Reprint from The Review of DIABETIC STUDIES Vol 7 No 1 2010

The Review of DIABETIC STUDIES

ORIGINAL DATA

Dendritic Cell-Targeted Pancreatic β-Cell Antigen Leads to Conversion of Self-Reactive CD4+ T Cells Into Regulatory T Cells and Promotes Immunotolerance in NOD Mice

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Manuscript submitted February 22, 2010; resubmitted April 21, 2010; accepted May 3, 2010

■ Abstract

Studies employing T cell receptor transgenic T cells have convincingly shown that selective delivery of non-self model antigens to DEC-205+ dendritic cells (DCs) in the steady-state can induce Foxp3-expressing CD4+CD25+ regulatory T (Treg) cells from conventional CD4+CD25-Foxp3- T cells. Although of considerable clinical interest, the concept of DC-targeted *de novo* generation of antigen-specific Treg cells has not yet been evaluated for self-antigens and self-reactive CD4+ T cells in the non-obese diabetic (NOD) mouse model of type 1 diabetes (T1D). Here, we show in proof-of-principle experiments that targeting a mimotope peptide to the endocytic receptor DEC-205 on DCs in NOD mice induces efficient conversion of pancreatic β-cell-reactive BDC2.5 CD4+ T cells into long-lived Foxp3+ Treg cells. Of note, conversion efficiency in normoglycemic and hyperglycemic mice with early diabetes onset was indistinguishable. While *de novo* generation of BDC2.5 Treg cells did not interfere with disease progression, anti-DEC-205-mediated targeting of whole proinsulin in prediabetic NOD mice substantially reduced the incidence of diabetes. These results suggest that promoting antigen-specific Treg cells *in vivo* might be a feasible approach towards cellular therapy in T1D.

Keywords: type 1 diabetes · NOD mouse · autoimmunity · immune regulation · regulatory T cell · FoxP3 · dendritic cell · DEC-205 · BDC2.5 mimotope · proinsulin

Introduction

Type 1 diabetes (T1D) is a chronic disease manifested by the loss of functional insulin-producing β-cells of pancreatic islets. The pathogenesis of T1D is characterized by islet-infiltrating autoreactive CD4+ and CD8+ T cells and T cell-mediated autoimmune destruction of pancreatic β-cells [1]. Studies on T1D are facilitated by animal models such as non-obese diabetic (NOD) mice, which show islet infiltration and destructive autoimmune insulitis as early as four weeks of age and spontaneously progress to overt diabetes in the adult [2]. This model recapitulates many aspects of human T1D. Studies in NOD mice attributed pancreatic β-cell autoimmunity to defects in thymic negative selection of islet antigen-specific T cells, and the failure to silence these autoreactive T cells in peripheral lymphoid organs [3, 4].

It is generally accepted that Foxp3-expressing CD4+CD25+ regulatory T (Treg) cells play an ac-

Abbreviations:

Ag - antigen
APC - antigen-presenting cell
BDC2.5 cell - autoreactive T cell clone isolated from NOD mice
BSA - bovine serum albumin
CD4 - cluster of differentiation 4 (glycoprotein expressed on lymphocytes)
CD11c - cluster of differentiation 11c (protein expressed on myeloid cells; adhesion molecule binding to fibrinogen/fibronectin)
CD25 - cluster of differentiation 25 (glycoprotein expressed on activated regulatory T cells)
CD62L - cluster of differentiation 62L (protein expressed on leukocytes; adhesion molecule that binds to CD34)
CFSE - 5,6-carboxy fluorescein succinimidyl ester
cDNA - complementary deoxyribonucleic acid
DC - dendritic cell
DEC-205 - membrane glycoprotein of 205 kDa (CD205; expressed on dendritic cells; cell receptor for endocytosis)
DNA - deoxyribonucleic acid
FACS - fluorescence activated cell sorting
FoxP3 - forkhead box P3 (transcriptional activator)
HBSS - Hank's balanced salt solution
HEK-293 cell - human embryonic kidney cell (produced by transformation of human embryonic kidney cells with sheared adenovirus 5 DNA)
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG1 - subclass 1 of immunglobulin G
IgH - immunglobulin heavy chain
Ig-κ - immunglobulin kappa locus
IgL - immunglobulin light chain
IgL-κ immunoglobulin kappa light-chain
IFN-γ - interferon gamma
IL-2 - interleukin 2
MHC - major histocompatibility complex
mLN - mesenteric lymph node
NIH #31M rodent diet - National Institutes of Health open-formula cereal-based diet
NLDC-145 - monoclonal antibody able to recognize DEC-205
NOD - nonobese diabetic
PBS - phosphate buffered saline
PE - Phycoerythrin
pLN - pancreatic lymph node
Rag1 - recombination activating gene product 1 (expressed on lymphocytes during development)
RPMI-1640 medium - Roswell Park Memorial Institute 1640 (cell culture medium for leukocytes)
SDS-PAGE - sodium dodecylsulfate polyacrylamide gel electrophoresis
sLN - subcutaneous lymph node
T1D - type 1 diabetes
TCR - T cell receptor
TGF-β - transforming growth factor beta
Thy1.1 - one of two alleles of the Thy1 gene in mice (encodes CD90 protein, also known as thymocyte antigen)
Thy1.2 - one of two alleles of the Thy1 gene in mice
Treg cell - regulatory T cell
% v/v - percent volume per volume
% w/v - weight per volume percentage

tive role in the maintenance of immune homeostasis and prevention of autoimmunity under physiological conditions [5]. It remains controversial whether reduced numbers or functional defects in Treg cells contribute to the pathogenesis of T1D [6-15]. Whereas, the important role of Foxp3$^+$ Treg cells in the control of autoimmune diabetes progression is exemplified by the observation that punctual ablation of Treg cells in NOD mice carrying a β-cell-reactive T cell receptor (TCR) as a transgene resulted in overt diabetes within 3 days (Petzold and Kretschmer, unpublished, and [16]). Intensive investigation is currently being directed to potential of strategies for promoting Treg cells, with known antigen (Ag) specificity, to protect pancreatic β-cells from autoimmune destruction. Interest in this strategy is due to its disease specificity, and its ability to reduce side effects without compromising desired protective host immune responses.

Multiple lines of investigation have shown that cellular therapy with induced Treg cells can be effective in the prevention, and even reversal, of early-onset diabetes in NOD mice. Investigated strategies have involved the *in vivo* application of *in vitro* expanded Ag-specific Treg cells [17, 18]. Another line of investigation showed that *in vitro* generated Ag-specific Treg cells either by ectopic expression of Foxp3 [19], or by TGF-β-mediated upregulation of Foxp3 expression in conventional CD4$^+$ T cells [20] can be effective in prevention or even reversal of early-onset diabetes in NOD mice. While cellular therapy with *in vitro* expanded Treg cells is limited to Treg cell antigen specificities preformed *in vivo*, extrathymic *de novo* generation of Treg cells from conventional CD4$^+$CD25$^-$ Foxp3$^-$ T cells offers the possibility of artificially induced Treg cells with any preferred antigen specificity [21]. *In vivo*, Ag-specific Treg cells can be induced from TCR transgenic CD4$^+$ T cells by recombinant anti-DEC-205 fusion antibody-mediated selective delivery of Ag to steady-state dendritic cells (DCs) [22-24]. In contrast to TGF-β-mediated *in vitro* generation of Foxp3$^+$ cells [25, 26], Treg cells that have been generated extrathymically by DC targeting *in vivo* exhibited complete demethylation of CpG motifs within the noncoding part of the Foxp3 gene that is associated with Treg cell stability. Consequently, these cells survived in mice for extended periods of time in the absence of the inducing Ag [25]. Another important observation was that the cells maintained a stable Foxp3$^+$ suppressor phenotype under immunogenic conditions. This is the prerequisite for

