Title: Islet-reactive CD8+ T-cell frequencies in the pancreas but not blood distinguish type 1 diabetes from healthy donors

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Abstract: The human leukocyte antigen (HLA)-A2-restricted zinc transporter (ZnT8)_{180-194} and other islet epitopes elicit interferon-γ secretion by CD8^+ T cells preferentially in type 1 diabetes (T1D) patients compared with controls. Here, we show that clonal ZnT8_{186-194}-reactive CD8^+ T cells express private T-cell receptors and display equivalent functional properties in T1D and healthy subjects. Ex-vivo analyses further revealed that CD8^+ T cells reactive to ZnT8_{180-194} and other islet epitopes circulate at similar frequencies and exhibit a predominantly naïve phenotype in age-matched T1D and healthy donors. Higher frequencies of ZnT8_{186-194}-reactive CD8^+ T cells with a more antigen-experienced phenotype were detected in children vs. adults, irrespective of disease status. Moreover, some ZnT8_{186-194}-reactive CD8^+ T-cell clonotypes were found to cross-recognize a Bacteroides stercoris mimotope. While ZnT8 was poorly expressed in thymic medullary epithelial cells, variable thymic expression levels of islet antigens did not modulate the peripheral frequency of their cognate CD8^+ T cells. In contrast, ZnT8_{180-194}-reactive cells were enriched in the pancreata of T1D donors vs. non-diabetic and type 2 diabetic controls. Thus, islet-reactive CD8^+ T cells circulate in most individuals, but home to the pancreas preferentially in T1D patients. We conclude that the activation of this common islet-reactive T-cell repertoire and progression to T1D likely require defective peripheral immunoregulation and/or a pro-inflammatory islet microenvironment.

One Sentence Summary: Islet-reactive CD8^+ T cells are common in the periphery, but home to the pancreas preferentially in the context of type 1 diabetes.
Introduction

In the setting of type 1 diabetes (T1D), insulitic lesions are enriched for CD8$^+$ T cells, which are held as the final mediators of islet destruction. Concordantly, preproinsulin (PPI)-reactive CD8$^+$ T-cell clones can lyse β cells \textit{in-vitro} (1), and β-cell-reactive CD8$^+$ T cells infiltrate the islets of T1D patients (2). Autoimmune CD8$^+$ T cells may therefore provide new biomarkers for disease staging complementary to auto-antibodies (aAbs). While interferon (IFN)-γ-secreting CD8$^+$ T cells detected by enzyme-linked immunospot (ELISpot) distinguish T1D patients from healthy donors (3), the situation is more complex when non-functional human leukocyte antigen (HLA) Class I multimer (MMr) assays are used. Although MMr$^+$CD8$^+$ T cells were often (4), but not invariably (5, 6), found at similar frequencies in both T1D and healthy subjects, they have been reported to exhibit more differentiated effector/memory phenotypes (4, 6) in T1D patients. A rather enigmatic state of ‘benign’ autoimmunity therefore exists in healthy individuals.

We therefore aimed to determine the key features of islet-reactive CD8$^+$ T cells that associate with T1D. We focused our efforts on well-defined HLA-A$^*$02:01 (HLA-A2)-restricted immunodominant epitopes derived from PPI, glutamic acid decarboxylase (GAD), insulinoma-associated protein-2 (IA-2), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (3). and on a highly immunoprevalent zinc transporter (ZnT)8;286-194 epitope that we recently described (7). The results indicate that incomplete central tolerance mechanisms allow the survival of an islet-reactive CD8$^+$ T-cell repertoire, which in some individuals may be primed in the presence of defective peripheral immunoregulation and/or a pro-inflammatory islet microenvironment to progress toward T1D.
Results

ZnT8_{136-144}-reactive CD8\(^+\) T-cell clones from T1D and healthy donors display equivalent functionality

Given that ZnT8_{136-144}-reactive IFN-γ responses are highly prevalent in T1D patients (7), we started by generating ZnT8_{136-144}-reactive CD8\(^+\) T-cell clones from a new-onset T1D patient (D222D) (7). Following in-vitro expansion with the ZnT8_{136-144} peptide (8), HLA-A2 MMrs cells were labeled with two different fluorochromes (9) and sorted (Fig. 1A). The 3 clones thus generated stained uniformly positive with ZnT8_{136-144} MMrs (Fig. 1B) and responded to ZnT8_{136-144} peptide stimulation by secreting tumor necrosis factor (TNF)-α (Fig. 1C), IFN-γ, interleukin (IL)-2 and, to a lesser extent, macrophage inflammatory protein (MIP)-1β in a dose-dependent fashion (Fig. S1A-C). Cytotoxicity was then tested against an HLA-A2\(^+\) Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell line (LCL) pulsed with the cognate ZnT8_{136-144} peptide. Increasing numbers of clonal CD8\(^+\) T cells led to the complete disappearance of ZnT8_{136-144}-pulsed but not control-pulsed targets, with >90% lysis at a 1/2 effector/target (E/T) ratio (Fig. 1D-F and S1D-F). This lytic activity was mostly perforin-mediated (Fig. 1F), as it was inhibited significantly by concanamycin A, marginally by brefeldin A (suppressing cytokine secretion) and not at all by a blocking anti-FasL mAb (suppressing Fas-dependent cytotoxicity), and it was associated with CD107a upregulation (Fig. 1G).

Additional ZnT8_{136-144}-reactive CD8\(^+\) T-cell clones were generated from other T1D patients and healthy donors. The final panel comprised 16 clones (9 clones from 5 T1D patients and 7 clones from 5 healthy donors), most of which were isolated directly ex-vivo (Table S1). All but one clone (H328C 9B3) stained with HLA-A2 MMrs loaded with ZnT8_{136-144} (VAANIVLTV), and several of them also with a longer ZnT8_{136-144} variant (AVAANIVLTV) reported to exhibit similar immunoprevalence (Fig. S2A) (10). Higher staining intensities were observed with ZnT8_{136-144} MMrs for all clones barring D010R 1D3. Concordantly,
ZnT8₁₈₆-₁₉₄ bound to recombinant HLA-A2 molecules with higher affinity (Kᵩ 15 nM vs. 207 nM) and similar stability (t₁/₂ 1.8 h vs. 2.3 h) relative to the longer ZnT8₁₈₅-₁₉₄ peptide (Fig. S2B-C). The ZnT8₁₈₆-₁₉₄ epitope was therefore retained for subsequent experiments, except for clone D010R 1D3.

Clones from T1D and healthy donors were first compared for the intensity of ZnT8₁₈₆-₁₉₄ MMr staining. The tyrosine kinase inhibitor dasatinib, which stabilizes T-cell receptor (TCR) interactions with peptide-HLA complexes (7), enhanced MMr staining, particularly for MMr<sup>low</sup> clones (Fig. 2A). Heterogeneous staining patterns were observed when comparing normalized MMr fluorescence intensities (Fig. 2B). While dasatinib reduced variability, even in its absence no difference was apparent between the T1D and healthy group.

Next, we performed in-vitro recall assays with increasing ZnT8₁₈₆-₁₉₄ peptide concentrations. Representative data are shown in Fig. 2C and results summarized in Fig. 2D-E. In line with the MMr staining profiles, the half maximal effective peptide concentration (EC₅₀) required to elicit cytokine responses (TNF-α, IFN-γ, IL-2) and the maximal cytokine responses were again heterogeneous, but not significantly different between T1D and healthy clones (Fig. 2D-E), with one exception noted for the lower MIP-1β EC₅₀ of T1D clones. Clones obtained after in-vitro expansion displayed an equivalent range of antigen (Ag) sensitivities, either high (D222D, H1017N) or low (H328C), arguing against a bias compared with clones isolated directly ex-vivo. Moreover, the polyfunctionality index, which reflects the number of cells secreting multiple cytokines (12), was similar for T1D and healthy clones (Fig. 2F). As expected, EC₅₀ values correlated negatively with MMr staining and polyfunctionality indices (Fig. S3).

Collectively, these results show that ZnT8-reactive CD₈<sup>+</sup> T cells isolated from T1D and healthy subjects exhibit similar Ag avidity, sensitivity and polyfunctionality.
ZnT8[186-194]-reactive CD8$^+$ T-cell clones from T1D and healthy donors display equivalent β-cell cytotoxicity

To determine if ZnT8[186-194]-reactive CD8$^+$ T cells can recognize naturally processed ZnT8 epitopes, we performed cytotoxicity assays using ZnT8-transduced K562-A2 targets (K562-A2/ZnT8). High avidity ZnT8[186-194]-reactive clones lysed unpulsed K562-A2/ZnT8 targets almost as efficiently as targets pulsed with the ZnT8[186-194] peptide, while unpulsed K562-A2 control targets remained largely intact (Fig. 3A-B). In contrast, low avidity ZnT8[186-194]-reactive clones were unable to lyse K562-A2/ZnT8 targets in the absence of exogenous ZnT8[186-194] peptide (Fig. 3C).

We then evaluated cytotoxicity against T1D-relevant targets by employing HLA-A2$^+$ ECN90 and control HLA-A2$^+$ EndoC-βH2 human β-cell lines. Although the ECN90 but not the EndoC-βH2 line expressed HLA Class I in the unstimulated state, expression levels were similarly upregulated by pretreatment with different cocktails of inflammatory cytokines, without inducing significant β-cell death (Fig. S4A-B). IFN-γ was chosen as the single cytokine that upregulated HLA Class I expression to comparable levels in both lines, and pretreatment was carried out for 18 h prior to a real-time cytotoxicity assay. As observed with K562-A2/ZnT8 targets, high avidity clones lysed unpulsed HLA-A2$^+$ ECN90 cells presenting naturally processed ZnT8-derived epitopes (Fig. 3D-E), while low avidity clones displayed marginal lytic activity (Fig. 3F). Cytotoxicity increased in all cases with the addition of ZnT8[186-194] peptide, suggesting more limited natural presentation compared with ZnT8-transduced targets. Lysis of HLA-A2 EndoC-βH2 cells was negligible, and a control clone reactive to the melanocyte self-epitope MelanA[26-35] only lysed ECN90 cells in the presence of exogenous MelanA[26-35] peptide (Fig. 3G). Microscopic inspection confirmed the lysis measured in real-time cytotoxicity assays (Fig. S4C). β-cell lysis was not different between T1D and healthy clones, either in the absence or presence of exogenous ZnT8[186-194] peptide (Fig. 3H). Moreover, IFN-γ-pretreatment of HLA-A2$^+$ ECN90 cells neither upregulated ZnT8
protein expression (Fig. S4D) nor increased the functional activation of co-incubated CD8\(^+\) T-cell clones (Fig. S4E-F). Collectively, these results demonstrate that ZnT8\(_{186.194}\)-reactive CD8\(^+\) T cells display similar cytotoxicity in T1D and healthy subjects and that Zn18 expression is not modulated by inflammation.

