Multipeptide-coupled nanoparticles induce tolerance in ‘humanised’ HLA-transgenic mice and inhibit diabetogenic CD8⁺ T cell responses in type 1 diabetes

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Abstract

Aims/hypothesis Induction of antigen-specific immunological tolerance may provide an attractive immunotherapy in the NOD mouse model but the conditions that lead to the successful translation to human type 1 diabetes are limited. In this study, we covalently linked 500 nm carboxylated polystyrene beads (PSB) with a mixture of immunodominant *HLA-A*02:01*-restricted epitopes (peptides-PSB) that may have high clinical relevance in humans as they promote immune tolerance; we then investigated the effect of the nanoparticle–peptide complexes on T cell tolerance.

Methods PSB-coupled mixtures of *HLA-A*02:01*-restricted epitopes were administered to HHD II mice via intravenous injection. The effects on delaying the course of the disease were verified in NOD. $\beta 2m^{null}$ HHD mice. The diabetogenic *HLA-A*02:01*-restricted cytotoxic lymphocyte (CTL) responses to treatment with peptides-PSB were validated in individuals with type 1 diabetes.

Results We showed that peptides-PSB could induce antigen-specific tolerance in HHD II mice. The protective immunological mechanisms were mediated through the function of CD4⁺CD25⁺ regulatory T cells, suppressive T cell activation and T cell anergy. Furthermore, the peptides-PSB induced an

activation and accumulation of regulatory T cells and CD11c⁺ dendritic cells through a rapid production of CD169⁺ macrophage-derived C-C motif chemokine 22 (CCL22). Peptides-PSB also prevented diabetes in ‘humanised’ NOD. $\beta 2m^{null}$ HHD mice and suppressed pathogenic CTL responses in people with type 1 diabetes.

Conclusions/interpretation Our findings demonstrate for the first time the potential for using multipeptide-PSB complexes to induce T cell tolerance and halt the autoimmune process. These findings represent a promising platform for an antigen-specific tolerance strategy in type 1 diabetes and highlight a mechanism through which metallophilic macrophages mediate the early cell–cell interactions required for peptides-PSB-induced immune tolerance.

Keywords Antigen-specific tolerance · Humanised mice · Immunotherapy · Nanoparticles · Type 1 diabetes

Abbreviations

Ag-SP	Antigen-conjugated apoptotic splenocyte
APC	Antigen-presenting cell
CCL22	C-C motif chemokine 22
CCR4	C-C chemokine receptor type 4
CFA	Complete Freund’s adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic lymphocyte
DC	Dendritic cell
ECDI	Ethylene carbodiimide
HIV	Human immunodeficiency virus
IFA	Incomplete Freund’s adjuvant
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
MARCO	Macrophage receptor with collagenous structure

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MΦ	Macrophage
MMΦ	Metallophilic macrophage
MZ	Marginal zone
MZMΦ	Marginal zone macrophage
PBMC	Peripheral blood mononuclear cells
PSB	Polystyrene beads
SFC	Spot-forming cells
Th	T helper
Treg	Regulatory T cell
ZnT8	Zinc transporter-8

Introduction

CD8⁺ T lymphocytes represent a proportion of the final effectors of beta cell destruction in type 1 diabetes [1]. Numerous immunotherapies aimed at inactivating T cells have been investigated. Non-specific systemic immune suppression that targets T cells, co-stimulatory molecules and cytokines does not confer long-term protection, is usually associated with severe systemic side effects and can increase the susceptibility to the development of cancer [2, 3]. Thus, induction of antigen-specific tolerance may represent a desirable diabetes intervention approach in humans [4], including free epitopes [5], high-affinity mimotopes [6] and altered peptide ligand

Fig. 1 Coupling of peptides to 500 nm PSB. **(a)** Release of peptide mixtures coupled to particles as a percentage of the total loading, $n = 3$ per peptide. Green circles, IGRP-PSB; blue squares, ZnT8-PSB; purple triangles, ZnT8/IGRP-PSB. **(b)** Release of each peptide-coupled particle as a percentage of the total loading, $n = 3$ per peptide. Black circles, IGRP-1-PSB; blue squares, IGRP-2-PSB; green triangles, ZnT8-3-PSB; purple inverted triangles, ZnT8-6-PSB; dark purple diamonds, ZnT8-9-PSB. ZnT8-PSB includes ZnT8-6, ZnT8-3 and ZnT8-9; IGRP-PSB includes IGRP-1 and IGRP-2; ZnT8/IGRP-PSB includes ZnT8-6, ZnT8-3, ZnT8-9, IGRP-1 and IGRP-2. Data are representative of three independent experiments

