

A distinct immunogenic region of glutamic acid decarboxylase 65 is naturally processed and presented by human islet cells to cytotoxic CD8 T cells

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Introduction

Type 1 diabetes is a prototypical autoimmune disease involving the specific targeting and destruction of β cells within the islets of Langerhans. The autoimmune processes involved are becoming better defined [1], and there is strong evidence that CD8 T cells play a key role [2–7]. For example, CD8 T cells are the predominant leucocyte present in islet infiltrates close to diagnosis [8,9]. In addition, CD8 T cell clones recognizing preproinsulin peptides kill β cells *in vitro* and CD8 T cells with the same epitope specificity can be detected in infiltrated islets in patients studied close to diagnosis [3,4,8]. Finally, several studies link disease susceptibility to inheritance of human leucocyte antigen class I (HLAI) genes, with odds ratios second only to those for HLAII genes [10]. Notably, susceptibility is linked to *HLA-A*2402* and *HLA-B*3906*, and while multiple studies have

Summary

CD8 T cells specific for islet autoantigens are major effectors of β cell damage in type 1 diabetes, and measurement of their number and functional characteristics in blood represent potentially important disease biomarkers. CD8 T cell reactivity against glutamic acid decarboxylase 65 (GAD65) in *HLA-A*0201* subjects has been reported to focus on an immunogenic region 114–123 (VMNILLQYVV), with studies demonstrating both 114–123 and 114–122 epitopes being targeted. However, the fine specificity of this response is unclear and the key question as to which epitope(s) β cells naturally process and present and, therefore, the pathogenic potential of CD8 T cells with different specificities within this region has not been addressed. We generated human leucocyte antigen (HLA)-A*0201-restricted CD8 T cell clones recognizing either 114–122 alone or both 114–122 and 114–123. Both clone types show potent and comparable effector functions (cytokine and chemokine secretion) and killing of indicator target cells externally pulsed with cognate peptide. However, only clones recognizing 114–123 kill target cells transfected with *HLA-A*0201* and *GAD2* and *HLA-A*0201*⁺ human islet cells. We conclude that the endogenous pathway of antigen processing by *HLA-A*0201*-expressing cells generates GAD65_{114–123} as the predominant epitope in this region. These studies highlight the importance of understanding β cell epitope presentation in the design of immune monitoring for potentially pathogenic CD8 T cells.

Keywords: autoimmunity, CD8 T cell clones, GAD65, peptide-processing, type 1 diabetes

examined *HLA-A*0201* subjects, a prevalent allele among European and worldwide populations [11], it is not actually disease-linked [12].

Given this background, and the increasing number of β cell epitopes being reported as targets of autoreactive CD8 T cells [13,14], there is a strong rationale for focusing on these effector cells in the peripheral blood as potential biomarkers of immune-mediated damage. Indeed, recent proof-of-concept for this strategy in the setting of an immunotherapy trial has now been gained [15].

Several technological advances have been highly enabling for this biomarker strategy. These include peptide elution studies [3,4], the wide availability of peptide binding algorithms for common HLA I molecules [16] and the creation of mice transgenic for human *HLAI* genes [17], each of which facilitates epitope discovery. These have been allied with the development of highly sensitive assays to examine

antigen-specific CD8 T cell number (peptide–HLA multimers) [18] and functional responses (enzyme-linked immunoassays) [4]. However, as we have discussed previously [19,20], it is important to appreciate the imperfections in these approaches. Notably, peptides identified via algorithms may bind HLA I and stimulate CD8 T cell responses, but this does not provide evidence that they are generated via endogenous and/or cross-presentation pathways of antigen processing, or that they are displayed on the surface of β cells. In the absence of these features, the disease relevance of epitopes and the CD8 T cells targeting them remains unclear.

