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SHORT COMMUNICATION

A selective CD28 antagonist and rapamycin synergise to protect against spontaneous autoimmune diabetes in NOD mice

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Abstract

Aims/hypothesis The CD28/B7 interaction is critical for both effector T cell activation and forkhead box P3 (FOXP3)$^+$ regulatory T cell (Treg) generation and homeostasis, which complicates the therapeutic use of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)–immunoglobulin fusion protein (CTLA-4Ig) in autoimmunity. Here, we evaluated the impact of a simultaneous and selective blockade of the CD28 and mammalian target of rapamycin (mTOR) pathways in the NOD mouse model of type 1 diabetes.

Methods NOD mice were treated with PEGylated anti-CD28 Fab′ antibody fragments (PV1-polyethylene glycol [PEG], 10 mg/kg i.p., twice weekly), rapamycin (1 mg/kg i.p., twice weekly) or a combination of both drugs. Diabetes incidence, pancreatic islet infiltration and autoreactive T cell responses were analysed.

Results We report that 4 week administration of PV1-PEG combined with rapamycin effectively controlled the progression of autoimmune diabetes in NOD mice at 10 weeks of age by reducing T cell activation and migration into the pancreas. Treatment with rapamycin alone was without effect, as was PV1-PEG monotherapy initiated at 4, 6 or 10 weeks of age. Prolonged PV1-PEG administration (for 10 weeks) accelerated diabetes development associated with impaired peripheral Treg homeostasis. This effect was not observed with the combined treatment.

Conclusions/interpretation CD28 antagonist and rapamycin treatment act in a complementary manner to limit T cell activation and infiltration of pancreatic islets and diabetes development. These data provide new perspectives for the treatment of autoimmune diabetes and support the therapeutic potential of protocols combining antagonists of CD28 (presently in clinical development) and the mTOR pathway.

Keywords CD28 antagonist · Combination therapy · NOD mice · Rapamycin · Type 1 diabetes

Alix Besançon and Tania Goncalves contributed equally to this work.

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Abbreviations

APC	Antigen-presenting cell
CIA	Collagen-induced arthritis
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLA-4Ig	Cytotoxic T lymphocyte-associated antigen 4–immunoglobulin fusion protein
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
ELISpot	Enzyme-linked immunospot
FOXP3	Forkhead box P3
IGRP	Islet-specific glucose-6-phosphatase catalytic-subunit-related protein
mTOR	Mammalian target of rapamycin
PEG	Polyethylene glycol
PI	Proinsulin
PI3K	Phosphoinositide 3-kinase
PV1-PEG	PEGylated CD28-specific Fab′ fragments

Research in context

What is already known about this subject?

- The CD28/B7 interaction is critical for both effector T cell (Teff) activation and forkhead box P3 (FOXP3)$^+$ regulatory T cell (Treg) generation and homeostasis
- Administration of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)–immunoglobulin fusion protein or rapamycin does not provide significant protective effects for type 1 diabetes in the NOD mouse model or individuals with type 1 diabetes
- Selective antagonism of CD28 has shown efficacy in preventing graft rejection and antigen-induced autoimmunity in mouse and non-human primate experimental models

What is the key question?

- Can simultaneous blockade of CD28 and mammalian target of rapamycin (mTOR) pathways impact the development of autoimmune diabetes?

What are the new findings?

- Short-term treatment with selective CD28 antagonist and rapamycin prevents autoimmune diabetes in NOD mice
- The combination therapy inhibits T cell activation and migration into the pancreas
- Prolonged administration of selective CD28 antagonist alone accelerates autoimmune diabetes by targeting Tregs

How might this impact on clinical practice in the foreseeable future?