Fig. 2 ZnT8-PSB and IGRP-PSB inhibit *HLA-A*02:01*-restricted diabetogenic CD8⁺ T cell responses. **(a–c)** Lymphocytes within the draining popliteal lymph nodes of HHD II mice that were immunised by each of the ZnT8 peptides were enriched and co-cultured in vitro with PSB bearing an ECDI-coupled cocktail of the *HLA-A*02:01*-restricted ZnT8-3, ZnT8-6 and ZnT8-9 epitopes (ZnT8-PSB) for 24 h and were then co-cultured for 36 h with 10 $\mu\text{mol/l}$ of each of the individual ZnT8 peptides; **(a)** ZnT8-3; **(b)** ZnT8-6; **(c)** ZnT8-9. Antigen reactivity was assessed by ELISpot analyses of IFN- γ production. Each well shown is representative of the response observed in triplicate of wells for mice (without basal subtraction). Each peptide was assayed in triplicate. **(d, e)** HHD II mice were i.v. injected with ZnT8-PSB or IGRP-PSB 7 days after two s.c. immunisations with a mixture of five ZnT8 and IGRP peptides in IFA in the presence of 140 μg of the HBcAg_{128–140} helper peptide. After 5 days of treatment, different T cell subsets were evaluated. **(f–j)** Five days after the PSB treatment, mice were footpad-primed with a cocktail containing 20 μg each of the five *HLA-A*02:01*-restricted ZnT8/IGRP peptides. Ten days after priming, lymphocytes within the draining popliteal lymph nodes were co-cultured for 36 h with 10 $\mu\text{mol/l}$ of each of the individual ZnT8 or IGRP peptides. Antigen reactivity was assessed by ELISpot analyses of IFN- γ production. Each peptide was assayed in triplicate. ELISpot results were represented for the individual assay after subtraction of the mean basal spot number. The basal level was determined by co-culture with negative control peptide (CMV pp65_{495–503}), ranging from 0 to 15 spot-forming cells (SFC)/10⁶. Graphs show data from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

forms of auto-antigens [7]. Although these approaches have been successful in animal models, their translation to the human clinical setting has been largely disappointing [8, 9].

Previous reports have shown that intravenous administration of antigen-conjugated apoptotic splenocytes (Ag-SPs) safely and efficiently induce antigen-specific T cell tolerance in autoimmune diseases. Ag-SPs can deliver peptides to antigen-presenting cells (APCs) and avoid the complications associated with a high dose of intravenous peptides [10–14]. This method has shown promise in clinical trials involving individuals with multiple sclerosis [15]. However, the challenge of separating splenocytes and covalently linking peptides through the use of good manufacturing practices may impede the clinical application of this immunotherapy. Recent studies have shown that the use of Ag-SPs for tolerance induction can treat and protect against experimental autoimmune encephalomyelitis (EAE) [16, 17], transplantation [18] and allergies in animal models [19]. Meanwhile, a number of microparticle-based therapeutics have been approved by the US Food and Drug Administration [20].

However, limited nanoparticle approaches have been identified as targets to attenuate the activity of diabetogenic CD8⁺ T cells, particularly those that recognise *HLA-A*02:01* restricted epitopes, which may have a high clinical relevance in humans. Here, we aimed to evaluate whether nanoparticles coupled with immunodominant *HLA-A*02:01*-restricted zinc transporter-8 (ZnT8_{107–116}, ZnT8_{115–123}, ZnT8_{153–161}) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP_{215–223} and IGRP_{293–301}) epitopes could treat T cell-based autoimmune disorders and suppress the

development of diabetes in humanised mice. Furthermore, we assessed whether this approach could inhibit antigen-specific T cell reactions in individuals with type 1 diabetes.

Methods

Participants Individuals with type 1 diabetes ($n = 24$; 13 male participants, median age 26 years [range 13–48 years]; 11 female participants, median age 25 years [range 9–64 years]) were recruited. The participant characteristics are shown in electronic supplementary material (ESM) Table 1. Individuals with an acute onset of symptoms requiring permanent insulin treatment from the time of diagnosis were diagnosed with type 1 diabetes [21]. All participants were *HLA-A*02:01* (*HLA-A*02:01*) by genotyping and provided written informed consent. The protocol was approved by the ethics committee of The First Affiliated Hospital of Nanjing Medical University and was carried out in agreement with the Declaration of Helsinki as revised in 2008.

Mice Six- to eight-week-old female or male *HLA-A*02:01*-transgenic $\beta 2m^{-/-}$ HHD mice (HHD II mice) were obtained as a gift from F. Lemonnier (Institute Pasteur, Paris) and have been described previously [22]. Female NOD.129P2(B6)-*B2m^{tm1Unc}* Tg(*HLA-A/H2-D/B2M*)1Dvs/DvsJ mice (also called NOD $\beta 2m^{null}$ HHD mice) were from the Jackson Laboratory (Bar Harbor, Maine, USA) [23]. NOD $\beta 2m^{null}$ HHD mice express a monochain chimeric *HLA-A*02:01* molecule consisting of a human $\beta 2$ -microglobulin covalently linked to the $\alpha 1$ and $\alpha 2$ domains of *HLA-A*02:01*, followed by the $\alpha 3$ transmembrane and cytoplasmic portions of H-2D^b [23]. Mice were housed in groups of 4–5 per individually ventilated cage in a pathogen-free facility under a 12 h light/dark cycle and were fed with a standard mouse chow diet. Mice were maintained according to the protocols approved by the Nanjing Medical University Institutional Animal Care and Use Committee.

Peptides Full details of the peptides used in this study are provided in the ESM [Methods](#).

Antibodies and flow cytometry Full details of the antibodies used in this study and the flow cytometry procedures are provided in the ESM [Methods](#).

Particle characterisation The amount of peptide released from the peptide-coupled or peptide-encapsulated nanoparticles was observed. Here, 10 mg of the polystyrene beads (PBS) were washed, resuspended in 1 ml PBS and incubated at 37°C with agitation. At the different time points, the nanoparticles were centrifuged and the peptide in the supernatant fraction was detected using the micro bicinchoninic acid (BCA) protein assay (Thermo Scientific, Waltham, MA, USA).

Immunisation in HHD II mice HHD II mice were immunised by s.c. injection (100 μ l) of 100 μ g ZnT8 and/or IGRP peptides emulsified in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, St Louis, MO, USA) in the presence of 140 μ g of the HBcAg_{128–140} helper peptide. A booster injection of 100 μ g ZnT8 and/or IGRP peptides emulsified in IFA was administered by s.c. injection 14 days later.

