid were prepared as described previously.[22] All the other chemicals were purchased from commercial sources and used without further purification. (*S*)-(–)-Chloropropionic acid was purchased from Alfa Aeser. (*S*)-2-Chloro-4-methylvaleric acid was purchased from TCI America. TentaGel R RAM resin was purchased from Rapp Polymere. All the Fmoc-protected amino acids and Knorr Amide MBHA resin were purchased from Novabiochem. All the other chemicals were obtained from Sigma-Aldrich, Acros Organics, or Oakwood Chemical. Commercial sources of materials required for screening are described in the screening section. All MALDI-TOF and TOF/TOF mass spectra were obtained using 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems). Cyano-4-hydroxycinnamic acid was used as a matrix for the mass analysis. Phosphate-buffered saline (PBS) solutions were diluted from a 10X stock PBS solution (Corning). Tris-buffered saline (TBS) solutions were diluted from a 20X TBS stock (Thermo Scientific). The following section details experimental procedures for serum preparation and autoantibody binding measurements. A descriptive experimental flow for library synthesis is provided in the supplementary information.

Library synthesis. The OBOC library was synthesized on TentaGel R RAM resin (0.18 mmol/g, 2 g, 5,720,000 beads) following one-bead-one-compound split and pool techniques.[23, 24] Bromoacetic acid was coupled to the resin by mixing 5.7 mL of 2 M bromoacetic acid in DMF and 5.7 mL of 1 M DIC in DMF in a 50 mL tube and pre-incubating for 5 min. The solution was applied to the beads and the reaction was carried out at 37 °C for 10 min with continuous shaking. The beads were washed with DMF three times. For amine displacement, the beads were split into eleven reaction vessels (143 mg each). The beads were incubated with 1 mL of 1 M amine in DMF at 37 °C for 1 h with continuous shaking. The beads were washed with DMF three times. For piperazine displacement, 20 equivalents of piperazine (78 mg) was dissolved in 900 μL of *N*-methyl-2-pyrrolidone and applied to the beads. The reaction was carried at 60 °C overnight with continuous shaking. The beads were washed with DMF three times. For

methylamine displacement, 500 μL of 2 M methylamine solution in water was diluted with 4.5 mL of DMF. The amine solution was added to the beads and reaction was carried at 37 °C overnight with continuous shaking. Amino acids were coupled to the beads by distributing resin into three reaction vessels (143 mg each). 8 equivalents of amino acid (Fmoc-Ala-OH, Fmoc-Phe-OH, or Fmoc-Glu(O-2-PhiPr)-OH) and 8 equivalents of oxyma (29 mg) were dissolved in 1 mL of DMF. 8 equivalents of DIC (32 μL) was added and the solution was pre-incuated at RT for 5 min. The solution was applied to the beads and the reaction vessel was shaken at 37 °C for 2 hrs. The beads were washed with DMF three times and the beads were pooled together. The Fmoc group on the resin was deprotected by exposing the beads to 3 mL of 20% piperidine in DMF for 3 min and, after washing with DMF once, to 3 mL of 20% piperidine in DMF for 12 min. The beads were washed with DMF three times. Haloacids, including (*R*)-(+)-2-bromopropionic acid (R-BPA), deuterated (*S*)-(−)-2-bromopropionic acid (S-BPA-d_4), (*S*)-2-Chloro-3-phenylpropanoic acid (S-CPhA) and (*S*)-2-Chloro-4-methylvaleric acid (S-CVA), were coupled to the beads by first washing the resin with DCM and anhydrous tetrahydrofuran (THF) three times each. The beads were swelled in 2 mL of anhydrous THF and 21 equivalents of diisopropylamine (DIEA) (439 μL). 3 equivalents of bis(trichloromethyl) carbonate (BTC) (107 mg) was dissolved in 5.5 mL of anhydrous THF. 9 equivalents of R-BPA (97 μL) was added to the BTC solution and the solution was cooled at −20 °C for 15 min. The cooled solution was mixed with 27 equivalents of 2,4,6-trimethylpyridine (TMP) (428 μL) and applied to the swelled beads. The reaction was carried out at RT with continuous shaking for 2 h (R- and S- BPA) or 3 h (CPA, CPhA and CVA). The beads were washed with THF, DCM, and DMF three times each. To construct the 2-oxopiperizine moiety, 10 equivalents of *N*-Alloc-1,4-ethylenediamine hydrochloride (279 mg) was dissolved in 3.1 mL of DMF and 20 equivalents of DIEA (538 μL) was added to the solution. The solution was applied to the beads and the amine displacement reaction was carried out at 60 °C overnight with continuous shaking. The beads were washed with DMF and DCM three times each. The beads were distributed into three reaction vessels (286 mg each) to these aliquots was coupled CPA, CPhA or CVA, as described above. The beads

