Type 1 diabetes results from T cell–mediated β-cell destruction. The HLA-A*2402 class I gene confers significant risk of disease and early onset. We tested the hypothesis that HLA-A24 molecules on islet cells present preproinsulin (PPI) peptide epitopes to CD8 cytotoxic T cells (CTLs). Surrogate β-cell lines secreting proinsulin and expressing HLA-A24 were generated and their peptide ligandome examined by mass spectrometry to discover naturally processed and HLA-A24–presented PPI epitopes. A novel PPI epitope was identified and used to generate HLA-A24 tetramers and examine the frequency of PPI-specific T cells in new-onset HLA-A*2402+ patients and control subjects. We identified a novel naturally processed and HLA-A24–presented PPI signal peptide epitope (PPI13-11; LWMRLPILL). HLA-A24 tetramer analysis reveals a significant expansion of PPI13-11–specific CD8 T cells in the blood of HLA-A*2402+ recent-onset patients compared with HLA-matched control subjects. Moreover, a patient-derived PPI13-11–specific CD8 T cell clone shows a proinflammatory phenotype and kills surrogate β-cells and human HLA-A*2402 islet cells in vitro. These results indicate that the type 1 diabetes susceptibility molecule HLA-A24 presents a naturally processed PPI signal peptide epitope. PPI-specific, HLA-A24–restricted CD8 T cells are expanded in patients with recent-onset disease. Human islet cell process and present PPI13-11, rendering themselves targets for CTL-mediated killing. Diabetes 61:1752–1759, 2012

The hallmark of type 1 diabetes is the destruction of insulin-producing β-cells in the pancreas. The processes and immune cells involved are not fully understood as yet, but there is an emerging view that CD8 T cells have a major role in β-cell death. Evidence for this includes the fact that CD8 T cells are the predominant population of lymphocytes infiltrating the islets in newly diagnosed type 1 diabetic patients (1). Moreover, their frequency is inversely correlated with insulin positivity, and once insulin content within an islet is lost, the number of CD8 T cells declines (2). Several studies show an increased expression of HLA class I on islet cells in type 1 diabetic patients (1,3,4), which implies an enhanced susceptibility to CD8 T cell–mediated killing. Finally, in a previous study, we recapitulated the CD8 T-cell pathway of selective β-cell death in vitro by showing that T-cell clones generated from the blood of a patient, which recognize a peptide of preproinsulin (PPI) presented by HLA-A24*0201, can mediate specific β-cell killing (5).

More recent, further weight has been added to the importance of the CD8 T-cell autoantigen/HLA class I pathway by the discovery in a large-scale single nucleotide polymorphism study of allelic forms of HLA class I genes that confer significant type 1 diabetes risk (6). Within these alleles, HLA-A*2402 has a strong disease-predisposing effect (odds ratio ∼1.5) and also is significantly associated with a younger age of diabetes onset (6). The HLA-A*2402 super-type is present in 12–20% of Caucasian and ∼60% of Japanese populations, with HLA-A*2402 being the most common variant (7,8).

We hypothesized that the presence of HLA-A24 molecules confers risk for type 1 diabetes through presentation of epitopes from key β-cell autoantigens and that recognition of these by CD8 cytotoxic T cells (CTLs), when displayed on the β-cell surface, contributes to β-cell death. We therefore examined the HLA-A24 peptide ligandome of a surrogate human β-cell line generated by transfection of HLA-A*2402 and INS genes into an inert HLA-negative carrier cell. We elected to study PPI as the index autoantigen, since there is evidence that proinsulin is the major target of the autoimmune response in children developing type 1 diabetes (9). In the current study, we identify a new epitope naturally processed and presented from the signal peptide of PPI and examine the prevalence of circulating PPI-specific CD8 T cells in newly diagnosed, HLA-A*2402 type 1 diabetic patients. Of import, we show the generation of CD8 T-cell clones specific for the HLA-A24–PPI complex and use cytotoxicity assays to examine the natural processing and presentation of PPI by human islet cells. These data highlight the importance of the PPI signal peptide in shaping the β-cell–specific autoreactive T-cell repertoire in type 1 diabetics and elucidate potential mechanisms through which HLA class I molecules might contribute to the pathogenesis of the disease.

