# Supporting Information

## Yang et al. 10.1073/pnas.1416864111

### SI Materials and Methods

**Peptide Binding Measurements.** For peptide binding assays, increasing concentrations of each nonbiotinylated test peptide were incubated in competition with 0.04 μM biotinylated GAD65 253–265$^{R255F}$ peptide (IAFFKMFPEVKEK) in wells coated with HLA-DQ8 protein, as previously described (1, 2). Europium-conjugated streptavidin (PerkinElmer) was used to label biotinylated peptide bound to the HLA-DQ protein and was quantified using a Victor2 multilabel time-resolved fluorometer (PerkinElmer). Binding curves were fitted by nonlinear regression with a sigmoidal dose–response curve model using Prism software (v5.0, GraphPad Software).

**CD4 T-Cell Isolation, in Vitro Stimulation, and Tetramer Staining.** Peripheral blood mononuclear cells (PBMC) and negatively enriched CD4 cells were isolated and expanded in vitro as described previously (1, 3). Briefly, $2.5 \times 10^6$ CD4 cells in a 48-well plate were stimulated with 6 μM of either B:11-23 or modified B:11-23$^{R22E}$ peptide, in separate, duplicate wells. After about 14 d of in vitro stimulation, tetramer staining was performed, and positivity was defined as the presence of a distinct population of CD4 bright cells at a percentage more than twofold above background (staining of cells from the unstimulated well, set to 0.1% for most experiments).

**Insulin-Specific CD4$^+$ T-Cell Cloning and Proliferation Assays.** Single tetramer-positive T cells were cloned and expanded as previously described (1). Their antigen specificity was confirmed by tetramer staining. To assess the proliferative response of T-cell clones to peptide and denatured insulin, $5 \times 10^4$ quiescent T cells of a desired clone and $1.0 \times 10^5$ irradiated HLA-DQ8+PBMC in 200 μL of T-cell culture medium [RPMI 1640 with 10% (vol/vol) pooled human serum] were stimulated in duplicate wells with 10-fold of serial dilutions or peptide or protein. After 48-h stimulation, each well was pulsed with 1 μCi of $^3$H-thymidine (Amersham Biosciences) for 16 h. Peptide stimulatory activity was determined as the absolute radioactive $^3$H cpm, or stimulation index (the ratio of $^3$H cpm in the presence of peptide verses no-peptide).

**Western Blot Analysis of DQ8/Ins Complexes.** Intact B6K10 cells ($3 \times 10^6$) were incubated with anti HLA-DQ8 monoclonal antibody SPVL3 for 1 h on ice. After washing to remove unbound antibody, cells were lysed in buffer containing 1% Triton X-100, protease inhibitors and 10 mM iodoacetamide, and immune complexes recovered using protein G Sepharose. The bound complexes were eluted from the beads using SDS/PAGE sample buffer with or without 10 mM DTT, and separated by SDS/PAGE. After transfer to nitrocellulose membranes DQ8 β-chains were detected using an anti-FLAG monoclonal antibody M2 (Sigma), followed by goat anti-mouse IgG1-HRP (Jackson Immunoresearch), and ECL-Plus (Pierce).

1. Yang J, et al. (2008) CD4+ T cells from type 1 diabetic and healthy subjects exhibit different thresholds of activation to a naturally processed proinsulin epitope. *J Autoimmun* 31(1):30–41.
2. Ettinger RA, Papadopoulos GK, Moustakas AK, Nepom GT, Kwok WW (2006) Allelic variation in key peptide-binding pockets discriminates between closely related diabetes-protective and diabetes-susceptible HLA-DQB1*06 alleles. *J Immunol* 176(3):1988–1998.
3. Yang J, James EA, Sanda S, Greenbaum C, Kwok WW (2013) CD4+ T cells recognize diverse epitopes within GAD65: Implications for repertoire development and diabetes monitoring. *Immunology* 138(3):269–279.