were pooled together and swelled in DCM for 15 min. 3 equivalents of tetrakis(triphenylphosphine)palladium(0) (535mg) and 12 equivalents of phenylsilane (228 μL) in 15 mL of DCM was added to the beads and the reaction vessel was shaken at RT for 30 min. The beads were washed with DCM, DMF, and 10% DIEA in DMF three times each. The beads were shaken at 37 °C overnight and washed with DMF three times. After library synthesis, the library was preincubated with DCM at 4 °C for 30 min. DCM was removed and the side-chain protecting groups were deprotected by treating the beads with 1% trifluoroacetic acid (TFA) in DCM for 3 min six times. The library beads were washed with DCM and dried under vacuum. A few hundreds of beads were transferred to a 96 microwell plate to assess the quality of the synthesis (Supplementary Figure S2). The deprotected library was washed with DCM (6 x 3 min), was neutralized using 10% DIEA in DMF for 10 min.

Library screening. The library was washed with DMF (10 x 10 mL) and equilibrated in DMF for 3 h. The library was then washed with ultrapure deionized water (10 x 10 mL) and equilibrated in water overnight at room temperature. The beads were then equilibrated in TBS-T for 1 h and blocked in Starting Block™ containing 0.05% Tween 20 (herein referred to as screening buffer). StartingBlock™ is a proprietary mixture of proteins containing an antimicrobial reagent in PBS. Pooled Swiss mouse sera derived from ten mice at 8 weeks of age was diluted to a concentration of 250 μg mL^{-1} in screening buffer and 3.5 mL of the diluted serum sample was added to the library. The library beads were incubated in the control serum overnight at 4 °C after which the library was washed with TBS-T (5 x 5 mL). A 1:100 dilution of anti-mouse IgG-conjugated to biotin (Immunoreagents, Inc.) in screening buffer was incubated with the library for 1 h at RT and washed with TBS-T (5 x 5 mL). Next, the library was suspended in a 1:100 dilution of Dynabeads® M-280 Streptavidin (Life Technologies) in screening buffer and incubated at room temperature for 1 h. Hits were isolated using a magnetic pull-down technique described previously.[10] Hits were discarded and the non-hit (e.g. non-magnetized) beads were carried on for further screening since hit beads at this step represent