In the present study, we focused on CD8 T cell responses targeting glutamic acid decarboxylase (GAD65), a major autoantigen in type 1 diabetes. In a groundbreaking 1995 study, a potentially important, HLA-A*0201-restricted 10-mer peptide of glutamic acid decarboxylase (GAD65) (VMNILLQYVV, representing residues 114–123) was identified as a CD8 T cell target [21]. Short-term T cell lines from patients with type 1 diabetes generated against GAD65_{114–123} were capable of killing target cell lines (lymphoblastoid B lymphocytes) transfected with GAD2. Although this study provided the first evidence of CD8 T cell targeting directed against any β cell autoantigen, it fell short of demonstrating the pathogenic (i.e. β cell destructive) potential of these responses or their disease specificity. Subsequent studies have highlighted the potential disease association of CD8 T cell responses to both GAD65_{114–123} and a nested 9-mer peptide, GAD65_{114–122} [22–25]. The 9-mer peptide was selected for study because of its higher predicted HLA-A*0201 binding than the 10-mer, and because of difficulties encountered in refolding 10-mer-HLA-A*0201 for use in peptide–HLA multimers [22,23]. Given the findings of our recent study, namely that any given $\alpha\beta$ T cell receptor exhibits an explicit preference for a single HLA I-peptide length [26], we considered it important to clarify the nature of the cytotoxic CD8 T cell response to the peptides nested in this immunogenic region of GAD65. Specifically, in the present study, we sought to establish whether human β cells process endogenously and present both or either of these target peptides to cytotoxic CD8 T cells, and thereby provide support for the rationale for studying these reactivities *ex vivo*.

Materials and methods

Cell lines

C1R and K562 transfected with HLA-A*0201 (designated C1R-A2 and K562-A2) were maintained in RPMI-1640 (Gibco, Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Life Technologies, Carlsbad, CA, USA). GAD65-target cells were generated by cloning GAD2 (Accession number: BC126327; Genome

Cube, Source Bioscience, Nottingham, UK) into the retroviral plasmid MigR1-eGFP (gift from Nathan Sherer, Wisconsin University). MigR1-GAD65-eGFP, VSVG plasmid (vesicular stomatitis virus G protein) and cytosine-phosphate–guanosine (CpG) plasmid (encoding gag and pol proteins) were transfected into 293T cells using polyethylenimine (PEI; Sigma-Aldrich, Gillingham, Dorset, UK) to produce non-replicative retrovirus particles (NRRPs). The K562-A2 target cell line was spinoculated at 300 g for 2-h with GAD2-containing NRRPs, and after 48 h GFP-positive clones were sorted and analysed by Western blot for protein expression. Previously generated preproinsulin_{15–24}-specific cloned CD8 T cells (3F2) were used as a positive control in functional assays [4].

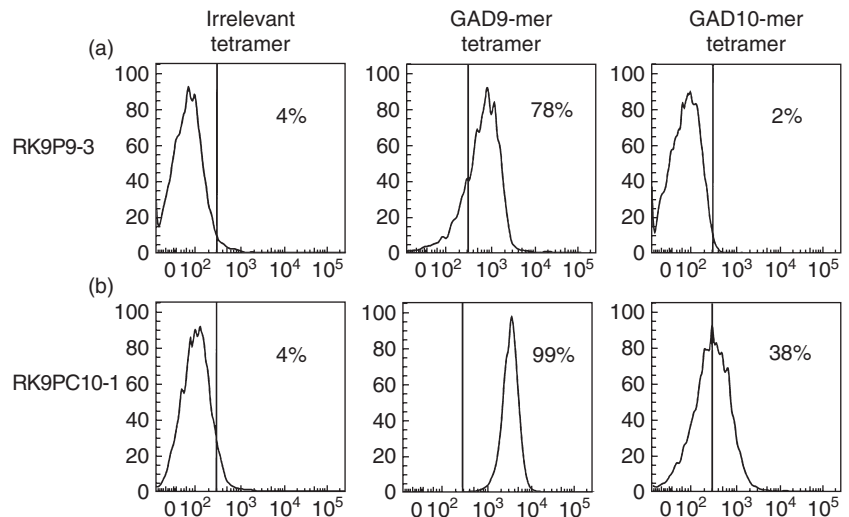
GAD65-specific CD8 T cell clone generation and isolation

GAD65-specific CD8 T cell clones were generated using our previously described method and patient collections [4,27]. Cell lines were maintained in X-Vivo 15/5% AB serum/interleukin (IL)-7 (10 ng/ml), IL-15 (0.1 ng/ml; Peprotech, Rocky Hill, NJ, USA) and 2.5% Cellkine (ZeptoMetrix, Buffalo, NY, USA). Single-cell clones were isolated by tetramer-guided sorting into wells containing irradiated feeder peripheral blood mononuclear cells (PBMCs) and phytohaemagglutinin (PHA)-M (500 μ g/ml; Sigma Aldrich).

