



# CTLs are targeted to kill $\beta$ cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope

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**The final pathway of  $\beta$  cell destruction leading to insulin deficiency, hyperglycemia, and clinical type 1 diabetes is unknown. Here we show that circulating CTLs can kill  $\beta$  cells via recognition of a glucose-regulated epitope. First, we identified 2 naturally processed epitopes from the human preproinsulin signal peptide by elution from HLA-A2 (specifically, the protein encoded by the *A\*0201* allele) molecules. Processing of these was unconventional, requiring neither the proteasome nor transporter associated with processing (TAP). However, both epitopes were major targets for circulating effector CD8<sup>+</sup> T cells from HLA-A2<sup>+</sup> patients with type 1 diabetes. Moreover, cloned preproinsulin signal peptide-specific CD8<sup>+</sup> T cells killed human  $\beta$  cells in vitro. Critically, at high glucose concentration,  $\beta$  cell presentation of preproinsulin signal epitope increased, as did CTL killing. This study provides direct evidence that autoreactive CTLs are present in the circulation of patients with type 1 diabetes and that they can kill human  $\beta$  cells. These results also identify a mechanism of self-antigen presentation that is under pathophysiological regulation and could expose insulin-producing  $\beta$  cells to increasing cytotoxicity at the later stages of the development of clinical diabetes. Our findings suggest that autoreactive CTLs are important targets for immune-based interventions in type 1 diabetes and argue for early, aggressive insulin therapy to preserve remaining  $\beta$  cells.**

## Introduction

The final molecular and cellular pathways leading to  $\beta$  cell destruction in the human autoimmune disease type 1 diabetes are not known. Any resolution of this question must address the mechanism through which insulin-producing  $\beta$  cells, but not adjacent, non- $\beta$  endocrine cells ( $\alpha$ ,  $\delta$ , and PP cells secreting the hormones glucagon, somatostatin, and pancreatic polypeptide), are singled out for killing. Two distinct hypotheses have emerged. The first proposes that  $\beta$  cells die as a result of their selective susceptibility to inflammatory mediators, such as cytokines, nitric oxide, and oxygen free radicals (1), released by islet-infiltrating immune cells. The second states that CD8<sup>+</sup> CTLs, recognizing  $\beta$  cell-specific peptides presented by HLA class I molecules, have the pivotal role in selective  $\beta$  cell death (2). In support of a CTL mechanism, postmortem studies of patients performed close to diabetes onset show numerical dominance of CD8<sup>+</sup> T cells in the characteristic islet mononuclear cell infiltrate (3–5). Genetic susceptibility to disease is linked to inheritance of selected class I HLA molecules, including HLA-A2 (*A\*0201*) (6). In animal models, efficient adoptive transfer of disease typically requires CD8<sup>+</sup> T cells (2), and dia-

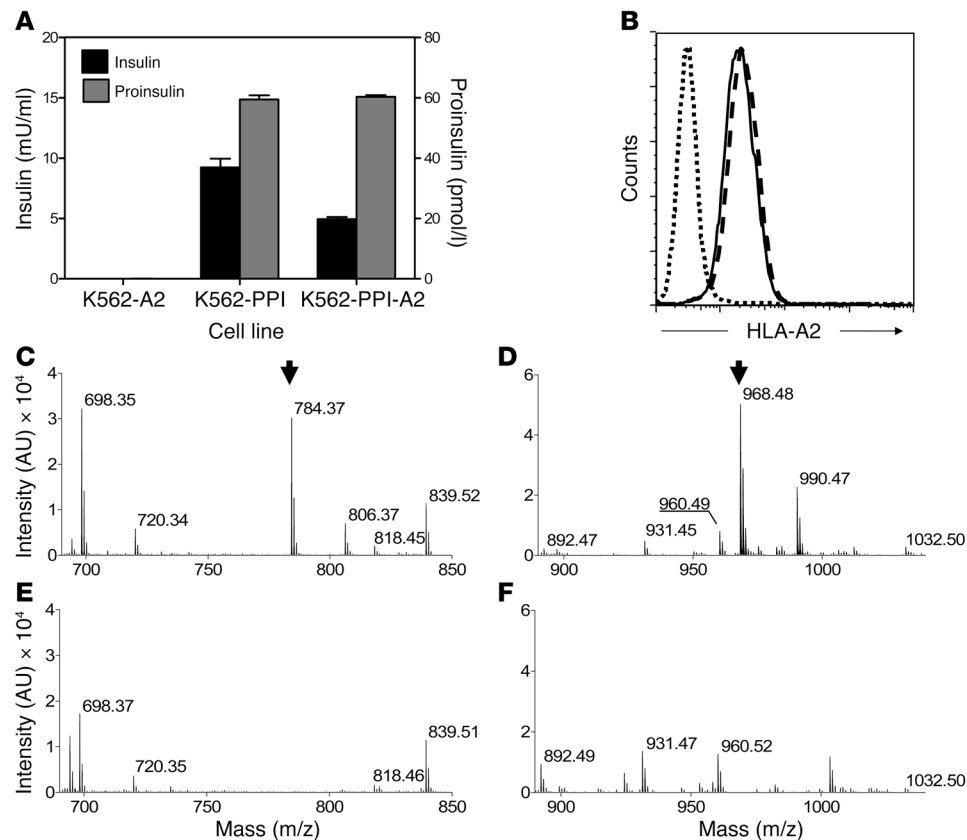
betes is prevented by genetic modifications that abrogate MHC class I molecule expression (7). Moreover, transgenic introduction of *HLA-A2\*0201* into diabetes-prone NOD mice markedly accelerates disease development (8).

Despite these pieces of circumstantial evidence, however, to date there are no published reports demonstrating CTL killing of human  $\beta$  cells. This gap in our knowledge is a result of many constraints on such studies. Most importantly, islet-infiltrating T cells from patients with diabetes are very rarely available for expansion and cloning to examine their CTL potential, while the search for such cells trafficking in the peripheral blood requires prior knowledge of the autoantigens targeted and the epitopes and HLA-presenting molecules involved. To address these requirements, we chose to create surrogate  $\beta$  cells expressing a single autoantigen and HLA class I molecule and investigate their naturally displayed peptide repertoire. This uncovered the signal peptide (SP) as a source of preproinsulin (PPI) epitopes that constitute major targets of CD8<sup>+</sup> T cell responses in HLA-A2<sup>+</sup> patients with type 1 diabetes. We show that expanded clones of PPI SP-specific CD8<sup>+</sup> T cells kill human HLA-A2-expressing  $\beta$  cells in vitro, especially when  $\beta$  cells are exposed to high glucose concentrations. It has long been speculated that  $\beta$  cells, stressed by the need to control blood glucose levels as diabetes develops, could become enhanced targets for the immune system. Our study provides evidence of a mechanism through which this can occur.

**Nonstandard abbreviations used:** MS, mass spectrometry; PPI, preproinsulin; SP, signal peptide; TAP, transporter associated with processing; TFA, trifluoroacetic acid.

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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**Figure 1**

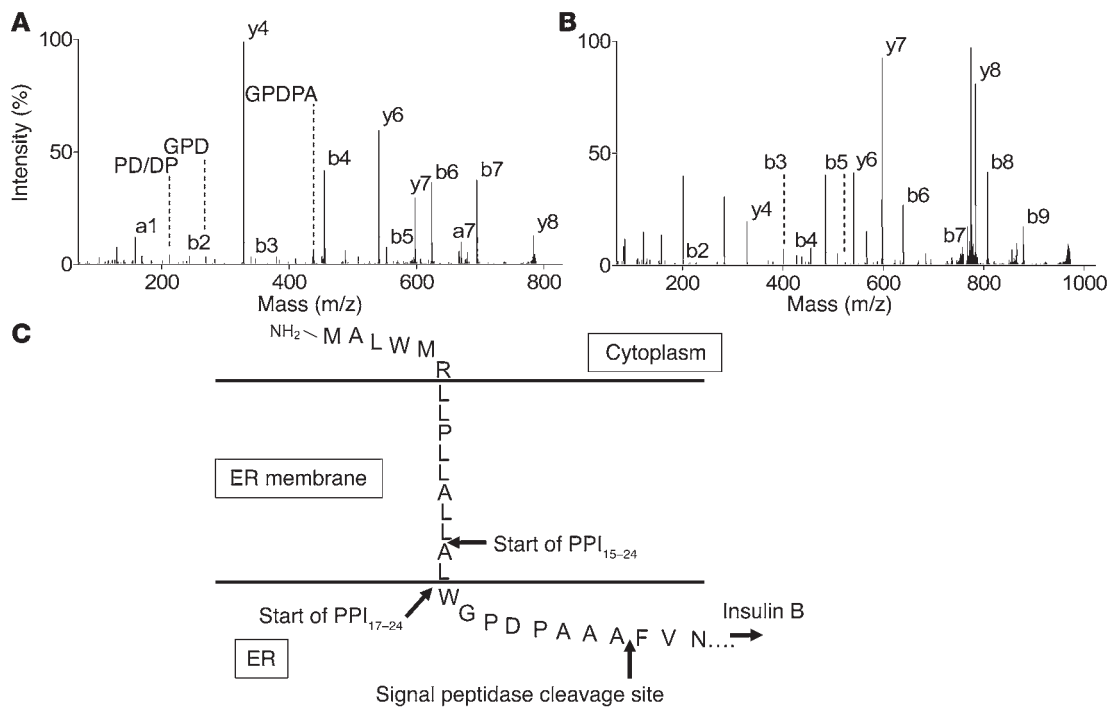
Generation of surrogate  $\beta$  cell lines and examination of their naturally processed and presented peptide repertoire. The chronic myelogenous leukemia cell line K562 was variously transfected with the genes for PPI and *HLA-A\*0201*. (A) This yielded cell lines (denoted K562-PPI and K562-PPI-A2) that secrete proinsulin (gray bars) and immunoreactive insulin species (black bars) into cell culture supernatants. No proinsulin or immunoreactive insulin is secreted by single-transfected K562-A2 cells. Bars represent mean levels present in cell supernatants and error bars the SEM. (B) Surface HLA-A2 expression was examined by flow cytometry using the allele-specific mAb BB7.2, showing comparable HLA-A2 levels on the K562-A2 (solid line) and K562-PPI-A2 (dashed line) cells compared with absence of staining on K562-PPI cells (dotted line). Iso-type control staining was similar to K562-PPI staining on all cell lines, and similar results were obtained with the pan-HLA-A,B,C-staining mAb W6/32. K562-PPI-A2 and K562-A2 cell lines were grown in large cultures and the natural peptide repertoire extracted and resolved by RP-HPLC, and fractions were compared by MS to identify masses unique to PPI-expressing cells. (C and D) MS analysis of HPLC fractions 55 and 65, respectively, from K562-PPI-A2 cells. Arrows indicate masses unique to these cells (784.37 and 968.48 m/z, respectively) that are not found in the equivalent (or adjacent) fractions from K562-A2 cells (E and F) or K562-PPI cells (data not shown).