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successful induction of Ag-specific dominant tolerance. It relies on immunosuppression of neighboring $CD4^+$ and $CD8^+$ T effector cells by $Foxp3^+$ Treg cells resident in antigen-draining lymph nodes and autoimmune tissue infiltrates [21]. However, Ag-specific conversion of conventional $CD4^+CD25^-$ $Foxp3^-$ T cells into Foxp3-expressing $CD4^+CD25^+$ Treg by DEC-205$^+$ DCs *in vivo* has been unambiguously shown only with non-self antigens such as hemagglutinin [22, 23] or ovalbumin [24]. Here, we report on our attempts to elucidate whether these observations made with model antigens can be extended to pancreatic β-cell-derived self-antigen and β-cell-reactive $CD4^+$ T cells in the NOD mouse model of T1D.

Materials and methods

Mice

NOD, NOD-Thy1.1, NOD-Rag1$^{-/-}$, and NOD-BDC2.5 mice were purchased from Jackson Laboratories (Bar Harbor, USA). Experimental colonies were maintained at the Experimental Center (Dresden University of Technology, Germany) under specific pathogen-free conditions. Thy1.2 NOD-BDC2.5 mice were crossed to Thy1.1 NOD congenic mice to obtain Thy1.1 NOD-BDC2.5 mice. Mice were fed with NIH #31M rodent diet (Altromin, Germany). All experiments were performed in accordance with the German law on care and use of laboratory animals.

Recombinant fusion antibody production

Eukaryotic expression vectors encoding the IgH chain cDNA of cloned anti-DEC-205 NLDC-145, and III/10 isotype control, and their respective Ig-κ L chain cDNA, were produced in the Nussenzweig laboratory at Rockefeller University [27, 28]. Double-stranded DNA fragments encoding the BDC2.5 mimotope peptide 1040-63 (hereafter referred to as m63) with the amino acid sequence RTRPLWVRME [29] were added in frame to the C terminus of anti-DEC-205 and III/10 as described previously [23], using the following oligonucleotides: m63-1for, 5'-TAG CGA CAT GGC CAA GAA GGA GAC AGT CTG GAG GCT CGA GGA GTT CGG TAG GTT CAC AAA CAG GCG C-3'; m63-1rev, 5'-GCG GGT GCG CCT GTT TGT GAA CCT ACC GAA CTC CTC GAG CCT CCA GAC TGT CTC CTT CTT GGC CAT GTC G-3'; m63-2for, 5'-ACC CGC CCG CTG TGG GTG CGC ATG GAA TAT TAT GAC GGT AGG ACA TGA TAG GC-3'; and m63-2rev, 5'-GGC CGC CTA TCA TGT CCT ACC GTC ATA ATA TTC CAT GCG CAC CCA CAG CGG-3'. Plasmid vectors of anti-DEC-205 and III/10 fused to proinsulin 2 (GenBank accession number NM008387) were generated using cDNA from whole pancreas and the primers PIns-for 5'- AGC TAG CGA CAT GGC CAA GAA GGA GAC AGT CTG GAG GCT CGA GGA GTT CGG TAG GTT CAC AAA CAG GTT TGT CAA GCA GCA CCT T-3' and PIns-rev 5'-CGC GGC CGC CTA TCA TGT CCT ACC GTC ATA ATA GTT GCA GTA GTT CTC CAG CTG GTA GAG GGA-3'.

Recombinant antibodies were produced using FreeStyle™ 293 Expression System (Invitrogen) according to the manufacturer's protocol. Briefly, HEK-293 cells were grown as suspension cultures in serum-free FreeStyle™ 293 medium and transiently co-transfected with the respective IgH and IgL chain plasmids using FreeStyle™ MAX reagent. The produced antibodies were purified on HiTrap™ Protein G HP columns (GE Healthcare). Protein concentrations were determined spectrophotometrically at 280 nm and the integrity of the produced fusion antibodies was verified by SDS-PAGE with an IgG1/IgL-κ antibody as reference.