Tetramer staining and functional responses of cloned CD8 T cells

Tetramer staining of isolated CD8 T cell clones and measurement of specific human islet-specific cytotoxicity were measured as described previously [28]. Peptide pulsing (10 μ g/ml) of target cells was performed for 1 h at 37°C followed by washing off peptide excess. Cytokines and chemokines secreted during 18-h cultures of 2×10^4 CD8 T cell clones in 96-well plates containing 2×10^4 target cells were measured by Luminex (Milliplex Mag Kit; Millipore, Darmstadt, Germany). Intracellular cytokine analysis was performed on 18-h cultures of 2×10^5 CD8 T cell clones and 2×10^5 target cells, using Cytoperm/Cytofix (BD Biosciences, San Jose, CA, USA). ViViD was used to distinguish live populations, co-stained with anti-CD3 and anti-CD8 antibodies. Under identical conditions, degranulation (as a surrogate for cytotoxicity) was examined over 4 h using fluorochrome-conjugated monoclonal anti-CD107a antibody. Non-peptide pulsed target cells, or cells pulsed with an irrelevant peptide, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP_{265–273}), were used as negative controls and ionomycin/phorbol myristate acetate (PMA) as positive controls (1 μ g/ml and 50 ng/ml, respectively).

Fig. 1. CD8 T cell clones display differential staining with tetramer loaded with either glutamic acid decarboxylase 9 (GAD9)-mer or GAD10-mer peptide. CD3⁺CD8⁺ clone cells raised against the immunogenic 114–123 region of GAD65 were stained with an irrelevant human leucocyte antigen (HLA)-A*0201 tetramer (loaded with preproinsulin 15–24), GAD9-mer tetramer (GAD_{114–122}) or GAD10-mer tetramer (GAD_{114–123}). (a) Clone RK9P9-3 stains with the GAD9-mer tetramer only. (b) Clone RK9C10-1 stains with both GAD9-mer and GAD10-mer tetramers. Histograms are displayed as % of maximum for 10 000 live events, the line indicative of positive staining represents the fluorescence-minus-one control.



Measuring the thermal stability of HLA-A2–GAD peptide complexes

Thermal stability of HLA-A2 complexes was assessed by circular dichroism (CD) spectroscopy monitoring the change of ellipticities Θ at 218 nm where the CD spectra exhibit a minimum. Data were collected on an Aviv Model 215 spectropolarimeter (Aviv Biomedical Inc., Lakewood, NJ, USA) equipped with a Peltier thermostatted cell holder using a 0.1-cm quartz cell. Proteins were dissolved in 75 mM NaCl, 20 mM PO₄⁻, pH 7.5, at concentrations of 5–7 μ M as determined by absorbance at 280 nm using extinction coefficients calculated from the amino acid composition [29]. Melting curves were recorded in 0.5°C intervals from 4°C up to a maximum temperature, when protein aggregation was observed with settings resulting in an average heating rate of ca. 30°C/h. Values were corrected to a calibration curve recorded with the temperature measured in the cell. After subtraction of a buffer baseline, Θ -values were converted to molar ellipticities $[\Theta]MRW = \Theta \times MRW / (c \times d)$, where MRW denotes the mean residue weight $Mr/(n-1)$ with n indicating the number of amino acid residues, c the concentration in mg/ml and d the cell path length in mm.