hits to healthy Swiss mouse antibodies. The previous control screen was repeated using 250 μg mL^{-1} MOG-immunized mouse serum using serum collected at week 7 (Fig S6). Next, pooled NOD mouse serum collected at 8 weeks of age was diluted to 125 μg/mL in 3.5 mL screening buffer and incubated with the library overnight at 4 °C. The library was washed several times in TBS-T (5 x 5 mL). The library was incubated with a 1:100 dilution biotin-conjugated secondary antibody in screening buffer for 1 h at room temperature, washed with TBS-T (5 x 5 mL), and suspended in a 1:100 dilution of Dynabeads® M-280 Streptavidin beads in screening buffer for 1 h at room temperature. Hit beads were removed using the magnetic pull-down method described above, paying close attention not to miss any of the isolated hits. The hit beads were saved, transferred to a fritted syringe, and bound protein was stripped by washing the beads in 8 M guanidinium hydrochloride (pH adjusted to 7.2) for 30 min, followed by extensive washing with water (5 x 10 min), DMF (5 x 10 min), and equilibrating the beads in DMF for 3 h. The beads were washed with DCM 10 times and equilibrated in DCM overnight. Finally, the beads were washed with DMF 5 times, equilibrated into DMF for 3 h and then washed with water 10 times, wherein the final water wash was shaken gently on a plate shaker overnight. The hit beads were re-equilibrated in TBS-T for 1 h followed by screening buffer for 1 h at room temperature. A secondary control screen was performed by adding 500 μL of pooled Swiss mouse serum (125 μg mL^{-1}) to the stripped hit beads in screening buffer. The mixture was incubated overnight at 4 °C and washed with TBS-T (3 x 2 mL). The beads were hybridized with a 1:200 dilution of QDot® 655-conjugated secondary antibody in screening buffer. After washing with TBS-T (4 x 2 mL), beads that bound significant antibodies were visualized by the appearance of a red halo under a DAPI filter on an inverted fluorescent microscope. “Hits” were manually removed using a micropipette and discarded. The remainder of the hit beads were subjected to a second control screen in which they were suspended in 500 μL of MOG-immunized mouse serum at a concentration of 125 μg/mL in screening buffer. The beads were incubated overnight at 4 °C, washed in TBS-T (4 x 2mL) and re-suspended in a 1:200 dilution of QDot® 655-conjugated secondary antibody was hybridized to the beads, washed with TBS-T (4 x 2 mL) and “hits” were

removed under a fluorescent microscope. Finally, the remaining hit beads were incubated overnight at 4 ºC with 500 μL NOD mouse serum diluted to 100 μg mL^{-1} in screening buffer. The bead were washed in TBS-T (4 x 2mL) and re-suspended in a 1:200 dilution of QDot® 655-conjugated secondary antibody was hybridized to the beads, washed with TBS-T (4 x 2 mL) and "hits" were removed under a fluorescent microscope. The beads were stripped of bound protein, separated individually into the wells of a 96-well microtiter plate, where compounds were cleaved from the resin and their identity deconvulated using MALDI-TOF and –TOF/TOF as described above.

Batch resynthesis of ligands: Resynthesized oligomers **1**–**3** were synthesized on 100 mg of Knorr Amide MBHA resin (0.44 mmol/g). Compounds **1** and **2** were constructed using stoichiometries outlined above. Deuterated reagents were not employed during ligand resynthesis. Compound **3** was synthesized using a previously described procedure [10].

Animal use and serum preparation. Animal experiments were performed under a protocol approved by the Scripps Florida Institutional Animal Care and Use Committee (IACUC). Female mice were used exclusively in this study, since hyperglycemia is more prevalent in female NOD mice. Blood was collected bi-weekly *via* the submandibular bleeding procedure described previously. To prepare serum, whole blood was incubated undisturbed at room temperature for 30 minutes and pelleted at 2,000 x g for 10 minutes at 4 ºC in a refrigerated centrifuge to remove the clot. The resulting supernatant was transferred to a fresh tube and stored at -80 ºC for further analysis. Immediately prior to use, the serum was thawed on ice for 60 min and diluted 15 times into phosphate-buffered saline (PBS). The diluted serum was centrifuged at 10,000 x *g* for 10 minutes at 4 ºC and the supernatant was removed for analysis. Final concentration of serum total protein was determined using a NanoDrop 2000 UV-vis spectrophotometer, and typically found to be 1–2 mg mL^{-1} total protein. Non-fasting blood glucose was monitored weekly from tail nicks using the OneTouch® Ultra® blood glucose

meter. Mice were sacrificed if hyperglycemia (> 200 mg/dl) persisted for more than 4 weeks. The remaining animals were sacrificed after 30 weeks. For MOG(35–55)-immunized mouse serum, C57BL/6 mice were immunized at 7-10 weeks of age with mouse/rat MOG(35–55) peptide (sequence: MEVGWYRSPFSRVVHLYRNGK, Anaspec) conjugated to mariculture keyhole limpet hemocyanin (mKLH) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and Titermax® Gold as the adjuvant for antibody production. Serum was collected from these mice pre-immunization and every two weeks post-immunization. Anti-MOG antibody titers were determined using the SensoLyte® Anti-MOG(35-55) mouse/rat IgG Quantitative ELISA Kit (Anaspec).