Flow cytometry and cell sorting

Single cell suspensions of spleen, mesenteric (mLNs), pancreatic (pLNs), or a pool (*Lnn. mandibularis, Lnn. cervicales superficiales, Lnn. axillares et cubiti, Lnn. inguinales superficiales,* and *Lnn. subiliaci*) of subcutaneous lymph nodes (sLNs) were prepared using 70 μm cell strainers (BD). Monoclonal antibodies to CD4 (RM4-5), CD11c (HL3), CD25 (PC61), CD62L (MEL-14), Vβ4-TCR (KT4), Thy1.1 (OX-7), and Thy1.2 (53-2.1) as well as APC-, Pacific Blue-, and PE-conjugated streptavidin were obtained from eBioscience or BD. The autoMACS™ magnetic cell separation system and streptavidin-conjugated magnetic microbeads (Miltenyi Biotec) were used to enrich $CD11c^+$ DCs (see below) labeled with biotinylated anti-CD11c antibodies, or prior to FACS to enrich $CD4^+$ T cells labeled with biotinylated anti-CD4 antibodies.

For analysis of intracellular Foxp3 expression, the monoclonal antibody Foxp3 (FJK-16s) and the Foxp3 staining buffer set (both eBioscience) were used according to the manufacturer's protocol. The samples were analyzed using a FACS Calibur or LSRII or sorted on a FACS Aria (all BD). Data were analyzed with the FlowJo software (Tree Star).