## Results

**Identification of PPI epitopes.** We elected to study PPI as a target autoantigen in type 1 diabetes because of its  $\beta$  cell specificity; the fact that we and others have shown its importance as a target of CD4<sup>+</sup> T cell responses in clinical diabetes in humans (9–11); and in view of its prominent involvement in murine diabetes models (12). Since access to sufficient numbers of human  $\beta$  cells to study the peptide repertoire is not feasible, we elected to generate a “surrogate  $\beta$  cell.” The HLA-negative chronic myelogenous leukemia cell line K562 was selected as an appropriate carrier cell and transfected with PPI and *HLA-A\*0201* genes to derive a surrogate human  $\beta$  cell (termed K562-PPI-A2) that secretes proinsulin and expresses HLA-A2 (Figure 1, A and B). Peptides were acid eluted from the surface HLA-A2 molecules, and using a subtractive approach, we identified peptide species unique to K562-PPI-A2 cells and not present in either the control K562-PPI or K562-A2 single gene-transfected cell lines (Figure 1, C–F). Two prominent peptides were identified by mass spectrometry

(MS) and sequenced by MS/MS and collision-induced dissociation, matching to PPI<sub>17–24</sub> (WGPDPAAA) and PPI<sub>15–24</sub> (ALWGPDPAAA) (Figure 2, A and B).

**Disease relevance of SP epitopes of PPI.** The finding that the major peptide species presented from PPI-expressing cells are located in the SP (Figure 2C) was unexpected and led us to examine their disease relevance. Circulating IFN- $\gamma$ -producing CD8<sup>+</sup> T cells specific for these epitopes were detected by ELISPOT assay (9) in approximately 50% of patients with new-onset type 1 diabetes who possess the *HLA-A\*0201* genotype (Figure 3, Table 1, and Supplemental Table 1; supplemental material available online with this article; doi:10.1177/JCI35449DS1). CD8 responsiveness was confirmed by disappearance of ELISPOT reactivity after immunomagnetic depletion of CD8<sup>+</sup> T cells, as described previously (9). In contrast, this reactivity was rare in HLA-A2-negative type 1 diabetes patients (2 of 12 patients [17%] positive to either peptide;  $P < 0.05$  versus HLA-A2<sup>+</sup> patients). Responses to PPI<sub>15–24</sub>, either or both epitopes were significantly more frequent in HLA-A2<sup>+</sup> patients than in



**Figure 2**

Mass spectrometry analysis of unique eluted masses. MS/MS analysis of the unique masses using collision-induced dissociation (CID) reveals their identity. Plots show fragmentation patterns of the 784.37 m/z (A) and 968.48 m/z (B) species under CID in atmospheric gas, revealing a series of ions (y, b, and a) and fragments (PD/DP, GPD, and GPDPA) that identify the parent ions as WGPDPAAA (PPI<sub>17-24</sub>, predicted monoisotopic mass, 784.3624 Da) and ALWGPDPAAA (PPI<sub>15-24</sub>, predicted monoisotopic mass, 968.4836 Da), respectively. These sequences map to the SP of PPI. These results, from the starting cellular material onward, were replicated in a further 2 independent experiments. (C) Representation of the SP region of PPI and beginning of the B chain of insulin. Both eluted peptides terminate at residue 24, the signal peptidase cleavage site. The transmembrane region of SP is shown, as predicted from SignalP-HMM (51) and Kyte-Doolittle (52) hydrophobicity plots, and indicates that residue 17 is immediately after the transmembrane segment, while the NH<sub>2</sub> terminus of PPI<sub>15-24</sub> commences in the intramembrane segment.

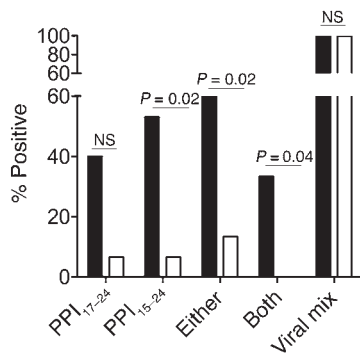
HLA-matched nondiabetic control subjects (Figure 3). The presence of circulating effector CD8<sup>+</sup> T cells recognizing these epitopes is therefore specific for disease and HLA-A2. Indeed, we were able to conclude that SP epitopes constitute major targets of the CD8<sup>+</sup> T cell response against PPI in type 1 diabetes, since the frequencies of responses we detected to these epitopes were higher than those to a range of other putative HLA-A2-restricted epitopes from insulin/proinsulin that are currently being investigated in parallel studies in our laboratory (insulin B10–18 [refs. 13, 14], 27% of patients; B18–27 [ref. 15], 27%; C22–30 [ref. 13], 14%; C27–35 [ref. 13], 23%; proinsulin C31–A5 [ref. 13], 9%; and insulin A1–10 [ref. 13], 18%) (data not shown).

*Phenotype and target cell killing of PPI<sub>15-24</sub>-reactive CD8<sup>+</sup> T cells.* To examine the functional properties of PPI SP-reactive CD8<sup>+</sup> T cells, we established short-term CD8<sup>+</sup> T cell lines from HLA-A2<sup>+</sup> patients with type 1 diabetes who showed appropriate ELISPOT reactivity, focusing on the PPI<sub>15-24</sub> epitope because of its relative immunodominance (Figure 3). From these lines, cells staining with PPI<sub>15-24</sub>-loaded HLA-A2 tetramers (PPI<sub>15-24</sub>-Tmr) were sorted by flow cytometry and expanded, yielding 5 PPI<sub>15-24</sub>-Tmr<sup>+</sup>CD8<sup>+</sup> T cell clones from a single patient (Figure 4, A and B). Three of these clones (1E6, 3F2, and 1C8) have been characterized in detail at a functional level (see below), and each is known to express TCR β variable chain gene *TRBV12-4* and 2 productively rearranged TCR α chain genes, *TRAV12-3* and *TRAV13-2*. Interestingly, a clone with the same *TRBV*

and *TRAV* expression, and *TRBV* CDR3 sequence identical to 1E6, was isolated from fresh blood obtained from the same patient 1.5 years after the first sample, in a “honeymoon” phase of the disease, during which insulin treatment was temporarily not required. Thus, while it is difficult to be certain whether the clones we originally obtained are independent or duplicate cloning events, our data show that their ancestral clone is long-lived and likely to be dominant in this patient. Future studies will be required to address whether similar clones are found in other patients and what additional specificities are responsible for their ELISPOT reactivity.

Clones showed HLA-A2-restricted responses to PPI<sub>15-24</sub>, either when PPI<sub>15-24</sub> peptide was presented after pulsing of HLA-A2-autologous antigen-presenting cells (Figure 4, C and D) or when it was presented naturally by K562-PPI-A2 cells (Figure 4E). PPI<sub>15-24</sub>-specific T cell clones did not recognize PPI<sub>17-24</sub>. In contrast, using an identical approach, we were unable to isolate PPI<sub>15-24</sub>-specific CD8<sup>+</sup> T cell clones from healthy HLA-A2<sup>+</sup> nondiabetic donors (*n* = 3).

After antigenic stimulation, PPI<sub>15-24</sub>-specific CD8<sup>+</sup> T cell clones predominantly produced TNF-α (Figure 4, C and E), in addition to IFN-γ, and expressed the chemokine receptors CXCR3 and CCR4, both of which have been shown to be important in CD8<sup>+</sup> T cell participation in autoimmune models of type 1 diabetes (16, 17) (Supplemental Figure 1). Clones also expressed surface markers characteristic of an effector CD8<sup>+</sup> T cell phenotype (Supple-



**Figure 3**

Circulating effector CD8<sup>+</sup> T cells that recognize PPI SP epitopes are present in patients with type 1 diabetes. IFN- $\gamma$  ELISPOT PBMC reactivity to PPI<sub>15-24</sub>, either PPI<sub>17-24</sub> or PPI<sub>15-24</sub>, or both peptides was significantly more frequent in patients with type 1 diabetes and *HLA-A\*0201* (black bars) than in HLA-matched nondiabetic control subjects (white bars). Responses to PPI<sub>17-24</sub> were not significantly different in this series ( $P = 0.08$ ), but see Supplemental Table 1 showing a similar analysis of an extended, independent series of cases in which the prevalence of responses to PPI<sub>17-24</sub> is significantly higher in patients with type 1 diabetes than in control subjects ( $P = 0.004$ ). In contrast, the prevalence of responses to the mixture of viral peptides was similar in the 2 groups (see also Supplemental Table 1).

mental Figure 1). The killing potential of PPI<sub>15-24</sub>-specific CD8<sup>+</sup> T cell clones was revealed by expression of cytolytins including granzyme B, perforin, and TNF-related apoptosis-inducing ligand (TRAIL; Supplemental Figure 1).