Antigen surrogate conjugation onto encoded microspheres. Beads were encoded with Pacific Orange and Pacific Blue as previously described.[20] After dye encoding, either glutathione or the antigen surrogate was conjugated to the beads. [2-(2-(Fmoc-amino)ethoxy)ethoxy]acetic acid (5 equiv) was pre-incubated with HBTU (5 equiv), HOBt (5 equiv) and DIEA (10 equiv) in 300 μL DMF and added to the beads. The mixture was shaken continuously overnight at room temperature. Fmoc was removed by washing with 20% piperidine in DMF (2 x 10 min) and the deprotected terminal amine was activated by addition of 2 M bromoacetic acid in DMF followed by 2.5 M DIC in DMF. The beads were mixed for 10 min at room temperature. The beads were pelleted and the supernatant was removed. The pelleted resin was resuspended in DMF, mixed thoroughly and pelleted once more. This preceding step was repeated a total of 4 times to wash the beads. 2.5 mg mL^{-1} of the sulfhydryl-bearing ligand dissolved in a 50:50 mixture of PBS/DMF at pH 7.4 was added to each population and mixed constantly overnight at 37 °C. The beads were washed (3 x 500 μL DMF) and transferred to a MultiScreen™ Solvinert PTFE filter plate (EMD Millipore). The DMF was evacuated and the beads were washed with water (10 x 300 μL) followed by an overnight water wash. The following day the beads were quenched with 150 mM 2-mercaptoethanol diluted in PBS, washed with PBS (10 x 300 μL), TBS-T (3 x 300 μL)

and transferred to a 500 μL centrifuge tube. The suspension of beads was diluted to ~10 mg mL^{-1} in TBS-T, blocked with 0.5% BSA and stored at 4 °C.

GAD65 immobilization onto TentaGel microspheres. GAD65 was conjugated to glutathione-modified 10 μm TentaGel microspheres as described previously.[10] Briefly, the terminal amine on the TentaGel resin was primed with 2 M bromoacetic acid and 2. 5 M DIC for 10 min at room temperature. The beads were washed 3 x 500 μL in DMF. In a separate vial, 20 eq of reduced glutathione was dissolved in PBS and the pH was adjusted to 7.4. The glutathione stock was diluted 1:1 into DMF and added to the primed TentaGel beads. The mixture was mixed vigorously and placed on a rotator overnight. Following thioalkylation, the beads were washed with DMF (3 x 500 μL) and transferred to a MultiScreen™ Solvinert PTFE filter plate (EMD Millipore). The DMF was evacuated and the beads were washed with water (10 x 300 μL). After an overnight water wash, the beads were quenched with 150 mM 2-mercaptoethanol in PBS for 30 min and washed extensively with PBS (10 x 300 μL). 0.5 mg (~1 x 10^6) of the beads was suspended in 25 mM tris buffer containing 75 μg murine GAD65 containing an N-terminal GST tag (Life Technologies). The suspension was transferred to a 10,000 molecular weight cut-off Slide-A-Lyzer® Mini Dialysis unit (Pierce Biotechnology) and dialyzed overnight in 4 L of tris-buffered saline (TBS) at 4 °C. The TBS was replaced and the dialysis was continued for an additional 24 h. This last step was repeated one more time and the remaining beads were transferred from the dialysis unit into a 96-well Solvinert hydrophilic filter plate. The beads were washed with TBS-T (5 x 300 μL) and blocked in ~150 μL PBS containing 0.5% BSA and 0.05% Tween 20 for 1 h at room temperature. The beads were used immediately in serum binding assays described below.

Antibody binding assays. For serum titration experiments, 100 μL of a stock serum solution was prepared in PBS buffer containing 0.5% BSA and 0.05% Tween 20 (herein referred to as binding buffer) in a 0.5 mL microcentrifuge tube at a concentration of 750 μg mL^{-1} total protein.