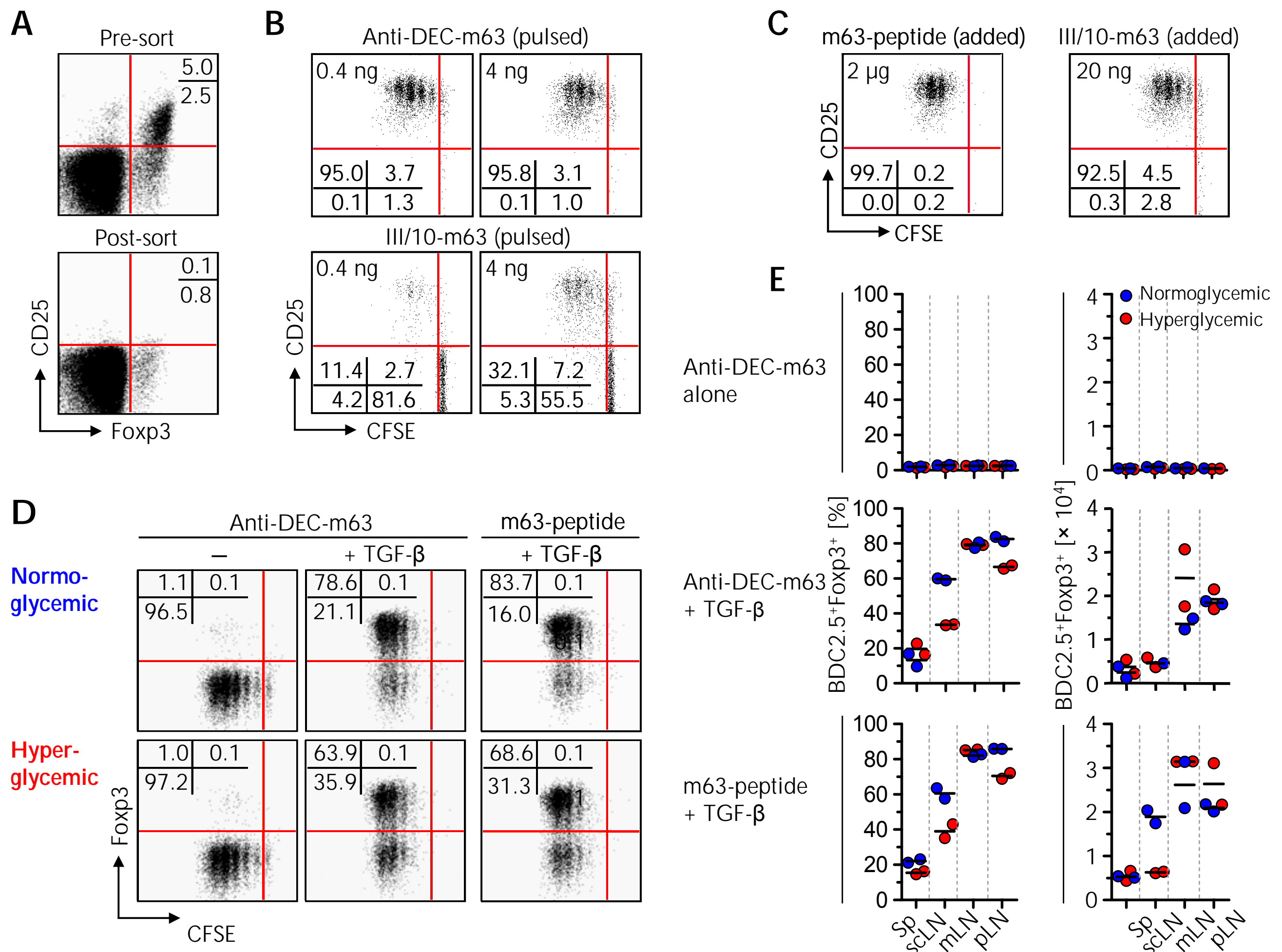

Figure 1. DEC-205-mediated delivery of a mimotope peptide to DCs efficiently activates β-cell-reactive BDC2.5 T cells *in vitro*. A: Representative flow cytometry of CD25 and Foxp3 expression in BDC2.5 T cells prior (top panel) and after (bottom panel) FACS purification of $CD4^+V\beta4^+CD62L^{high}CD25^-$ cells from NOD-BDC2.5 donor mice. Numbers in plots indicate percentages of $Foxp3^+$ cells. **B, C**: Flow cytometry of CD25 expression and cell division of $CD4^+$-gated BDC2.5 T cells at day 3 of co-cultures with CFSE-labeled naïve BDC2.5 T cells (6×10^4) and $CD11c^+$ DCs (5×10^3). **B**: Prior to culture, DCs were pulsed with 100 ng/ml (20 ng/well, corresponding to ~0.4 ng/well m63 peptide), or 1 µg/ml (200 ng/well, corresponding to ~4 ng/well m63 peptide) anti-DEC-m63 (top panels), or equivalent amounts of III/10-m63 (bottom panels). **C**: As control, 10 µg/ml (2 µg/well) synthetic m63 peptide (left), or 5 µg/ml (1 µg/well, corresponding to ~20 ng m63 peptide) III/10-m63 (right) were directly added to cultures. Numbers in plots indicate percentages of cells within respective quadrants. **D**, **E**: Foxp3 expression and cell division of $CD4^+$-gated BDC2.5 T cells at day 3 of co-cultures with 5×10^4 CFSE-labeled naïve BDC2.5 T cells and 2×10^5 total cells from single cell suspensions of pancreatic LNs as APCs of indicated organs. For T cell stimulation, APCs were either pulsed with anti-DEC-m63 (100 ng/ml and 20 ng/well, corresponding to ~0.4 ng/well m63 peptide) or synthetic m63 peptide (10 µg/ml, 2 µg/well) was directly added to the cultures, in the absence or presence of 5 ng/ml TGF-β (1 ng/well), as indicated. Normoglycemic or hyperglycemic NOD donor mice are indicated by blue and red circles, respectively. The results of two independent preparations of APCs from lymphoid organs of individual normo- and hyperglycemic mice are shown. Numbers in dot plots indicate percentages of cells within respective quadrants.