Melting curves were analysed assuming a two-state trimer-to-monomer transition from the native (N) to unfolded (U) conformation $N_3 \leftrightarrow 3U$ with an equilibrium constant $K = [U]^3/[N_3] = F/[3c_2(1-F)^3]$, where F and c are the degree of folding and protein concentration, respectively. Data were fitted as described [30] using the non-linear least-squares routine of Origin version 7.5 (OriginLab, Northampton, MA, USA). Fitted parameters were the melting temperature T_m , at which 50% of proteins are in the folded and unfolded state, van't Hoff's enthalpy ΔH_{vH} at the transition mid-point and the slope and Θ -intercept of the native baseline assumed as a linear function of the temperature. As all protein complexes aggregated

to various degrees upon unfolding, the ellipticity of the unfolded state was set as a constant of $-4500 \text{ deg cm}^2 \text{ dmol}^{-1}$ [31]. For all peptides, the coefficient of determination for fitted curves versus measurements was $r^2 > 0.99$.

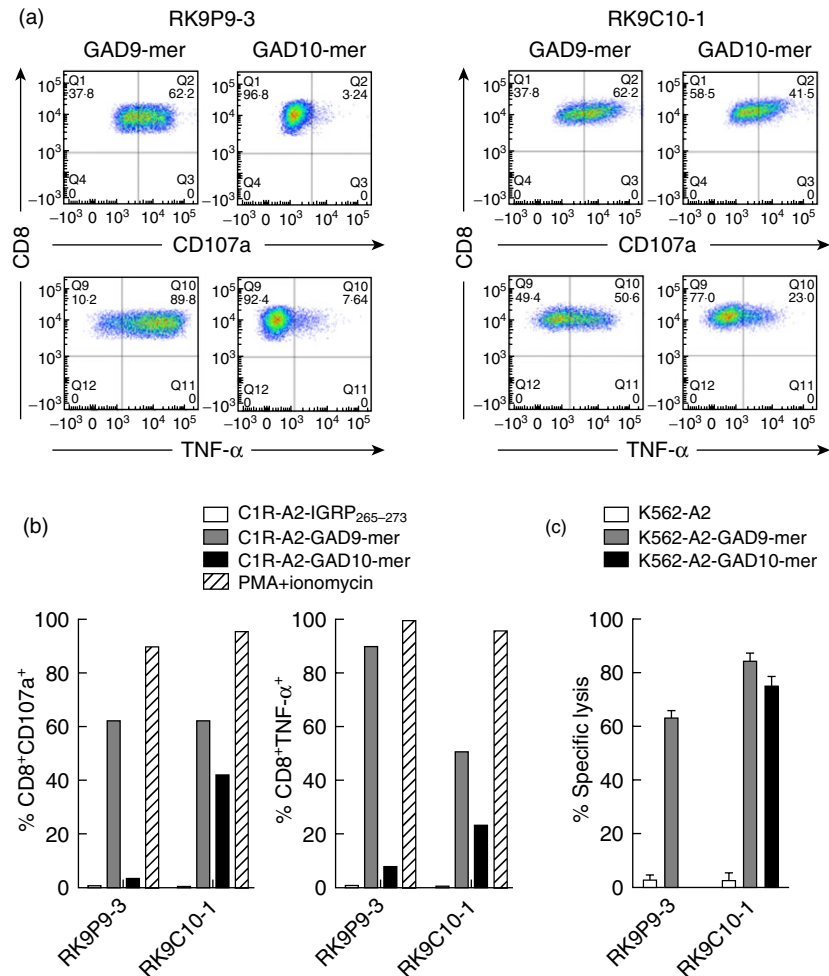
Results

Generation of GAD65-specific CD8 T cell clones

We successfully isolated and expanded a series of GAD65-specific CD8 T cell clones (Supporting information, Fig. S1). Two of these are used here to exemplify the specificities we observed and to address processing and presentation of GAD65 by human HLA-A*0201 β cells.