ZnT8\(_{186.194}\)-reactive CD8\(^+\) T cells display private TCR gene usage but public CDR3\(\beta\) aminoacid sequences

Molecular analysis of expressed TCR\(\alpha\) (TR\(\alpha\)) and TCR\(\beta\) (TRB) gene transcripts revealed that no sequences were shared (public) among ZnT8\(_{186.194}\)-reactive clones isolated from T1D or healthy subjects (Fig. S5). Of note, the 3 clones from patient D222D harbored an identical TCR. However, this observation did not affect our previous functional comparisons, because the measured parameters were even more similar between T1D and healthy donors when only one D222D clone was considered.

We then interrogated an in-silico database of TRB sequences compiled by high-throughput sequencing of naïve/terminal effector (CD45RA\(^-\)CD45RO\(^+\)) or central memory (CD45RO\(^-\)CD45RA\(^-\)CD62L\(^{hi}\)) CD4\(^+\) and CD8\(^+\) T cells obtained from HLA-A2\(^+\) recent-onset T1D patients, at-risk aAb\(^+\) and healthy subjects (Table S2). The D010R 1E2, H328C 8E8 and H034O 141B9 complementarity-determining region (CDR3\(\beta\) aminoacid sequences were found among CD8\(^+\) and CD4\(^+\) T cells isolated from several individuals (Fig. 4A-C), mostly within the CD45RA\(^-\)CD45RO\(^+\) pool for CD8\(^+\) T cells. Several different TRBV genes were used in conjunction with these identical CDR3\(\beta\) loops to generate 'mosaic' sequences. The same CDR3\(\beta\) aminoacid sequences were detected in-silico among the polyclonal TCR repertoire compiled from conventional and regulatory CD4\(^+\) and CD8\(^+\) T cells isolated from pancreatic lymph node (P1.N) and spleen samples by the Network for Pancreatic Organ Donors (nPOD) (Fig. 4D: n=67 identical CDR3\(\beta\), n=6 identical TCR\(\beta\)). Most of the CD8\(^+\) T-
cell hits in PLNs (11/15, 73%) were from HLA-A2⁺ subjects, but with no obvious association with T1D.

To extend these findings, we developed D010R 1E2, H328C 8E8 and D222D TRA and TRB clonotype-specific TaqMan real-time quantitative PCR (qPCR) assays (Fig. S6A-B). Applied to CD4⁺ and CD8⁺ T-cell cDNA preparations from two independent cohorts of T1D (n=97 and n=53) and healthy subjects (n=97 and n=38), these assays detected the D010R and H328C TRB among CD8⁺ T cells from a single HLA-A2⁺ T1D patient in each case (Fig. S6C-D).

Collectively, these results show that ZnT8₁₆₅.₁₉₄ recognition is mediated primarily by private clonotypes, some of which share CDR3β aminoacid sequences among individuals to form "mosaic" TCRs.

*Circulating islet-reactive CD8⁺ T cells display similar ex-vivo frequencies and a predominantly naive phenotype in T1D and healthy subjects*

In further experiments, we used ex-vivo combinatorial HLA-A2 MMr assays (9) to analyze ZnT8₁₆₅.₁₉₄-reactive CD8⁺ T cells in 39 HLA-A2⁺ recent-onset T1D patients (16 children, 23 adults) and 39 age/sex-matched healthy donors (17 children, 22 adults) (Table S3, Fig. S7). Control specificities included MelanA₂₅-₃₅, which is recognized by a large naive pool in humans (13), and Flu MP₅₈-₆₆, to which most individuals harbor Ag-experienced CD8⁺ T cells reflecting prior viral exposure. Donors yielding <3×10⁵ total CD8⁺ T cells or <5 MMr⁺ cells were excluded from the analysis to avoid undersampling. Parallel ELISpot assays confirmed that ZnT8₁₆₅.₁₉₄-reactive IFN-γ responses were more frequent in T1D vs. healthy donors (Fig. S8) (7). In contrast, similar frequencies of ZnT8₁₆₅.₁₉₄ and MelanA₂₅-₃₅ MMr⁺CD8⁺ T cells were detected in age-stratified T1D and healthy donors (Fig. S5A). Healthy children and adults displayed higher frequencies of Flu MP₅₈-₆₆ MMr⁺CD8⁺ T cells compared with their age/sex-matched T1D counterparts, while children displayed ~4-fold higher
frequencies of ZnT8\textsubscript{186-194} and MelanA\textsubscript{26-35} MMr\textsuperscript{+}CD8\textsuperscript{+} T cells compared with adults, irrespective of T1D status.

Among ZnT8\textsubscript{186-194} MMr\textsuperscript{+}CD8\textsuperscript{+} cells, the Ag-experienced fraction (CD45RA\textsuperscript{+}CCR7\textsuperscript{−} or CD45RA\textsuperscript{−}CCR7\textsuperscript{−}/−) was similarly limited (generally ≤25\% of all ZnT8\textsubscript{186-194} MMr\textsuperscript{+} events) in T1D and healthy adults (Fig. S5B). In contrast, higher proportions of Ag-experienced ZnT8\textsubscript{186-194} MMr\textsuperscript{+} cells were present in T1D children vs adults, although these values were not different in healthy children. T1D children also harbored significantly higher proportions of Ag-experienced ZnT8\textsubscript{186-194} vs MelanA\textsubscript{26-35} MMr\textsuperscript{+} cells. MelanA\textsubscript{26-35}-reactive CD8\textsuperscript{+} T cells were mostly naïve, while Flu MP\textsubscript{38-48}-reactive CD8\textsuperscript{+} T cells were mostly Ag-experienced in all groups. Comparable results were obtained using the absolute frequencies of Ag-experienced MMr\textsuperscript{+}CD8\textsuperscript{+} T cells (Fig. 5C).

Single-cell gene expression analysis of sorted ZnT8\textsubscript{186-194} MMr\textsuperscript{+} cells (Fig. S9A) revealed few differences, with T1D patients displaying higher expression of the Th17-related aryl hydrocarbon receptor (AHR) and the mitotic aurora kinase A (AURKA) and lower expression of the transcriptional activator RAR related orphan receptor A (RORA). In line with our \textit{in-vitro} data, clonotypic analyses of individual ZnT8\textsubscript{186-194} MMr\textsuperscript{+}CD8\textsuperscript{+} T cells sorted \textit{ex-vivo} yielded unique CDR3\textalpha{} and CDR3\textbeta{} sequences (Fig. S9B). Of the 21 CDR3\textbeta{} aminoacid sequences obtained, 7 (33\%) were found in our \textit{in-silico} TRB database across all subject groups (Fig. S9C). Among CD8\textsuperscript{+} T cells, these 7 sequences were again most frequent within the CD45RA\textsuperscript{−}CD45RO\textsuperscript{−} compartment, and predominantly found in T1D vs. healthy subjects. Preferential usage of certain TRBV (mostly TRBV\textsubscript{19}, 25\%) and TRAV (mostly TRAV\textsubscript{12-2}, 38\%) genes was observed (Fig. S9D-E), with TRBV\textsubscript{19} also shared among the ZnT8\textsubscript{186-194}-reactive clones. However, only 8 out of 63 matching sequences (13\%) expressed the TRBV gene of the corresponding ZnT8\textsubscript{186-194}-reactive T cell. Thus, despite biased TRAV and TRBV gene usage, the \textit{ex-vivo} data confirmed that ZnT8\textsubscript{186-194} recognition is mediated primarily by private clonotypes expressing 'mosaic' TCRs.
Next, we used an extended combinatorial MMr panel to compare ZnT8_{186-194}-reactive CD8+ T cells with those recognizing other immunodominant β-cell epitopes in adult donors (Fig. S10A). Assay reproducibility across panels was confirmed using separate blood draws from the same individuals (Fig. S10B). As observed for ZnT8_{186-194}, other β-cell-reactive CD8+ T-cell populations displayed similar frequencies (typically 1-50 MMr7 cells/10^6 CD8+ T cells) in T1D and healthy adults (Fig. S1D). An exception was noted for PPI_{15-24}-reactive CD8+ T cells, whose frequencies were lower than for other β-cell-reactive fractions, and higher in T1D vs. healthy donors. In all instances, the Ag-experienced fraction among MMr1'CD8+ T cells was limited in both subject groups (Fig. S5E-F). The few children included in this extended analysis harbored β-cell-reactive CD8+ T cells with a more Ag-experienced phenotype. CD45RA-CCR7- islet-reactive cells were *bona fide* naïve, as they were largely CD27-CD28+ and CD95+ (Fig. S11).

Collectively, these results show that circulating CD8+ T cells reactive to ZnT8_{186-194} and other β-cell epitopes occur at similar frequencies and exhibit a predominantly naïve phenotype in T1D and healthy adults, while higher frequencies of total and Ag-experienced ZnT8_{186-194}-reactive CD8+ T cells are present in children, irrespective of T1D status.

*Poor promiscuous ZnT8 gene expression in human thymic medullary epithelial cells (mTECs)*

The combined observations that CD8+ T cells reactive to ZnT8_{186-194} and other β-cell epitopes are predominantly naïve and circulate at similar frequencies in T1D and healthy donors are compatible with frequent escape from thymic deletion due to poor islet Ag expression in mTECs (14). We tested this hypothesis by examining ZnT8 (SLC30A8) gene expression in total or immature (AIRE- HLA Class II^{low}) vs. mature (AIRE- HLA Class II^{high}) human mTECs from 5 children undergoing heart surgery. As mis-initiated mRNA transcription is described in mTECs (13, 15), forward primers were designed to identify potential alternative start sites via hybridization with exons 5, 6, 7 and 8 of the SLC30A8 gene (Fig. 6A). The ZnT8_{186-194}
region is encoded by exons 7-8, with the exon 8 primer located just downstream. Combinations of these forward primers with reverse primers located either in exon 11 (Fig. 6B) or in the 3'-UTR (Fig. 6C) yielded the expected bands using human islet mRNA. However, mTECs did not express SLC30A8. One sample (#64) displayed a faint band amplified with the exon 8 forward primer, which matched SLC30A8 by sequencing, suggesting low-level mis-initiated transcription starting at exon 8 (i.e. downstream of the ZnT8_{180-194} region). Collectively, these results demonstrate that ZnT8 is poorly expressed in mTECs, and that SLC30A8 transcription is limited to a mis-initiated mRNA isoform skipping the ZnT8_{180-194} sequence.

Islet-reactive CD8 T cells circulate at similar frequencies irrespective of thymic expression of their cognate epitopes

In contrast to ZnT8, other β-cell Ags are expressed by mTECs, including PPI (16, 17), IA-2 (17, 18) and IGRP (19), or by thymic Ag-presenting cells, including GAD65 (20). Moreover, circulating PPI_{1-14}-reactive CD8 T cells displayed higher frequencies than PPI_{15-24}-reactive CD8 T cells, despite the fact that the complete INS transcript incorporating both epitopes was detected in the thymus (Fig. 6D-E). In conjunction with the finding that circulating CD8 T cells reactive to ZnT8_{180-194} and other islet epitopes also occur at similar frequencies (Fig. 5D), these observations suggest a limited role of thymic Ag gene expression in setting such frequencies.