- These data offer new perspectives for the treatment of autoimmune diabetes as the human CD28 antagonist FR104 has recently been tested in healthy individuals

SFU	Spot-forming units
TCR	T cell receptor
Teff	Effector T cell
Treg	Regulatory T cell
VPD	Violet Proliferation Dye

Introduction

Targeting costimulatory molecules to inhibit T cell activation has emerged as a promising approach to tackle the aberrant immune response to self-antigens that occurs in autoimmunity. In type 1 diabetes, treatment of recently diagnosed individuals with the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)–immunoglobulin fusion protein (CTLA-4Ig) abatacept has had mixed results. It slowed the decline of beta cell function, but insulin needs remained similar to those of placebo-treated individuals [1]. CTLA-4Ig therapy has several drawbacks as it antagonises the CTLA-4/B7 interactions that deliver inhibitory signals to T cells and that are key for CD4$^+$ forkhead box P3 (FOXP3)$^+$ regulatory T cell (Treg) functions, as evidenced in NOD mice [2, 3].

Understanding and exploiting the role of CD28 in T cell biology remains an active area of experimental and clinical investigation [4]. We recently showed in non-human primates that monovalent PEGylated antagonistic forms of a human CD28 antibody (FR104), promoted cardiac and kidney allograft survival in association with immunosuppressive drugs [5, 6] and prevented the development of acute experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) [7, 8]. In addition, FR104 treatment prolonged human skin graft survival in humanised mice [9]. Here, we evaluated the impact of a short-term course of PEGylated anti-CD28 Fab′ antibody fragment (PV1-polyethylene glycol [PEG]) in adult NOD mice and its therapeutic potential on disease development when combined with rapamycin.

Methods

Mice Wild-type NOD and CD28$^{-/-}$ NOD mice [2] were bred in our animal facility under specific pathogen-free conditions and with free access to food and water. CD28$^{-/-}$ NOD mice were provided by J. Bluestone (University of California, San Francisco, CA, USA). Blood glucose levels were measured using an Accu-Chek Performa glucometer (Roche Diabetes Care, Meylan, France). A value >13.9 mmol/l signalled diabetes onset. Four to 10 week-old female mice were used in this study. The experiments were approved by the French Ministry of Education and Research (number 04463.02).

In vivo treatments PV1-PEG was produced by OSE Immunotherapeutics (Nantes, France). Fab′ fragments were obtained from hamster anti-mouse CD28, clone PV1, and subsequently conjugated to a 40 kDa PEG moiety to prolong its half-life in vivo. PV1-PEG was administered to NOD mice at 10 mg/kg i.p. (in PBS), twice weekly for 4 or 10 consecutive weeks. Rapamycin (Sigma-Aldrich, Lyon, France) was reconstituted according to the manufacturer's instruction and injected i.p. at 1 mg/kg twice weekly for 4 weeks. These treatments were randomly allocated to each group of mice.

Flow cytometry Antibodies to CD4 (GK1.5, 1/300), CD8 (53-6.7, 1/300), T cell receptor (TCR) Vβ (H57-597, 1/400), CD44 (IM7, 1/400), CD62L (MEL-14, 1/300) and CD19 (1D3, 1/400) were obtained from BD Biosciences (Le Pont de Claix, France). Antibody to FOXP3 (FJK-16S, 1/200) was obtained from eBioscience (Life Technologies, Saint-Aubin, France). All antibodies were titrated before use. Detection of autoantigen-specific $CD8^+$ T cells was performed using allophycocyanin-labelled MHC class I ($H\text{-}2K^d$) tetramers carrying the proinsulin $(PI)_{15–23}$ or islet-specific glucose-6-phosphatase catalytic-subunit-related protein $(IGRP)_{206–214}$ peptides (NIH Tetramer Core Facility, Atlanta, GA, USA). Cells were analysed on a FACSCanto II cytometer using FlowJo software (FlowJo, Ashland, OR, USA).

IFNγ enzyme-linked immunospot Polyvinylidene fluoride (PVDF) plates (Merck Millipore, Guyancourt, France) were coated with an IFNγ capture antibody (U-CyTech, Utrecht, the Netherlands). Splenocytes were cultured at 2.5×10^5/well with $PI_{15–23}$ or $IGRP_{206–14}$ peptides (7 μmol/l). CD3 antibody (145 2C11, 1 μg/ml) was used as a positive control. After a 20 h culture period, IFNγ was detected using biotinylated IFNγ antibody, streptavidin–horseradish peroxidase and Sigma*FAST* nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma-Aldrich). IFNγ spot readouts were expressed as spot-forming units (SFU)/10^6 cells.