As shown in Fig. 1, we used tetramers to identify clone cells with specificity for 9-mer peptide (two independent clones generated from a single donor: data shown for RK9P9-3; HLA-A*0201–GAD65_{114–122} tetramer-positive); and clones with specificity that enabled binding to both 9-mer and 10-mer peptide (two independent clones generated from a further donor; data shown for RK9C10-1, HLA-A*0201–GAD65_{114–122} and HLA-A*0201–GAD65_{114–123} tetramer-positive). The recognition pattern of these clones for these peptide–HLA-A*0201 complexes was confirmed by measuring tumour necrosis factor (TNF)- α production and degranulation in 18- and 4-h co-cultures, respectively, with peptide-pulsed C1R-A2 target cells. RK9P9-3 produces TNF- α and degranulates when co-cultured with target cells pulsed with 9-mer peptide GAD65_{114–122} to levels comparable to those achieved with the positive control. In contrast, responses to 10-mer peptide GAD65_{114–123} are similar to background. By comparison, RK9C10-1 produces TNF- α and degranulates in the presence of both 9-mer (GAD65_{114–122}) and 10-mer (GAD65_{114–123}) pulsed target cells (Fig. 2a,b). Final confirmation that effector function of these clones was restricted in this specific fashion was derived by measuring killing of peptide-pulsed K562-A2 target cells

Fig. 2. Differential recognition of peptide-pulsed human leucocyte antigen (HLA)-A*0201⁺ cell lines by CD8 T cell clones. Representative intracellular staining for tumour necrosis factor (TNF)- α and surface staining of CD107a (degranulation marker) of clone cells after 18 h co-culture with peptide-pulsed target cell lines. (a) RK9P9-3 shows a high percentage of events positive for TNF- α production and degranulation following co-culture with glutamic acid decarboxylase 9 (GAD9)-mer-pulsed target cells, but minimal positivity using GAD10-mer-pulsed target cells. In contrast, RK9C10-1 displays a cross-reactive profile to both GAD9-mer- and GAD10-mer peptide-pulsed cell lines. (b) These responses contrasted against a negative control peptide stimulation (IGRP₂₆₅₋₂₇₃) and the positive control of phorbol myristate acetate/ionomycin stimulation. (c) Specific lysis of GAD9-mer-pulsed K562-A2 target cells by both RK9P9-3 and RK9C10-1 and killing of GAD10-mer-pulsed target cells by RK9C10-1 only; 10 000 live CD3⁺CD8⁺ events were recorded for flow cytometry analyses; cytotoxicity and functional assays are representative data performed at an effector : target ratio of 25:1 for 4 h. Data shown are representative single experiments.



(Fig. 2c). Thus we derived and characterized two CD8 T cell clones specific for the immunogenic region 114–123 region of GAD65. These clones offer distinct specificities that enable their use in examining natural processing and presentation of GAD65. A difference in the level of response by clone RK9C10-1 was observed in assays measuring either cytokine production or killing, in conditions using either GAD9-mer- or GAD10-mer-expressing target cells (Fig. 2). One explanation for the reduced response to 10-mer-expressing targets, compared to 9-mer-pulsed cells, would be reduced stability of 10-mer-bound HLA-A2 complexes, which could contribute to a reduced activation signal. To determine empirically *in-silico* prediction algorithms reporting weaker binding of HLA-A*0201 by GAD10-mer [32,33], circular dichroism was performed on HLA-A2–GAD10-mer and HLA-A2–GAD9-mer complexes to assess their respective thermostability (Supporting information, Fig. S2). The results show that GAD9-mer peptide forms a more stable complex with HLA-A2 than GAD10-mer, as it requires more energy to become denatured. The observed difference in GAD–peptide–HLA stability therefore provides an explanation for the different quality of response observed.

GAD9-mer restricted CD8 T cells do not recognize endogenously processed and presented GAD65