Serial dilutions were prepared by making 1:2 dilutions into binding buffer so that the total volume for each measurement was 50 μL. To each serum sample was added 1 μL of a 3 mg mL^{-1} TentaGel bead suspension. Binding took place overnight at 4 ºC. The bead suspension was transferred to a 96-well MultiScreen™ Solvinert hydrophilic filter plate and washed with TBS-T (2 x 300 μL). To each well of the filter plate was added 150 μL of a 1:200 dilution of Alexa Fluor 647-conjugated goat anti-mouse antibody in TBS-T containing 0.5% BSA. After mixing each well by aspiration with a micropipette, the secondary antibody was hybridized for 1 h at room temperature. The beads were washed (2 x 300 μL), taken up in 200 μL of TBS-T and analyzed on a BD™ LSRII flow cytometer (BD Biosciences). The mean fluorescence intensity from the 100 beads was averaged across 3 independent experiments, and reported as the average MFI ± standard deviation of the three runs. For binding measurements at a single concentration, serum was diluted to 500 μg mL^{-1} in 50 μL of binding buffer and agitated gently for 2 h at room temperature. The bead suspension was transferred to a filter plate for washing and secondary antibody hybridization as described above. Analysis was performed using a flow cytometer to monitor fluorescence intensity of each bead. To determine the five-sigma threshold for compound **1**, the MFI from all serum samples tested was averaged and to this value was added five standard deviations. The assay cutoff MFI was set at the 99.7th percentile of 127 control samples for five-sigma threshold and 99.9th percentile for a five-sigma threshold

Competition binding analysis. Serum was diluted to 500 μg mL^{-1} in binding buffer containing 250 nM of compound **1**, compound **3** or GAD65 (as the GST fusion construct). After a 5 min incubation at room temperature, 1 μL of a 3 mg mL^{-1} TentaGel bead suspension containing bead-linked ligand **1** and ligand **3** was added to each sample and binding was evaluated using the cytometry assay.

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References

1. A. G. Ziegler, M. Rewers, O. Simell, T. Simell, J. Lempainen, A. Steck, C. Winkler, J. Honen, R. Veijola, M. Knip, E. Bonifacio and G. S. Eisenbarth, *J. Am. Med. Assoc.*, 2013, **309**, 2473–2479.
2. E. Liu and G. S. Eisenbarth, *Clinical Immunology*, 2007, **125**, 120–126.
3. L. Yu, D. T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen and G. S. Eisenbarth, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 1701–1706.
4. E. Bonifacio, V. Lampasona, L. Bernasconi and A.-G. Ziegler, *Diabetes*, 2000, **49**, 202–208.
5. C. Greenbaum, J. Palmer, B. Kuglin and H. Kolb, *J. Clin. Endocrinol. Metab.*, 1992, **74**, 1040–1044.
6. D. Miao, K. M. Guyer, F. Dong, L. Jiang, A. K. Steck, M. Rewers, G. S. Eisenbarth and L. Yu, *Diabetes*, 2013, **62**, 4174–4178.
7. L. Yu, D. Miao, L. Scrimgeour, K. Johnson, M. Rewers and G. S. Eisenbarth, *Diabetes*, 2011, **61**, 179–186.
8. B. Zhang, R. B. Kumar, H. Dai and B. J. Feldman, *Nat. Med.*, 2014, **20**, 948–953.
9. T. Kodadek, *Chem. & Biol.*, 2014, **21**, 1066-1074.
10. T. M. Doran, S. Simanski and T. Kodadek, *ACS Chem. Biol.*, 2015, **10**, 401-412.
11. B. Raveendra, W. Hao, R. Baccala, M. M. Reddy, J. Schilke, J. L. Bennett, A. N. Theofiliopolous and T. Kodadek, *Chem. & Biol.*, 2013, **20**, 350-359.
12. M. M. Reddy, R. Wilson, J. Wilson, S. Connell, A. Gocke, L. Hynan, D. German and T. Kodadek, *Cell*, 2011, **144**, 132-142.
13. M. Sarkar, Y. Liu, J. Morimoto, H. Peng, C. Aquino, C. Rader, N. Chiorazzi and T. Kodadek, *Chem. & Biol.*, 2014, **111**, 1670-1679.
14. K. S. Lam, S. E. Salmon, E. M. Hersh, V. J. Hruby, W. M. Kazmierski and R. J. Knapp, *Nature*, 1991, **354**, 82-84.
15. G. M. Figliozzi, R. Goldsmith, S. C. Ng, S. C. Banville and R. N. Zuckermann, *Methods Enzymol.*, 1996, **267**, 437-447.
16. R. N. Zuckermann, J. M. Kerr, S. B. H. Kent and W. H. Moos, *J. Amer. Chem. Soc.*, 1992, **114**, 10646-10647.
17. R. J. Simon, R. S. Kania, R. N. Zuckermann, V. D. Huebner, D. A. Jewell, S. Banville, S. Ng, L. Wang, S. Rosenberg, C. K. Marlowe and et al., *Proc Natl Acad Sci U S A*, 1992, **89**, 9367-9371.
18. M. K. Racke, *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al*, 2001, **Chapter 9**, Unit9 7.
19. T. M. Doran, Y. Gao, K. Mendes, S. Dean, S. Simanski and T. Kodadek, *ACS Comb. Sci.*, 2014, **16**, 259-270.
20. T. M. Doran and T. Kodadek, *ACS Chem Biol*, 2013, **9**, 339-346.