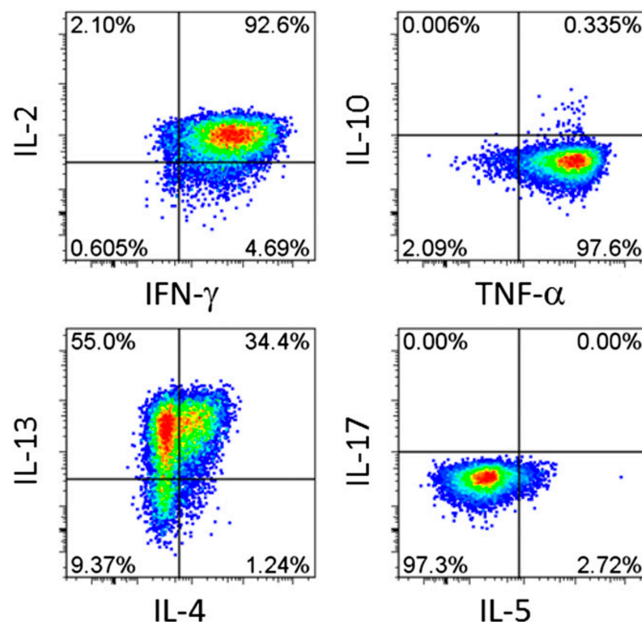


**Fig. S1.** Intracellular cytokine analysis for a representative insulin specific T-cell clone. A representative T-cell clone T1D#10 C2 was stimulated with PMA and ionomycin and stained intracellularly with anti–IL-2, IFN-γ, IL-10, TNF-α, IL-10, IL-4, IL-5, and IL-17. Each panel shown is gated on live tetramer-positive cells and depicts staining for one pair of cytokines.