In vitro suppression assay $CD4^+CD25^-$ Teffs and $CD4^+CD25^+$ Tregs were isolated by magnetic sorting (Miltenyi Biotec, Paris, France). Violet Proliferation Dye (VPD)450-labelled Teffs from untreated NOD mice were incubated with Tregs (5×10^4 cells/well each) isolated from treated NOD mice. Cells were stimulated with CD3 antibody and antigen-presenting cells (APCs: irradiated syngeneic splenocytes, 10^5 cells/well) for 3 days. Proliferation was evaluated by VPD450 dilution.

Inhibition of proliferation (%) = [1 − (%Teffs that divided more than three times in the presence of Tregs)/(% $CD4^+CD25^-$ that divided more than three times alone)] ×100.

Analysis of pancreatic T cell infiltrate Pancreases were perfused via the pancreatic duct with collagenase P (Roche Diagnostics, Mannheim, Germany), recovered and digested for 8 min at 37°C. After washing with Hank's balanced salt solution (HBSS) + 10% (vol./vol.) FBS and vigorous shaking to allow islet disruption, pellets were resuspended in PBS + 2% FBS before staining.

Statistical analysis All statistical analyses were performed using GraphPad Prism 6 software (Graphpad Software, La Jolla, CA, USA). The occurrence of diabetes was plotted using the Kaplan–Meier method. Statistical comparison between the curves was performed using the logrank (Mantel–Cox) test. When appropriate, results were analysed using the Mann–Whitney test.

Results

PV1-PEG monotherapy exacerbates diabetes The inhibitory effect of PV1-PEG was first tested in vitro on T cell proliferation. The proportion of VPD450-labelled $CD4^+$ and $CD8^+$ T cells that divided following CD3 antibody stimulation significantly decreased in the presence of PV1-PEG in a dose-dependent manner (electronic supplementary material [ESM] Fig. 1a, b). The impact of CD28 blockade was further analysed on $CD8^+$ T cell responses to beta cell-derived antigens using IFNγ enzyme-linked immunospot (ELISpot). A modest inhibition of IFNγ response to MHC I-restricted $PI_{15–23}$ and $IGRP_{206–214}$ peptides was observed only with high doses of PV1-PEG (50 μg/ml) (ESM Fig. 1c).

We next administered PV1-PEG, 10 mg/kg twice weekly for 4 weeks, to 4, 6 or 10 week old NOD mice to test whether transient CD28 blockade altered the development and/or activation of diabetogenic T cells. The diabetes incidence in all treated groups was similar to that in control mice (Fig. 1a). $CD8^+$ T cell IFNγ responses to $IGRP_{206–214}$ and $PI_{15–23}$ peptides were comparable in all groups (Fig. 1b). Similarly, pancreatic lymph nodes of NOD mice treated with PV1-PEG at 6 weeks of age displayed proportions of $PI_{15–23}$- or $IGRP_{206–214}$-specific $CD8^+$ T cells similar to those found in untreated NOD mice (Fig. 1c, d).

When administered for 10 consecutive weeks, starting at 6 weeks of age, PV1-PEG accelerated diabetes development: 91.6% vs 40% at 20 weeks of age (Fig. 1e). The frequency and absolute number of Tregs were significantly reduced in PV1-PEG-treated mice, which presented an intermediate phenotype between wild-type and $CD28^{-/-}$ NOD mice, exhibiting exacerbated diabetes resulting from a drastic decline in natural Tregs [2, 3, 10] (Fig. 1f, g).