RESEARCH DESIGN AND METHODS

Transfected cell lines. PPI cDNA was inserted between the BamHI and EcoRI sites in pcDNA6/myc-His B vector (Invitrogen, Paisley, Scotland, U.K.) for expression in mammalian cell lines. HLA-A*2402 was cloned from Epstein
Both cell lines; gray the K562-A24 cells (dashed line). Isotype control staining was similar in molecules on K562-A24-PPI cells (solid line) was slightly higher than in antibody W6/32. Expression of major histocompatibility complex I here detected by fl

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of overnight cultures by ELISA (DRG International, Marburg, Germany). Next, molecules was con

supplemented with 10% heat-inactivated FCS and 1% penicillin/streptomycin and

HLA-A*24+

patients with recent-onset type 1 diabetes and 10

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FIG. 3. A: Tandem mass spectrometry analysis of collision-induced dissociation revealing the tandem mass spectrum of a peptide of mass (577.86 m/z) in fraction 33. B: The correct identity of the peptide was proven by tandem mass spectrometry of the synthetic compound. C: Amino acid sequence of the peptide with the expected b- and y-fragment ions. Observed fragment ions are underlined. This confirms PPI11 as naturally processed and presented by HLA-A24 on K562-A24-PII cells. (A high-quality color representation of this figure is available in the online issue.)
molecules on their surface (Fig. 1). We then affinity purified HLA-A24 from bulk cultures of these cells and acid eluted the peptide ligandome. Using stringent mass spectra matching (MASCOT scores ≥40), we identified 179 peptides of 8–12 amino acid length that represent variants of 165 unique peptides, with the vast majority (60%) being 9-mers. In accordance with earlier reports (15,16), we found a strong preference for an aromatic residue in position P2 (90% Tyr or Phe) and a nonhydrophobic amino acid at the c-terminus (89% Leu, Phe, or Ile) (Fig. 2). Within the peptide pool, we identified a sequence of PPI signal peptide (PPI3–11) (Fig. 3A) that was confirmed by the tandem mass spectrometry profile of the synthetic compound (Fig. 3B). It is intriguing that PPI3–11 has a Trp at P2; only 1.9% of 9-mer peptides we identified had a Trp at this position, and Trp at P2 is highly unusual in published algorithms of HLA-A24 binding peptides (15,16). Within our peptide pool of 9-mers, there were no peptides with nucleophilic (Ser, Thr, and Cys), acidic (Asp and Glu), or basic (His, Lys, and Arg) amino acids in P2. There was a strong preference for hydrophobic amino acids at P9, although amino acids with neutral side chains (Trp and Tyr) were also tolerated (Fig. 2). Although at other positions certain amino acids were significantly more frequently found (e.g., Ile, Lys, and Asn in P5), there were no or very few amino acids that were not tolerated at those positions. Overall, only P2 and P9 showed a restrictive amino acid pattern.

Detection of PPI3–11-specific CD8 T cells by HLA-A24 tetramer analysis. We used PPI3–11- and CMV-AYA–loaded HLA-A24 tetramers to assess the frequencies of circulating autoreactive and antiviral CD8 T cells, respectively, in recent-onset HLA-A*24+ patients with type 1 diabetes and in non-diabetic HLA-A*24+ control subjects (Table 1). To address the issue of signal to noise being unfavorable in the detection of rare autoreactive T cells, we used a dual-labeled tetramer approach for identifying PPI3–11-specific CD8 T cells similar to that used in the quantum dot studies of Velthuis et al. (17) for quantification of HLA-A2–restricted autoreactive T cells. Dual-stained PPI3–11 and CMV-AYA tetramer-positive CD8 T cells were clearly distinguishable (Fig. 4A and B and Supplementary Fig. 1). Tetramer-positive CD8 T cells reactive to PPI3–11 were significantly more frequent in recent-onset patients with type 1 diabetes compared with control subjects \((P = 0.02)\) (Fig. 4C). In contrast, CMV-AYA–specific CD8 T cells were detected at similar levels in patients and control subjects \((P = NS)\) (Fig. 4D). There was no significant correlation between the frequencies of PPI3–11- and CMV-AYA–specific CD8 T cells in patients and control subjects.