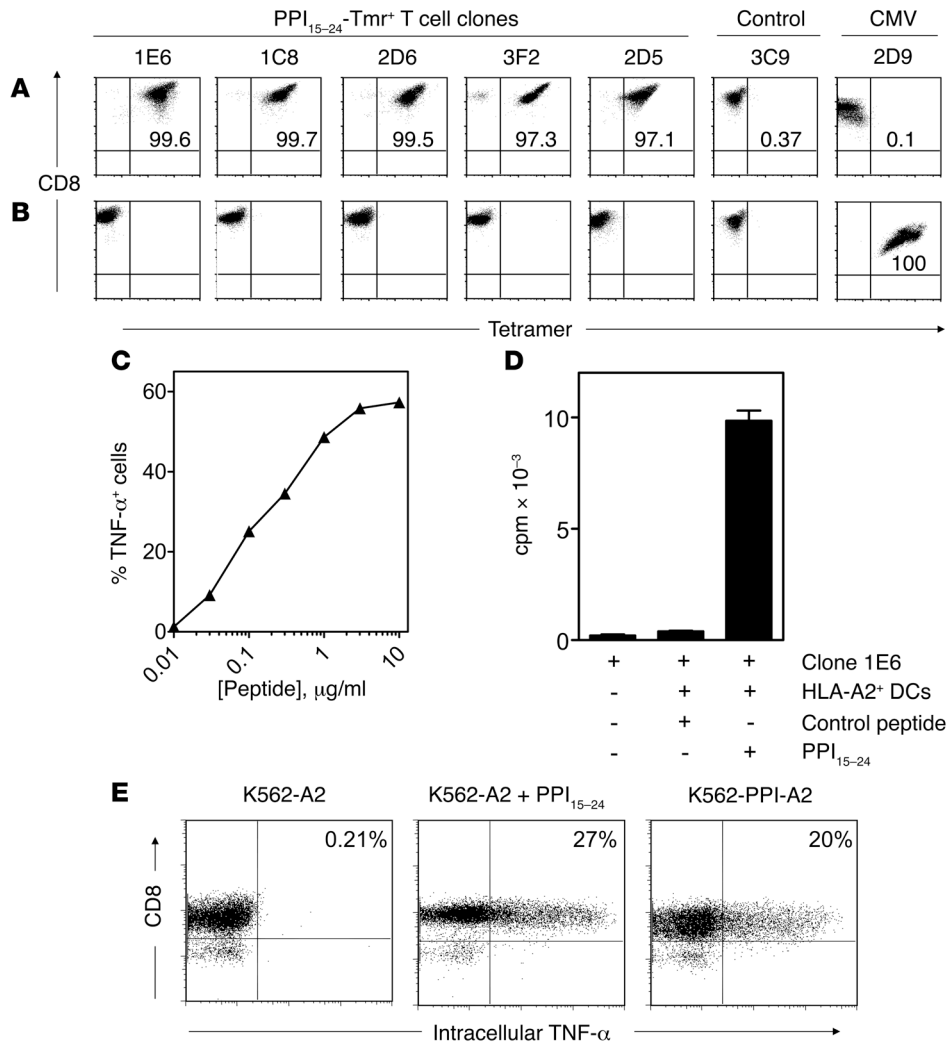
To examine antigen-specific CTL activity of PPI<sub>15-24</sub>-reactive CD8<sup>+</sup> T cells, we first used K562-PPI-A2 cells as targets in a conventional cytotoxicity assay. Killing was highly efficient and restricted to K562 cells expressing both PPI and HLA-A2 (Figure 5A).

**Table 1**

IFN- $\gamma$  ELISPOT response to PPI peptides in HLA-A2<sup>+</sup> type 1 diabetes patients and HLA-A2<sup>+</sup> nondiabetic control subjects

Clinical details				Raw counts (spots/million PBMCs)						Stimulation index					
Case	Age (yr)	Sex	Bgd	A2 <sub>140-149</sub>	CSP <sub>334-342</sub>	PDC <sub>159-167</sub>	CEF	PPI <sub>17-24</sub>	PPI <sub>15-24</sub>	A2 <sub>140-149</sub>	CSP <sub>334-342</sub>	PDC <sub>159-167</sub>	CEF	PPI <sub>17-24</sub>	PPI <sub>15-24</sub>
<b>T1D</b>															
1	30	M	10	15	13	15	63	19	29	1.5	1.3	1.5	<b>6.3</b>	1.9	<b>2.9</b>
2	21	M	5	1	0	1	199	28	19	0.2	0.0	0.2	<b>39.8</b>	<b>5.6</b>	<b>3.8</b>
3	38	M	3	5	3	5	31	5	5	1.7	1.0	1.7	<b>10.3</b>	1.7	1.7
4	31	F	1	1	0	0	10	3	0	1.0	0.0	0.0	<b>10.0</b>	<b>3.0</b>	0.0
5	20	M	4	2	4	6	19	6	5	0.5	1.0	1.5	<b>4.8</b>	1.5	1.3
6	21	F	5	5	5	5	105	5	9	1.0	1.0	1.0	<b>21.0</b>	1.0	1.8
7	23	M	10	5	3	3	237	12	56	0.5	0.3	0.3	<b>23.7</b>	1.2	<b>5.6</b>
8	32	F	3	3	5	3	216	12	9	1.0	1.7	1.0	<b>72.0</b>	<b>4.0</b>	<b>3.0</b>
9	24	F	2	3	8	4	7	9	6	1.5	4.0	2.0	<b>3.5</b>	<b>4.5</b>	<b>3.0</b>
10	21	M	2	1	2	1	31	11	5	0.5	1.0	0.5	<b>15.5</b>	<b>5.5</b>	<b>2.5</b>
11	18	F	4	2	0	1	17	4	5	0.5	0.0	0.3	<b>4.3</b>	1.0	1.3
12	35	F	11	14	13	11	221	35	36	1.3	1.2	1.0	<b>20.1</b>	<b>3.2</b>	<b>3.3</b>
13	18	F	3	5	1	1	15	2	2	1.7	0.3	0.3	<b>5.0</b>	0.7	0.7
14	35	M	11	10	11	10	279	11	26	0.9	1.0	0.9	<b>25.4</b>	1.0	<b>2.4</b>
15	30	M	9	12	6	5	51	8	8	1.3	0.7	0.6	<b>5.7</b>	0.9	0.9
<b>NCs</b>															
C1	32	F	11	5	13	4	192	2	20	0.5	1.2	0.4	<b>17.5</b>	0.2	1.8
C2	23	M	7	1	0	1	144	3	1	0.1	0.0	0.1	<b>20.6</b>	0.4	0.1
C3	28	F	1	0	1	0	30	1	0	0.0	1.0	0.0	<b>30.0</b>	1.0	0.0
C4	33	F	15	13	10	10	44	18	10	0.9	0.7	0.7	<b>2.9</b>	1.2	0.7
C5	36	F	12	6	11	21	115	13	8	0.5	0.9	1.8	<b>9.6</b>	1.1	0.7
C6	33	F	13	23	15	9	241	15	6	1.8	1.2	0.7	<b>18.5</b>	1.2	0.5
C7	29	M	12	5	17	10	173	11	7	0.4	1.4	0.8	<b>14.4</b>	0.9	0.6
C8	23	F	4	4	2	1	91	3	7	1.0	0.5	0.3	<b>22.8</b>	0.8	1.8
C9	34	M	8	15	24	27	38	24	13	1.9	<b>3.0</b>	<b>3.4</b>	<b>4.8</b>	<b>3.0</b>	1.6
C10	27	F	8	5	13	4	183	3	20	0.6	1.6	0.5	<b>22.9</b>	0.4	<b>2.5</b>
C11	29	F	6	3	1	2	231	6	1	0.5	0.2	0.3	<b>38.5</b>	1.0	0.2
C12	34	M	2	3	3	3	282	3	0	1.5	1.5	1.5	<b>141.0</b>	1.5	0.0
C13	31	M	16	7	12	3	40	6	6	0.4	0.8	0.2	<b>2.5</b>	0.4	0.4
C14	20	M	2	0	1	1	29	0	0	0.0	0.5	0.5	<b>14.5</b>	0.0	0.0
C15	29	M	12	6	13	2	96	4	1	0.5	1.1	0.2	<b>8.0</b>	0.3	0.1

Bgd, background (DMSO) spots; control peptides A2<sub>140-149</sub> from HLA-A2; CSP<sub>334-342</sub> from *Plasmodium falciparum* circumsporozoite; and PDC<sub>159-167</sub> from the E2 pyruvate dehydrogenase complex. Bold indicates positive response (stimulation index,  $\geq 2.0$ ). CEF, mix of viral peptides as positive control; NCs, nondiabetic control subjects; T1D, type 1 diabetes patients.



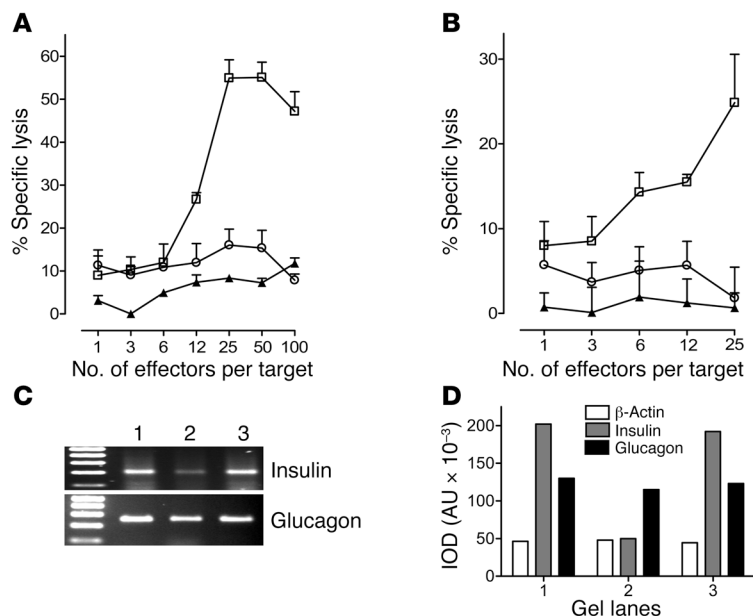
**Figure 4**

Generation of CD8<sup>+</sup> T cell clones specific for PPI SP epitope PPI<sub>15-24</sub>. (A) Five clones isolated from a patient with type 1 diabetes stained with anti-CD8 and HLA-A2 tetramers loaded with PPI<sub>15-24</sub>. Tetramer-stained cells were detected in the upper-right quadrant. A CD8<sup>+</sup> T cell clone obtained in the same expansion that was negative for PPI<sub>15-24</sub>-Tmr (3C9) and a clone (2D9) raised against the CMV pp65 epitope NLVPMVATV are shown. (B) Staining of the same clones with HLA-A2 tetramers loaded with the CMV pp65 epitope NLVPMVATV. (C) PPI<sub>15-24</sub>-specific T cell clones produce TNF- $\alpha$  in a dose-dependent fashion, shown here as the percentage of 1E6 clone cells staining for intracellular cytokine in response to HLA-A2<sup>+</sup> PBMCs pulsed with varying concentrations of PPI<sub>15-24</sub>. (D) 1E6 clone cells proliferate in response to PPI<sub>15-24</sub> peptide-pulsed monocyte-derived DCs, as measured by [<sup>3</sup>H]thymidine incorporation. No response was observed using antigen-presenting cells lacking HLA-A2 (data not shown). (E) 1E6 clone cells also recognize the PPI<sub>15-24</sub> epitope when naturally processed by K562-PPI-A2 cells, since no TNF- $\alpha$  was produced after coculture with K562-A2 cells, but in the presence of PPI<sub>15-24</sub> peptide-pulsed K562-A2 (1  $\mu\text{g/ml}$ ) or K562-PPI-A2 cells, copious amounts of cytokine were produced. Isotype control staining was similar to control-activated clone cells. Proliferation assays were performed in triplicate; bars represent means, error bars are SEMs. Values inside flow cytometry quadrants indicate percentage of positive cells. Data are representative of more than 5 individual experiments.