Tolerance induction with antigen-coupled microparticles in NOD. $\beta 2m^{null}$ HHD mice Tolerance was induced by an i.v. injection of carboxylated PSB coupled with peptides as described previously [16]. Briefly, carboxylated PSB with a 500 nm diameter were purchased from Polysciences (Warrington, PA, USA). Peptides were linked to the PSB using ethylene carbodiimide (EDCI; Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions (12.5 mg of polystyrene microparticles and 500 μ g of peptide in the presence of 10 mg/ml EDCI). Approximately 9×10^9 microparticles comprising 15–20 μ g of the peptide, depending on the sequence used in the coupling reaction, were i.v. infused in the mice. Two injections were given at 2 week intervals, followed by a treatment once every 3 weeks. The mice were monitored for the development of autoimmune diabetes.

Assessment of diabetes and development of insulinitis Blood glucose levels and insulinitis were analysed as described previously [24]. Further details are provided in the ESM [Methods](#).

ELISpot assay IFN- γ and IL-10 ELISpot assays were performed according to the manufacturer's instructions. Details are provided in the ESM [Methods](#).

Chemotaxis assay To assay chemotaxis, sorted CD8⁺ T cells from immunised HHD II mice were incubated with peptides-PSB for 8 h. Then, the sorted CD169⁺ M Φ s were incubated with apoptotic CD8⁺ T cells at a 5:1 ratio for 24 h. The culture supernatant fractions were seeded in the lower chambers of 96-well plates after collecting and preparing per the manufacturer's instructions for the QCM chemotaxis 5 μ m cell migration assay (Millipore, MA, USA). The upper chambers were seeded with sorted CD4⁺CD25⁺ regulatory T cells (Tregs), CD103⁺CD8⁺ dendritic cell (DCs) or CD8⁺ DCs in a normal culture medium in the presence or absence of the C-C chemokine receptor type 4 (CCR4) antagonist (39 nmol/l C-021 dihydrochloride) (Millipore, MA, USA), C-C motif chemokine 22 (α CCL22) blocking antibody (2 μ g/ml; R&D Systems, Minneapolis, MN, USA) or the isotype control, and the cells were allowed to migrate for 6 h. The plate was then processed and read at 485 nm (excitation)/530 nm (emission). The migration index was determined per the manufacturer's directions.

Fig. 3 Response of antigen-specific T cells to the tolerance induction with peptides-PSB. **(a)** HHD II mice i.v. injected with ZnT8-PSB or IGRP-PSB 7 days after the two s.c. immunisations with a mixture of five ZnT8 and IGRP peptides in IFA in the presence of 140 μ g of the HBcAg_{128–140} helper peptide. After 5 days of treatment, Treg cells were evaluated. Data shown are representative of three separate experiments. **(b–f)** Five days after the PSB treatment, mice were footpad-primed with a mixture of the five ZnT8/IGRP peptides. Ten days after priming, lymphocytes within the draining popliteal lymph nodes were co-cultured for 36 h with 10 μ mol/l of each of the individual ZnT8 or IGRP peptides. Antigen reactivity was assessed by ELISpot analyses of IL-10 production. Each peptide was assayed in triplicate. ELISpot results were represented for the individual assay after subtraction of the mean basal spot

number. The basal level was determined by co-culture with negative control peptide (CMV pp65_{495–503}), ranging from 0 to 5 SFC/10⁶. Graphs show data from two independent experiments. **(g)** HHD II recipient mice exposed to i.v. ZnT8-PSB or CMV-PSB 48 h after being transferred with CD8⁺ T cells sorted from ZnT8-3, ZnT8-6 or ZnT8-9-immunised donor lymph nodes (left) and spleens (right) labelled with CFSE. Flow cytometric analyses of the CFSE dilution 5 days after the priming in mice additionally treated with ZnT8-PSB or CMV-PSB 5 days after the initial treatments and then primed with ZnT8-3, ZnT8-6 or ZnT8-9 plus CFA. Three separate mice were analysed in each group and representative plots are shown. Percentages in the graphs reflect the per cent of T cells that have divided. Data shown are representative of three separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 4 Peptides-PSB induces CCL22 expression in splenic CD169⁺ MMΦs. **(a–d)** HHD II mice immunised with a ZnT8 cocktail in the presence of 140 µg of the HBcAg_{128–140} helper peptide were infused with ZnT8-PSB or PSB alone. *Marco*, *Scarf-1*, *Sign-r1* and *Siglec-1* expression was measured by quantitative PCR 18 h after the infusion. **(e)** At 4 h after the ZnT8-PSB infusion, which was performed as in **(a)**, phagocytes were sorted based on the indicated markers by FACS and the *Ccl22* expression was measured by quantitative PCR; white bars, PSB; black bars, ZnT8-PSB. pos, positive; neg, negative. **(f)** At 4 h after the ZnT8-PSB injection, which was performed as in **(a)**, CD169⁺ MΦs were sorted by FACS and the chemokine expression for the species indicated was measured by quantitative PCR; white bars, PSB; black bars, ZnT8-PSB. Data are representative of three independent experiments. **p* < 0.05

Quantitative PCR For details on the PCR procedures see ESM Methods.