Combining PV1-PEG with rapamycin prevents diabetes development In transplantation, combining CD28 antagonists with

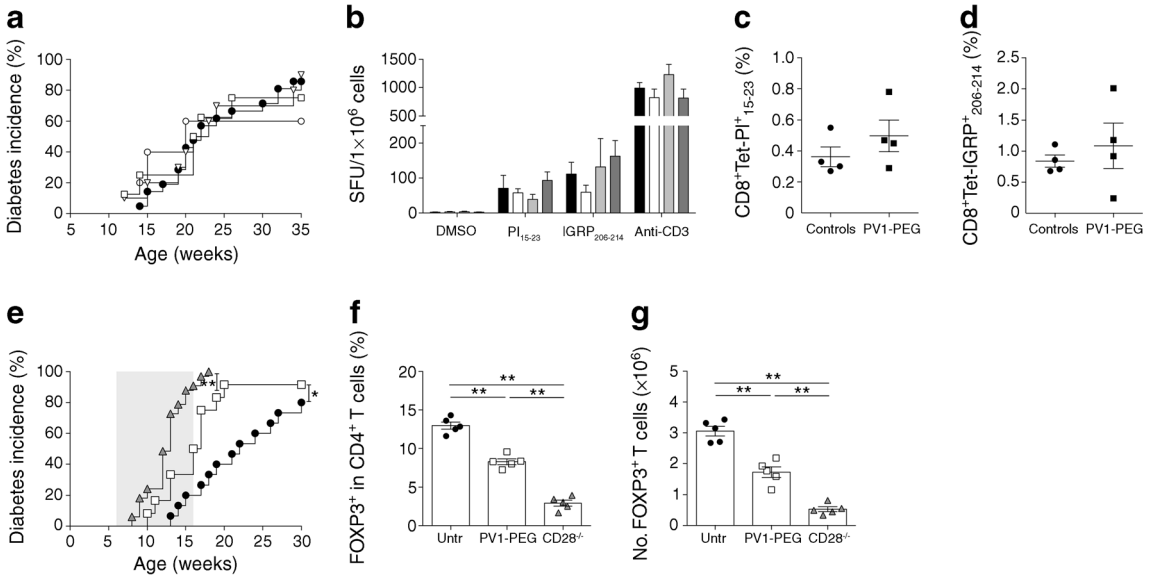

Fig. 1 Short-term treatment with PV1-PEG does not protect from diabetes development. PV1-PEG, 10 mg/kg, was administered twice weekly for 4 consecutive weeks to NOD mice at 4, 6 and 10 weeks of age. (**a**) Diabetes incidence was monitored and compared with that of untreated NOD mice. Black symbols, untreated mice ($n = 21$). White symbols, treated mice: circles, age 4 weeks ($n = 10$); squares, age 6 weeks ($n = 8$); and triangles, age 10 weeks ($n = 10$). (**b**) Autoantigen ($IGRP_{206–214}$ and $PI_{15–23}$)-specific $CD8^+$ T cell responses (IFNγ ELIspot assay) was measured in the spleens of NOD mice treated with PV1-PEG at different ages (black, control, $n = 8$; white, 4 weeks of age, $n = 4$; light grey, 6 weeks of age, $n = 4$; and dark grey, 10 weeks of age, $n = 4$); responses were analysed 1 week after treatment ended. Data are expressed as SFU/10^6 cells (mean ± SEM). (**c**, **d**) Frequency of $CD8^+$ T cells specific for $PI_{15–23}$ or $IGRP_{206–214}$ peptides (evaluated by MHC class I tetramer staining in $CD8^+$ T cells) in pancreatic lymph nodes of NOD mice treated with PV1-PEG at 6 weeks of age and analysed 1 week after treatment. (**e**) Prolonged PV1-PEG monotherapy accelerated autoimmune diabetes development: at 6 weeks of age, NOD mice ($n = 12$) were treated with PV1-PEG, 10 mg/kg twice weekly for 10 consecutive weeks (grey shading) (white squares), or not treated (black circles). Diabetes incidence was monitored and compared with that of CD28-deficient NOD mice ($n = 10$, grey triangles) (*$p < 0.05$, **$p < 0.01$, logrank Mantel–Cox test). (**f**, **g**) Proportion and absolute numbers of spleen $CD4^+$ $FOXP3^+$ Tregs analysed at the end of the 10 week treatment or at diabetes onset compared with control or $CD28^{-/-}$ NOD mice (mean ± SEM, $n = 5$/group, **$p < 0.01$, Mann–Whitney test). No., number; untr, untreated