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T cell proliferation measurement

Where indicated, CD4$^+$ T cells were labeled with 1 or 10 μM 5,6-carboxy fluorescein succinimidyl ester (CFSE, Invitrogen) for *in vitro* and *in vivo* proliferation measurements, respectively, by incubation for 10 min at 37°C in the dark at a density of ≤10^7 cells/ml in 0.1% BSA in PBS (w/v).

In vitro T cell activation

Prior to CD11c-bead enrichment, single cell suspensions from spleen were prepared with 0.2 mg/ml Dispase I, 0.2 mg/ml Collagenase D and 25 μg/ml DNase I (Roche) in 1 × HBSS supplemented with 5% (v/v) FCS and 10 mM HEPES (Invitrogen). FACS-purified CD4$^+$Vβ4$^+$ CD62L^{high}CD25$^-$ BDC2.5 T cells were cultured in 96-well round-bottom plates (Greiner) and RPMI 1640 medium supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate, 1 mM HEPES, 2 mM glutamax, 100 U/ml penicillin-streptomycin, 0.1 mg/ml gentamycin, 0.1 mM non-essential amino acids, and 0.55 mM β-mercapto-ethanol (all Invitrogen).

For antigen-specific activation, BDC2.5 T cells were co-cultured for 72 h with CD11c$^+$ DCs at 37°C and 5% CO_2. As indicated, synthetic BDC2.5 mimotope peptide (RTRPLW VRME, Peprotech, Hamburg) or III/10-m63 isotype control antibody was either added directly to the co-cultures, or DCs were incubated prior to culture with anti-DEC-m63 or III/10-m63 isotype control antibody for 30 min on ice, followed by extensive washing to remove unbound antibodies. For induction of Foxp3 expression *in vitro*, BDC2.5 T cells were co-cultured for 72 h with total cells of peripheral lymphoid organs obtained from normo- or hyperglycemic NOD donors, in the presence of anti-DEC-m63 antibody or m63 peptide, either alone or supplemented with 5 ng/ml TGF-β, as indicated.

In vivo T cell activation, studies on diabetes prevention, and adoptive cell transfer model of diabetes

Synthetic BDC2.5 mimotope peptide or recombinant antibodies fused to pancreatic β-cell antigens were administered by intraperitoneal injection. In some experiments, FACS-purified