21. T. M. Doran, Y. Gao, S. Simanski, P. McEnaney and T. Kodadek, *Bioorg. Med. Chem. Lett.*, 2015, **Submitted**.
22. Y. Gao and T. Kodadek, *Chemistry & biology*, 2013, **20**, 360-369.
23. S. Suwal and T. Kodadek, *Org. Biomol. Chem.*, 2013, **11**, 2088-2092.
24. Y. Gao and T. Kodadek, *Chem. Biol.*, 2013, **20**, 360–369.

Table 1. Incidence of above-threshold autoantibody binding to 1 for NOD and Swiss mice

	Mouse Strain	
Mouse ID	SWR/J (Swiss)[a]	NOD/ShiLtJ[a]
1		+
2		
3		
4		
5		
6		
7		+
8		
9		
10		+
11		
12		+
13		
14		
15		
16		+
17		+
18		+
19		+
20		

[a] + indicates MFI greater than threshold (5-sigma) value of 2250 arbitrary

Table 2. Incidence of autoantibody binding to compound 1, compound 3 and GST-GAD65 for individual Swiss and NOD mice aged 5–8 weeks old.

Mouse ID	Strain	**1**[a]	**3**[b]	GAD65[c]
1	SWR/J (Swiss)			
2	SWR/J (Swiss)			
3	SWR/J (Swiss)			
4	SWR/J (Swiss)			
5	SWR/J (Swiss)			
6	SWR/J (Swiss)			
7	SWR/J (Swiss)			
8	SWR/J (Swiss)			
9	SWR/J (Swiss)			
10	SWR/J (Swiss)			
11	SWR/J (Swiss)			
12	SWR/J (Swiss)			
13	SWR/J (Swiss)			
14	SWR/J (Swiss)			
15	SWR/J (Swiss)			
16	SWR/J (Swiss)			
17	SWR/J (Swiss)			
18	SWR/J (Swiss)			
19	SWR/J (Swiss)			
20	SWR/J (Swiss)			
1	NOD/ShiLtJ	+		+
2	NOD/ShiLtJ			+
3	NOD/ShiLtJ	+		+
4	NOD/ShiLtJ	+	+	+
5	NOD/ShiLtJ		+	+
6	NOD/ShiLtJ			+
7	NOD/ShiLtJ	+	+	
8	NOD/ShiLtJ		+	
9	NOD/ShiLtJ		+	+
10	NOD/ShiLtJ	+	+	+
11	NOD/ShiLtJ			+
12	NOD/ShiLtJ	+		+
13	NOD/ShiLtJ			+
14	NOD/ShiLtJ	+		+
15	NOD/ShiLtJ			+
16	NOD/ShiLtJ			+
17	NOD/ShiLtJ	+		
18	NOD/ShiLtJ	+		+
19	NOD/ShiLtJ	+		+
20	NOD/ShiLtJ			+