Immunofluorescence and immunohistochemistry Mice were infused with PBS or ZnT8-PSB. After 18 h, the spleen was dissected and fixed in paraformaldehyde. The detailed

Fig. 5 ZnT8-PSB exposure drives rapid Treg and DC migration into the splenic follicle. For the experiments in which CCR4 was inhibited, HHD II mice immunised with a ZnT8 cocktail in the presence of 140 µg of the HBcAg_{128–140} helper peptide were injected i.p. with 1.5 µg of the CCR4 antagonist C-021 dihydrochloride in 100 µl of PBS 6 h before the i.v. injection of ZnT8-PSB. Four hours after ZnT8-PSB administration, the spleen was collected for analysis. **(a)** Splenocytes were analysed by flow cytometry to quantify CD4⁺FoxP3⁺ Treg accumulation after exposure to ZnT8-PSB. **(b)** Representative immunofluorescence staining of splenic sections to determine localisation of CD11c⁺ DCs (red) and FoxP3⁺ Tregs (green) 4 h after the ZnT8-PSB challenge. Scale bars, 200 µm. **(c)** Image analysis of the number of FoxP3⁺ cells per follicle in splenic sections from mice treated as described in **(b)**. **(d, e)** Image analysis of splenic sections from mice treated as described in **(b)** for the semiquantitative analysis of follicular CD11c⁺ DC accumulation and Treg–DC interactions after the ZnT8-PSB challenge. **(f)** Representative FACS analysis histograms showing the surface expression of the markers indicated in the CD25⁺FoxP3⁺ Tregs 4 h after the ZnT8-PSB challenge. Green lines, nil; red lines, ZnT8-PSB; blue lines, CCR4 antagonist (anti) + ZnT8-PSB. PD-1, programmed cell death-1. **(g)** FACS-purified splenic CD169⁺ MΦs were incubated with apoptotic CD8⁺ T cells at a 5:1 ratio. After 24 h, the supernatant fractions from the cultures were analysed for chemotactic activity against sorted CD4⁺CD25⁺ Tregs. In some wells, the neutralising αCCL22 antibody (ab) or the CCR4 antagonist was added to confirm the dependence of the migration on CCL22-dependent mechanisms as described in Methods. **(h)** Histogram analysis of CCR4 expression in the DC populations indicated (i.e. CD11c⁺CD8a⁺CD103⁺). **(i)** Chemotaxis of DCs in response to the apoptotic cell/MMΦ-conditioned (cond.) media was performed as described in **(g)** using FACS-purified DCs with the phenotype indicated. White bars, CD103[−] DC; black bars, CD103⁺ DC. Bars represent the mean value of triplicate samples **(g, i)** of four or more mice per group **(h)** ± SD. Images in **(b)** are representative of five or more mice and are at ×200 magnification. Experiments were repeated at least three times with similar results. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

procedures for the immunohistochemical, morphological analyses are described in ESM Methods.

Isolation of pancreatic lymphocytes Pancreatic lymphocytes were isolated as described previously [25]. Details are provided in the ESM Methods.

Statistical analyses The data were evaluated using Prism 5 software (GraphPad, La Jolla, CA, USA). Data are expressed as mean ± SEM. The log rank test was used to compare the diabetes incidence curves. The differences in the antigen-reactive CD8⁺ T cell numbers between different groups were analysed using a nonparametric unpaired test. Experimenters were blind to outcome assessment but not for group assignment. No data were excluded from analysis.

Results

Coupling of the ZnT8 and IGRP peptides to the PSB particles Initially, we characterised the loading of the CD8-encoded peptides onto the 500 nm PSB. We selected five

*HLA-A*02:01*-restricted antigenic epitopes that may have a high clinical relevance in type 1 diabetes (ESM Table 2) [26]. Peptides containing a mixture of three ZnT8 peptides (ZnT8-PSB), two IGRP peptides (IGRP-PSB) or a cocktail of these five peptides (ZnT8/IGRP-PSB) were bound to the nanoparticles using ECDI. The coupling efficiencies ranged from 65.27% to 90.48%. Between these three groups, the

amount of coupled peptides was comparable and ranged from 26.11 µg/mg to 36.19 µg/mg PSB (ESM Table 3). In vitro release studies demonstrated that low levels of the peptide are released at 2 h for IGRP, ZnT8 and ZnT8/IGRP-PSB (12.21%, 23.37% and 15.05%, respectively). However, a greater amount of IGRP-PSB was released at 72 h in vitro compared with that coupled to ZnT8-PSB or ZnT8/IGRP-PSB (Fig. 1a).

Fig. 6 CCR4 inhibition promotes the PSB-driven proinflammatory cytokine response in the spleen. (**a–d**) Immunised HHD II mice were pretreated with the CCR4 antagonist as described in Fig. 5. After 12 h, the mice were challenged with i.v. ZnT8-PSB and 18 h after ZnT8-PSB administration the spleens were collected and the cytokine protein concentrations in the whole spleen lysate were determined by ELISA. (**e–l**) HHD II mice immunised with the ZnT8 peptide cocktail were treated with

the CCR4 antagonist (anti) and injected with ZnT8-PSB as described in (**a–d**). At 4 h after the ZnT8-PSB challenge, CD169⁺ MMΦs and CD8a⁺CD11c⁺ DCs were sorted by FACS and the cytokine expression for the indicated species was determined by quantitative PCR. White bars, nil; black bars, ZnT8-PSB. Data are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Individual peptide-PSB had similar coupling efficiencies to that of the mixture peptides-PSB, ranging from 52.83% to 93.19% with comparable amounts of peptide (21.13–37.28 $\mu\text{g}/\text{mg}$ PSB) (ESM Table 4). Approximately half of the loaded peptide was released from ZnT8 or IGRP peptide-coupled nanoparticles by 48 h in vitro, with higher amounts of the peptide released by 72 h (Fig. 1b).