Potential confounders in this scenario include the efficiency of epitope presentation and the odds of encountering cognate peptide-HLA complexes in the thymic environment. Accordingly, we compared the frequencies of islet-reactive CD8 T cells in age/sex-matched HLA-A2+ and HLA-A2- healthy adults, reasoning that thymic deletion cannot occur in the absence of the appropriate HLA-A2 restriction (21). To exclude promiscuous presentation by non-HLA-A2 molecules, donors were selected based on HLA-A and HLA-B molecules.
incapable of binding the selected islet epitopes (Table S4). Moreover, all subjects were seronegative for Ebola (EboV) and hepatitis C virus (HCV). Representative flow cytometry plots are shown in Fig. S12. Except for PPL6-14, HLA-A2-restricted islet-reactive CD8\(^+\) T cells occurred at largely equivalent frequencies in HLA-A2\(^+\) and HLA-A2\(^-\) donors (Fig. 6F). Although higher overall relative to most islet specificities, the frequencies of MMr\(^+\)CD8\(^+\) cells recognizing the HLA-A2-restricted foreign epitopes EboV NP\(_{202-210}\) and HCV P1406-1415 were also similar between HLA-A2\(^+\) and HLA-A2\(^-\) groups. No significant phenotypic differences were observed between groups for any of these MMr\(^+\)CD8\(^+\) T-cell populations (Fig. 6G). As expected, control Flu MP\(_{58-66}\) MMr\(^+\)CD8\(^+\) cells were more prevalent and more Ag-experienced in HLA-A2\(^+\) donors.

Collectively, these findings suggest that thymic presentation of these islet epitopes does not trigger significant clonal deletion in HLA-A2\(^+\) donors.

*ZnT8\(_{186-194}\)-reactive CD8\(^+\) T cells cross-recognize a Bacteroides stercoris mimotope*

Although islet-reactive CD8\(^+\) T cells were predominantly naïve in T1D and healthy adults, substantial Ag-experienced fractions were noted in some individuals, irrespective of disease status (Fig. 5B-5E). Moreover, CD8\(^+\) T-cell frequencies correlated with the size of the Ag-experienced fraction for some individual (ZnT8\(_{186-194}\), IGRP\(_{205-273}\)) and pooled islet epitopes (Fig. S13A-C), consistent with the patterns observed for Flu MP\(_{58-66}\)-reactive CD8\(^+\) T cells (Fig. S13D). These observations raise the possibility of cross-priming by unrelated homologous epitopes (mimotopes). Indeed, sequence homology was observed between the ZnT8\(_{186-194}\) epitope (VAANIVLTV) and a peptide (KAANIVLTV) derived from protein WP_060386636.1 of the intestinal commensal *Bacteroides stercoris*. To assess potential cross-reactivity, we performed *ex-vivo* MMr assays on duplicate PBMC samples. One sample was stained with pairs of ZnT8\(_{186-194}\) MMrs, while the other was stained with one ZnT8\(_{186-194}\) MMr and one *B. stercoris* MMr. Similar frequencies were detected for ZnT8\(_{186-194}\)-ZnT8\(_{186-194}\)
and ZnT8_{186-194}-B. stercoris double-MMr⁺CD8⁺ T cells in 3 of 4 donors (Fig. 7A). As a negative control, ZnT8_{186-194}-EboV NP_{202-210} double-MMr⁺CD8⁺ T cells were undetectable (Fig. 7B), while EboV NP_{202-210}EboV NP_{202-210} double-MMr⁺CD8⁺ T cells were present at similar frequencies in duplicate samples (Fig. 7C). Cross-reactivity with the B. stercoris mimotope was confirmed for 1 of 4 ZnT8_{186-194}-reactive CD8⁺ T-cell clones via MMr co-staining (Fig. 7D) and in-vitro recall (Fig. 7E), with the B. stercoris mimotope displaying stronger agonist potency than the native ZnT8_{186-194} peptide. Collectively, these results show that ZnT8_{186-194}-reactive CD8⁺ T cells can cross-recognize a bacterial mimotope.

ZnT8_{186-194}-reactive cells are enriched in the pancreas of T1D donors

To reconcile the finding that equivalent frequencies of predominantly naive islet-reactive CD8⁺ T cells circulate in most individuals, we hypothesized that the T1D-relevant fraction may be sequestered in the pancreas. We therefore performed in-situ ZnT8_{186-194} MMr staining on frozen pancreatic sections from HLA-A2⁺ T1D (n=9), aAb⁺ (n=9), non-diabetic (n=11) and type 2 diabetes (T2D) cases (n=3) from nPOD (Table S5). Representative images are shown in Fig. 8A-E, with scattered ZnT8_{186-194} MMr⁺ cells either within islets or the exocrine tissue. Consecutive sections from ZnT8_{186-194} MMr⁺ pancreata were probed with MelanA_{26-35} MMrs, which, in conjunction with positive control vitiligo skin sections, confirmed staining specificity (Fig. 8F). Donor-matched PLN sections were stained in parallel (Fig. 8G-I). While ZnT8_{186-194} MMr⁺ cells were significantly more abundant than MelanA_{26-35} MMr⁺ cells in T1D, aAb⁺ and non-diabetic cases, ZnT8_{186-194} MMr⁺ cells were enriched in the pancreata of T1D vs non-diabetic and T2D cases (Fig. 8I). In contrast, ZnT8_{186-194} MMr⁺ cell were present at similar densities across all groups in PLN sections (Fig. 8J). Several of these nPOD cases were previously analyzed in-silico for the presence of ZnT8_{186-194} CDR3β sequences in PLNs (Fig. 4D). These sequences were present in 4/5 cases with ZnT8 MMr⁺ pancreata and 3/5 cases with ZnT8 MMr⁺ PLNs (Table S5). A donor with chronic pancreatitis (#6288) and
very high ZnT8_{186-191}-reactive CD3R8 counts among spleen and PLN CD8^{+} T cells also displayed high densities of ZnT8 MMr^{+} cells in the pancreas. Collectively, these results show that ZnT8-reactive cells are preferentially enriched in the pancreas of T1D donors.
Discussion

In this study, we found that ZnT8\textsubscript{186-194}-reactive CD8\textsuperscript{+} T-cell clones exhibited heterogeneous functional profiles, but no consistent differences between T1D and healthy subjects. Most ZnT8\textsubscript{186-194}-reactive clones originated from naïve precursors and expressed private TCRs. Ex-vivo analyses on larger donor cohorts revealed that the frequency of circulating ZnT8\textsubscript{186-194}-reactive CD8\textsuperscript{+} T cells was similar in age-matched T1D vs. healthy donors, but higher in children vs. adults, irrespective of T1D status. A similar pattern was noted for CD8\textsuperscript{+} T cells recognizing the extra-pancreatic self-epitope MelanA\textsubscript{26-35}, but the corresponding ZnT8\textsubscript{186-194}-reactive populations were more Ag-experienced in T1D children. Thus, while most children harbor a larger autoimmune repertoire not restricted to islet Ags, the activation of the islet-reactive fraction occurs preferentially in T1D children, which may reflect a more aggressive islet autoimmunity leading to earlier disease onset. On the other hand, some Ag-experienced β-cell-reactive CD8\textsuperscript{+} T cells were invariably detected in healthy donors, supporting the possibility that foreign epitopes may prime autoreactive clonotypes expressing promiscuous TCRs (22-24). Indeed, some ZnT8\textsubscript{186-194}-reactive CD8\textsuperscript{+} T-cell clonotypes were able to cross-recognize a B. stercoris mimotope. It is noteworthy that B. stercoris belongs to the Bacteroidetes phylum, which is enriched in the gut microbiome of T1D (25) and at-risk aAb\textsuperscript{+} subjects (26). Circulating CD8\textsuperscript{+} T cells reactive to other HLA-A2-restricted β-cell epitopes were also detected at equivalent frequencies (1-50 MMR\textsuperscript{+} cells/10\textsuperscript{6} CD8\textsuperscript{+} T cells) and with a predominantly naïve phenotype in T1D and healthy adults. This coherent pattern across islet specificities suggests that Ag-driven recruitment involves a limited fraction of naïve precursors, which fits with the paucity of public clonotypes found for ZnT8\textsubscript{186-194}-reactive CD8\textsuperscript{+} T cells, as reported for PPI\textsubscript{15,24} (4). Interestingly, the lower frequency of Flu-reactive CD8\textsuperscript{+} T cells observed in T1D vs. healthy donors may reflect impaired anti-viral responses (27).
One strength of our *ex-vivo* studies is the highly specific and reproducible quantification of MMr$^+$ cells. The observed frequencies of β-cell-reactive CD8$^+$ T cells fall below previous estimates obtained without enrichment (4, 5), but mirror those described using stringent MMr-based magnetic enrichment (22). Although higher frequencies of β-cell-reactive CD8$^+$ T cells have been observed in T1D vs. healthy donors (5), our data align with another study reporting no difference (4). Importantly, this study validates comparisons based on dasatinib-enhanced MMr staining. Although the T1D children in our cohort had a longer disease duration than T1D adults, comparable age-related differences were observed in healthy donors, and similar trends remained when restricting the analysis to more recently diagnosed T1D children. Other studies reporting higher Ag-experienced β-cell-reactive CD8$^+$ T-cell fractions in T1D vs. healthy donors are potentially limited by undersampling and single MMr labeling (4). Moreover, such differences were not observed for all islet epitopes (4), and significant naïve fractions (~40%) were also present in T1D patients (4, 6).

T-cell precursors can escape thymic negative selection due to ‘blinds spots’ that result from poor or incomplete expression of certain tissue Ags (13, 17, 28–30), due to alternative splicing and promoter usage and mis-initiated transcription (13–15, 30). These features favor the generation of truncated transcripts lacking certain T-cell epitopes, as observed for ZnT8. However, ZnT8$^{180–194}$-reactive CD8$^+$ T cells circulated at similar frequencies relative to other islet-reactive populations. Together with the finding that HLA-A2-restricted islet-reactive CD8$^+$ T cells circulated at similar frequencies in HLA-A2$^+$ and HLA-A2$^-$ donors, these results show that the thymus does not eliminate all autoreactive CD8$^+$ T cells. Nonetheless, thymic self-Ag expression may ‘prune’ autoreactive clonotypes bearing high-affinity TCRs (22, 37).

Perhaps the most outstanding question raised by our findings relates to the ‘benign’ state of islet autoimmunity ‘licensed’ by incomplete central tolerance. Evidence for such thymic defects in human T1D has been limited to INS polymorphisms, which rank second among
T1D susceptibility loci after DQB1 and modulate thymic INS expression (16). Given the ongoing debate surrounding the role of thymic negative selection for autoreactive T cells (22, 32, 33), it is not altogether surprising that even this insulin paradigm does not fully explain T1D development. Indeed, homozygous INS susceptibility alleles are present in ~55% of Caucasians (34), yet very few develop T1D. We propose that incomplete clonal deletion of autoreactive T cells involves several other β-cell Ags besides insulin and is not restricted to T1D patients.

So what distinguishes benign from pathogenic autoimmune T cells? The evidence suggests that neither their blood frequency nor their phenotype are at play. However, naïve T cells circulate perpetually, in contrast to memory T cells. The body load of Ag-experienced autoreactive T cells may therefore be underestimated in the blood of diseased individuals due to tissue sequestration (35). In line with this, the number of ZnT8186-194-reactive cells was higher than for Melan-A28,33-reactive ones in the pancreas, similar between aAb+ and non-diabetic donors, and enriched in the pancreas of T1D donors. Moreover, ZnT8186-194-reactive CD8+ T-cell clones displayed potent lytic activity, much higher than other islet specificities (I). While these findings suggest a pathogenic role for ZnT8-reactive CD8+ T cells, their precise localization and Ag-experienced status within the pancreas and whether their numbers are inversely correlated with age as observed in the blood remain to be verified.