[a] + indicates MFI greater than threshold (3-sigma) value of 1590 arbitrary fluorescence units
[b] + indicates MFI greater than threshold (3-sigma) value of 1960 arbitrary fluorescence units
[c] + indicates MFI greater than threshold (3-sigma) value of 20,000 arbitrary fluorescence unit

Figures

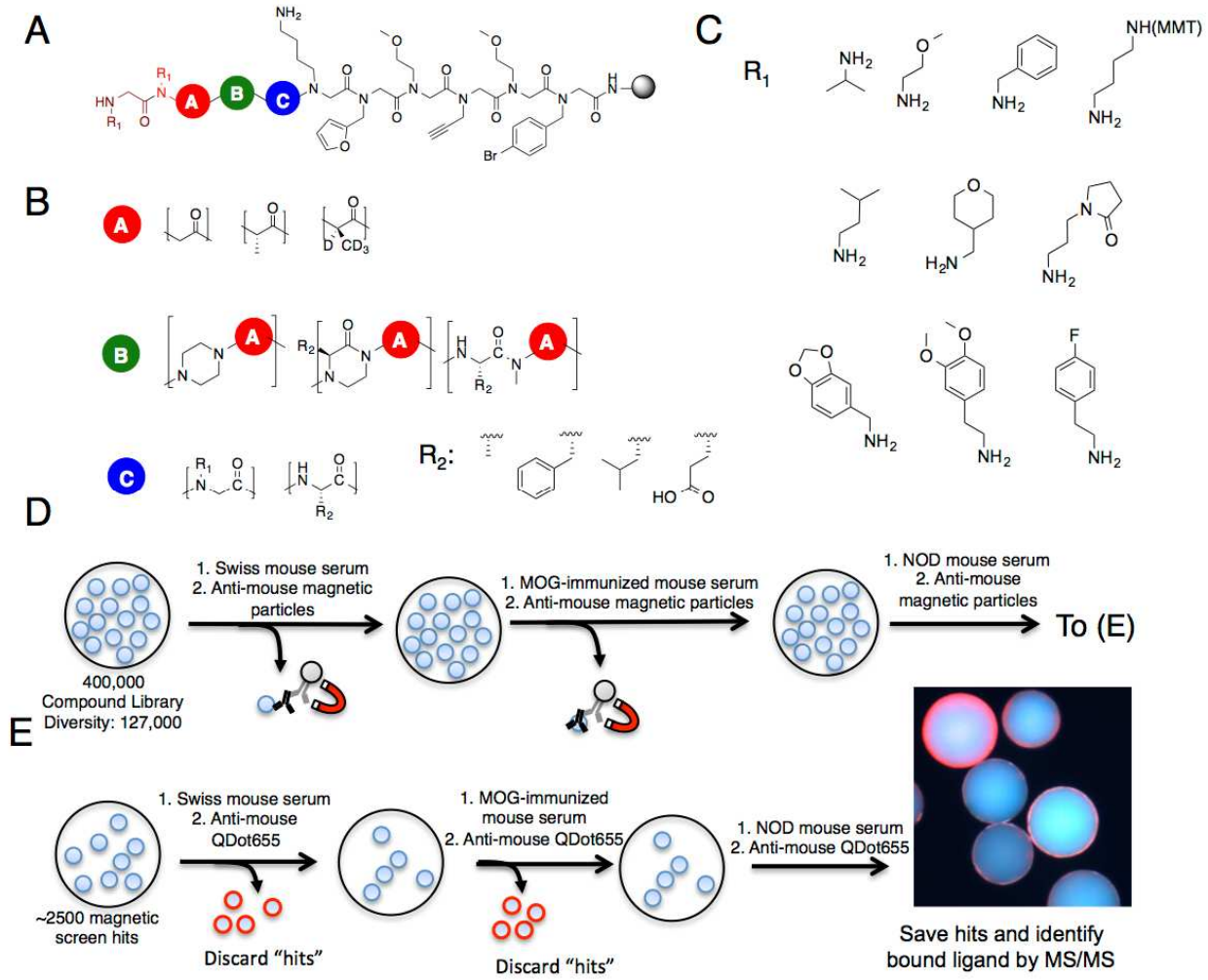

Figure 1. OBOC library design and screening. A) A OBOC library was synthesized on 90 µm TentaGel macrobeads. The variable positions included an N-terminal peptoid at position 1 (brown), a PTA or peptoid at position 2 (red circle "A"), heterocyclic rings or amino acids at position 3 (green circle "B") followed immediately by another PTA or peptoid monomer (red circle A). PTA units were incorporated as either the R or S stereoisomer, with their isomerism encoded isotopically. The last position was designated for amino acids or peptoids (blue circle "C"). B) R1 represents the amines used to generate the peptoid or PTA amide side chains. C) R2 represents amino acid side chains used in position "C". D) Library screening schema showing the sequential comparative screening process. E) Illustration