Peptides-PSB ablate antigen-specific CD8⁺ T cell responses in HHD II mice We have previously demonstrated that the novel epitopes of ZnT8-3, ZnT8-6 and ZnT8-9 could generate antigen-specific CD8⁺ T cells in HHD II mice, and all of these epitopes are dominant in humans [26]. Thus, we measured the capability of 500 nm PSB coupled with these immunodominant epitopes (ZnT8-PSB) to prevent ZnT8-

specific T cell responses in vitro in HHD II mice. Antigen-specific CD8⁺ T cells were significantly increased in all groups with the related ZnT8-peptide stimulation (Fig. 2a–c). Compared with control mice, CD8⁺ T cells from HHD II mice incubated with ZnT8-PSB displayed significantly decreased responses to the re-stimulation induced by the ZnT8-3, ZnT8-6 and ZnT8-9 epitopes in vitro (Fig. 2a–c). Representative IFN- γ ELISpot results are shown in ESM Fig. 1. A previous study has indicated that Ag-SPs are differentially responsible for the diabetes-protective effects [13]. Hence, we chose to couple ZnT8 and two other immunodominant antigen epitopes (IGRP-1 and IGRP-2), which were demonstrated in our human participants, with PSB to determine whether the peptides-PSB complexes were responsible for the different protective immunity effects in vivo. Because peptides-PSB could induce long-

term T cell tolerance [16], ZnT8-PSB and IGRP-PSB (mixture of IGRP-1 and IGRP-2) were administered intravenously to HHD II mice immunised with an adjuvant mixture of the five ZnT8 and IGRP epitopes. Compared with control mice that received cytomegalovirus (CMV)-PSB, the frequencies of both CD4⁺ and CD8⁺ T cells were slightly reduced in the mice treated with ZnT8-PSB and IGRP-PSB. In addition, the number of T helper (Th) 1 cells was decreased in the HHD II mice treated with PSB. However, no significant differences were found in the number of Th17 cells in these treated mice (Fig. 2d). Treatment with ZnT8-PSB and IGRP-PSB also significantly decreased the activated CD4⁺ T cell (CD62L^{low}CD44^{high}) responses. Although the activated CD8⁺ T cell (CD62L^{low}CD44^{high}) decreased in the mice treated with peptides-PSB, no statistically significant differences were found among the groups. Conversely, the concentration of naive CD4⁺ and CD8⁺ T cells (CD62L^{high}CD44^{low}) was significantly higher in the mice treated with peptides-PSB than in their control mice (Fig. 2e).

In the HHD II mice treated with ZnT8-PSB or IGRP-PSB, antigen-specific CD8⁺ T cell responses to both the HLA-A*02:01-restricted ZnT8 and IGRP peptides were significantly decreased compared with control mice (Fig. 2f–j). Interestingly, treatment with ZnT8-PSB showed a more significant reduction in CD8⁺ T cell responses to the IGRP-1 peptide than treatment with IGRP-PSB (Fig. 2f). These in vivo assays further demonstrated that the HHD II mice treated with peptides-PSB are tolerant to the peptides, as shown by reduced T cell frequencies and IFN- γ secretion following a challenge with the ZnT8 and IGRP peptides.

Peptides-PSB induce Tregs and IL-10 production and abort T cell proliferation A previous study showed that the induced T cell tolerance was partially dependent on the production of antigen-specific Tregs and IL-10 [16]. The FoxP3⁺ Tregs were significantly increased after treatment with either ZnT8-PSB or IGRP-PSB in immunised HHD II mice (Fig. 3a). Treatment with ZnT8-PSB and IGRP-PSB resulted in a significantly higher increases in IL-10 cytokines in response to the IGRP or ZnT8 peptides (Fig. 3b–f). Representative IL-10 ELISpot results are shown in ESM Fig. 2. Altogether, these data indicate that both ZnT8-PSB and IGRP-PSB are significantly important for inducing antigen-specific CD8 T cell tolerance in HHD II mice.

To further investigate the effects of the peptides-PSB on T cell activation and proliferation, we immunised HHD II mice with the ZnT8-3, ZnT8-6 or ZnT8-9 epitope. Then, we adoptively transferred carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled CD8⁺ T cells from these immunised mice into naive HHD II mice. After 48 h, we administered 9×10^9 ZnT8-PSB or CMV-PSB via i.v. injection. After 5 days, we administered ZnT8-3, ZnT8-6 or ZnT8-9 plus complete Freund's adjuvant (CFA) via s.c. injection. The ZnT8-specific T cells isolated from both the spleen and lymph

nodes of the mice treated with ZnT8-PSB exhibited a significantly lower proliferative capacity than those from the control mice (Fig. 3g).

Peptides-PSB induces CCL22 expression specifically in CD169⁺ MM Φ Splenic function plays an important role in systemic tolerance by clearing apoptotic cells [27]. Splenic macrophages (M Φ s) are involved in the rapid phagocytosis of different pathogens and other particles [28]. Previous studies have also demonstrated that treatment with peptides-PSB increases the expression of some scavenger receptors [16]. We found that the scavenger receptors *Marco* and *Scarf-1* were upregulated in the spleen following the administration of ZnT8-PSB (Fig. 4a, b). However, no significant difference was found in the expression *Sign-r1* (Fig. 4c). The spleen cells containing ZnT8-PSB also induced the expression of *Siglec-1*, which is known as a metallophilic macrophage (MM Φ) marker (Fig. 4d). Overall, the data indicate that i.v. administration of peptides-PSB specifically localises to macrophage receptor with collagenous structure (MARCO)⁺ marginal zone macrophages (MZM Φ s) and MM Φ s. These M Φ s act as scavenger cells, developing anti-inflammatory responses.