The second possibility is that autoimmune T cells may be differentially regulated in T1D and healthy donors (33, 36), either intrinsically (e.g. anergy or exhaustion) or extrinsically by regulatory T cells. In support of this notion, T1D-specific islet-reactive CD8+ T cells have been repeatedly detected using functional IFN-γ ELISpot readouts (3, 7, 10), which employ unfractionated PBMCs, thus preserving regulatory networks. This observation also argues that peripheral blood can be informative under appropriate assay conditions. ZnT8186-194-reactive CD8+ T cells from T1D patients also expressed higher levels of aurora A kinase, which might hint at increased mitotic activity in the T1D setting. Mirroring this observation,
an anergic phenotype has been reported for self-reactive CD8\(^{+}\) T cells in non-autoimmune subjects (22, 36), which may be at least partially imprinted in the thymus (33, 37).

The third possibility is that the central diabetogenic ingredient may be enhanced β-cell vulnerability caused by islet inflammation in the face of similar autoimmune T-cell repertoires across individuals. In this scenario, tolerance to β-cell Ags may depend primarily on T-cell ignorance (32, 37). Three observations are noteworthy in this respect. First, the higher T1D risk at younger age contrasts with the lower risk of other autoimmune diseases, which may indicate greater β-cell vulnerability due to childhood stressors, such as islet-tropic enteroviruses and the metabolic demands imposed by growth. Second, high densities of ZnT8\(_{186-194}\) MMr\(^{+}\) cells were detected in the pancreas of an organ donor with chronic pancreatitis, suggesting that islet-reactive CD8\(^{+}\) T cells can expand under inflammatory conditions that are not autoimmune ab initio. Third, PPI\(_{15,24}\)-reactive CD8\(^{+}\) T cells were more frequent in T1D vs. healthy donors, with similar results reported for PPI\(_{2,10}\) (22). These epitopes map to the PPI leader sequence, which may undergo enhanced processing and presentation under metabolic stress (7).

Collectively, the present data pose new challenges toward development of circulating T-cell biomarkers for T1D staging and suggest novel therapeutic strategies based on mimicking ‘benign’ autoimmunity or complementing incomplete central tolerance (38).
Supplementary Materials:

Materials and Methods.

**Fig. S1.** Cytokine secretion and cytototoxicity of ZnT8_{186-194}-reactive CD8^+ T cells from T1D patient D222D.

**Fig. S2.** CD8^+ T-cell recognition and HLA-A2 binding of ZnT8_{186-194} and ZnT8_{185-194} epitope variants.

**Fig. S3.** Ag sensitivity correlates with Ag avidity and polyfunctionality in ZnT8_{186-194}-reactive CD8^+ T-cell clones.

**Fig. S4.** Modulation of HLA Class I and ZnT8 expression in human β-cell lines.

**Fig. S5.** TCR sequences of ZnT8_{186-194}-reactive CD8^+ T-cell clones.

**Fig. S6.** ZnT8_{186-194}-reactive clonotype-specific TaqMan assays.

**Fig. S7.** Gating strategy for the analysis of ZnT8_{190-194}, MelanA_{20-35} and Flu MP_{58-66} MMr^+CD8^+ T cells.

**Fig. S8.** IFN-γ secretion by ZnT8_{186-194}-reactive CD8^+ T cells.

**Fig. S9.** Gene expression in ex-vivo single-sorted ZnT8_{186-194} MMr^+CD8^+ T cells.

**Fig. S10.** Extended combinatorial MMr panel for the analysis of multiple islet-reactive CD8^+ T-cell populations, and reproducibility of ex-vivo MMr assays.

**Fig. S11.** CD27, CD28 and CD95 expression on ZnT8_{186-194}-reactive CD8^+ T cells.

**Fig. S12.** Representative MMr and CD45RA/CCR7 dot plots for HLA-A2^+ and HLA-A2^− healthy donors depicted in Fig. 6F-G.

**Fig. S13.** Correlation between the frequency of MMr^+CD8^+ T cells and the Ag-experienced fraction within the same MMr^+CD8^+ population.

**Table S1.** Summary of ZnT8_{186-194}-reactive CD8^+ T-cell clones.

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**Table S3.** Characteristics of HLA-A2^+ study subjects for ex-vivo MMr studies.

**Table S4.** Characteristics of HLA-A2^+ and HLA-A2^− healthy donors for ex-vivo MMr studies.

**Table S5.** Characteristics of nPOD cases for in-situ ZnT8_{186-194} MMr staining.

**Table S6.** Primers used for gene expression analysis of the individual ZnT8_{186-194} MMr^+CD8^+ T cells depicted in Fig. S9A.

Members of the ImMaDiab Study Group.

Excel file. Raw data from figure graphs.
References and Notes


38. S. Culina, N. Gupta, R. Boisgard, G. Afonso, M. C. Gagné, J. Dimitrov, T. Osterby, S. Justesen, S. Luce, M. Attias, B. Kyewski, S. Buus, F. S. Wong, S. Lacroix-


Acknowledgments: We thank C. Maillard, M. Scotto and S. Rozlan for technical assistance; Univercell Biosolutions for providing the ECN90 β-cell line; K. Kedzierska and her laboratory (University of Melbourne) for help with single-cell TCR sequencing; T. Brusko (University of Florida, Gainesville) for help with the nPOD TCR database; the CyBio platform of the Cochin Institute and DKFZ Flow Cytometry Core Facility for assistance with flow cytometry; T. Loukanov (University of Heidelberg) for providing human thymic tissue; K. Boniface and J. Seneschal (INSERM U1035, Bordeaux) for providing vitiligo skin sections; and S. You for reviewing the manuscript. Members of the ImMaDiab Study Group are listed in the Supplemental Acknowledgments. Funding: This work was performed within the Département Hospitalo-Universitaire (DHU) Authors, supported by the Programme Hospitalier de Recherche Clinique ImMaDiab, Lilly France, the INSERM-Transfert Proof-of-Concept program acDC, the Ile-de-France CORDDIM, and by grants from the JDRF (1-PNF-2014-155-A-V, 2-SRA-2016-164-Q-R), the Aviesan/Astra Zeneca ‘Diabetes and the Vessel Wall Injury’ program, the Société Francophone du Diabète, the Agence Nationale de la Recherche (ANR-2015-CE17-0018-01), and the Fondation pour la Recherche Médicale (Equipe FRM DEQ20140329520), to RM; a JDRF Biomarkers grant (17-2012-598), to KC; an NIH R01 grant (DK052068), to TWJ; Lilly and Fondation Bettencourt-Schueller funds, to RS; a European Research Council grant (ERC-2012-AdG), to BK; and funds from JDRF, the Wellcome Trust and the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre, to the JDRF/Wellcome Trust Diabetes and Inflammation Laboratory (CIMR, University of Cambridge), which provided PBMC samples from the JDRF D-GAP study. Samples from at-risk subjects were obtained through a TrialNet ancillary study to the TN-01 Pathway to Prevention study funded by NIH grants U1 DK061010, U1 DK061034, U01 DK061042, U01 DK061058, U01 DK085465, U01 DK085453, U01 DK085461, U01 DK085463, U01 DK085466, U01 DK085499, U01 DK085504, U01 DK085505, U01 DK085509, U01 DK103180, U01 DK103153, U01-
DK085476. U01-DK103266 and by JDRF. DAP is a Wellcome Trust Senior Investigator (100326Z/12/Z). This research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative T1D research project funded by JDRF. Organ Procurement Organizations partnering with nPOD to provide research resources are listed at www.jdrfnpod.org/our-partners.php. This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (INNODIA, no. 115797). This Joint Undertaking receives support from the Union’s Horizon 2020 research and innovation programme and the European Federation of Pharmaceutical Industries and Associations, JDRF, and The Leona M. and Harry B. Helmsley Charitable Trust. Author contributions: S.C. A.L., G.A., K.C., S.P., G.S., K.K., L.N., A.E., T.O., J.E.M. and M.K. performed experiments and analyzed data; A.M., K.I., E.L., J.P.B., A.L., V.A., H.W.D., S.B., D.A.P., E.B., M.B., S.C.Z., F.D. and R.S. provided critical reagents, experimental assistance and intellectual input; M.K. and R.M. performed statistical analyses; S.C., A.L., G.A., K.C., S.P., D.A.P., B.K. and R.M. designed and interpreted experiments, and wrote the paper; the ImMaDiab Study Group recruited patients and performed clinical phenotyping. Competing interests: The authors declare that no conflict of interest exists. Some T-cell receptor sequences described herein are covered by Inserm-Transfert patent WO/2017/046335. Data and materials availability: available materials will be provided with the appropriate material transfer agreement.
Fig. 1. ZnT8186-194-reactive CD8\(^+\) T-cell clones from patient D222D. (A) Frozen-thawed PBMCs were cultured with ZnT8186-194 or no peptide and stained with PE/APC-labeled ZnT8186-194 MMRs. (B) ZnT8186-194 and control MMR stains for one clone obtained from single-sorted ZnT8186-194/ZnT8186-194 double-MMR\(^+\) cells. (C) Percent intracellular TNF-\(\alpha\)-D222D clone 1 cells stimulated for 6 h with K562-A2 cells pulsed with ZnT8186-194 or Flu MP34-66 peptide. (D) Percent lysis of FarRed-labeled LCL targets pulsed with ZnT8186-194 (top) or Flu MP34-66 peptide (bottom) and cultured for 24 h with CFSE-labeled D222D clone 3 at increasing E/T ratios. (E) Percent lysis of LCL targets cultured with D222D clones 1, 2 or 3 (mean±SEM; each clone is depicted in Fig. S1D-F). (F) Lysis of cognate peptide-pulsed LCLs cultured for 4 h with D222D clone 2 or a MelanA25-35-reactive clone (E/T 1:1) in the presence of concanamycin A (CMA), brefeldin A (BFA), CMA and BFA, anti-FasL or control IgG1. *\(p=0.015\), **\(p=0.009\), ***\(p<0.001\) by Student’s \(t\) test. Results are mean±SEM of triplicate measurements from one of three experiments. (G) Percent surface CD107\(\alpha\)-D222D clone 1 cells stimulated as in (C). For panels A, C, G, gate is on viable CD8\(^+\) cells.