K562 cells stably expressing HLA-A*0201 were transduced to express GAD2 (K562-A2–GAD65) and intracellular GAD65 production confirmed by Western blot (not shown). To determine whether these cells processed and presented the 9-mer GAD65_{114–122} complexed with HLA-A*0201, they were co-cultured with RK9P9-3 (GAD65_{114–122}-specific) and the chemokine response examined in culture supernatants. Clone RK9P9-3 responds to peptide-pulsed K562-A2 cells, but not K562-A2–GAD65 (Fig. 3a). In contrast, RK9C10-1 (9-mer/10-mer; GAD65_{114–122/123}-specific) responds comparably to K562-A2–GAD65 target cells and to K562-A2 target cells pulsed with the 9-mer (GAD65_{114–122}) peptide (Fig. 3b) or 10-mer (data not shown and see Fig. 2b). These data show that clone RK9P9-3 does not engage cognate ligand when cultured with cell lines expressing GAD65 and HLA-A*0201, indicating that the 9-mer peptide GAD65_{114–122} is not naturally processed and presented by K562 cells. In contrast, clone RK9C10-1 engages cognate ligand when cultured with the same cell lines, indicating that endogenously expressed GAD65 is naturally

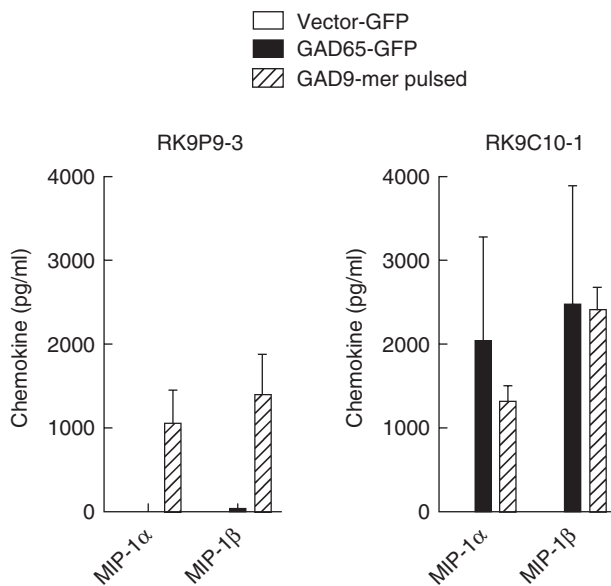


Fig. 3. CD8 T cell clone recognition of endogenously presented glutamic acid decarboxylase 65 (GAD65) by transduced target cells. CD8 T cell clones were co-cultured with *GAD2* transduced human leucocyte antigen (HLA)-A2 expressing K562 cells (K562-A2-GAD65) for 18 h, and supernatants assessed for macrophage inflammatory protein (MIP)-1 α and MIP-1 β production. Both RK9P9-3 and RK9C10-1 respond similarly to stimulation with GAD9-mer peptide-pulsed cells. Similarly, neither respond to the empty vector control cell line. RK9C10-1 produced high levels of chemokines when cultured with K562-A2-GAD65 target cells, but RK9P9-3 did not respond. These data indicate that only RK9C10-1 recognizes GAD65 after endogenous processing, and that the naturally processed and presented epitope is GAD65_{114–123}. Cells were incubated at an effector : target ratio of 1:1. Bars show triplicate means and error bars standard errors of the mean of representative experiments.

processed by K562 cells, but that only the 10-mer GAD65_{114–123} is presented.

HLA-A0201⁺ human islet cell presentation of GAD65

Once characterized for their specificity, these clones provided robust reagents for examining processing and presentation of this immunogenic region of GAD65 by human islet cells. In keeping with our findings in assays performed using K562-A2-GAD65 cells (endogenously expressing *GAD2*), no reactivity was observed when RK9P9-3 clone was co-cultured with human islet cells (Fig. 4a). In contrast, RK9C10-1 clone cells killed disaggregated human HLA-A*0201 + islet cell preparations (Fig. 4b) and secreted chemokines when co-cultured with these targets over 18 h (Fig. 4a).

Discussion

We describe the generation of CD8 T cell clones specific for an immunogenic region of GAD65, which we have used to

examine the natural processing and presentation of endogenously expressed GAD65 in human islet cells. We provide the first evidence that human islet cells can be targeted and destroyed via recognition of GAD65, and show that for the immunogenic region we studied, it is the 10-mer peptide GAD65_{114–123} that is naturally processed and presented by HLA-A*0201.