In the spleen, MZM Φ s and marginal zone (MZ) DCs actively phagocytose apoptotic cells from circulation [29]. After activation, both M Φ s and DCs can produce CCL22 [30]. CCL22 also plays a key role in Treg recruitment and immune suppression [31]. When FACS-sorted splenic phagocytes (see ESM Fig. 3) were examined, only CD169⁺ MM Φ s and CD8⁺ DCs showed an increase in the expression of *Ccl22* (Fig. 4e). In CD169⁺ MM Φ s, the CCL22 response is specific and unique to apoptotic cell exposure [32]. Our data also confirm that peptides-PSB did not induce significant expression of C-C motif chemokines other than *Ccl22* in the CD169⁺ MM Φ s (Fig. 4f).

Peptides-PSB induce rapid DC and Treg accumulation in the MZ Apoptotic cell-driven MZM Φ activation potently stimulated Treg migration. This response was dependent on CCL22-mediated recruitment via CCR4 [33]. To determine whether this mechanism is relevant to the administration of ZnT8-PSB, we challenged mice with an injection of a CCR4 antagonist after immunisation with a ZnT8 cocktail and then examined FoxP3⁺ Treg accumulation 4 h after pretreatment with ZnT8-PSB. Mice injected with ZnT8-PSB exhibited significantly more splenic FoxP3⁺ Tregs than the control mice, and this effect was completely inhibited by a treatment with the CCR4 antagonist (Fig. 5a). Treg recruitment primarily occurred in the splenic MZ of the mice treated with ZnT8-PSB (Fig. 5b), suggesting that CCL22 might increase Treg chemotaxis to the spleen, promoting the increased expression of Tregs in the organ. Pretreatment with the CCR4 antagonist prevented this increase in Tregs, resulting in splenic FoxP3 staining that was similar to that in the control groups (Fig. 5b, c). Meanwhile, the CCL22- and CCR4-dependent follicular

accumulation of CD11c⁺ cells was recruited from the MZ rather than systemically (Fig. 5b, d). Moreover, image analysis showed that there was a significant increase in follicular Treg–DC interactions in the follicle in the mice treated with ZnT8-PSB (Fig. 5b, e). In response to peptides-PSB, splenic Tregs rapidly upregulated surface cytotoxic lymphocyte (CTL) A-4, whereas CD103 and programmed cell death-1 (PD-1) expression was not changed (Fig. 5f). Moreover, pre-treatment with the antagonist prevented surface CTLA-4 expression (Fig. 5f). Furthermore, when the MMΦs were cultured with apoptotic CD8⁺ T cells treated with ZnT8-PSB, the

conditioned media induced Treg migration and this effect was inhibited by adding either a CCL22-neutralising antibody or the CCR4 antagonist (Fig. 5g). CD8⁺CD103⁺ DCs preferentially present apoptotic cell-associated antigen in the spleen and induce Treg differentiation and promote T cell tolerance [34, 35]. We found that CCR4 was significantly more highly expressed in the CD103⁺ DCs than in the CD103[−] DCs in the mice treated with ZnT8-PSB (Fig. 5h), suggesting that CD8⁺CD103⁺ DCs may preferentially migrate in response to CCL22 production. Consistent with this result, we found that interactions between apoptotic CD8⁺ T cells and MMΦs

Fig. 7 Treatment with peptides-PSB prevents diabetes in NOD. $\beta 2m^{null}$ HHD mice. **(a)** The incidence of diabetes in peptides-PSB treated mice over the treatment period. Female NOD. $\beta 2m^{null}$ HHD mice were treated with ZnT8-PSB, IGRP-PSB and ZnT8/IGRP-PSB with two injections at 2 week intervals starting at 8 weeks of age and the incidence of diabetes was monitored until 28 weeks of age compared with the incidence in the CMV-PSB control group. **(b)** Survival curves in peptides-PSB treated mice over the treatment period, including mice with and without diabetes. Female NOD. $\beta 2m^{null}$ HHD mice were treated with IGRP-PSB, ZnT8-PSB and ZnT8/IGRP-PSB with two injections at 2 week intervals starting at 8 weeks of age. Survival analysis was observed from 8 to 28 weeks of age. There was a significant increase in the survival time of mice treated with peptides-PSB compared with mice treated with CMV-PSB. Graph shows pooled data from two independent experiments. In **(a, b)**, black circles, CMV-PSB ($n = 16$); blue squares, PSB ($n = 16$); brown triangles, mixture of IGRP peptides ($n = 15$); green diamonds, mixture of ZnT8 peptides ($n = 15$); orange circles, mixture of ZnT8 and IGRP peptides ($n = 15$); red squares, ZnT8/IGRP-PSB ($n = 16$); dark purple diamonds, ZnT8-PSB ($n = 16$); purple hexagon, IGRP-PSB ($n = 16$). **(c)** Representative pictures of the islets in the 28-week-old ‘non-diabetic’ mice from **(a)**. Scale bars, 100 μm . **(d)** Infiltration of pancreatic islets in 28-week-old ‘non-diabetic’ mice from **(a)** quantified as percentages of islets showing the indicated histological scores. Ten sections per pancreas were blindly scored for insulinitis (0 = no infiltrate, 1 = 0–25%, 2 = 25–75%, 3 = > 75%). White bars, 0; light grey bars, 1; dark grey bars, 2; black bars, 3. **(e)** Pancreatic lymphocytes from mice receiving IGRP-PSB, ZnT8-PSB or ZnT8/IGRP-PSB were isolated from the pancreas at the end of the observation time (28 weeks). Foxp3⁺ Treg cells were evaluated. Data are representative of three independent experiments. **(f)** Consecutive pancreas tissue sections obtained from mice receiving IGRP-PSB, ZnT8-PSB or ZnT8/IGRP-PSB at the end of the observation time (28 weeks) were stained for insulin, CD4 and CD8. Scale bars, 100 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

mediated a significant migration of CD8⁺CD103⁺ DCs in a CCL22- and CCR4-dependent manner (Fig. 5i). In contrast, CD8⁺CD103[−] DCs were independent of CCL22/CCR4 and did not show an apoptotic cell-dependent migratory capacity (Fig. 5i).