Isolation of CD8 T-cell clones. At two different time points, we attempted to isolate PPI3–11-specific CD8 T-cell clones from the same index patient with type 1 diabetes. On the first occasion, short-term peptide-stimulated PBMC lines were generated. Single cell sorting of dual PPI3–11 tetramer-positive cells on day 9, followed by expansion in vitro, yielded three clones of sufficient number to identify as dual PPI3–11 tetramer positive (1D5, 1D10, and 2B3) (Supplementary Fig. 2). Cell pellets were stored for TCR clonotyping, but these clones failed to expand further. On the second occasion, 6 months later, sufficient PBMCs from two coordinated blood draws were available to enable

FIG. 4. Representative dual tetramer staining (PE- and APC-labeled) of PBMCs from a patient with type 1 diabetes. Gated, live CD3+CD8 cells are shown stained with PPI3–11-loaded (A) and CMV-AYA–loaded (B) tetramers. C: Percentage of CD8 T cells that are dual tetramer positive when stained with PPI3–11-loaded reagents in 10 patients with recent onset type 1 diabetes and 10 control subjects, all bearing HLA-A*24. Horizontal lines represent medians, and levels are significantly higher in patients than in control subjects. D: Staining with dual tetramers loaded with CMV-AYA. Percentage dual tetramer-positive cells is similar in patients and control subjects.
use of our conventional cloning strategy (5) in which autologous matured DCs pulsed with PPI3–11 are cultured with purified peripheral blood CD8 T cells. Using this strategy, a further three dual PPI3–11 tetramer-positive clones were established (4C6, 3E7, and 1C11) (Fig. 5A–C) and expanded to sufficient quantity for functional analysis. PPI3–11-specific clones did not bind irrelevant tetramer (CMV-AYA) (Supplementary Fig. 3). In addition, a CMV-AYA–specific CD8 T-cell clone was obtained by direct ex vivo single cell sorting of dual CMV-AYA HLA-24 tetramer-positive cells (Fig. 5D). TCR clonotyping established that 1D5, 1D10, and 2B3 from the first cloning and 4C6 and 3E7 (1C11 not tested) from the second cloning 6 months later all expressed the same clonotype: the TCR α-chain gene TRAV5*01 with CDR3-α sequence CAEPSGNTGKLIF and β-chain gene TRBV7–9*03 with CDR3-β sequence CASSLHHEQYF.

Cytotoxic potential of PPI3–11-reactive CD8 T-cell clones. We examined the antigen-specific cytotoxic potential of PPI3–11-reactive CD8 T-cell clone 4C6 initially against K562-A24-PPI cells, which according to the elution studies, naturally process and present PPI3–11, and also against K562-A24 cells pulsed with relevant peptide. Using a range of effector-to-target ratios, killing was specific and approached a maximal ratio of 12:1 when K562-A24 cells were pulsed with PPI3–11 peptide (Fig. 6A). K562-A24-PPI cells, naturally processing and presenting PPI3–11, were also killed, albeit to a lesser extent (Fig. 6A). The specificity of 4C6 for PPI3–11 is indicated by the very low level of killing seen upon coculture of clone with K562-A24 cells pulsed with peptide only (open bars, Fig. 6A) or pulsed with an irrelevant peptide (CMV-AYA) (Fig. 6B). HLA-A24–restricted CD8 T-cell clone cells (1G8) specific for CMV-AYA did not kill K562-A24-PPI cells to any significant degree (Fig. 6C), and this low-level killing lacked specificity since it was similar to that observed when K562-A24 (unpulsed) or K562-A24-PPI cells were used as targets (Fig. 6B and C).

We next examined the cytotoxic potential of the PPI3–11 CD8 T-cell clone on HLA-A*24+ human islet cells. Islet cell numbers were limiting and, therefore, a restricted range of effector-to-target ratios were used selected based on the K562 cell line killing experiments. Islet cells were killed by PPI3–11–specific CD8 T-cell clone 4C6; in contrast, CD8 T-cell clone 1G8 specific for CMV-AYA failed to kill (Fig. 6D). Both PPI3–11– and CMV-AYA–specific clones killed HLA-A*24+ islet cells to a comparable and much higher degree when the targets were pulsed with cognate peptide. The lower level of PPI3–11–mediated killing with unpulsed human islet cells is likely to reflect the heterogeneous nature of the islet cell preparations and the fact that levels of surface peptide-HLA complexes achieved via endogenous processing and presentation do not reach that attained via exogenous pulsing. Only background levels of killing were observed when HLA-A24 PPI3–11 CTL 4C6 or HLA-A24 CMV-AYA CTL 1G8 were cocultured with human HLA-A*0201+ (HLA-A*24+) islet cells (Fig. 6E).