These results indicate that circulating autoreactive CD8<sup>+</sup> T cells in patients with type 1 diabetes recognize  $\beta$  cell-specific targets and have cytotoxic capability.

*PPI-specific CD8<sup>+</sup> T cells kill human  $\beta$  cells.* We next labeled monodispersed preparations of human islet cells that were HLA-A2<sup>+</sup>, isolated from pancreatic islets obtained from organ donors, to examine the key question of  $\beta$  cell killing. PPI<sub>15-24</sub>-specific CD8<sup>+</sup> T cells killed human islet cells in an HLA-A2-restricted manner (Figure 5B and Supplemental Figure 2). Killing of human islet cells appeared to require cell-cell contact mechanisms, such as cytolysin release direct-

ly onto target cell membranes, since the supernatants from CTLs activated with cognate peptide had no cytotoxic effects on human islet cells or K562-PPI-A2 (data not shown). Moreover, the killing of human islet cells by PPI<sub>15-24</sub>-specific CD8<sup>+</sup> T cell clones was selective for the  $\beta$  cell. Semiquantitative RT-PCR analysis of mRNA for insulin (specific for  $\beta$  cells) and glucagon (specific for  $\alpha$  cells, which are not destroyed in type 1 diabetes) in human islet cells after exposure to PPI<sub>15-24</sub>-specific CTLs revealed a marked reduction in insulin expression (Figure 5, C and D, lane 2) when compared with islet cells cultured alone (lane 1) or with an irrelevant (HLA-A2-restricted



**Figure 5**

CD8<sup>+</sup> T cell clones specific for PPI SP epitope PPI<sub>15-24</sub> are cytotoxic and kill human  $\beta$  cells. (A) Percent specific lysis of K562-PPI-A2 cells by 1E6 PPI<sub>15-24</sub>-specific T cell clone (open squares) but not K562-A2 (triangles) or K562-PPI cells (data not shown). Control CTL 2D9 recognizing A2-presented CMV pp65<sub>495-503</sub> does not kill K562-PPI-A2 cells (open circles). Both clones kill K562-A2 and K562-PPI-A2 cells prepulsed with respective cognate peptide (lysis >90% at 25:1 effector/target ratio; data not shown). (B) Lysis of human HLA-A2<sup>+</sup> (open squares) islet cells by 1E6. A2-negative islet cells (triangles) were not killed, and there was no killing by 2D9 (open circles). A2<sup>+</sup> (but not HLA-A2<sup>-</sup>) islet cells prepulsed with PPI<sub>15-24</sub> were killed (lysis >90% at 25:1; data not shown). Representative of 5 assays for K562 and 3 for human islets (including 2 different donors), performed in triplicate (data are presented as mean, with error bars representing SEMs). (C) Representative (from 3 independent experiments) agarose gel resolution of semiquantitative RT-PCR amplification from human islet cell cytotoxicity assays using insulin- and glucagon-specific primers. (D) PCR product density relative to  $\beta$ -actin (IOD, integrated optical density). A greater than 4-fold reduction in insulin mRNA when islet cells were cultured with 1E6 PPI<sub>15-24</sub>-specific CTLs is seen (lane 2; IOD, 50.0  $\times$  10<sup>3</sup>), compared with no added clone (lane 1; IOD, 202.0  $\times$  10<sup>3</sup>). No reduction in insulin expression is seen in the presence of 2D9 (lane 3). Glucagon expression is similar under all conditions, indicating that killing of human islet cells by 1E6 PPI<sub>15-24</sub>-specific CTLs is  $\beta$  cell specific.

CMV peptide-specific) CTL clone (lane 3). Under similar conditions, glucagon expression remained unchanged (Figure 5, C and D), indicating that  $\beta$  cells were being targeted selectively for killing in these assays. Importantly, these studies confirm that the PPI SP epitope PPI<sub>15-24</sub> is naturally processed and presented by human  $\beta$  cells, as well as their engineered surrogates.

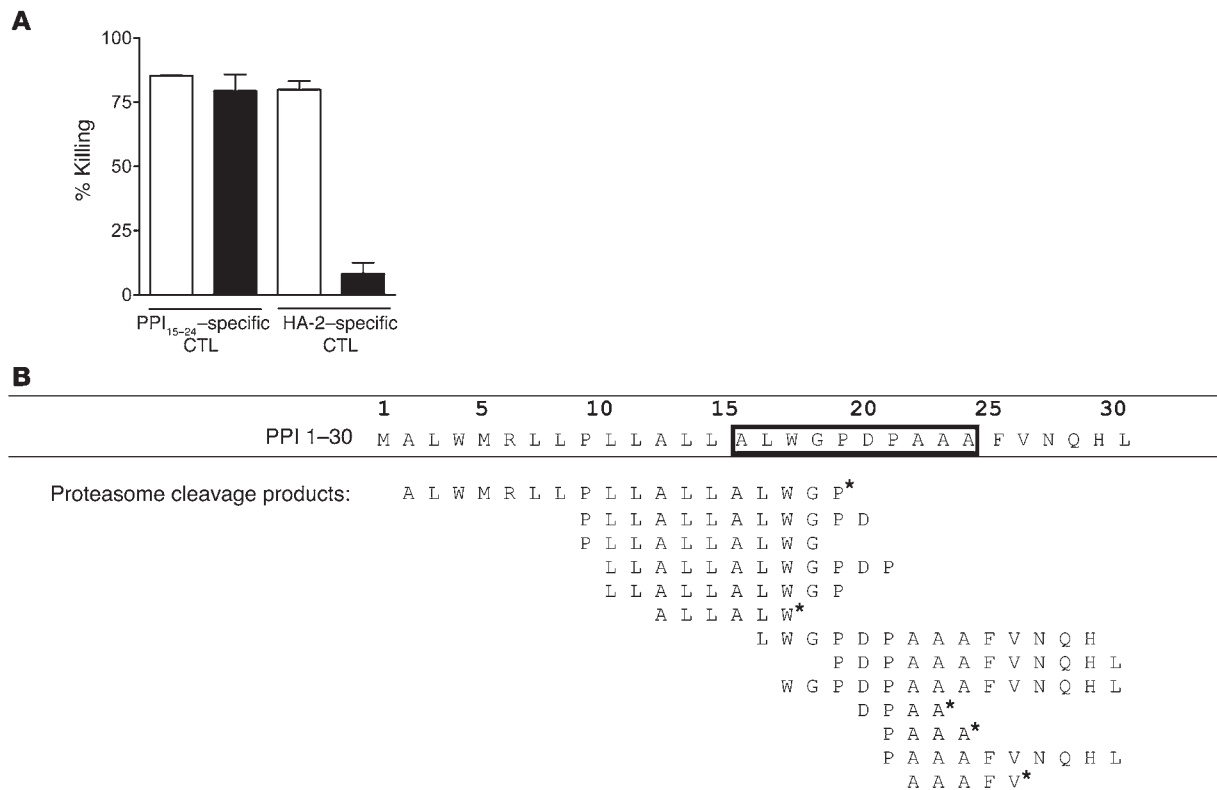
**Processing and presentation of PPI SP epitopes.** HLA class I molecule presentation of SPs is unusual, being most typically observed in the absence of functioning transporter associated with processing (TAP) (18). However, this seemed an unlikely explanation for our observation, since K562 cells are TAP replete. Moreover, processing and presentation of PPI<sub>15-24</sub> by K562-PPI-A2 cells is not affected by cotransfection with the gene encoding the varicellovirus-derived TAP inhibitor protein UL49.5 (19) (Figure 6A). In addition, digestion of a PPI<sub>1-30</sub> peptide by both constitutive and immunoproteasomes did not identify any products matching to NH<sub>2</sub>-terminal extensions of peptides with COOH terminus

at residue 24 and of appropriate length. Table 2 shows average masses for putative NH<sub>2</sub>-terminally extended peptide species with a COOH terminus at residue 24 and length of 8-mer or greater. None of these species was detected after proteasome digestion, although several other fragments of PPI<sub>1-30</sub> were found (Figure 6B). Since conventional generation of HLA class I epitopes requires that the correct COOH terminus is generated by the proteasome (since COOH-terminal trimming does not occur), this makes it unlikely that PPI<sub>15-24</sub> and PPI<sub>17-24</sub> are derived by proteasomal cleavage. The lack of requirement for TAP transport or proteasomal cleavage in the generation and loading of PPI<sub>17-24</sub> and PPI<sub>15-24</sub> suggests that they are generated within the ER, rather than via retrograde translocation of PPI fragments into the cytoplasm for conventional processing. Indeed, we demonstrate that intact PPI<sub>17-24</sub> is generated by soluble ER proteases and that PPI<sub>15-24</sub> is likely to require a combination of these and intramembrane proteolysis (see Supplemental Results). Overall, these data indicate an unconventional processing route for PPI<sub>17-24</sub> and PPI<sub>15-24</sub>, which includes signal peptidase activity, soluble ER proteases, and intramembrane-cleaving proteases (I-CLiPs), the nature and specificity of which remain to be established.

**Effects of glucose concentration on  $\beta$  cell killing.** The unconventional nature of processing of the PPI SP epitopes is reminiscent of that reported for Jaw1, which is inserted into ER membranes and from which C-terminal (luminal) peptides undergo class I processing and presentation via TAP- and proteasome-independent mechanisms (20). It has been speculated that such processing is explained by the protrusion of domains into the ER lumen, facilitating direct loading into nascent class I molecules (21). This led us to hypothesize that the efficiency of HLA class I presentation of PPI SP epitopes would be directly influenced by the availability of substrate, namely newly synthesized PPI. To explore this, human islet cells were cultured at 5.6, 11, and 20 mM glucose for 16 hours before exposure to PPI<sub>15-24</sub>-specific T cells and measurement of TNF- $\alpha$  production by the T cell clone. As glucose concentration increased, more insulin mRNA was expressed by  $\beta$  cells (Figure 7,

A and B), and in concert TNF- $\alpha$  production by PPI<sub>15-24</sub>-specific T cells increased significantly ( $P < 0.01$ ) and linearly (test for linear trend,  $P < 0.005$ ; Figure 7C). In contrast, TNF- $\alpha$  production was not influenced by glucose concentration when islet cells were prepulsed with PPI<sub>15-24</sub> peptide ( $P = \text{NS}$ ; Figure 7D).