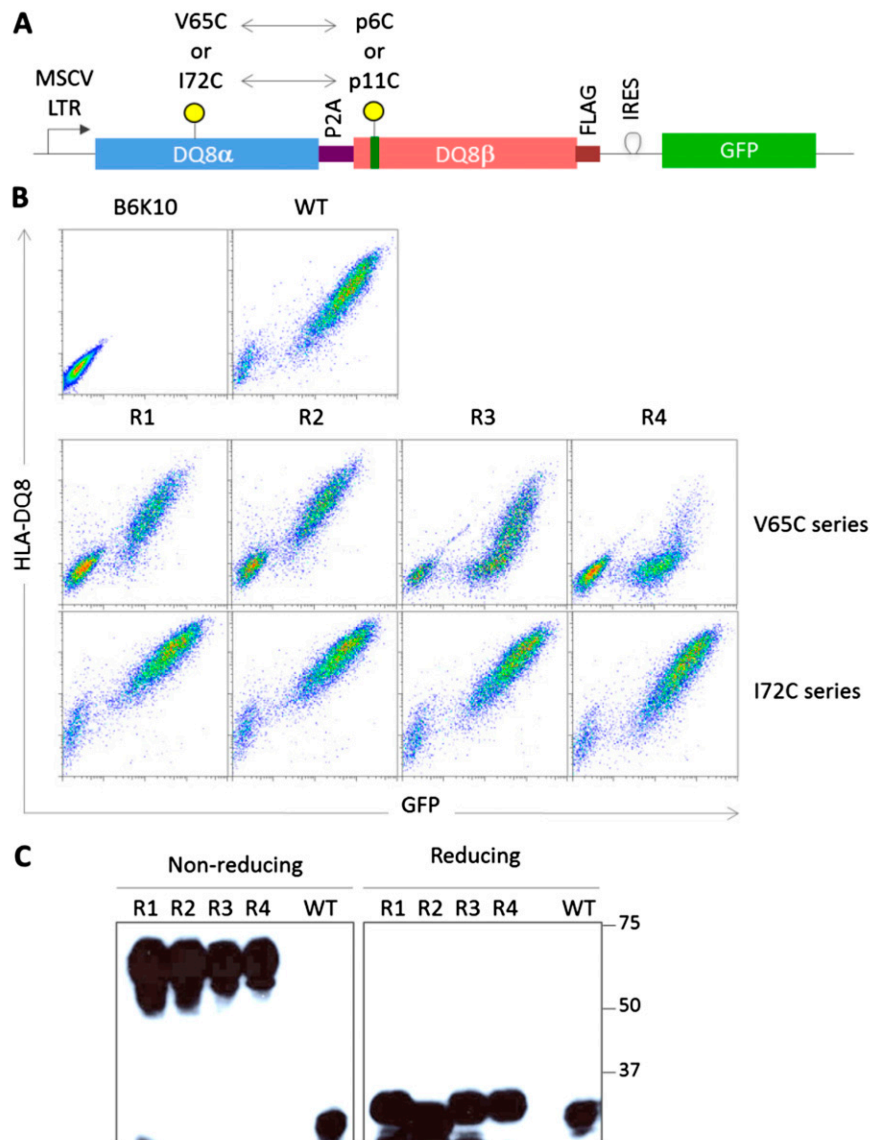


**Fig. S2.** Characterization of B6K10 cells expressing register fixed DQ8/Ins complexes. (*A*) Diagram of the murine stem cell virus-based retroviral constructs encoding HLA-DQ8 with variants of the B:11-23 peptide covalently attached to the mature N terminus of the DQ8 β-chain. The position of introduced cysteine residues in the α-chain (V65C or I72C) and antigenic peptide (at p6 or p11 of the designed binding register, respectively) are indicated. The β-chain is also tagged at its C terminus with a FLAG epitope for immunoblotting. Internal ribosome entry site-driven expression of GFP indicates the level of integrated viral DNA. (*B*) Expression level of the various HLA-DQ8 molecules with the linked and register-fixed peptides. The DQ8 molecules expressed on the cell surface of B6K10 transductants were stained with a PE-conjugated anti–HLA-DQ monoclonal antibody and analyzed by flow cytometry. (*C*) Analysis of disulfide bond formation. Disulfide bonding of surface-expressed DQ8 molecules was analyzed as described in *SI Materials and Methods*. The register-fixed constructs (R1, R2, R3, and R4) contain a pair of cysteines that form a disulfide bond linking the DQ8 α-chain to the DQ8 β-chain through the antigenic peptide, and therefore are detected as an αβ dimer under nonreducing conditions by the epitope tag in the β-chain. This resolves into the individual monomers upon reduction. The wild-type construct contains only a single native cysteine in the peptide and provides a negative control for the nonreduced samples.

**Table S1. Subject information**

| Subject | Sex | Age at draw | Days after dx | Insulin Ab | HLA-DQB1 (1) | HLA-DQB1 (2) | Insulin VNTR |
|---|---|---|---|---|---|---|---|
| Controls | | | | | | | |
| Healthy#1 | F | 28 | NA | Negative | *03:02 | *05:01 | |
| Healthy#2 | F | 32 | NA | Negative | *03:02 | *05:02 | |
| Healthy#3 | M | 39 | NA | Negative | *03:02 | *05:01 | |
| Healthy#4 | M | 27 | NA | Negative | *03:02 | *06:02 | |
| Healthy#5 | F | 27 | NA | Negative | *03:02 | *03:01 | |
| Healthy#6 | M | 27 | NA | Negative | *03:02 | *02:01 | |
| Healthy#7 | F | 31 | NA | Negative | *03:02 | *02 | |
| Healthy#8 | M | 22 | NA | Negative | *03:02 | *05:01 | |
| Healthy#9 | M | 27 | NA | Negative | *03:02 | *06:04 | |
| Healthy#10 | F | 22 | NA | Negative | *03:02 | NA | |
| T2D#11 | M | 60 | 25 y | Negative | *03:02 | *03:02 | |
| T2D#12 | M | 68 | 23 y | Negative | *03:02 | *06:02 | |
| Patients | | | | | | | |
| T1D#1 | M | 14 | 1,156 | Negative | *03:02 | *02:01 | 1/1 |
| T1D#2 | F | 14 | 367 | Positive | *03:02 | *05:01 | 1/3 |
| T1D#3* | M | 13 | 497 | Positive | *03:02 | *05:01 | 1/1 |
| T1D#4* | M | 21 | 212 | Positive | *03:02 | *05:01 | 1/1 |
| T1D#5 | F | 11 | 1,116 | Positive | *03:02 | *03:03 | 1/1 |
| T1D#6 | M | 16 | 937 | NT | *03:02 | *05:01 | 1/1 |
| T1D#7 | F | 15 | 970 | Positive | *03:02 | *03:03 | 1/1 |
| T1D#8* | M | 16 | 468 | Negative | *03:02 | *03 | 1/1 |
| T1D#9 | F | 9 | 859 | NT | *03:02 | *03:01 | 1/3 |
| T1D#10* | M | 13 | 1,531 | Positive | *03:02 | *05:01 | 1/1 |
| T1D#11 | M | 24 | 568 | Positive | *03:02 | *05:01 | 1/3 |
| T1D#12 | M | 11 | 897 | Positive | *03:02 | *02:01 | 1/1 |
| T1D#13 | M | 38 | 539 | Positive | *03:02 | *02:01 | 3/3 |
| T1D#14 | M | 30 | 977 | Negative | *03:02 | *03 | 1/1 |
| T1D#15* | M | 28 | 637 | Negative | *03:02 | *03 | 1/3 |
| T1D#16* | M | 14 | 953 | Positive | *03:02 | *02 | 1/1 |