When cocultured with HLA-A*24+ human islets, the PPI3–11–specific CD8 T-cell clone 4C6 secreted effector cytokines,
most notable, macrophage inflammatory protein (MP)-1β and IFN-γ when compared with resting levels (Fig. 7), as well as TNF-α (data not shown). In contrast, the 1G8 CMV-AYA-specific CD8 clone failed to produce cytokine in response to coculture with human islets. Both clones show maximal MIP-1β and IFN-γ secretion when cocultured with cognate peptide-pulsed HLA-A*24+ islets or when stimulated with PMA/ionomycin (Fig. 7).

**DISCUSSION**

The underlying basis for HLA class I gene–associated risk in type 1 diabetes is not known. In an attempt to elucidate this, in the current study we manufactured an HLA class I negative human cell line to express the type 1 disease-risk allele HLA-A24 and also PPI, a major β-cell–specific autoantigen targeted in the disease. We used sensitive mass spectrometry to examine the HLA-associated peptides that these cells display. A 9-mer sequence from the signal peptide region of PPI, LWMRLLPLL, was identified as naturally processed and presented by HLA-A24 under these conditions. This PPI epitope was confirmed as being recognized by CD8 T cells in the peripheral blood using HLA-A24 tetramers loaded with LWMRLLPLL. Moreover, circulating CD8 T cells recognizing this peptide were significantly more frequent in patients with type 1 diabetes than in control subjects matched for HLA-A*24. It is important that disease relevance was substantiated by the demonstration that a CD8 T-cell clone specific for LWMRLLPLL presented by HLA-A*24+ human islet cells to comparable, high levels when targets are pulsed with cognate peptide (black and cross-hatched, respectively). Results are a single experiment, and error bars are SEMs from triplicate wells. E: There is robust, high-level killing of human HLA-A*0201+ islets by the previously characterized CTL clone 3F2, specific for PPI15–24 when presented by HLA-A2 (5) either when the islet cells are pulsed with PPI15–24 (gray bar) or when islets are naturally presenting PPI15–24 (open bar). In contrast, killing of the same HLA-A*2+ islets (unpulsed) is only at background levels in the presence of HLA-A24–restricted 4C6 PPI3–11 CTLs (black bar) and HLA-A24 1G8 CMV-AYA CTLs (cross-hatched bar). Effector-to-target ratio 50:1. Experiments represented in B, C, and E were performed in parallel to one another with the same passage of CTL.
It is intriguing that LWMRLLPLL derives from the signal peptide region that acts as a presequence targeting proinsulin to the endoplasmic reticulum. Signal peptide epitopes are unusual in relation to human disease, but this is the second time that this phenomenon has been observed in the context of type 1 diabetes. Other signal peptide epitopes have also been suggested to play a role in type 1 diabetes, including the prediction that PPI\textsubscript{8-16} would bind DQB1*0602-DQA1*0102 with high affinity (18). In a previous study that uses a similar approach, we showed that the signal peptide epitope PPI\textsubscript{15-24} is naturally presented by HLA-A2 (5). In that study, we also provided evidence that epitope presentation from the signal peptide may not follow a conventional processing route (i.e., it does not require proteasome cleavage and import into the endoplasmic reticulum via transporter associated with processing). Future studies will be required to establish whether the same holds for LWMRLLPLL. For both PPI\textsubscript{15-24} and PPI\textsubscript{3-11}, we confirmed presentation by human islet cells, using CTL lines as probes and cytotoxicity as the readout. This finding rules out the possibility that the discovery of signal peptide epitopes is solely an artifact of using a surrogate β-cell line in the discovery process.

This is the first time that PPI\textsubscript{3-11} has been described as being naturally processed and presented by HLA-A24. It is important that LWMRLLPLL derives from the signal peptide region, which acts as a presequence targeting proinsulin to the endoplasmic reticulum. Signal peptide epitopes are unusual in relation to human disease, but this is the second time that this phenomenon has been observed in the context of type 1 diabetes. Other signal peptide epitopes have also been suggested to play a role in type 1 diabetes, including the prediction that PPI\textsubscript{8-16} would bind DQB1*0602-DQA1*0102 with high affinity (18). In a previous study that uses a similar approach, we showed that the signal peptide epitope PPI\textsubscript{15-24} is naturally presented by HLA-A2 (5). In that study, we also provided evidence that epitope presentation from the signal peptide may not follow a conventional processing route (i.e., it does not require proteasome cleavage and import into the endoplasmic reticulum via transporter associated with processing). Future studies will be required to establish whether the same holds for LWMRLLPLL. For both PPI\textsubscript{15-24} and PPI\textsubscript{3-11}, we confirmed presentation by human islet cells, using CTL lines as probes and cytotoxicity as the readout. This finding rules out the possibility that the discovery of signal peptide epitopes is solely an artifact of using a surrogate β-cell line in the discovery process.