Fig. 2. Ag avidity, Ag sensitivity and polyfunctionality of ZnT8186-194-reactive CD8\(^+\) T-cell clones. (A) ZnT8186-194 MMR staining in the absence (light grey) or presence (dark grey) of dasatinib. The dotted profile indicates the unstained control. (B) ZnT8186-194 MMR median fluorescence intensity (MFI) for the indicated clones in the absence (left) or presence (right) of dasatinib. Bars indicate median values. Results are representative of two separate experiments. (C) The indicated clones were stimulated for 6 h with ZnT8186-194-pulsed K562-A2 cells and percent cytokine\(^+\) cells out of viable CD8\(^+\) cells calculated. Results are representative of three independent experiments. (D-E) Half maximal effective peptide concentration (EC50; D) and maximal cytokine response (percent cytokine\(^+\) cells at optimal
peptide concentrations; E) for clones stimulated as above. Bars indicate median values. Results are representative of two to four separate experiments. *p=0.014 by Mann-Whitney test. (F) Polyfunctionality distribution of T1D (left) and healthy clones (right). Percent T cells producing 0 to 4 cytokines among TNF-α, IFN-γ, IL-2 and MIP-1β upon exposure to ZnT8186.194-pulsed K562-A2 cells (100 μM) are shown.

Fig. 3. Target cell lysis by ZnT8186.194-reactive CD8⁺ T-cell clones. (A-C) Lysis of K562-A2 cells transfected (open triangles) or not (open circles) with a full-length ZnT8 plasmid and cultured for 24 h with clones D222D 2 (A), H017N A1 (B) or H314C 6C4 (C). Filled symbols indicate ZnT8186.194-pulsed target cells (10 μM). Results are presented as mean±SEM of triplicate wells from two separate experiments. (D-G) Real-time cytotoxicity for the indicated clones vs. HLA-A2⁺ ECN90 (white triangles) or control HLA-A2⁻ EndoC-βH2 β-cell targets (white circles) (E/T 2/1). Black and grey symbols indicate the corresponding targets pulsed with 10 μM ZnT8186.194 or GAD111-122 peptide, respectively. Mean±SEM of triplicate measurements are shown at each time point. Results are representative of at least two separate experiments. (H) Percent maximal HLA-A2⁺ ECN90 and HLA-A2⁻ EndoC-βH2 β-cell lysis by the indicated clones (T1D, grey symbols; healthy, white symbols; control, horizontal dotted line) in the absence or presence of ZnT8186.194 peptide. Bars indicate median values. Lysis was calculated from the cytotoxicity profiles as in panels D-G.

Fig. 4. In-silico search for CDR3β aminoacid sequences from ZnT8186.194-reactive CD8⁺ T-cell clones. (A-C) Prevalence of the CDR3β aminoacid sequences from clones D010R 1E2 (A), H328C 8E8 (B) and H1034O 141B9 (C) among HLA-A2⁺ T1D (n=5), aAb⁺ (n=5) and healthy subjects (n=10), as assessed by in-silico analysis of TCRβ repertoires obtained from the indicated CD8⁺ and CD4⁺ T-cell subsets. (D) In-silico search for the same CDR3β
aminoacid sequences in the repertoire of CD8\(^+\), conventional CD4\(^+\) (Tconv; CD127\(^+\)) and regulatory CD4\(^+\) (Treg; CD25\(^+\)/CD127\(^-\)) T cells obtained from nPOD PLN, spleen and inguinal lymph node (ILN) samples via the online database http://clonesearch.jdrfnpod.org. For each cell type and tissue, the first, second and third columns refer to clones D010R 1E2, H034O 141B9 and H328C 8E8, respectively. Dark and light grey cells indicate negative and positive samples, respectively. Frequencies per 10\(^5\) TCRs are annotated, and underlining indicates samples with a nucleotide sequence match. White cells indicate unavailable samples. Pancreatic NET, neuro-endocrine tumor.

Fig. 5. Ex-vivo frequencies and Ag-experienced phenotypes of circulating islet-reactive CD8\(^+\) T cells. (A) ZnT8\(_{180,194}\), MelanA\(_{26,35}\) and Flu MP\(_{58,66}\) MMr\(^+\)CD8\(^+\) cells were stained ex-vivo and counted (see Fig. S7). Frequencies out of total CD8\(^+\) T cells are depicted for TID adults (red circles), TID children (crossed red circles), age/sex-matched healthy adults (blue circles) and children (crossed blue circles). \(*p<0.05\), \(**p<0.002\), \(***p<0.0003\). (B) Percent Ag-experienced cells out of total MMr\(^+\) cells. \(p<0.03\), \(**p<0.004\), \(***p<0.0007\). (C) Absolute frequencies of the corresponding Ag-experienced fractions. \(p<0.03\), \(**p<0.01\), \(***p<0.0001\). (D) MMr\(^+\)CD8\(^+\) cells reactive to the indicated islet epitopes were stained ex-vivo and counted (see Fig. S10A). Frequencies out of total CD8\(^+\) T cells are depicted as in panel A. \(*p=0.02\). (E) Percent Ag-experienced cells out of total MMr\(^+\) cells. (F) Absolute frequencies of the corresponding Ag-experienced fractions. Bars display median values. The median number of MMr\(^+\) events and total CD8\(^+\) T cells analyzed are indicated for each distribution. Significance was determined using the Mann-Whitney test. For panels A and D, data-points with <300,000 CD8\(^+\) T cells and <5 MMr\(^+\) cells were excluded. For panels B-C and E-F, data-points with <5 MMr\(^+\) cells were excluded.
Fig. 6. SLC30A8 and INS gene expression in mTECs and circulating islet-reactive CD8+ T-cell frequencies in HLA-A2+ and HLA-A2− healthy donors. (A) SLC30A8 RT-PCR strategy. Forward primers spanned exons 5 to 8, reverse primers spanned either exon 11 or the 3′-UTR. The position of the ZnT8185-194-coding region is shown. (B) SLC30A8 expression in mTECs, using the indicated forward primers and the exon 11 reverse primer. (C) SLC30A8 expression in mTECs from donor #64 (previously testing positive with the exon 8 forward primer) and #211 (previously testing negative), using the 3′-UTR reverse primer. (D) INS RT-PCR strategy. Forward primers spanned both or neither of the PPI6-14 and PPI15-24 regions, reverse primers spanned either the 3′-UTR or exon 2 (PCR products 1 and 2, respectively). (E) INS expression in thymuses pooled from 5-8 donors. (F) Ex-vivo MMr+CD8+ cell frequencies in age/sex-matched, EboV- and HCV-seronegative HLA-A2+ and HLA-A2− healthy donors. (G) Percent Ag-experienced MMr+ cells. Bars indicate median values. The median number of MMr+ events is indicated, with a median of 1x10^6 total CD8+ T cells analyzed. *p<0.03, **p=0.008, ***p<0.0004 by Mann-Whitney test. For panels G, data points with <5 MMr+ cells were excluded. NA, not available.

Fig. 7. ZnT8185-194-reactive CD8+ T cells cross-recognize a B. stercoris mimotope. (A-C) Four donors with sizable ZnT8185-194 MMr+CD8+ T-cell fractions were selected. A first PBMC aliquot received PE/BV786-labeled ZnT8185-194 MMr+ and PE/BV711-labeled EboV NP202-210 MMr+. For the second aliquot, PE-labeled B. stercoris MMr+ replaced the PE-labeled ZnT8185-194 MMr+. (A) Overlay of ZnT8185-194/B. stercoris MMr+ cells (red) and ZnT8185-194/B. stercoris MMr+ cells (red). (B) Negative control staining of ZnT8185-194/EboV NP202-210 MMr+ cells. (C) Positive control staining of EboV NP202-210/EboV NP202-210 MMr+ cells from the first and second aliquot. The frequencies of MMr+ out of total CD8+ T cells are indicated. (D) Four ZnT8185-194-reactive CD8+ T-cell clones (D222D 2, D349D 178B9, H017N A1, H328C 9C8) were stained with BV786/PE-labeled ZnT8185-194, PE-labeled B. stercoris and
BV650-labeled MelanA<sub>26-35</sub> MMrs. The ZnT8<sub>186-194</sub>/B. stercoris cross-reactive clone H017N is shown, from left to right: ZnT8<sub>186-194</sub>/B. stercoris MMr<sup>+</sup>; ZnT8<sub>186-194</sub>/ZnT8<sub>186-194</sub> MMr<sup>+</sup>; and negative control ZnT8<sub>186-194</sub>/MelanA<sub>26-35</sub> and B. stercoris/MelanA<sub>26-35</sub> MMr<sup>+</sup> cells. (E) The H017N clone was stimulated with peptide-pulsed LCLs (0.1 μM, 6 h). Percent cytokine cells are shown as mean±SEM of two experiments. p<0.008 for Wilcoxon signed-rank comparison of pooled cytokine responses between B. stercoris and ZnT8<sub>186-194</sub>, MelanA<sub>26-35</sub> or no peptide, and between ZnT8<sub>186-194</sub> and MelanA<sub>26-35</sub> or no peptide.

**Fig. 8. In-situ ZnT8<sub>186-194</sub> MMr staining of unPOD pancreas and PLN sections.** (A-E) Representative pancreas images (20X magnification; scale bar 100 μm) from cases T1D #6161 (A), aAb<sup>+</sup> #6347 (B) and non-diabetic (ND) #6289 (C). Red arrows indicate MMr<sup>+</sup> cells and the dotted areas of panels A-B are magnified in panels D (scale bar 80 μm) and E (scale bar 50 μm), respectively. (F) Consecutive sections from ZnT8<sub>186-194</sub> MMr<sup>+</sup> pancreata were probed with negative control MelanA<sub>26-35</sub> MMrs. A representative image from T1D case #6211 is shown on the left, and a positive control staining on skin sections from a vitiligo patient is shown on the right (20X; scale bar 100 μm). (G) Representative PLN image (20X; scale bar 100 μm) from T1D case #6161. (H) Magnification of the dotted area of panel G (scale bar 40 μm). (I-J) Number of ZnT8<sub>186-194</sub> and MelanA<sub>26-35</sub> MMr<sup>+</sup> cells/mm<sup>2</sup> section area of pancreas (I) and PLNs (J). Each point represents an individual case (detailed in Table S5). Bars indicate median values. *p<0.05, **p<0.009 by Mann-Whitney test. NA, not assessed.
Figure 1.
Figure 2.
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Figure 4.
Figure 5.
Figure 7.
Figure 8.
S. Culina, A.I. Lalanne et al., Islet-reactive CD8 T-cell frequencies in the pancreas but not blood distinguish type 1 diabetes from healthy donors.

Supplementary Materials

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Members of the ImMaDiab Study Group.

Excel file. Raw data from figure graphs.
Materials and Methods

Study design. The objective of this study was to identify the features of islet-reactive CD8+ T cells that associate with T1D. Hypotheses were formulated on a prospective basis guided by the data. Based on a detailed analysis of ZnT18188-191-reactive CD8+ T-cell clones (listed in Table S1), we initially hypothesized that peripheral autoreactivity occurs independently of disease status. This hypothesis was substantiated using HLA-A2 MMs to quantify and characterize islet-reactive CD8+ T cells directly ex vivo (donors listed in Table S3). Next, we hypothesized that this widespread autoimmune repertoire stems from a universal leakiness of central tolerance, which was verified by thymic gene expression studies and by comparing HLA-A2-restricted Ag-reactive CD8+ T-cell population frequencies in HLA-A2+ vs. HLA-A2- donors (listed in Table S4). Finally, we hypothesized that the lack of distinguishing features in the periphery reflects sequestration of the disease-relevant subset in the pancreas. This hypothesis was confirmed by in situ MMr staining of pancreatic sections (donors listed in Table S3). Following power analysis, age/sex-matched, unblinded case-control sets were selected from donors recruited at affiliated Diabetology Units. Samples were processed in batch, and no outliers were excluded. All in vitro experiments were performed on at least two separate occasions. For ex vivo MMr analyses, undersampled data points were excluded, as detailed in Fig. 5-6.