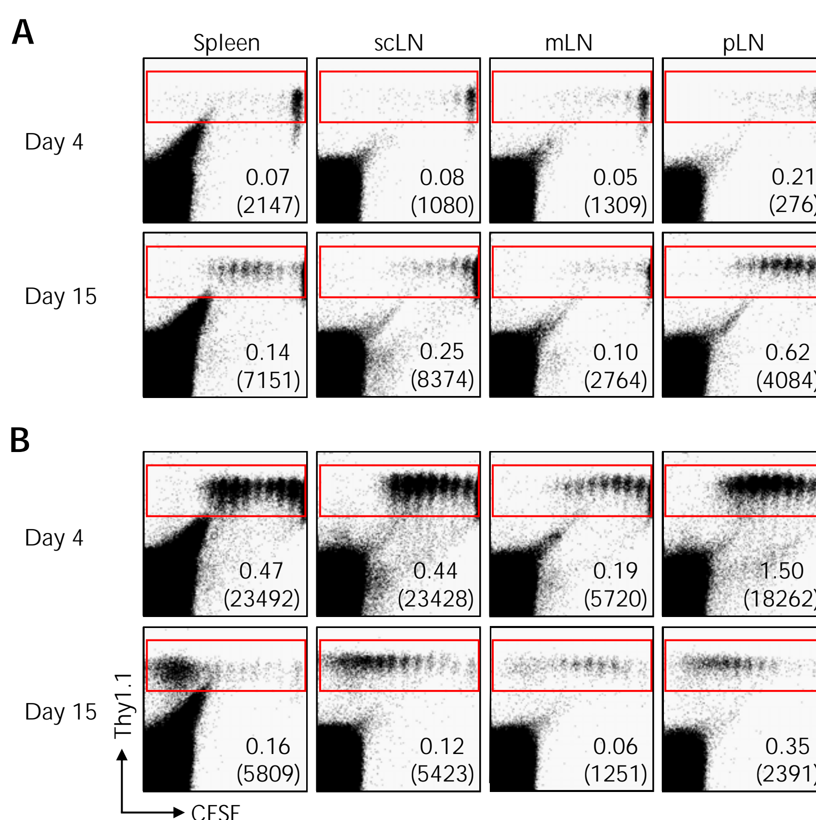

Figure 2. Proliferation of BDC2.5 T cells stimulated by their cognate self-Ag can be enhanced by DC-targeted mimotope peptide *in vivo*. FACS-purified Thy1.1$^+$ BDC2.5 T cells (~2 x 10^6) were CFSE-labeled and adoptively transferred into congenic NOD-Thy1.2 hosts. Recipient mice were either left untreated (**A**), or injected with 50 ng recombinant anti-DEC-m63 antibody (~1 ng m63 peptide) the next day (**B**). Spleen, scLNs, mLNs, and pLNs were collected at day 4 (top panels) or day 15 (bottom panels) after cell transfer, and were analyzed by flow cytometry. Numbers in dot plots indicate percentages of Thy1.1$^+$ BDC2.5 T cells among gated CD4$^+$ T cells. Numbers in brackets indicate absolute numbers of Thy1.1$^+$ BDC2.5 T cells recovered from indicated lymphoid organs.