This finding prompted us to examine whether increased activation of PPI<sub>15-24</sub>-specific CTLs by islet cells exposed to high glucose concentration could result in enhanced  $\beta$  cell killing. As shown in Figure 8A, stepwise increases in ambient glucose concentration resulted in significant ( $P < 0.005$ ) and linear ( $P < 0.005$ ) increases in killing by the CTL clone 1E6 and other clones (Supplemental Figure 2).  $\beta$  Cell specificity was confirmed by a stepwise decline in levels of insulin mRNA as CTL killing increased (Figure 8, B and C). Moreover, the increased killing was not a result of increased fragility of  $\beta$  cells, since killing of PPI<sub>15-24</sub> peptide-pulsed islet cells was not significantly altered by high ambient glucose ( $P = \text{NS}$ ; Figure 8D) and was not  $\beta$  cell specific (Figure 8, E and F). Moreover,



**Figure 6** Generation of SP epitopes of PPI is independent of TAP. **(A)** To examine TAP dependency of PPI<sub>15-24</sub> presentation by K562 cells, the CTL clone (1E6) recognizing PPI<sub>15-24</sub> restricted by HLA-A2 was used in cytotoxicity assays in the presence of the varicellovirus-derived TAP inhibitor UL49.5 (19). As a control, we used the HA-2 CTL clone 5H17 (53), specific for an endogenously expressed, TAP-dependent minor histocompatibility antigenic epitope (sequence YIGEVLVSV derived from a diallelic gene encoding a novel human class I myosin protein) presented by HLA-A2 (54). Target cells for clone 1E6 were K562-PPI-A2 cells or K562-PPI-A2 cells additionally transfected to express UL49.5. Target cells for clone 5H17 were K562-A2 cells or K562-A2 cells additionally transfected to express UL49.5. Data are expressed as percent killing (see Methods for calculation of specific cytotoxicity using DELFIA assay); bars represent means and error bars, SEMs. In the presence of the 1E6 PPI<sub>15-24</sub>-specific CTLs, killing of K562-PPI-A2 cells cotransfected with UL49.5 (black bars) was comparable to that of untransfected K562-PPI-A2 cells (white bars), indicating that TAP inhibition by UL49.5 does not reduce PPI<sub>15-24</sub> presentation. In contrast, killing of target cells by the HA-2-specific CTL 5H17 that recognizes a TAP-dependent epitope is markedly inhibited in the presence of UL49.5 (black bars). **(B)** To examine proteasome dependency of PPI<sub>15-24</sub> generation, MS analyses of immuno- and constitutive proteasome digests of PPI<sub>1-30</sub> were performed. Results are shown in Table 2. In the same experiment, several other fragments of PPI<sub>1-30</sub> were generated by proteasome digestion. Sequences marked with an asterisk were generated by the constitutive proteasome only. For reference, the 10-mer PPI<sub>15-24</sub> peptide eluted from surrogate β cells is indicated by the black rectangle.

over a 16-hour period in the presence of high glucose (20 mM), islet killing peaked when insulin mRNA transcripts were most abundant, rather than when insulin secretion was at its highest (Figure 9). Taken together, these data show that when β cells are induced to increase PPI synthesis, there is a concomitant increase in PPI SP epitope presentation. In the presence of specific CTLs, such β cells are subjected to a greater β cell killing efficiency. Prolonged exposure to high glucose concentrations was required to see the effect, since killing was not enhanced when islets were exposed to 20 mM glucose for 30 minutes before the cytotoxicity assay (data not shown).

*Cross-presentation of PPI SP epitopes.* Having established the importance of the PPI<sub>15-24</sub> epitope in β cell targeting, we questioned how specific effector CD8<sup>+</sup> T cells could be primed to recognize this epitope during the pathogenesis of type 1 diabetes. CTL priming requires cross-presentation, a process in which professional antigen-presenting cells such as DCs take up, process, and present antigens as peptide-HLA class I molecule complexes to naive

CD8<sup>+</sup> T cells. This process is a necessary component of immunity, for example, against viruses that do not infect antigen-presenting cells, but its involvement in autoimmune disease is relatively unexplored. Moreover, in this particular context, it is clear that intact PPI, from which PPI<sub>15-24</sub> is derived, is ER membrane inserted and not directly available for extracellular uptake as a soluble protein. Tang and colleagues have shown that in the NOD mouse model of autoimmune diabetes, whole β cells are ingested by DCs and transported to the pancreatic lymph node (22). We therefore examined whether DCs cultured with soluble PPI or with K562-PPI cells could cross-present PPI<sub>15-24</sub> to specific T cell clones. Our results show that DCs are efficient at cross-presentation of PPI<sub>15-24</sub> from soluble PPI and also from intact PPI-expressing cells (Figure 10).

**Discussion**

The novelty of this study is 4-fold. First, it provides evidence that human autoreactive CD8<sup>+</sup> T cells can be responsible for the selective β cell destruction that is the hallmark of clinical type 1 diabe-

**Table 2**MS analysis of immuno- and constitutive proteasome digests of PPI<sub>1-30</sub> shows proteasome-independence of PPI<sub>15-24</sub> generation

PPI peptides searched for:	Mass	1 <sup>+</sup> (m/z)	2 <sup>+</sup> (m/z)	3 <sup>+</sup> (m/z)	4 <sup>+</sup> (m/z)	Proteasome	
						Constitutive	Immuno-
WGPDPA AAA	783.84	784.84	392.92	262.28	196.96	ND	ND
LWGPDP AAA	897.00	898.00	449.50	300.00	225.25	ND	ND
ALWGPDP AAA	968.08	969.08	485.04	323.69	243.02	ND	ND
LALWGPDP AAA	1,081.24	1,082.24	541.62	361.41	271.31	ND	ND
LLALWGPDP AAA	1,194.40	1,195.40	598.20	399.13	299.60	ND	ND
ALLALWGPDP AAA	1,265.48	1,266.48	633.74	422.83	317.37	ND	ND
LALLALWGPDP AAA	1,378.63	1,379.63	690.32	460.54	345.66	ND	ND
LLALLALWGPDP AAA	1,491.79	1,492.79	746.90	498.26	373.95	ND	ND
PLLALLALWGPDP AAA	1,588.91	1,589.91	795.46	530.64	398.23	ND	ND
LPLLALLALWGPDP AAA	1,702.07	1,703.07	852.04	568.36	426.52	ND	ND
LLPLLALLALWGPDP AAA	1,815.23	1,816.23	908.62	606.08	454.81	ND	ND
RLLPLLALLALWGPDP AAA	1,971.42	1,972.42	986.71	658.14	493.86	ND	ND
MRLLPLLALLALWGPDP AAA	2,102.62	2,103.62	1,052.31	701.87	526.66	ND	ND
WMRLLPLLALLALWGPDP AAA	2,288.83	2,289.83	1,145.42	763.94	573.21	ND	ND
LWMRLLPLLALLALWGPDP AAA	2,401.99	2,402.99	1,202.00	801.66	601.50	ND	ND
ALWMRLLPLLALLALWGPDP AAA	2,473.07	2,474.07	1,237.54	825.36	619.27	ND	ND
MALWMRLLPLLALLALWGPDP AAA	2,604.27	2,605.27	1,303.14	869.09	652.07	ND	ND

MS analyses of immuno- and constitutive proteasome digests of PPI<sub>1-30</sub> as average masses for putative NH<sub>2</sub>-terminally extended peptide species with a COOH terminus at residue 24 and length of 8-mer or greater. The table lists m/z values of possible ionization species (1+ to 4+) for each of the hypothetical peptide products in the search. None of these species was detected after proteasome digestion, indicating a very low probability that the proteasome is able to generate the PPI<sub>15-24</sub> and PPI<sub>17-24</sub> SP epitopes. ND, not detected.

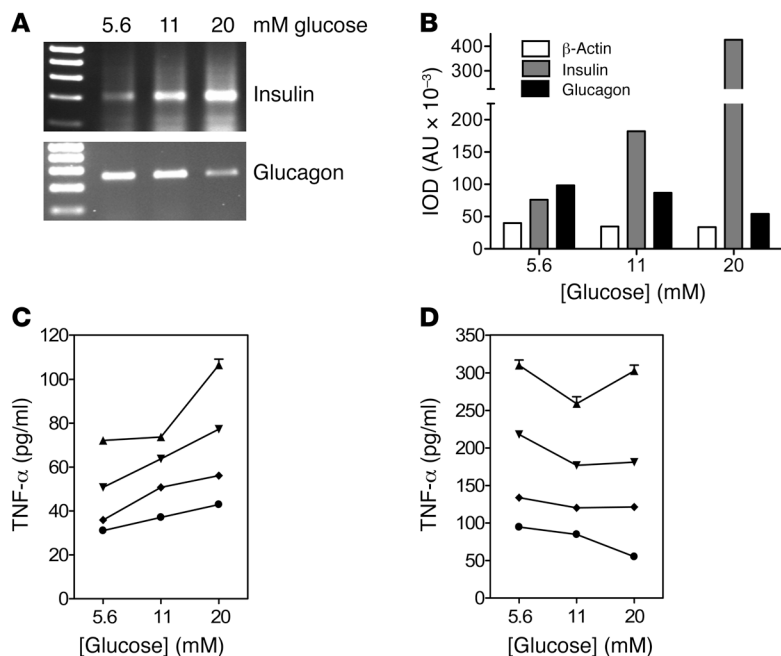
tes. Second, it also reveals that a relatively unstudied portion of the SP of PPI is a major source of CTL targets. Third, these epitopes are generated by an unconventional processing pathway that is influenced by the ambient glucose concentration. Finally,  $\beta$  cells can therefore unwittingly be active players in their own demise, being targeted more efficiently as they are induced to secrete more insulin. These findings may have important disease implications. In prediabetic subjects *in vivo*, it is likely that individual  $\beta$  cells are required to increase insulin secretion, as the total  $\beta$  cell mass diminishes during the asymptomatic prodrome of the disease. Thus, the microenvironmental conditions prevailing in islets of Langerhans as clinical type 1 diabetes approaches, which include the well-documented  $\beta$  cell hyperexpression of HLA class I molecules (3, 5, 23) coupled with increased secretion of insulin, provide optimal killing conditions for CTLs that may be present.