Study approval. All subjects provided written informed consent. Ethical approval was granted by the Comité de Protection des Personnes Ile de France 1-2 (AOR10049, K091101, A01094-53) and by the Institutional Review Boards of the Cambridge Royal Free Hospital (08/H0720/25), the Benaroya Research Institute (7109-147), the University of Heidelberg (367/2002) and the University of Florida Health Center (nPOD Project). The ImMaDiab study is registered at www.clinicaltrials.gov (NCT01747967).

Peptides, MMs and HLA-A2 binding measurements. Peptides ZnT18188-191 (VAANIVLTV) and its B. stercoris WP-060386636.1 mimotope (KAANIVLTV), ZnT18185-194 (AVANAIVLTV), MelanA25-35 (A27L variant; FLAGeth35TV), Flu MP58-66 (GILGFVFTL), PP1(2-12) (RLPPLLALL), PP1(3-24) (ALWGPDPAAA), GAD11-22 (VMNLILQYV), IA-2RI5-113 (VIVMLTPIV), IGRP196-213 (VLFQFLGFA), EboV NP199-216 (RLMRFNL) and HCV P1(66-115) (KLVALGINAV) were synthesized at >85% purity (ChinaPeptides). Peptide-HLA-A2 affinity and stability assays were performed as detailed in Fig. S2. HLA-A2 MMs were produced as described (39), and staining was performed in the presence of 50 nM dasatinib (11). For Fig. 2B, MMr MFIs were normalized to that of D222D clone 2 in the presence of dasatinib.

Cloning of ZnT18188-191-reactive CD8+ T cells. Frozen-thawed PBMCs (2-10x10^6) were stained with dual fluorochrome-labeled ZnT18188-191 MMs, either directly ex vivo or after 10 days of acDC culture (9) in the absence or presence of 1 μM peptide (ZnT18188 or ZnT18185). Double-positive events were then sorted as single cells into individual wells of a U-bottom 96-well plate. Each sort well contained 200,000 irradiated PBMCs, 5% Cellkines (Zeptometrix), 200 IU/ml Proleukin, 25 ng/ml IL-15, 1 μg/ml phytohemagglutinin (PHA)-I (Sigma), penicillin/streptomycin and amphotericin B. Medium was replenished every 3 days.
without PHA-L. Expanding clones were selected by visual inspection, transferred into 48-well plates for specificity testing and restimulated as above every 2-3 weeks.

**Antigen recall assays.** Peptide-pulsed HLA-A2<sup>+</sup> LCLs or K562 cells transduced with HLA-A2, CD80 and 4-1BBL (a kind gift from Dr. J. Riley, University of Pennsylvania, Philadelphia, PA) were labeled with CellTrace Violet (Life Technologies) and incubated with T cells at an E/T ratio of 2/1 for 6 h in the presence of 10 μg/ml brefeldin A. Intracellular cytokine staining was performed using BD Cytofix/Cytoperm reagents and analyzed using a BD LSR Fortessa cytometer. CD107a staining was performed with a FITC-labeled mAb (clone H4A3, BD). Polyclonality indices were calculated as described (12).

**Cytotoxicity assays.** LCL, K562-A2 or K562-A2/ZnT8 target cells were labeled with CellTrace FarRed (Life Technologies), dispensed into 96-well flat-bottom plates at 10<sup>5</sup> cells/well and co-cultured with different numbers of CFSE-labeled T cells for 6-24 h. After staining with Live/Dead Aqua (Life Technologies) and fixation, a set number of CompBeads (BD) was added to each well. Flow cytometric analysis was performed by counting the numbers of CFSE-FarRed<sup>+</sup>Live/Dead<sup>+</sup> targets for each condition, normalized to equal numbers of CompBeads. Percent lysis was calculated as 100 × (live targets cultured alone) - (live targets in the presence of T cells) / (live targets cultured alone). Blocking experiments were conducted with concanamycin A (100 nM; Sigma), brefeldin A (5 μg/ml; Sigma) and the anti-Fasl antibody NOK-1 (5 μg/ml; BD).

The EndoC-βH2 cell line (HLA-A*B*01:03, -B*07:08, -C*07:07) was described previously (40), and the ECN90 cell line (HLA-A*B*02:01:03, -B*40:49, -C*03:07) was derived from a human neonatal pancreas using similar protocols. Real-time cytotoxicity assays on β-cell lines were performed using the xCELLigence system (ACEA Biosciences). Briefly, β cells were dispensed into 96-well E-plates and pretreated as indicated. After resting for 20 h and pulsing with 10 μM peptide or DMSO for 2 h, T cells were added at an E/T ratio of 2/1, and impedance was recorded every 5-15 min for 4 h. Cell indices were normalized to values at the time of T-cell addition (t=0) and transformed to percent lysis.

**TCR sequencing, in-silico analyses and clonotype-specific TaqMan assays.** TRA and TRB gene expression was analyzed using a template-switch anchored RT-PCR (41) for T-cell clones and a multiplex nested PCR (42) for single-sorted cells. Gene usage was determined according to the ImMunoGeneTics (IMGT) nomenclature.

The TRB database (Adaptive Biotechnologies) used for in-silico analyses was derived from the donors listed in Table S2. TaqMan assays (Life Technologies; Fig. S6) were applied to cDNA samples from naïve (CD4<sup>+</sup>CCR7<sup>+</sup>) and Ag-experienced (CD4<sup>+</sup>CCR7<sup>+</sup> or CD4<sup>+</sup>CCR7<sup>+</sup>, CCR7<sup>-</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T cell bulk-sorted from age/sex-matched cohorts incorporating 83 TID patients [age 34 years (17-59), 51% females, T1D duration 8 years (0.1-55), 51% HLA-A2<sup>+</sup>] and 93 healthy donors [age 34 years (17-60), 47% females, 41% HLA-A2<sup>+</sup>]. cDNA samples were amplified using clonotype-specific TaqMan primers for 18 cycles, followed by real-time qPCR using clonotype-specific TaqMan assays on Fluidigm 96.96 microfluidic chips with a BioMark HD qPCR system. Amplification curves for
individual assays were examined and compared with curves from a TRB constant region assay as a control for TRB templates in each reaction.

**Ex-vivo analysis of ZnT8156.19-transient CD8⁺ T cells.** Cryopreserved PBMCs from T1D and healthy donors (Table S3) were magnetically depleted of CD8⁺ cells (StemCell Technologies), stained with the combinatorial MMr panels (9) detailed in Fig. S7 and S10 and acquired using a BD FACSAria III cytometer. IFN-γ ELISpot assays were performed as described (7). Single-cell gene expression analysis is detailed in Fig. S9A and Table S6.

**Gene expression in human mTECs.** Human thymus samples were obtained from children undergoing corrective cardiac surgery at the University of Heidelberg, Germany, mTECs were purified as described (13). Sorted total, immature and mature mTECs (CD45 EpCAM⁺CDR2⁺) were independently validated for gene expression of the tissue-restricted Ags β-casein and MelanA (13). Amplified bands were sequences to confirm identity with the expected SLC30A8 and INS regions. SLC30A8 exons were annotated with reference to Ensembl ID ENST00000427715. The INS PCR covered all INS transcripts except ENST00000512523 (product 1) and ENST00000421783 (product 2).

**In-situ ZnT8156.19 MMr staining.** In-situ staining was performed as described (2). Briefly, unfixed, frozen sections were dried for 2 h, loaded with 1 μg of MMrs overnight at 4°C, washed gently with phosphate-buffered saline and fixed in 2% paraformaldehyde for 10 min. After a further wash, endogenous peroxidase activity was blocked with 0.3% H₂O₂. Sections were then incubated serially with rabbit anti-phycoerythrin, horseradish peroxidase-conjugated swine anti-rabbit and 3,3'-diaminobenzidine tetrahydrochloride substrate (Thermo Scientific). After a final wash, sections were counterstained with hematoxylin, dehydrated via sequential passages in 95-100% ethanol and xylene, mounted and analyzed using a Nikon Eclipse Ni microscope with NIS-Elements Analysis D software v4.40.