Our work was prompted by numerous studies documenting type 1 diabetes-related CD8 T cell responses directed against the region of GAD65 encompassing residues 114–123. Attention to this sequence was drawn originally by Sinigaglia's group, who showed that CD8 T cells with cytotoxic potential could be generated against GAD65_{114–123}, although their work fell short of demonstrating that human islet cells are susceptible to killing through this pathway [21]. Our report is the first to show islet cell killing via targeting of the GAD65 sequence 114–123. Subsequent studies have also provided strong evidence that a focused cytotoxic response against GAD65_{114–123} may be important in disease pathogenesis, as CD8 T cells with this specificity have been identified in insulinitic lesions in type 1 diabetes patients studied close to diagnosis using *in-situ* tetramer staining [8]. Taken together with our findings, these data indicate that tracking circulating CD8 T cells specific for GAD65_{114–123} in HLA-A*0201 individuals with, or at risk of, type 1 diabetes has a robust experimental basis as a biomarker strategy.

In contrast, our study of HLA-A*0201-restricted CD8 T cells with specificity for GAD65_{114–122} presents a more nuanced set of questions. First, we show clearly that this 9-mer peptide is not displayed by target cells transduced to express GAD65, or by human islet cells that express this protein constitutively. This is of interest, as algorithms (<http://www.syfpeithi.de/> and <http://www.cbs.dtu.dk/services/HLArestrictor/> [32,33]) predict that this shorter peptide binds with higher affinity to HLA-A*0201 than the 10-mer GAD65_{114–123}, the naturally presented species. In our study, this prediction was corroborated by studies using circular dichroism to examine the thermal stability of the relevant peptide–HLA complexes. The reduced stability of HLA-A2–GAD10-mer compared to GAD9-mer complexes most probably explains the reduced response of RK9C10-1 to GAD10-mer expressing target cells. However, the affinity of RK9C10-1 T cell receptor (TCR) for stable peptide–HLA complexes may also be a factor in the observed differential response. It should be noted that the relative instability of HLA–GAD10-mer complexes reported here could also account for the difficulty in tetramer production, and thus the original rationale for investigating GAD-specific CD8 T cell responses with more stable GAD9-mer-containing tetramers [22,23]. As we have stated previously [19,20], this argues for caution in the use of algorithms to predict epitopes, as they promote a focus on high-affinity peptides that may not be generated by the target cell.