of the second phase of the screening process using red Qdot655-conjugated secondary antibodies to visualize the beads containing ligands that retained significant antibodies from serum, which are shown in the photomicrograph as blue beads containing red halos.

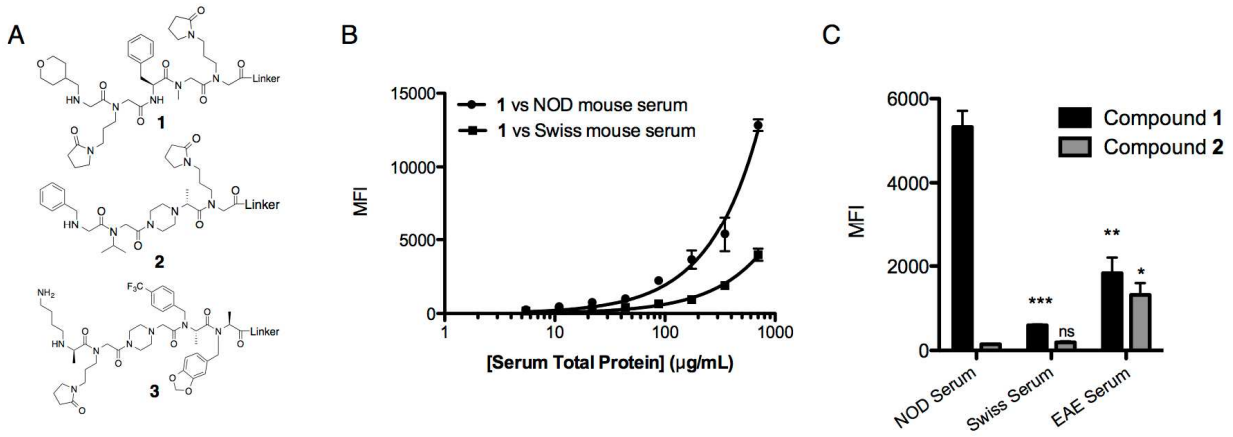

Figure 2. Binding characterization of antigen surrogate 1. A) Chemical structures of compounds used in this study. B) Binding isotherms generated using **1** as a probe for NOD or Swiss mouse serum autoantibodies. C) Binding of serum autoantibodies from NOD, Swiss or MOG-immunized (EAE) mice to compound **1** (black bars) or compound **2** (gray bars). Statistical difference significance is indicated for each ligand separately, using NOD antibody binding as a benchmark. Statistical significance was determined using an unpaired t-test: ns, not significant; $^{*}P < 0.01$; **P < 0.05; ***P < 0.001

Graphical Abstract

A flow cytometer is used to measure autoantibody binding to antigens and antigen surrogates displayed on a multiplexed liquid array. Collectively, these biomarkers predict diabetes in NOD mice with high specificity.