**Statistical analysis.** Data are shown as median (range) or mean±SEM. Significance was assessed using two-tailed tests with a cut-off value of α=0.05, as detailed for each figure.
Fig. S1. Cytokine secretion and cytotoxicity of ZnT8<sub>186-194</sub>-reactive CD8<sup>+</sup> T cells from T1D patient D222D. (A-C) D222D clone 1 (A), 2 (B) or 3 (C) were stimulated for 6 h with K562-A2 cells pulsed with the indicated peptide concentrations. Graphs display percent intracellular cytokine<sup>+</sup> cells calculated after gating on viable CD8<sup>+</sup> cells, as shown in Fig. 1C. The following antibodies were used: anti-TNF-α-APC (clone MAb11, BD), anti-IFN-γ-PE (clone 4S.B3, eBioscience), anti-IL-2-PE/Cy7 (clone MQ1-17H12, eBioscience) and anti-MIP-1β-FITC (clone 24006, R&D). (D-F) FarRed-labeled HLA-A2<sup>+</sup> ICl<sub>2</sub> target cells were pulsed with the indicated peptides and cultured with CFSE-labeled D222D clones 1 (D), 2 (E) or 3 (F) at increasing E/T ratios. Live FarRed<sup>+</sup> target cells were counted at 24 h and normalized to a fixed number of beads added to each well. Percent lysis is plotted for each graph, calculated as 100 x (live targets cultured alone) / (live targets cultured alone). Results are representative of three independent experiments. For panels A-C, results are shown from a separate experiment than depicted in Fig. 1C. For panels D-F, results are shown from the three separate clones, with raw data for D222D clone 3 depicted in Fig. 1D, and pooled data for all three clones depicted in Fig. 1E.
Fig. S2. CD8⁺ T-cell recognition and HLA-A2 binding of ZnT8186-194 and ZnT8185-194 epitope variants. (A) The indicated clones were stained with HLA-A2 MMRs loaded with either ZnT8186-194 (VAANIVLTV; blue profiles) or ZnT8185-194 (A VAANIVLTV; red profiles) in the presence of dasatinib. MMR fluorescence intensities registered in separate runs were made comparable by using the same flow cytometer settings, by recalibrating the cytometer for each run using Spherotech Rainbow Calibration particles, and by including a reference D222D clone (thawed cryovials from the same freeze) in all experiments. The MMR fluorescence registered in each run for the reference D222D clone was also used to further normalize the median fluorescence intensities plotted in Fig. 2B. (B) In-vitro HLA-A2 binding affinity measurements for the ZnT8186-194 (white diamonds) and ZnT8185-194 peptides (black diamonds). Recombinant HLA-A2 (0.7 nM) was mixed in 96-well polypropylene plates (Nunc) with β2-microglobulin (β2M; 25-50 nM) and the indicated peptide (5-fold dilutions) in phosphate-buffered saline supplemented with 0.1% Triton X-100 and allowed to form complexes at 18°C for 48 h. Amounts of each peptide-HLA complex were determined using an AlphaScreen assay (Perkin Elmer) with streptavidin-conjugated donor beads and W6/32 anti-HLA Class I antibody-conjugated acceptor beads. Peptide-HLA complexes (10 μl) were transferred to 384-well OptiPlates (Perkin Elmer) in duplicates, mixed with 30 μl of streptavidin-conjugated donor beads and W6/32-conjugated acceptor beads, and
incubated in the dark for 6-8 h at room temperature. Plates were analyzed using an Envision reader (Perkin Elmer). (C) In-vitro HLA-A2 stabilization assays with the ZnT8188-194 and ZnT8185-194 peptides. Recombinant HLA-A2 molecules were incubated with the indicated peptides and the corresponding dissociation rates were monitored over time. Briefly, biotinylated recombinant HLA-A2 (30 nM) was mixed with peptide (10 nM) and 125I-labeled β2M (25,000 cpm/well), transferred to a 96-well FlashPlatePlus (Perkin Elmer), and allowed to form complexes overnight at 18°C. Dissociation was initiated by adding unlabeled β2M at a final concentration of 360 nM and monitored by consecutively reading the microplates in a TopCount NXT scintillation counter (Perkin Elmer) at 37°C for 15 h. Kd affinity (B) and half-life (t1/2) values (C) are displayed, as calculated using a non-linear regression fit (GraphPad Prism 5). Results are representative of three independent experiments.
Fig. S3. Ag sensitivity correlates with Ag avidity and polyfunctionality in ZnT8_{186-194} reactive CD8⁺ T-cell clones. (A-D) The indicated CD8⁺ T-cell clones generated from T1D and healthy subjects (white and black symbols, respectively, as in Fig. 2) were compared for normalized MMr MF1 in the absence of dasatinib (from Fig. 2B; x-axis) vs. ZnT8_{186-194} peptide EC50 for the indicated cytokine responses (from Fig. 2D; y-axis). EC50 values are plotted from higher to lower, corresponding to increasing Ag sensitivity. (E-H) The same clones were compared for polyfunctionality index (from Fig. 2F; x-axis) vs. ZnT8_{186-194} peptide EC50 (as in panels A-D). The corresponding Spearman r and p values are displayed for each panel.
Fig. S4. Modulation of HLA Class I and ZnT8 expression in human β-cell lines. (A-B) Surface HLA Class I expression (anti-HLA-A, B, C antibody W6/32, labeled in-house with AlexaFluor647) and Live/Dead Red viability of HLA-A2⁺ ECN90 (A) and HLA-A2⁻ EndoC-BH2 cell lines (B) with and without exposure to the indicated cytokines for 18 h. The following cytokine cocktails were used: TNF-α alone (1,100 U/ml); IFN-γ alone (500 U/ml); IFN-γ alone (500 U/ml); TNF-α, IFN-γ and IL-1β (1,100 U/ml, 2,000 U/ml and 1,000 U/ml, respectively); TNF-α, IFN-α, IFN-γ and IL-1β (2,500 U/ml, 1,000 U/ml, 500 U/ml and 50 U/ml, respectively). Results are representative of three independent experiments. (C) Representative optical microscopy images (10X magnification) of wells in which ZnT8₁₈₆-₁₉₁-reactive D222D clones or MelanA₂₅-₃₃-reactive clones were co-cultured with HLA-A2⁺ ECN90 or HLA-A2⁻ EndoC-BH2 cells for the cytotoxicity assays depicted in Fig. 3D-G. T cells were removed by gentle washing and the remaining cells were stained with Trypan Blue. Two independent experiments were performed. (D) ZnT8 expression in ECN90 and EndoC-BH2 β-cell lines pretreated with or without IFN-γ (500 U/ml, 18 h), as assessed by Western blot using an anti-ZnT8 antibody (clone 17H2.4, produced in-house). K562-A2 cells transduced or not with a full-length ZnT8 plasmid are shown as positive and negative controls, respectively. α-tubulin expression is shown as a loading control. (E) IFN-γ recall
assay for D222D clone 2 on ECN90 cells pretreated with or without IFN-γ (500 U/ml, 18 h), washed and pulsed with DMSO or 10 μM ZnT8136-154 peptide for 2 h. A fixed number of T-cell effectors was co-cultured with increasing numbers of ECN90 targets for 6 h. Percent IFN-γ⁺ cells was calculated from plots gated on viable CD8⁺ cells. (F) Real-time cytotoxicity assays for D222D clone 2 in the presence of ECN90 cells (E/T ratio 2/1) pretreated with or without the indicated cytokines for 18 h. Results in panels D-F are representative of at least two independent experiments.
Fig. S5. TCR sequences of ZnT8<sub>196-199</sub>-reactive CD8<sup>+</sup> T-cell clones. Clones isolated from T1D patients are shown on the left and clones isolated from healthy donors are shown on the right. Nucleotide sequences rearranged from the indicated V (green), D (black) and J (blue) genes are shown for each clone. Nucleotide additions at the V-D, D-J and V-J junctions are shown in red and the corresponding N-region length is indicated (right column).
Fig. S6. ZnT8|8|9-194-reactive clonotype-specific TaqMan assays. (A) TaqMan assays were designed for the ZnT8|8|9-194-reactive CDR3 regions of the rearranged TRA (top) and TRB genes (bottom) from clones D010R 1E2, H528C 8E8, and D222D by placing forward primers in the V region and reverse primers in the J region of each transcript (black fonts). A 6-carboxyfluorescein (FAM)-labeled minor groove binder (MGB) probe was designed to cover the unique N(D)N region of each chain. For TRBV19 CDR3β regions (clones D010R and D222D), two unique nucleotide sequences were screened using two different probes (TRB19#1 and TRB19#2). Sequences corresponding to the V, D, N and J regions are shown in green, black, red and blue, respectively. The example shows TaqMan assays for the D010R clonotype. (B) Clonotype-specific TaqMan assays were validated using synthetic DNA templates (Life Technologies) for clonotype-specific or irrelevant TRA and TRB sequences. Ten-fold dilutions of the templates (from 10^-3 to 10^-7; left panel, purple profiles) were spiked into naïve CD8+ T-cell cDNA and amplified by qPCR. As shown for the D010R TRB19#2 probe, the sensitivity was 10^-5, equivalent to 100 copies of template (equivalent to -1 T cell). The right panel shows that non-specific DNA templates and no-template controls (colored profiles) were not amplified. The blue line marks the detection threshold, set at a change in normalized reporter value (ΔRn) of 0.2. (C) Clonotype-specific TaqMan assays were used to screen cDNA from pooled naïve (CD45RA+CCR7+) or Ag-experienced (CD45RA+CCR7-)
CD45RA<sup>+</sup>CCR7<sup>+</sup> CD4<sup>+</sup> or CD8<sup>+</sup> T cells isolated from T1D or age/sex-matched healthy donors. Samples were pre-amplified with the pooled clonotype assays, and the products were used for real-time qPCR on a Fluidigm platform. Representative results are shown for the D010R TRA and TRB probes depicted in panels A-B, along with a TRBC probe included as a positive control for TRB templates in each sample. Results are shown for T1D patient xPb73638725 testing positive on memory and negative on naïve CD8<sup>+</sup> T cells. NTC, no-template controls. (D) Summary of the results obtained by screening T1D and control (C) healthy donors (number of subjects indicated in parentheses) with the D222D ZnT8 and control GAD clonotype-specific probes (experiment 1, top panel) and with the H328C and D010R clonotype-specific probes (experiment 2, bottom panel; only HLA-A2<sup>+</sup> subjects were analyzed). The number of subjects with ≥1 positive sample for each of the indicated assays is reported.
Fig. S7. Gating strategy for the analysis of ZnT8<sub>186-194</sub>, MelanA<sub>26-33</sub> and Flu MP<sub>26-66</sub> MMr<sup>+</sup>CD8<sup>+</sup> T cells. (A) After magnetic depletion of CD8<sup>+</sup> cells in frozen-thawed PBMCs from T1D patient #D011W, cells were sequentially gated on small lymphocytes, singlets, live cells (Live/Dead Red<sup>+</sup>), CD8<sup>+</sup> T cells (FITC-CD14/CD19<sup>+</sup>, AlexaFlour700-CD8<sup>+</sup>; clones 61D3, HIB19, RPA-T8, respectively; eBioscience) and total PE<sup>+</sup>, BV650<sup>+</sup>, APC<sup>+</sup> and BV421<sup>+</sup> MMr<sup>+</sup> T cells. ZnT8<sub>186-194</sub> MMr-PE/BV421<sup>+</sup>, MelanA<sub>26-33</sub> MMr-PE/APC<sup>+</sup> and Flu MP<sub>26-66</sub> MMr-APC/BV650<sup>+</sup> events were visualized using the gating strategy previously detailed for combinatorial MMr staining (9) and FlowJo v10 software (Tree Star). The staining panel also included anti-CD45RA-BV785 (clone HI100, BioLegend) and anti-CCR7-BV711 (clone 150503, BD). (B) The final readout obtained for T1D patient #D011W after gating out events positive for <2 or >2 MMr fluorochromes is shown, with events corresponding to each epitope-reactive population depicted in different colors within each plot, and MMr events depicted in grey. (C) Percent naïve (CD45RA<sup>+</sup>CCR7<sup>+</sup>) cells is shown after gating on the corresponding MMr fractions, with the distribution of total CD8<sup>+</sup> T cells shown for comparison. Percent Ag-experienced fractions displayed in Fig. 5 included all cells not falling into the naïve gate (i.e. CD45RA<sup>+</sup>CCR7<sup>-</sup> and CD45RA<sup>-</sup>CCR7<sup>+</sup>). (D-E) The final readout of MMr<sup>+</sup> cells and naïve fractions is shown for healthy donor #H020W.