immunosuppressive drugs prevents acute rejection of renal allografts and alloantibody production and induces long-term kidney graft survival in non-human primates [5, 6]. We applied the same combination in our NOD model: 10 week old NOD mice were treated with PV1-PEG, rapamycin or both drugs combined for 4 consecutive weeks. Results showed that, while rapamycin or PV1-PEG monotherapy did not confer any protection, the combination treatment significantly delayed diabetes onset; only 2/10 of treated mice were diabetic at 25 weeks of age compared with 60%, 70% and 80% in the untreated, PV1-PEG-only and rapamycin-only groups of mice, respectively (Fig. 2a).

Combination treatment did not modify the proportion of spleen $CD4^+$ and $CD8^+$ T cells (Fig. 2b, c), but it decreased the frequency of $CD44^{high}CD62L^{low}$ activated Teff cells in both subsets (Fig. 2d, e). Within pancreatic islets, both $CD4^+$ and $CD8^+$ T cell numbers were significantly reduced in mice treated with rapamycin alone or in association with PV1-PEG (Fig. 2f–h). However, expression of CD44 and CD69 was decreased in islet-infiltrating T cells from combination-treated NOD mice compared with the mice treated with rapamycin only (Fig. 2i–l). $FOXP3^+$ Treg proportion and suppressive capacities were similar between all groups (Fig. 2m–o).

Discussion

Administration of a selective CD28 antagonist combined with rapamycin for only 4 weeks controlled the development of autoimmunity in NOD mice. The two drugs acted in a complementary manner to significantly delay diabetes by reducing the activation and migration of effector T cells to pancreatic islets. Treatment with each individual drug had no effect. This contrasted with data collected in other mouse and non-human primate models of autoimmunity, such as experimental autoimmune uveitis (EAU), EAE and CIA, where selective CD28 blockade effectively altered effector T cell responses and reduced disease severity [7, 8, 11]. A major difference between these models and the type 1 diabetes NOD mouse model is that they are induced in naive animals on administration of antigen with adjuvant. In contrast, spontaneous diabetes in NOD mice results from a chronic process characterised by the progressive destruction of beta cells, leading to overt hyperglycaemia. The present data suggest that, while CD28 antagonists interfere with priming of antigen-specific T cells and development of an acute reaction in a highly inflammatory environment, they are less efficient at modulating differentiated Teffs in the context of an ongoing immune response. Similar findings were