Subjects marked with an asterisk have DQ8-restricted insulin responses. F, female; M, male; NT, not tested; T1D and T2D, type 1 and 2 diabetes, respectively.

**Table S2. Cytokine secretion profiles of insulin-specific T-cell clones**

| Clone | IFN-γ | IL-2 | TNF-α | IL-10 | IL-13 | IL-4 | IL-5 | IL-17A |
|---|---|---|---|---|---|---|---|---|
| T1D#15 C1 | 89.9 | 80.9 | 95.2 | 12.6 | 57.9 | 12.9 | 0.8 | 0.0 |
| T1D#15 C2 | 76.1 | 95.2 | 98.5 | 19.0 | 93.7 | 29.0 | 0.2 | 0.1 |
| T1D#10 C1 | 90.0 | 96.7 | 93.9 | 2.4 | 95.5 | 70.9 | 5.3 | 0.0 |
| T1D#10 C2 | 97.3 | 94.7 | 97.9 | 0.3 | 89.4 | 35.6 | 2.7 | 0.0 |
| T1D#10 C3 | 71.3 | 97.6 | 96.0 | 0.3 | 91.5 | 60.0 | 0.6 | 0.0 |
| T1D#10 C4 | 91.4 | 96.9 | 90.6 | 1.6 | 91.9 | 78.4 | 0.4 | 0.0 |
| T1D#10 C5 | 10.0 | 94.0 | 80.9 | 0.0 | 99.1 | 73.2 | 12.6 | 0.0 |
| T1D#3 C1 | 96.1 | 93.4 | 99.1 | 17.8 | 52.0 | 3.4 | 0.3 | 0.0 |
| T1D#3 C2 | 99.5 | 60.3 | 85.2 | 68.8 | 26.0 | 2.6 | 0.0 | 0.0 |
| T1D#3 C3 | 100.0 | 91.2 | 99.8 | 3.6 | 88.4 | 41.5 | 4.0 | 0.0 |

T cells clones were isolated from T1D patients, expanded, and stimulated with PMA and ionomycin in the presence of Brefeldin A to induce cytokine production. The value listed in each column is the percentage of cells that stained positive for each respective cytokine.

**Table S3. Sequences from microbial antigens that share homology with Ins B:11-23**

| Source organism | Accession no. of protein | Sequence* | Source |
|---|---|---|---|
| Human | AEG19452 | LVEALYLVCGEEG | |
| *Bacteroides salanitronis* | YP_004259638 | GNALFLLCGEEVKI | (1) |
| *Clostridium acetobutylicum* | NP_348681.1 | IKELYLICGEDKW | (2) |
| *Treponema phagedenis* | ZP_08037042.1 | SEADALYLVSSEEVLY | (3) |
| *Microscilla marina* | ZP_01690034.1 | VNALYLVSGDEVQI | (4) |

*The shared homologous region is underlined in each sequence.

1. Gronow S, et al. (2011) Complete genome sequence of *Bacteroides salanitronis* type strain (BL78). *Stand Genomic Sci* 4(2):191–199.
2. Nölling J, et al. (2001) Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. *J Bacteriol* 183(16):4823–4838.
3. Trott DJ, et al. (2003) Characterization of *Treponema phagedenis*-like spirochetes isolated from papillomatous digital dermatitis lesions in dairy cattle. *J Clin Microbiol* 41(6):2522–2529.
4. Hopkinson BM, Roe KL, Barbeau KA (2008) Heme uptake by *Microscilla marina* and evidence for heme uptake systems in the genomes of diverse marine bacteria. *Appl Environ Microbiol* 74(20):6263–6270.