Fig. S8. IFN-γ secretion by ZnT8186-194-reactive CD8+ T cells. (A) T1D and healthy donors previously analyzed by ex-vivo MMr staining for whom sufficient PBMCs remained were further analyzed by IFN-γ ELISpot as described (7). Briefly, unfraccionated PBMCs (3x10^5/well) were plated in triplicate in anti-IFN-γ antibody-coated ELISpot PVDF plates in the presence of 10 μM ZnT8186-194 or Flu MP58-66 peptide or DMSO vehicle diluted in AIM-V medium supplemented with 0.5 U/ml IL-7. After 18 h, plates were revealed with biotin-conjugated anti-IFN-γ antibodies (U-CyTech), alkaline phosphatase-conjugated streptavidin and NBT-BCIP substrate, and counted on a BioSys Bioreader 5000 Pro-SF. Results are expressed as frequencies of epitope-reactive T cells out of total PBMCs after subtraction of background responses in the presence of DMSO alone (which were ≤10^3 in all cases). The dotted line represents the median cut-off for a positive response, which was set at 3 SDs above the average background for each individual, as previously determined by receiver-operator characteristics analysis (3). *p<0.03 by Mann-Whitney test. (B) The corresponding raw IFN-γ ELISpot counts. All values, including basal + nSD cutoffs, are expressed as spot-forming cells/10^6 PBMCs with baseline subtraction. Non-subtracted basal values (reactivities to DMSO) are shown in the last row of each column. Reactivities are ranked as low (between 3 and 4 SD, yellow), intermediate (between 4 and 5 SD, orange), and high (>5 SD, red). The percentage of T1D and healthy subjects positive for each epitope is indicated in the second and third column, respectively. PHA was used as a polyclonal positive control. +++++, off-scale ELISpot reading.
Fig. S9. Gene expression in ex-vivo single-sorted ZnT8186-194 MMr'CD8+ T cells. (A) Genes differentially expressed in individual ZnT8186-194 MMr'CD8+ T cells from T1D and healthy subjects. Single cells were sorted into empty PCR wells. cDNA synthesized with a Superscript VILO RT Kit (Invitrogen) was pre-amplified for 16 cycles with TATAA GrandMaster Mix and 61 primer pairs (Table S6) as follows: 1x [95°C 8 min], 16x [95°C 45 sec, 49°C 1 min (with 0.3°C increment/cycle), 72°C 1.5 min], 1x [72°C 7 min]. RT-PCR was carried out on a Fluidigm BioMark HD with the 96.96 Dynamic Array IFP, the GE 96x96 Fast PCR+ Melt protocol and SsoFast EvaGreen Supermix with Low ROX (Biorad), with 5 μM primers per assay. Data were analyzed using Fluidigm Real-Time PCR software followed by KNIME 2.5.2 and R 3.2.2 (www.r-project.org). Pre-processing via the linear model to correct for confounding effects was performed as described (43). The semi-continuous Hurdle model was subsequently applied to account for bimodal gene expression in single cells, allowing assessment of differential expression with respect to both the frequency of expression and the positive expression means via a likelihood ratio test. Violin plots display the density of expression (max.Ct-Ct) of genes that differ significantly between T1D and healthy donors (n=32 and n=25 ZnT8186-194 MMr'CD8+ T cells from 11 and 6 individuals, respectively). Blue shading illustrates the proportion of positive cells. p values were calculated with the Hurdle model. (B) TRB and TRA gene usage and the corresponding CDR3 aminoacid sequences of single-sorted cells from T1D (top, D-coded IDs) and healthy donors (bottom, H-coded IDs). Each line corresponds to an individual T cell. TRBV and TRAV genes shared with ZnT8186-194-reactive clones obtained from separate subjects are shown in bold; repeatedly used genes are shown in color. (C) Prevalence of the CDR3β aminoacid sequences obtained from single-sorted cells among HLA-A2+ T1D (n=5), aAb+ (n=5) and healthy subjects (n=10), as assessed by in-silico analysis of CDR3 repertoires obtained from the indicated CD8+ and CD4+ T-cell subsets. *p<0.04, **p=0.008 by Fisher's exact test. (D-E) Distribution of TRBV (D) and TRAV (E) gene usage among ZnT8186-194 MMr'CD8+ T cells. Color codes are matched to panel B.
Fig. S10. Extended combinatorial MMr panel for the analysis of multiple islet-reactive CD8+ T-cell populations, and reproducibility of ex-vivo MMr assays. (A) After magnetic depletion of CD8− cells, PBMCs were sequentially gated as depicted in Fig. S7 and the indicated MMr+ populations were visualized using the gating strategy previously detailed for combinatorial MMr staining (9) and the FlowJo v10 software. The staining panel also included Live/Dead Aqua, anti-CD3-APC-H7 (clone SK7, BD), anti-CD8-PE-Cy7 (clone SK1, eBioscience), anti-CD45RA-FITC (clone HI100, eBioscience) and anti-CCR7-BV421 (clone 150503, BD). The final readout obtained for T1D patient D322D after gating out events positive for <2 or >2 MMr fluorochromes is shown, with events corresponding to each epitope-reactive population depicted in different colors within each plot, and MMr+ events depicted in grey. (B) Reproducibility between the 3-MMr panel depicted in Fig. S7 and the extended 8-MMr panel presented here. Separate blood draws from 5 subjects (H1004N, green; H1315C, black; H1354C, red; H1355C, grey; H1356C, blue) were analyzed with both panels and the frequency counts were compared. The corresponding MMr+ and total CD8+ T-cell counts (x10^6) are indicated with the same color code below each distribution, together with the percentage of naïve (CD45RA+CCR7+) cells in each MMr+ fraction (for fractions ≥5 MMr+ cells).
Fig. S11. CD27, CD28 and CD95 expression on ZnT8_{186-194}-reactive CD8^+ T cells. (A) Representative CD27/CD28 staining of MMr^+CD8^+ T cells from subject H1372C. The first row depicts the fraction of MMr^+ cells for each of the indicated epitopes. The corresponding frequency out of the total 1.7x10^6 CD8^+ T cells acquired and the number of MMr^+ events counted are indicated. The second row shows the percent distribution of naive (CD45RA^+CCR7^+; grey), central memory (CM, CD45RA^+CCR7^+; blue), effector memory (EM, CD45RA^+CCR7^+; green) and terminally differentiated EM CD45RA^- cells (EMRA, CD45RA^-CCR7^-; red) within each MMr^+ fraction. The third row depicts CD27 (AlexaFluor700-labeled clone O323, eBioscience) and CD28 expression (BV421-labeled clone CD28.2, BioLegend) within each of these subsets, using the same color code, with total percent cells indicated in each quadrant. NA, not available (<5 MMr^+ events counted). (B) Representative CD95 staining (PE/Cl594-labeled clone DX2, BD) for MMr^+CD8^+ T cells from subject H004N. A total of 5.3x10^6 CD8^+ T cells was acquired, and data are represented as in panel A. Results are representative of 4 subjects tested.
Fig. S12. Representative MMr and CD45RA/CCR7 dot plots for HLA-A2⁺ and HLA-A2⁻ healthy donors depicted in Fig. 6F-G. (A) MMr (top row) and CD45RA/CCR7 staining (bottom row) for each of the indicated epitopes for HLA-A2⁺ case H015T. The corresponding frequency out of the total 0.9x10⁶ CD8⁺ T cells acquired and the number of MMr⁺ events counted are indicated in the top row. The second row shows the percent distribution of naïve (CD45RA⁺CCR7⁺; grey), central memory (CD45RA⁻CCR7⁺; blue), effector memory (CD45RA⁻CCR7⁺; green) and terminally differentiated EM CD45RA⁺ cells (CD45RA⁺CCR7⁺; red) within each MMr⁺ fraction, with percent cells indicated in each quadrant. NA, not available (<5 MMr⁺ events counted). (B) The same representation is shown for HLA-A2⁻ case H424C. A total of 1.3x10⁶ CD8⁺ T cells was acquired.
Fig. S13. Correlation between the frequency of MMr^CD8^+ T cells and the Ag-experienced fraction within the same MMr^CD8^+ population. (A) Correlation for ZnT8_186-194 MMr^CD8^+ T cells. (B) Correlation for IGRP_265-273 MMr^CD8^+ T cells. (C) Correlation for pooled MMr^CD8^+ T cells recognizing all islet epitopes analyzed (ZnT8_186-194, PPI_6-14, PPI_15-24, GAD_114-122, IA_2805-813, IGRP_265-273). (D) Correlation for Flu MP_58-66 MMr^CD8^+ T cells. Graphs were obtained by compiling data presented in Fig. 5 for T1D patients (adults, grey circles; children, crossed grey circles) and age/sex-matched healthy donors (adults, white circles; children, crossed white circles). Values obtained by Spearman correlation analysis are shown for each graph.
Table S1. Summary of ZnT8-reactive CD8+ T-cell clones. Clones in parentheses could not be stabilized in long-term cultures and underwent more limited characterization. For those clones obtained after ex vivo index sorting of ZnT8+ B cells, the surface phenotype of the source cell is indicated in the last column. NA, not applicable; ND, not determined; ODA, CAD antigens; IA-2A, IA-2A Abs; Zn18A, Zn18A Abs.
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Table S2. Characteristics of study subjects for in-silico TRB analyses. IAA, insulin aAbs; NA, not applicable.
Table S3. Characteristics of HLA-A*02 study subjects for ex-vivo MMr studies. Individual clinical profiles of TID pediatric subjects (top left; n=16), age/sex-matched healthy pediatric subjects (bottom left; n=17), TID adults (top right; n=23) and age/sex-matched healthy adults (bottom right; n=22) are shown along with their distribution within each group (median and range for numerical variables). ND, not determined; NA, not applicable.
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Table S4. Characteristics of HLA-A2' and HLA-A2' healthy donors for ex-vivo MMr studies. Predicted HLA Class I peptide binding affinities were calculated using NetMHC 4.0 (http://www.cbs.dtu.dk/services/NetMHC/). HLA-A2 (A*02:01) and predicted affinity values compatible with peptide binding to the indicated HLA allotypes are highlighted in red. NA, not available.
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Table S5. Characteristics of nPOD cases for in-situ ZnT8_{186-194} MMr staining. The clinical characteristics of each case are reported along with the counts ($\times 10^3$) of ZnT8_{186-194} and MelanA_{35-35} MMr$^+$ cells/mm$^2$ pancreas (Pa) and PLN sections area. Positive sections are marked in red. In several cases, the PLN CD8$^+$ T-cell repertoire was interrogated in-silico for the presence of immunodominant CDR3$\beta$ aminoacid sequences retrieved from 3 ZnT8_{186-194}-reactive CD8$^+$ T-cell clones (Fig. 4D). The results of this in-silico search are reported in the last column, with numbers indicating the frequency of the 3 selected sequences (D010R, E2, F1034O, 141B9 and H328C 8E8, respectively) per 10$^6$ TCRs, and positive counts indicated in red. Case #6287 (presenting a circumscribed neuroendocrine tumor in the pancreatic pan-
body region; pan-tail region analyzed here) was classified as a non-diabetic control, while non-diabetic case #6288 was classified as 'other' based on a history of non-alcoholic cirrhosis and histological findings of chronic pancreatitis. NA, not applicable or not available; mIAA, micro-insulin aAbs.
Table S6. Primers used for gene expression analysis of the individual ZnT8<sup>185-194</sup> MMr<sup>+</sup> CD8<sup>+</sup> T cells depicted in Fig. S9A.
Members of the ImMaDiab Study Group.
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Florence Travers, Michel Marre (Bichat Hospital, Paris);
Philippe Chanson, Claire Briet (Kremlin-Bicêtre Hospital, Paris);
Pierre-Jean Guillaume, Leila Ait-Bachir (La Raboisière Hospital, Paris);
Carole Collet, Frédéric Bezriaud, Gwenaelle Petit-Aubert (Versailles André Mignot Hospital, Le Chesnay);
Sophie Christin-Maitre, Bruno Fève, Camille Vatier (Saint Antoine Hospital, Paris);
Najiba Lahlou (Laboratory of Hormonology, Cochin Hospital, Paris);
Prissile Bakouboula, Caroline Elie, Hélène Morel, Jean-Marc Treluyer (Clinical Research Unit Paris Centre Descartes, Paris);
Marie-Claude Gagnerault, Claire Maillard, Anna Jones (INSERM U1016, Cochin Institute, Paris).