Although putative epitopes of insulin, proinsulin, and PPI have been identified through the use of predictive HLA class I binding algorithms (15, 24), immunization of HLA class I-transgenic mice (13), and proteasome digestion patterns (13–15, 25), none has been confirmed as being naturally processed by human  $\beta$  cells, and none has been identified as a target of CTLs. By focusing our epitope discovery effort onto the naturally presented repertoire, we identified 2 novel epitopes, both residing within the SP, and confirmed that at least one of these (PPI<sub>15-24</sub>) is naturally presented by human  $\beta$  cells. SP epitopes are unusual, and, to our knowledge, none of relevance to human disease have been described to date. We provide strong evidence that the processing of PPI SP for presentation by HLA class I molecules does not follow the conventional route (*i.e.*, via proteasome cleavage and TAP transport), requiring instead the activity of soluble and membrane-bound ER proteases.  $\beta$  Cell processing and presentation of the CTL epitope PPI<sub>15-24</sub> is enhanced by a prolonged increase in ambient glucose. We consider it most likely that this results from an increase in available PPI substrate entering

the ER membrane. Enhanced presentation will occur as the quantity of PPI mRNA increases and it undergoes augmented cotranslation alongside HLA-A2 mRNA, also upregulated on infiltrated islets and in our islet cultures (3). Such a rise in  $\beta$  cell PPI mRNA content requires exposure to high ambient glucose concentrations for prolonged periods *in vitro* (26), the corollary of which *in vivo* is likely to be prolonged hyperglycemia experienced, for example, in advanced, uncontrolled clinical diabetes. An alternative but not mutually exclusive explanation is that ER stress, known to be a consequence of high ambient glucose (27), could upregulate the requisite ER processing machinery for SP, leading to enhanced PPI<sub>15-24</sub> presentation.

The debate as to the mechanism of  $\beta$  cell death in type 1 diabetes has simmered for several years but has important clinical implications, since greater understanding in this area has the potential to guide novel immune intervention strategies. At present, none of the therapeutic approaches under evaluation in type 1 diabetes at phase I, II, or III stages is specifically designed to target autoreactive CD8<sup>+</sup> T cells (28). Our finding that circulating CTLs capable of killing  $\beta$  cells are present in a sizeable proportion of patients with type 1 diabetes legitimizes the development of CD8<sup>+</sup> T cell-specific strategies to halt  $\beta$  cell destruction, although the challenge will be to develop approaches that leave antiviral responses relatively intact. An example might be targeting of the NKG2D/MICA axis. The CTL clones we isolated from type 1 diabetes patients were NKG2D<sup>+</sup>, and blockade of the equivalent pathway in the NOD mouse prevents islet destruction and development of diabetes (29).

A second approach that might at least slow progression of  $\beta$  cell destruction would be to minimize endogenous insulin production through early and aggressive introduction of insulin therapy. The repeated observation that many patients who are insulin dependent at diagnosis then temporarily cease to require insulin injections in subsequent months (the so-called honeymoon period) is consistent with the hypothesis that the introduction of exogenous



**Figure 7** High ambient glucose increases  $\beta$  cell PPI synthesis and activation of PPI SP epitope PPI<sub>15-24</sub>-specific CTLs and the efficiency of human  $\beta$  cell killing. (A) Agarose gel showing resolution of products of semiquantitative RT-PCR amplification using insulin- and glucagon-specific primers from total RNA extracted from human islet cells cultured for 14 hours in the presence of 5.6, 11, and 20 mM glucose. Results show increasing insulin mRNA synthesis with increasing glucose concentration. (B) Expressed relative to the density of the  $\beta$ -actin gene PCR product, insulin expression is greater than 4-fold higher at 20 mM compared with 5.6 mM glucose. There is an expected reduction (~2-fold) in glucagon expression at 20 mM compared with 5.6 mM glucose due to the known suppression of glucagon secretion by high glucose and insulin concentrations (55–57). (C) Coculture of islet cells at different glucose concentrations with 1E6 PPI<sub>15-24</sub>-specific T cell clone results in a significant increase (1-way ANOVA,  $P < 0.01$ ) in TNF- $\alpha$  production by the clone, with a significant linear trend as glucose concentration increases ( $P < 0.005$ ), indicating an increase in epitope presentation at higher glucose concentration. (D) A similar analysis of islets precultured at different glucose concentrations and prepulsed with PPI<sub>15-24</sub> peptide shows that high glucose alone has no effect on T cell clone activation. Symbols indicate different effector/target ratios of 3:1 (circles), 6:1 (diamonds), 12:1 (inverted triangles), and 25:1 (triangles).

insulin therapy allows targeted  $\beta$  cells to reduce their synthetic activity and thus acquire a degree of protection from CTL attack. Indeed, previous studies, albeit on small numbers of subjects, have suggested that preservation of endogenous insulin reserve might benefit from administration of insulin in the prediabetic period (30) or exertion of tight metabolic control soon after diagnosis (31). Our study provides a rational basis for such an approach by identifying a possible mechanism through which it operates. Finally, the novel pathway through which PPI SP epitopes are generated implies the existence of I-CLiPs and ER proteases that are critical for CTL epitope display. Identification of the precise components involved in PPI SP processing could lead to the development of novel therapeutic agents that target these pathways.

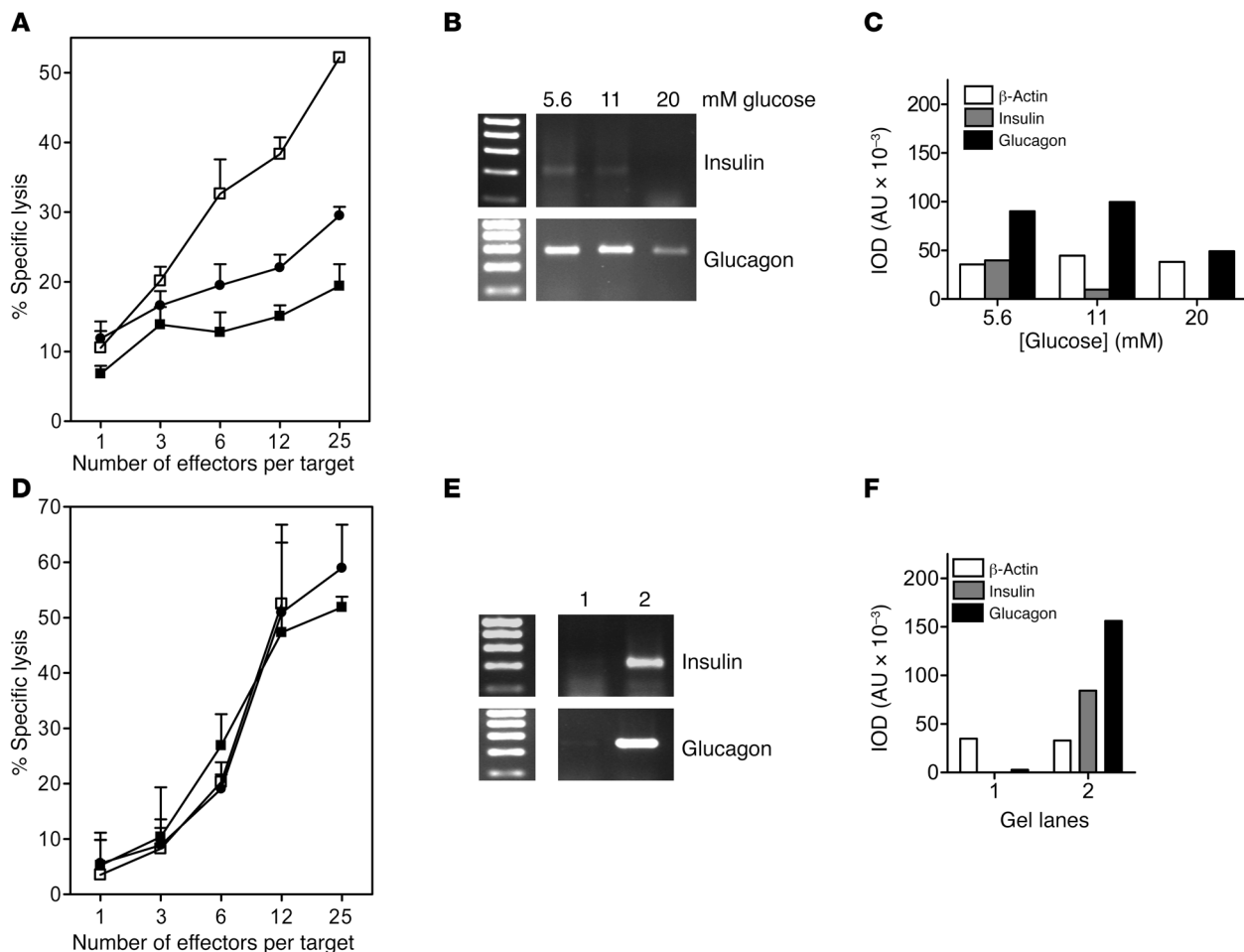
**Methods**

*Generation of surrogate  $\beta$  cells.* PPI cDNA was inserted between the BamHI and EcoRI sites in the pcDNA6/ myc-His B vector (Invitrogen) for expression in mammalian cell lines under blasticidin selection. The human chronic

myelogenous leukemia cell line K562 expressing HLA-A2 (K562-A2) under Geneticin resistance was obtained (32) from C. Britten (Johannes Gutenberg-University of Mainz, Mainz, Germany) and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin solution (all Invitrogen) at 37°C, 5% CO<sub>2</sub>. Geneticin (Invitrogen) concentration in the medium was maintained at 0.7 mg/ml. HLA-A2 was expressed by greater than 95% of cells. Transfection of the PPI gene was carried out using Effectene (QIAGEN), and K562-PPI-A2 cells were maintained in the same manner as K562-A2 cells with the addition of 10  $\mu$ g/ml blasticidin. After 2 weeks of culture under selection, proinsulin (DRG International Inc.) and immunoreactive insulin (Diagnostic Systems Laboratories Inc.) were detected in the supernatants by ELISA and RIA, respectively.

*Analysis of HLA-A2 peptide repertoire.* Peptides were extracted from the surface of cultured cells by citric acid elution (33). Briefly,  $4 \times 10^9$  cells were washed 3 times in PBS and resuspended in a total volume of 25 ml of pH 3.3 citrate-phosphate buffer (0.131 M citric acid, 0.066 M Na<sub>2</sub>HPO<sub>4</sub>) for 1 minute at room temperature. Cells were then pelleted and the supernatant citric acid solution (containing previously cell-bound HLA class I peptides) harvested and filtered through a 0.2- $\mu$ m syringe filter before resolution using 2 Sep-Pak C18 cartridges (Waters) connected in series and conditioned using 50 ml acetonitrile and then 50 ml citrate buffer. Peptides were eluted with 3 ml 80% acetonitrile per cartridge and vacuum dried to 50  $\mu$ l. This residue was resuspended by addition of a further 150  $\mu$ l citrate buffer. Peptides were additionally purified by ultrafiltration through a 3,000-molecular-weight-cutoff Centricon-3 ultrafiltration concentrator (Millipore) at 6,500 g for 6 hours at 18°C. The filtrate (approximately 100  $\mu$ l) was stored at -80°C prior to fractionation by reverse phase-HPLC (RP-HPLC) on a Symmetry C18 column (Waters) connected to a Waters 2690 Separations Module and 2487 Detector using water/0.05% trifluoroacetic acid (TFA) and acetonitrile/0.05% TFA with collection of 120 fractions. Each fraction was vacuum dried (SpeedVac vacuum concentrator; Thermo Scientific) at room temperature, to approximately 5–10  $\mu$ l, and stored at -80°C.

For matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis of peptides, 0.5  $\mu$ l sample was mixed with 0.5  $\mu$ l freshly prepared 2,5-dihydroxybenzoic acid matrix (MassPREP DHB matrix; Waters) or  $\alpha$ -cyano-4-hydroxycinnamic acid matrix (CHCA; Bruker Daltonics) prepared at 10 mg/ml in 1:1 (v/v) acetonitrile, 0.1% TFA. For internal calibration, a further 1  $\mu$ l MS calibrant mixture was added, containing 2.5 fmol leu-enkephalin (556.2771 m/z) and 5 fmol substance P (1347.73543 m/z). MS was performed using a Bruker Daltonics MALDI-TOF mass spectrometer. Samples from K562-PPI-A2, K562-A2, and K562-PPI cells were ionized using laser intensities in the range of approximately 5,000 V and approximately 30,000 spectra acquired and compared to identify unique masses in the K562-PPI-A2 extract as described previously (9, 34). Spectra were aligned with internal calibrants for maximum accuracy using linear calibration to enable matching of putative unique masses to linear sequences of PPI. Putative unique sequences, i.e., those found in K562-PPI-A2 but not control K562-A2 or K562-PPI fractions, could then be identified either manually or using computer algorithms. Unique masses were then characterized by MS/MS using a Voyager ABI 4700 (Applied Biosystems) to derive



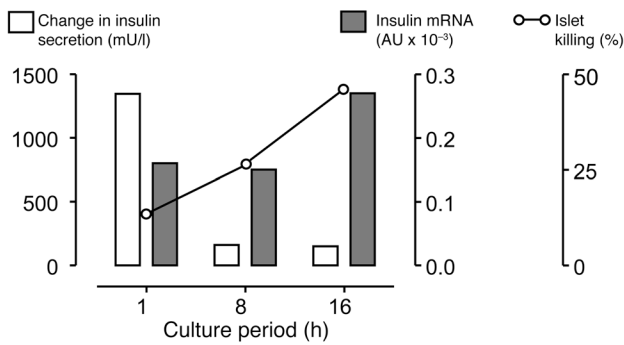
**Figure 8** High ambient glucose increases the efficiency of human  $\beta$  cell killing. **(A)** Specific lysis of human islet cells after 14 hours preculture at different glucose concentrations (5.6 mM, 11 mM, and 20 mM; filled squares, filled circles, and open squares, respectively). Higher glucose results in significantly enhanced CTL killing by 1E6 (1-way ANOVA,  $P < 0.005$ ; significant linear trend,  $P < 0.005$ ). Representative agarose gel of RT-PCR amplification using insulin- and glucagon-specific primers of total RNA from human islet cells after 14 hours at different glucose concentrations **(B)** and band density in the same gel **(C)**. Islet cells at 5.6, 11, and 20 mM glucose exposed to killing by 1E6 clone cells show increasing loss of insulin mRNA, declining from approximately 50,000 to approximately 0 counts. Glucagon expression is identical to that at 20 mM glucose in the absence of 1E6 clone cells (see Figure 7A). **(D)** In contrast, human islet cells pulsed with PPI<sub>15-24</sub> show no  $\beta$  cell specificity of killing and no additive killing at higher glucose concentrations (symbols as above). **(E)** Agarose gel of RT-PCR using insulin- and glucagon-specific primers after human islet cells were cultured for 14 hours at 20 mM glucose, either with PPI<sub>15-24</sub> peptide and exposed to killing by 1E6 (lane 1) or cultured alone (lane 2) and **(F)** graphical representation of band density. The results show loss of both insulin and glucagon expression, demonstrating a lack of  $\beta$  cell-specific killing of islets prepulsed with PPI<sub>15-24</sub>. Cytotoxicity assays were performed in triplicate; symbols represent means, and error bars represent SEMs.

partial sequence information under collision-induced dissociation (CID) using atmospheric gas. The MS/MS spectrum for each unique mass was submitted to a Mascot server (35), querying either the NCBI nr (nonredundant) (36) or a local database to identify the precursor sequence.

**Subjects.** Fresh heparinized and clotted blood samples were obtained from 56 HLA-A\*0201 patients of mixed European descent with type 1 diabetes (mean  $\pm$  SD age, 29 years  $\pm$  6.5; median duration of disease 10 weeks, range 3–14 weeks) with acute onset of symptoms requiring insulin from the time of diagnosis and 36 HLA-A\*0201 nondiabetic control subjects, also of mixed European descent, without history of first-degree relatives with type 1 diabetes and matched for age (mean  $\pm$  SD age, 31  $\pm$  5.7 years). All samples were screened initially for HLA-A2 by flow cytometry (using the BB7.2 mAb clone; BD Biosciences – Pharmingen), and HLA-A1\*0201 positivity was confirmed by PCR sequence-specific primer (PCR-SSP) genotyping.

Fresh PBMCs were isolated on density gradients (Lymphoprep; Nycomed) and washed in RPMI-1640 (Invitrogen) twice before use. These studies were carried out with the approval of King’s College Hospital Research Ethics Committee, and informed consent was obtained from all participants.

**ELISPOT for the detection of CD8<sup>+</sup> T cell responses.** Fresh PBMCs were precultured in single wells of 48-well plates at a density of  $1 \times 10^6$  in 0.5 ml TC medium (RPMI-1640, antibiotics from Invitrogen, and 10% human AB serum from PAA) containing test peptide to a final concentration of 10  $\mu$ M and incubated at 37°C, 5% CO<sub>2</sub>, tilted by 5°. Peptides were synthesized by standard Fmoc chemistry (Interactiva Biotechnologie GmbH) to at least 90% purity as confirmed using RP-HPLC and MS. Control wells contained TC medium with an equivalent concentration of DMSO or CEF (viral peptide mix, at a final concentration equivalent to 10  $\mu$ M; Mabtech). After 24 hours incubation, nonadherent cells were resuspended using pre-



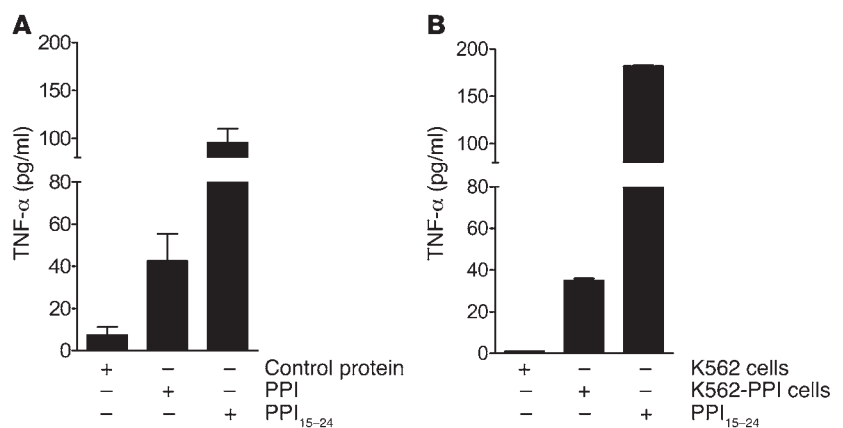
**Figure 9**

Enhanced islet killing at high glucose concentration is related to translation of insulin mRNA rather than insulin secretion. Analysis of islet cell killing in relation to insulin secretion and insulin mRNA expression. Human A2<sup>+</sup> islets were cultured for the specified time periods in the presence of 20 mM glucose, and at the end of that time, islet cells were removed into a cytotoxicity assay with clone 3F2. White bars show insulin secretion over the periods 0–1 hours, 1–8 hours, and 8–16 hours. Gray bars show insulin mRNA expression above baseline (designated 1,000 AU, measured as IOD) in islets harvested at the specified time points. The line graph with open circles shows killing of islets harvested at the specified time points. The results show that  $\beta$  cells exposed to high glucose degranulate rapidly but that this is not related to enhanced killing by PPI<sub>15–24</sub>-specific CTLs. The relationship between insulin mRNA levels and killing suggests that PPI<sub>15–24</sub> generation is related to levels of PPI transcription and translation rather than to insulin secretion.

warmed TC medium (2% AB serum), washed, and brought to a concentration of 10<sup>6</sup>/300  $\mu$ l, and 100  $\mu$ l was dispensed in triplicate into wells of 96-well ELISA plates (Nunc MaxiSorp) preblocked with 1% BSA in PBS and precoated with monoclonal anti-IFN- $\gamma$  (U-CyTech Biosciences). In some assays, harvested cells were depleted of CD8<sup>+</sup> T cells using immunomagnetic beads (Miltenyi Biotec). After cytokine capture at 37°C, 5% CO<sub>2</sub> for 15 hours, cells were lysed in ice-cold double-distilled water, plates washed in PBS/Tween-20 (0.05%), and spots developed according to the manufacturer's instructions. Plates were dried and spots of greater than 60  $\mu$ m counted in a BioReader 3000 (Bio-Sys). Triplicate values were pooled to provide total spots per 10<sup>6</sup> PBMCs, and values obtained in test wells were compared with the background (DMSO) wells to derive a stimulation index (SI). In preliminary studies using insulin/proinsulin peptides known to elicit responses in patients with type 1 diabetes (14, 15, 24, 37), the optimal operating conditions for the assay were established using a receiver-operator characteristic (ROC) curve as described previously (9), and samples were considered positive when the SI was at least 2. In a subset of patients and control subjects ( $n = 15$  per group), additional non-islet peptides were included as controls for non-specific reactivity: an immunodominant epitope of the E2 component of pyruvate dehydrogenase complexes, the major mitochondrial antigen in primary biliary cirrhosis residues 159–167: KLSEGDLLA (38); an epitope of the circumsporozoite protein of *Plasmodium falciparum* variant NF54 residues 334–342: YLNKIQNSL (39); and an HLA-A2-derived peptide eluted from HLA-A2 (B.O. Roep et al., unpublished observations) residues 140–149: YAYDGKDYIA. For the remaining subjects ( $n = 41$  patients and  $n = 21$  controls), the human islet amyloid polypeptide precursor protein 5–13 (IAPP<sub>5–13</sub>) (KLQVFLIVL) (40, 41) was used as a control for non-specific reactivity to SPs. Positive ELISPOT responses to IAPP<sub>5–13</sub> were present in 4 of 41 patients (9.7%) and 4 of 21 control subjects (19%;  $P = NS$ ).