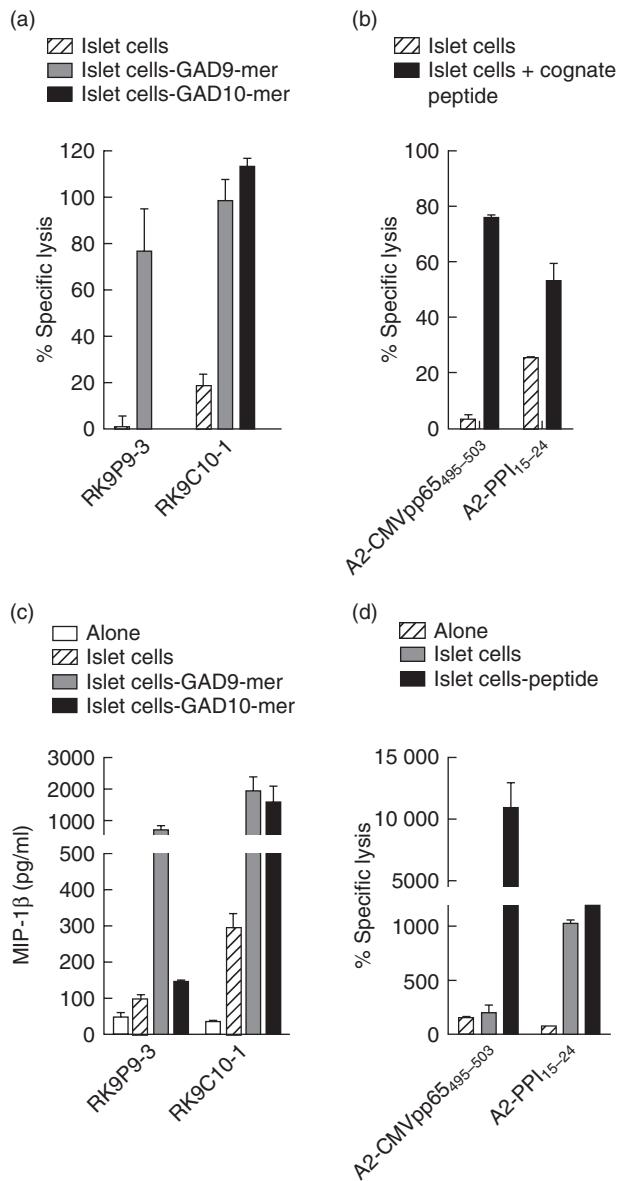


Fig. 4. CD8 T cell clone recognizing glutamic acid decarboxylase 65 (GAD65)₁₁₄₋₁₂₃ recognizes and kills human islet cells. (a) Cytotoxicity assays were performed using RK9P9-3 and RK9C10-1 clone cells and human leucocyte antigen (HLA)-A*0201⁺ human islet cells as targets. As shown in (a) RK9C10-1 robustly kills islet cells [comparable to the positive control (b), namely killing of islet cells from the same preparation by the preproinsulin (PPI)-specific CD8 T cell clone 3F2 specific for PPI₁₅₋₂₄], whereas RK9P9-3 displays background levels of specific lysis in the absence of cognate peptide. (b) Negative control, cytomegalovirus (CMV)-specific clone A2-CMVpp65₄₉₅₋₅₀₃, which does not kill islet cells in the absence of cognate peptide. (c) Analysis of supernatants of 18-h co-cultures of clone cells and islet cells for secretion of macrophage inflammatory protein (MIP)-1 β . RK9C10-1 showed robust production of MIP-1 β in response to islet cells, whereas RK9P9-3 production of MIP-1 β is similar to background. (d) Negative and positive control responses to the same islet cell preparations. Cytotoxicity assays were performed at an effector : target ratio of 25:1. Bars show means of triplicates and error bars the standard errors of the mean (data are representative of $n = 2$ experiments using the same islet donor).

tory process. As a result, 9-mer-specific CD8 T cells could indeed be induced, disease-related and represent putative biomarkers, in this case of an inflammatory process that may not have islet cell killing as its end-point ('frustrated autoimmunity').

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Disclosure

There are no potential conflicts of interest relevant to this article to report.

Author contributions

R. R. K., D. K., M. E. and A. S. designed and performed the experiments and with M. P. analysed data and wrote the manuscript. R. R. K., A. S. and M. P. conceived ideas and

oversaw research. G. D and A. K. S provided cellular material and input on experimental design. D. K. C. and K. B. performed CD experiments and analysis, and wrote the respective report. M. Z. and G. C. H. performed human islet experiments.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Tetramer staining of glutamic acid decarboxylase 65 (GAD65) peptide-specific clones derived from two donors. CD3⁺CD8⁺ clone cells raised against the immunogenic 114–123 region of GAD65 were stained with an irrelevant human leucocyte antigen (HLA)-A*0201 tetramer (loaded with preproinsulin 15–24), GAD9-mer tetramer (GAD114–122) or GAD10-mer tetramer (GAD114–123). Clones RK9P9-3 and RK9C10-1 were used as tools for further studies in this paper to decipher and ascribe relevant importance to putative GAD65 epitopes, due to their differential recognition of GAD peptides from the immunogenic 114–123 region.

Fig. S2. Glutamic acid decarboxylase 65 (GAD65) 10-mer binding to human leucocyte antigen (HLA)-A*0201 is less stable than the 9-mer. Left panel. Circular dichroism thermal denaturation curves recorded at 218 nm are shown for selected samples as indicated. Dots represent measured values fitted assuming a two-state trimer-to-monomer transition, as described in Materials and methods. Open symbols and dashed line for stabilization of GAD65 9-mer and closed circles/solid line for GAD65 10-mer. Right panel: bar graphs of the thermal stability with respect to melting temperature (upper) and van't Hoff's enthalpy of unfolding (lower panel). Error bars show standard deviation of the respective parameter based on fitting each set of measured data.