Because CCL22 expression increased rapidly after the peptides-PSB administration and was a driver of Treg accumulation, we hypothesised that regulatory cytokine production may be based on CCL22-mediated Treg recruitment. We found that ZnT8-PSB prominently increased splenic TGF- β and IL-10. When CCR4 was blocked, the induction of IFN- γ and IL-12 was significantly increased (Fig. 6a, b), and the induction of TGF- β and IL-10 was significantly decreased (Fig. 6c, d). These findings suggest that deficiency in the CCL22-dependent Treg recruitment led to the formation of a proinflammatory environment.

We further confirmed whether CCL22-mediated Treg recruitment could promote the prevention of inflammatory cytokine production and the acquisition of a regulatory phenotype in M Φ s and DCs following peptides-PSB treatment. *Tgf- β* transcription increased in CD169⁺ M Φ s ($p = 0.0038$) and CD8⁺ DCs ($p = 0.0194$) (Fig. 6g, k) after a ZnT8-PSB challenge. Similarly, the *Il-10* expression was increased in the CD169⁺ M Φ s ($p = 0.0006$) and CD8⁺ DCs ($p = 0.0017$)

following i.v. injection of ZnT8-PSB (Fig. 6h, l). However, when CCR4 function was inhibited by the pretreatment with an antagonist, the peptide-PSB-driven increases in *Tgf- β* and *Il-10* mRNA levels were blocked in both CD169⁺ M Φ s ($p = 0.0015$, $p = 0.0437$, respectively) and CD8⁺ DCs ($p = 0.0362$, $p = 0.0020$, respectively) (Fig. 6g, h, k, l) with a concomitant significant increase in *Il-12p40* and *Il-6* in CD169⁺ M Φ s ($p < 0.0001$, $p = 0.0059$, respectively) (Fig. 6e, f, i, j).

Peptides-PSB prevent autoimmune diabetes in NOD. $\beta 2m^{null}$ HHD mice

To further assess whether peptides-PSB would interfere with disease development, we administered ZnT8-PSB, IGRP-PSB and ZnT8/IGRP-PSB to 8-week-old NOD. $\beta 2m^{null}$ HHD mouse models of diabetes. In the control groups, the mice developed diabetes at 12 weeks of age. The incidence of diabetes gradually increased over the treatment period. In contrast, only 12.5–18.75% of mice receiving peptides-PSB developed diabetes during the treatment period (Fig. 7a). Moreover, treatment with ZnT8-PSB and ZnT8/IGRP-PSB significantly delayed the development of diabetes and improved survival rates (Fig. 7a, b). Although the mice treated with IGRP-PSB similarly reduced the incidence of diabetes, the difference was not statistically significant (Fig. 7a). Treatment with IGRP-PSB only improved survival rates (Fig. 7b). The protection observed in the animals treated with peptides-PSB was accompanied by diminished islet infiltration (Fig. 7c, d) as determined by histological examination. In contrast to control mice, the number of FoxP3⁺ Treg cells was significantly increased in the pancreas after treatment with peptides-PSB (Fig. 7e). The mice receiving peptides-PSB showed a reduced degree of CD4⁺ and CD8⁺ T cell infiltration (Fig. 7f). It must be noted here that only non-diabetic mice in these groups were analysed at 28 weeks of age. These results show that treatment with peptides-PSB is essential for ameliorating insulinitis and delaying disease progression in mouse models of type 1 diabetes.

Peptides-PSB ablate antigen-specific CD8⁺ T cell responses in individuals with type 1 diabetes

The peptides-PSBs were subsequently tested for antigen-specific T cell responses using peripheral blood mononuclear cells (PBMCs) from individuals with *HLA-A*02:01*-restricted new-onset type 1 diabetes who were previously positive for these epitopes. PBMCs were co-cultured in vitro with ZnT8-PSB, IGRP-PSB or ZnT8/IGRP-PSB, and antigen-specific CD8⁺ T cells for each individual epitope were detected by ELISpot assays. Compared with those exposed to the control human immunodeficiency virus (HIV)-PSB, CD8⁺ T cells co-cultured with ZnT8-PSB displayed significantly diminished responses to the re-stimulation with the ZnT8-3, ZnT8-6 and ZnT8-9 epitopes (Fig. 8a–c). Similarly, IGRP-PSB significantly decreased the ability of CD8⁺ T cells to respond to re-

stimulation with IGRP-1 and IGRP-2 (Fig. 8d, e). Furthermore, the co-incubation with ZnT8/IGRP-PSB diminished the CD8 T cell responsiveness to the IGRP, ZnT8-3 and ZnT8-9 epitopes (Fig. 8f). Although the ZnT8-6 epitope-specific T cell responses were reduced by ZnT8/IGRP-PSB treatment, the effect did not quite achieve statistical significance (Fig. 8f).