**Generation of CD8<sup>+</sup> T cell clones.** Monocyte-derived DCs were obtained as described previously (42) and used to generate mature DCs. DCs were pulsed with 10  $\mu$ g/ml PPI<sub>15–24</sub> peptide for 1 hour at 37°C in IMDM/AB medium (Invitrogen). After extensive washing, monocyte-derived DCs and autologous CD8<sup>+</sup> lymphocytes (purified from PBMCs using the CD8 Isolation Kit II; Miltenyi Biotec) were cultured at a 1:5 ratio in 24-well culture plates. Initial culture medium IMDM/AB was

supplemented with IL-7 (10 ng/ml; PeproTech). After 2 days, 5% Cellkine (ZeptoMatrix Corp.) and IL-15 (0.1 ng/ml; PeproTech) were added. After 7 days of culture, CTLs were restimulated with mature autologous DCs (pulsed with 10  $\mu$ g/ml PPI<sub>15–24</sub>) at a 1:5 ratio. On day 22, CD3<sup>+</sup>CD8<sup>+</sup> T cells staining with PPI<sub>15–24</sub>-Tmr and negative for 7-aminoactinomycin-D (live/dead discriminator) were flow sorted and seeded at 1 cell/well in 96-well plates, each well containing 1  $\times$  10<sup>5</sup> irradiated allogeneic PBMCs, in X-Vivo 15 (BioWhittaker) and 5% Cellkine, IL-7 (10 ng/ml), IL-15 (0.1 ng/ml), and PHA-M (5  $\mu$ g/ml; Sigma-Aldrich). Growing CD8<sup>+</sup> T cell clones were isolated and restimulated every 14 days as described above. Phenotypic analysis of clones was carried out as previously described (43) using the following mAbs for surface and intracellular staining (43): anti-CD3, anti-CD4, anti-CD56, anti-CD16, anti-CD8, anti-TCR- $\alpha\beta$ , anti-NKG2D, anti-CD28, anti-CD45RA, anti-CD45RO, anti-CXCR3, anti-TNF- $\alpha$ , anti-IFN- $\gamma$ , anti-granzyme B, anti-perforin, and anti-TRAIL (all from BD and BD Biosciences – Pharmingen); anti-CCR7 and anti-CCR4 (both from R&D Systems); anti-CD62L and anti-CD44 (both from Serotec Ltd).



**Figure 10**

PPI SP epitope PPI<sub>15–24</sub> is cross-presented by DCs from intact PPI and PPI-expressing cells. (A) DCs pulsed with soluble PPI and then matured induce TNF- $\alpha$  production by 1E6 PPI<sub>15–24</sub>-specific T cells. Cytokine production in the presence of a control protein (the fusion partner of recombinant human PPI) and PPI<sub>15–24</sub> peptide are shown for comparison. (B) DCs pulsed with freeze-thawed K562 cells expressing PPI (K562-PPI) and then matured induce TNF- $\alpha$  production by 1E6 clone cells. Cytokine production in the presence of control cells (nontransfected K562) and PPI<sub>15–24</sub> peptide are shown for comparison. Bars represent means from triplicate experiments, error bars SEMs; data are representative of 3 independent experiments.



Functional analysis of clones was carried out after stimulation with peptide-pulsed DCs, PBMCs, and variously transfected K562 cell lines, using [<sup>3</sup>H]thymidine incorporation, TNF- $\alpha$  secretion (measured by ELISA from ImmunoTools), or intracellular TNF- $\alpha$  and IFN- $\gamma$  expression (measured by flow cytometry). As a control, we used the same approach to clone CD8<sup>+</sup> T cells recognizing the immunodominant HLA-A2-restricted CMV pp65 peptide NLVPMVATV.

**Human islet isolation, culture, and cytotoxicity assays.** Human islet isolations were performed using a method described previously (44). Pancreata were retrieved with the consent of donors' relatives and permission of the Ethical Review Committee of King's College Hospital. Islet-enriched cell fractions were cultured in CMRL1066 containing 5.6 mM glucose, supplemented with 10% FBS and penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and L-glutamine (2 mmol/l) (Invitrogen) for 2 weeks, with medium changed every 2 days. Cells were allowed to become monolayer cultures and before cytotoxicity assays were cultured in the presence of cytokines (500 IU/ml IFN- $\gamma$ ; 50 IU/ml IL-1 $\beta$ ; 2,500 IU/ml TNF- $\alpha$  [Strathmann Biotech] and 1,000 IU/ml IFN- $\alpha$  [Roche Laboratories]) for 16–24 hours to upregulate HLA class I expression (cytokines and concentrations established in preliminary studies and selected for optimal upregulation).

Cytotoxicity was analyzed by a nonradioactive europium TDA (EuTDA) cytotoxicity assay using DELFIA Technology (PerkinElmer) according to the manufacturer's instructions (45). Briefly, either human islet cells ( $1 \times 10^6$ ) or K562-PPI-A2, K562-A2, or K562-PPI-A2-UL49.5 cell lines were incubated for 20 minutes at 37°C in 1 ml of RPMI/10% FBS with 3  $\mu$ l of fluorescence-enhancing ligand. After extensive washing with PBS (5 times), 50  $\mu$ l of labeled islet cells ( $5 \times 10^3$ ) were seeded in triplicate into V-shaped 96-well plates followed by 50  $\mu$ l of effector cells (CD8<sup>+</sup> T cell clone) at varying ratios in X-Vivo 15/5% AB serum/IL-7 (10 ng/ml), IL-15 (0.1 ng/ml), and 2.5% Cellkine. The cultures were incubated for 4 hours at 37°C, and 20  $\mu$ l of the supernatant transferred to a clear, flat-bottom microtitration plate, followed by addition of 200  $\mu$ l/well of enhancement solution. After 15 minutes at room temperature with shaking, the europium signal was measured using a VICTOR3 Time-Resolved Fluorescence Reader (PerkinElmer). To determine maximum lysis, islet cells alone ( $5 \times 10^3$  in 100  $\mu$ l) were treated with 20  $\mu$ l lysis buffer. Spontaneous release was measured from wells containing only labeled islet cells in 100- $\mu$ l volume. Specific cytotoxicity was calculated using the following formula: % specific release = (experimental - spontaneous release)  $\times$  100/(maximum - spontaneous release). In some experiments, islet cell monolayers were cultured for 14 hours before cytotoxicity assays in the presence of 11 mM or 20 mM glucose.

**Detection of insulin and glucagon mRNA.** Semiquantitative RT-PCR was performed using total RNA isolated using an RNA Miniprep Kit (Promega) and quantified using a spectrometer (GeneQuant II; Pharmacia Biotech) at 260-nm wavelength as described previously (44, 46, 47). Briefly, 100 ng of total RNA was converted into first-strand cDNA using RT (Invitrogen) in a volume of 20  $\mu$ l. The hot-start (QIAGEN) RT-PCR was used with the following parameters: 95°C for 15 minutes for 1 cycle; then various amplification cycles for individual genes. Human  $\beta$ -actin gene was amplified in parallel as an internal control. The PCR primers used were: human insulin-forward (5'-CATCAAGCACATTGTCC-3') and -reverse (5'-CTGGTTCAAGGCTTATTTC-3'); human glucagon-forward (5'-GCATTTACTTTGTGGCTGGGTC3') and -reverse (5'-AGTGATTTGGTCTGAATC-3'); human  $\beta$ -actin-forward (5'-CCCAGATCATGTTTGAGACC-3') and -reverse (5'-CCAACAGGAGTACTTGCCTCAG-3'). DNA relative quantity was analyzed using a gel program (version 3.0 computer analyzer of Media Cyber-

netics) and expressed relative to the density of the  $\beta$ -actin gene PCR product. For analyzing small quantities of RNA following cytotoxicity assays, RNA derived from 106 HEK 293 cells was added to each assay sample before cDNA synthesis (HEK 293 cells do not express insulin or glucagon).

**Analysis of processing of PPI SP epitopes.** In vitro proteasome-mediated digestions were carried out as described previously (14). In brief, 20S proteasomes were purified from an Epstein-Barr virus (immunoproteasome) and a HeLa cell line (constitutive proteasome) as described (48). Low-molecular-mass polypeptide 2 (LMP2) and LMP7 contents were confirmed by 2D immunoblotting. To assess kinetics, digestions were performed during different incubation periods. The PPI<sub>1-30</sub> synthetic peptide (20  $\mu$ g) was incubated with 1  $\mu$ g of purified proteasome at 37°C for 2 and 20 hours in 300  $\mu$ l of proteasome digestion buffer (49). TFA (1%; 30  $\mu$ l) was added to halt the digestion, and samples were stored at -20°C until MS analysis.

**Cross-presentation of PPI SP epitopes.** To examine cross-presentation of PPI SP epitopes by DCs, *FastDC* were generated from a HLA-A2<sup>+</sup> donor based on a method previously described (50) and pulsed with soluble antigen (PPI generated in *E. coli* as described [ref. 9] or purification tag alone as control, both at 20  $\mu$ g/ml) or heat-killed PPI-expressing cells (K562-PPI and K562 as control; 1:1 equivalent ratio with DCs) for 6 hours prior to addition of maturation factors (1,000 IU/ml TNF- $\alpha$  and 10 ng/ml IL-1 $\beta$ , both from Strathmann Biotech; and 1  $\mu$ M PGE<sub>2</sub> from Sigma-Aldrich). After overnight culture, DCs were harvested, washed twice, and cultured with PPI<sub>15-24</sub>-specific T cell clone at a 5:1 ratio (T/DC) for 24 hours, after which supernatants were removed and TNF- $\alpha$  measured by ELISA.

**Statistics.** Differences in frequencies of responses in study groups were compared using Fisher's exact test. ROC plots were generated as described previously (9). The effects of glucose concentration were analyzed by 1-way ANOVA with repeated-measures test and test for linear trend. Comparisons were carried out using GraphPad Prism software and considered significant when *P* was less than 0.05.

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