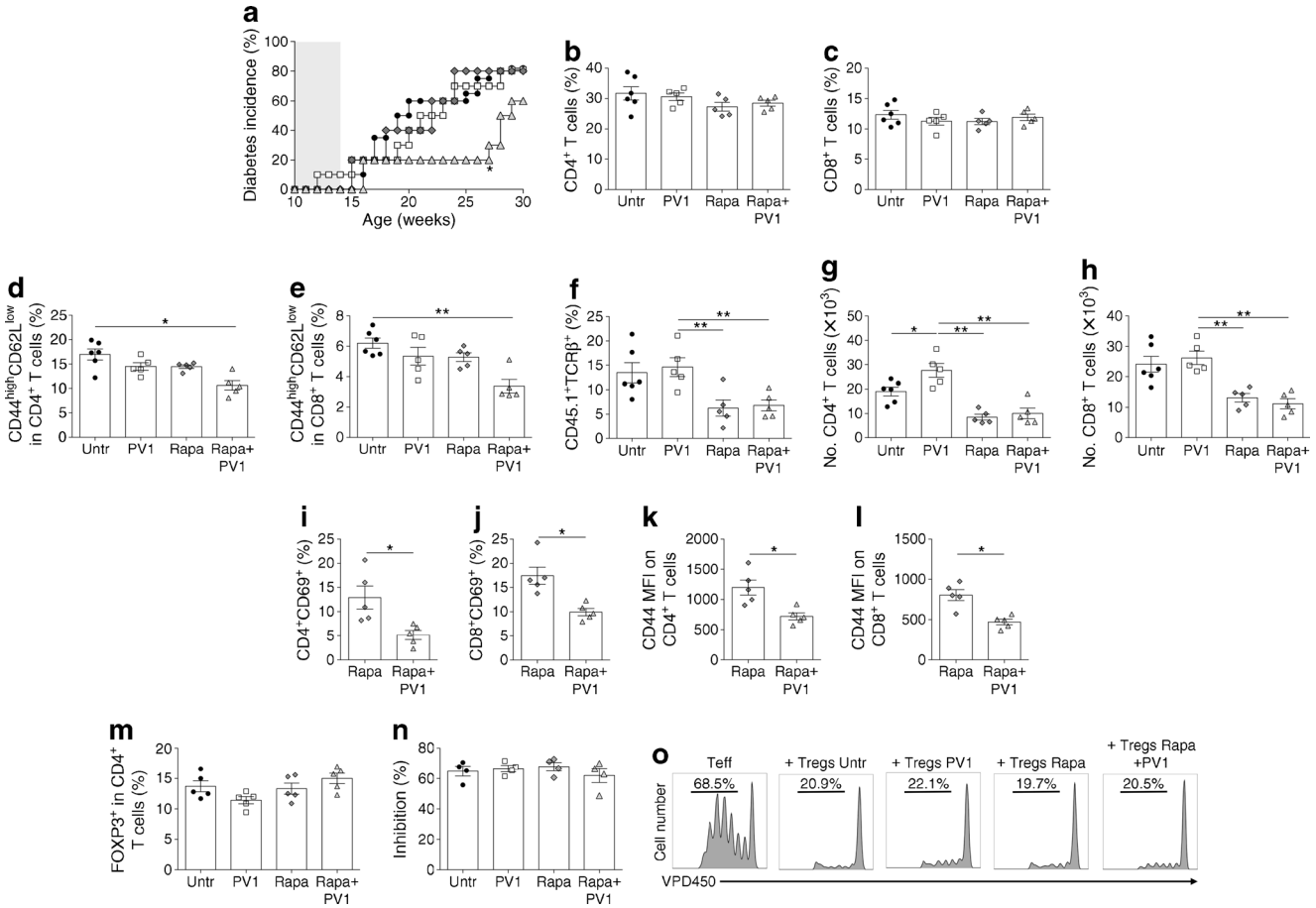

Fig. 2 Combination of PV1-PEG with rapamycin delays diabetes development in NOD mice. NOD mice aged 10 weeks were untreated ($n = 10$, black circles) or treated with PV1-PEG, 10 mg/kg twice weekly ($n = 10$, white squares), rapamycin, 1 mg/kg twice weekly ($n = 10$, dark grey diamonds) or PV1-PEG + rapamycin ($n = 10$, light grey triangles) for 4 consecutive weeks (grey shading). (**a**) Occurrence of diabetes was evaluated by weekly measurement of glycosuria and confirmed by detection of hyperglycaemia (*$p < 0.05$ PV1-PEG + rapamycin vs other groups, logrank Mantel–Cox test). (**b**, **c**) Proportions of $CD4^+$ and $CD8^+$ T cells in the spleen 1 week after treatment ended. (**d**, **e**) Frequency of $CD44^{high}CD62L^{low}$ activated effector T cells within the $CD4^+$ and $CD8^+$ compartment ($n = 6$ mice in the control group, $n = 5$ in the PV1-PEG, rapamycin and combination groups; *$p < 0.05$, **$p < 0.01$, Mann–Whitney test). (**f**–**h**) Proportion of $CD45.1^+$ $TCR\beta^+$ cells and absolute numbers of $CD4^+$ and $CD8^+$ T cells detected in the pancreatic islets of treated NOD mice 1 week after treatment ended ($n = 5$–6 mice/group, *$p < 0.05$, **$p < 0.01$, Mann–Whitney test). (**i**–**l**) CD69 expression and CD44 mean fluorescence intensity detected in islet-infiltrating $CD4^+$ and $CD8^+$ T cells recovered from NOD mice treated with rapamycin only and with PV1-PEG + rapamycin (*$p < 0.05$, Mann–Whitney test). (**m**) Proportion of $FOXP3^+$ Tregs present in the spleens of treated and untreated NOD mice at 6 weeks post-treatment ($n = 5$ mice/group). (**n**) $CD4^+CD25^+$ Tregs were isolated and tested in vitro for their capacity to inhibit CD3 antibody-induced proliferation of VPD450-labelled $CD4^+CD25^-$ Teffs purified from untreated animals. Percentage of inhibition of Teff proliferation (three or more cell divisions) is shown ($n = 4$). (**o**) Representative histograms of VPD450 dilution after 4 days of culture in the absence or presence of Tregs from the four groups of mice (cultured at a 1/1 ratio). Data for (**b**–**n**) were recovered from two independent experiments. MFI, mean fluorescence intensity; no., number; PV1, PV1-PEG; rapa, rapamycin; untr, untreated