Discussion

Our study has demonstrated for the first time that an i.v. injection of a PSB-coupled cocktail of *HLA-A*02:01*-restricted peptides can induce antigen-specific tolerance in HHD II mice, inhibit the development of diabetes in ‘humanised’ NOD. $\beta 2m^{null}$ HHD mice, and may have potentially high clinical relevance in humans. Furthermore, these peptides-PSB complexes could suppress antigen-specific CD8⁺ T cell responses in individuals with type 1 diabetes.

Recently, the use of nanoparticles for immune regulation has been a promising approach in animal models of diseases [20, 36]. Here, we found that only 1.32 μg peptide, which was administered i.v. twice, induced long-term tolerance. This dose is much lower than the 20 μg of soluble IGRP that was administered three times to the NOD. $\beta 2m^{null}$ HHD mice [13], suggesting that the nanoparticle form has a significantly higher efficiency. Tolerance induced by high levels of soluble peptide has been successful in animal models [37, 38]. However, a range of adverse effects have been occasionally

observed, such as an exacerbation of the existing disease and fatal anaphylaxis [14]. Owing to these safety problems, the nanoparticles platform is an attractive strategy for avoiding high concentrations of peptides in vivo. Niens et al found that insulin (INS)/IGRP or IGRP peptides coupled to splenocytes effectively inhibited IGRP-specific CD8⁺ T cell responses and the progression of diabetes in NOD. $\beta 2m^{null}$ HHD mice [13]. However, we found that the treatment effect of IGRP-PSB was not better than that of ZnT8-PSB or ZnT8/IGRP-PSB in either ablating antigen-specific CD8⁺ T cell responses or delaying the development of diabetes. A greater amount of IGRP peptides was released in vitro compared with that of the other peptides over a 72 h period at 37°C. The stability was suggested to be able to affect the nanoparticle recognition and uptake by APCs.

MΦs and DCs can selectively endocytose nanoparticles [39, 40]. Previous data have shown that MZMΦs have an important effect on immunological surveillance in the spleen, clearing apoptotic cells and suppressing immune responses immediately after antigen exposure. Significantly, DCs are functionally influenced by MZMΦs through the phagocytosis of apoptotic cells and the regulation of TGF- β production [27, 41]. Nanoparticles, as vehicles for peptide delivery, have been demonstrated to be able to be taken up by MZMΦs expressing the scavenger receptor MARCO and halt the disease process in a model of EAE [16]. Our data further showed that peptides-PSB complexes captured by MZ CD169⁺ MΦs and DCs rapidly produced the chemokine CCL22, which could promote the migration and activation of Tregs. Thus, MΦ–

Fig. 8 Peptides-PSB inhibit the in vitro responses of *HLA-A*02:01*-restricted ZnT8 and IGRP autoreactive CD8⁺ T cells from individuals with new-onset type 1 diabetes. PBMCs were co-cultured for 24 h with 20 $\mu\text{mol/l}$ of each of the individual ZnT8 or IGRP peptides. Antigen reactivity was assessed by ELISpot analyses of IFN- γ production. (a–e) T cell responses to ZnT8-PSB or IGRP-PSB from PBMCs from individuals with *HLA-A*02:01*-restricted new-onset type 1 diabetes who were previously positive for the ZnT8 or IGRP epitope. (f) T cell responses to

ZnT8/IGRP-PSB from PBMCs from individuals with *HLA-A*02:01*-restricted new-onset type 1 diabetes who were previously positive for the ZnT8 and IGRP epitopes. White bars, IGRP-1; black bars, IGRP-2; dark grey bars, ZnT8-3; light grey bars, ZnT8-6; diagonal stripe bars, ZnT8-9. The results are expressed as the mean IFN- γ SFC/10⁶ PBMCs and were background-subtracted. The basal level was determined by co-culture with negative control peptide (HIV_{gag77–85}), ranging from 0 to 17.5 SFC/10⁶. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

DC communication could be important for the initial generation of innate immune suppression and induction of a regulatory phenotype. Our data suggest that CD169⁺ MΦs and CD8⁺ DCs provide the link between innate immunity and adaptive tolerance in peptides-PSB immunotherapy. Additionally, previous studies have demonstrated that both CD4⁺ and CD8⁺ autoimmune T cells could be potentially suppressed by cross-presentation of apoptotic cell antigens [42, 43].

We also observed that MZ CD169⁺ MΦs and DCs produce CCL22 to mediate Treg chemotaxis and immune suppression. CCL22, also known as MΦ-derived chemokine [44], has been shown to induce chemotaxis in activated T cells, and CCR4 is a cognate receptor for CCL22 [31, 45]. Certain stimuli can also promote the expression of CCR4 by DCs [46]. Expression of CCL22 in splenic CD169⁺ MΦs promoted the accumulation of tolerogenic splenic DCs [32]. In addition, CD103⁺ DC promoted Foxp3⁺ Treg enrichment [47]. Importantly, antagonising CCL22 inhibited the tolerogenic DC maturation [32]. Here, we also showed that the inhibition of CCR4 had a major influence on CD103⁺ DC chemotaxis following peptides-PSB treatment.

In our study, we demonstrated that ZnT8-PSB and ZnT8/IGRP-PSB were more effective than IGRP-PSB in preventing the development of diabetes in NOD. $\beta 2m^{null}$ HHD mice. These results did not absolutely coincide with those from our human participants. Previous studies have demonstrated that the IGRP antigen could be a non-essential target for autoimmune diabetes in animal models [48, 49]. However, we found that several ZnT8-specific CD8⁺ T cell responses were also caused by treatment with ZnT8/IGRP-PSB in humans with type 1 diabetes. Thus, our data indicate that targeting the ZnT8 antigen may also be required to effect diabetes development in humans.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

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