This is the first time that PPI\textsubscript{3-11} has been described as being naturally processed and presented by HLA-A24 molecules. An earlier report by Chang et al. (19) identified PPI\textsubscript{3-11} as being capable of binding to HLA-B15, HLA-B8, and HLA-A2 using HLA class I assembly assays, although of interest, binding to HLA-A24 could not be demonstrated. Toma et al. (20) tested the binding affinity and CD8 T-cell response toward the 10-mer PPI\textsubscript{2-11}, which was identified as a potential epitope of HLA-A24 using a binding algorithm (20). Despite extensive searching for oxidized and nonoxidized forms, we did not identify this 10-mer as being naturally processed and presented by K562-A24-PEPI cells. Moreover, the same group only rarely detected responses to PPI\textsubscript{2-11} in newly diagnosed patients with type 1 diabetes using a sensitive IFN-γ enzyme-linked immunosorbent assay. It remains unclear, therefore, whether a nested set of peptides, similar to the combination of epitopes (PPI\textsubscript{15-24} and PPI\textsubscript{17-24}) that we described previously for HLA-A2, also exists in this region for HLA-A24. Although our approach requires a complex series of techniques that includes the challenge of cloning epitope-specific CD8 T-cells, the advantage of this strategy is that the novel HLA-A24-presented PPI\textsubscript{3-11} epitope is confirmed as naturally processed and presented by both surrogate β-cells and human HLA-A*24+ islet cells.

The disease relevance of the PPI\textsubscript{3-11} epitope is further bolstered by the increased number of circulating PPI\textsubscript{3-11}-specific CD8 T cells that we detected in recent-onset type 1 diabetic patients when compared with control subjects. Since both groups bear HLA-A*24+, there is a strong implication that this expansion is directly associated with either disease development or disease predisposition. The cytotoxicity studies that we conducted in vitro, although using a single CD8 T-cell clonotype from a single patient, offer the proof of concept that a CTL response to PPI\textsubscript{3-11}, in type 1 diabetic patients with HLA-A*24+, is a direct
contributor to β-cell death and, hence, to disease pathogenesis. Future studies on the HLA-A*24+ subset of patients and at-risk subjects will be able to use the PPIs-11-A24 tetramer reagents that we describe here to track, enumerate, and phenotype PPI-reactive CD8 CTLs and establish whether there are temporal links to decline in β-cell mass.

On two separate occasions, 6 months apart, we generated PPIs-11-specific T-cell clones with the same antigen-specific TCR clonotype. This evidence of prolonged existence of circulating, identical, disease-related clonotypes is of considerable interest. Again, the finding resonates with our previous work, in which we showed that PPIs-11-specific CD8+ T cells with identical TCR clonotypes could be isolated from fresh blood samples obtained from the same patient 1.5 years apart (5). The data imply that there may be ancestral clones that are long lived and likely to be dominant in the immune response of an individual patient. It is tempting to speculate that this quality may be a component of the mechanism(s) through which HLA-A24, and comparable disease-risk HLA molecules, render an individual susceptible to disease or an early age of onset. It is plausible that molecules such as HLA-A24 bias the selection of T-cell repertoires, either during positive selection in the thymus or during priming in the periphery, toward a configuration that favors presentation of autoantigenic epitopes that are in turn dominantly and abundantly presented by the β-cell. PPI signal peptide has properties that fit this description, being generated in tune with metabolic demands on the β-cell and being cotranslated with HLA class I molecules at the intracellular location that is critical for their peptide loading.

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D.K. designed and performed experiments, analyzed data, conceived ideas, oversaw research, and wrote the manuscript. R.R.K., M.E., R.J.E., M.G.K., A.d.R., M.E., and G.C.H. performed experiments. J.P. and C.M.D. recruited and characterized patients. A.S. performed experiments, conceived ideas, and oversaw research. P.A.V. designed and performed experiments, analyzed data, conceived ideas, and oversaw research. M.P. designed and performed experiments, analyzed data, conceived ideas, oversaw research, and wrote the manuscript. M.P. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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