reported for belatacept and its ability to control alloantigen or viral-specific T cell responses [12].

In NOD mice, combined CD28 and mammalian target of rapamycin (mTOR) blockade was required to efficiently downregulate T cell activation and reduce pancreatic islet invasion. Our data are in line with previous evidence that suggests that the phosphoinositide 3-kinase (PI3K)/Akt pathway is triggered on CD28 crosslinking and that CD28 crosslinking is essential for T cell-mediated cytotoxicity but dispensable for mTOR-mediated Teff cell metabolism e.g. glucose metabolism and glycolysis [13, 14]. Thus, both PI3K/Akt and mTOR converge to promote full effector function; the simultaneous blockade of these pathways resulted in an efficient inhibition of autoimmune responses in NOD mice.

Prolonged in vivo treatment of NOD mice with PV1-PEG monotherapy weakened $FOXP3^+$ Tregs by decreasing their number, accelerating diabetes onset. Such a reduction in Tregs was also recently reported in EAU and in the kidneys of New Zealand (NZ)B/NZW F1 mice after treatment with the same PV1-PEG antibody [11, 15]. All these findings support a

cell-intrinsic role of CD28 in peripheral Treg homeostasis; this is highlighted by the systemic inflammation observed in mice lacking CD28 exclusively in FOXP3+ T cells [16].

In conclusion, over the last two decades, targeting CD28 costimulation has been the focus of numerous investigations in autoimmune and inflammatory diseases. However, the complexity of CD28 function, which varies depending on the cell type and the immunological context and that likely underlies the failure and success of abatacept and belatacept in the clinic, has brought into question the safety/efficacy of CD28 costimulation blockade. In addition, prolonged treatment with rapamycin has been shown to impair glucose tolerance and beta cell homeostasis [17, 18]. Here, we provide evidence that a short-term course of selective CD28 antagonist can synergise with rapamycin to prevent autoimmune diabetes. These results have translational relevance as FR104, the human CD28 antagonist equivalent of the product used here, has recently been shown to be safe and well tolerated in healthy individuals (first-in-human study using escalating doses) [19].

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Data availability The data generated during the current study are available from the corresponding author on reasonable request.

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Duality of interest BV and CM are current employees of OSE Immunotherapeutics (formerly Effimune SAS). The pegylated anti-CD28 Fab′ antibody fragment (PV1-PEG) is the property of OSE Immunotherapeutics, Nantes, France. AB, FV, TG, LC and SY declare no commercial or financial conflict of interest. The study sponsor was not involved in: the design of the study; the collection, analysis or interpretation of data; writing the report; or the decision to submit the report for publication.

Contribution statement AB and TG designed experiments, acquired and analysed data and wrote part of the manuscript. FV and CM designed and performed experiments and analysed data. BV contributed to the conception and design of the study, provided PV1-PEG and reviewed the manuscript. LC contributed to the design of the study and interpretation of data, and helped write the manuscript. SY designed and directed the study, analysed the data and wrote the manuscript. All authors revised the manuscript and approved the final version to be published. SY is the guarantor of this work.

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