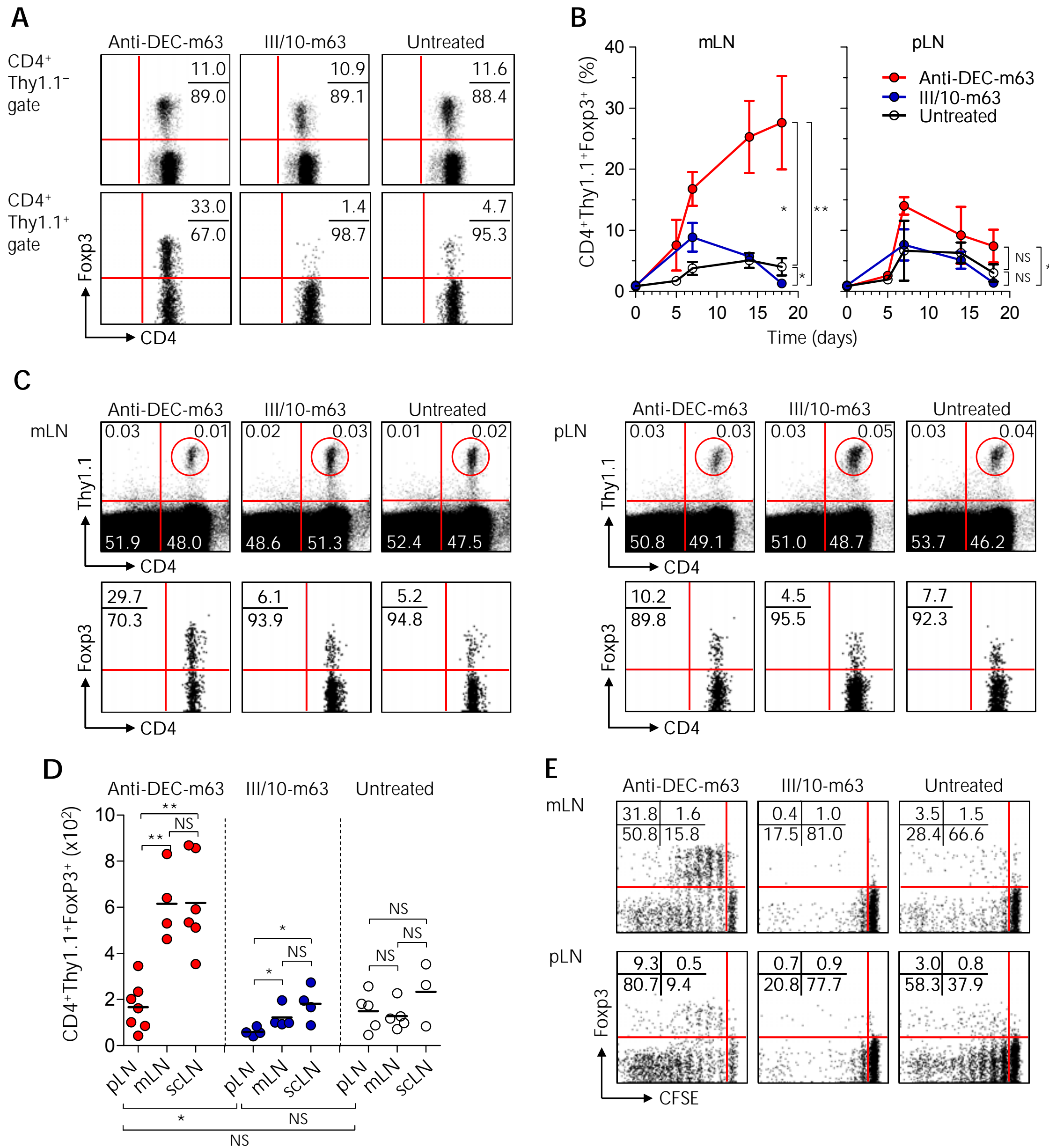

Figure 3. Minute amounts of DC-targeted mimotope peptide promote *in vivo* conversion of transferred CD4⁺Foxp3⁻ BDC2.5 T cells into CD4⁺Foxp3⁺ BDC2.5 T cells. NOD hosts were adoptively transferred with CFSE-labeled congenic marker⁺ BDC2.5 T cells as described in the legend of Figure 2. The next day recipient mice were either left untreated or injected with 50 ng anti-DEC-m63 or III/10-m63 recombinant antibodies, corresponding to ~1 ng m63 peptide. **A**: Representative flow cytometry of Foxp3 expression among endogenous CD4⁺Thy-1.1⁻ (top), or adoptively transferred Thy-1.1⁺ BDC2.5 T cells (bottom) recovered from mLNs of NOD recipient mice at day 18 after initiation of the experiment. **B**: Foxp3 expression among gated CD4⁺Thy-1.1⁺ BDC2.5 T cells at indicated time points after adoptive transfer into congenic recipient mice that were left untreated, or injected with 50 ng anti-DEC-m63 or III/10-m63 isotype control antibodies. Mean values and standard deviations of data from 3-5 mice for each time point and experimental condition are shown. Representative flow cytometry of Foxp3 expression among gated CD4⁺Thy-1.1⁺ BDC2.5 T cells in mLNs (left) and pLNs (right) at day 14 is shown in (**C**). Numbers of Foxp3-expressing cells (**D**) and representative CFSE dilution (**E**) among gated CD4⁺Thy-1.1⁺ BDC2.5 T cells recovered from indicated peripheral lymphoid organs at day 18 after adoptive transfer and recombinant antibody administration, as indicated. Untreated recipient mice were included as controls. Dots and horizontal lines in **D** indicate individual mice and mean values, respectively. Numbers in dot plots indicate the percentage of gated cells in the respective quadrant or gate. Statistical analysis was performed using Mann-Whitney-U test (** $p \leq 0.01$; * $p \leq 0.05$; NS: not significant).

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CD4$^+$Vβ4$^+$CD62L^{high}CD25$^-$ Thy1.1 or Thy1.2 BDC2.5 T cells (1-2 × 10^6) were adoptively transferred by injection into the lateral tail veins of congenic NOD recipients prior to recombinant antibody administration. If not stated otherwise, only female NOD mice were used.

To determine the establishment of tolerance, NOD-Rag1$^{-/-}$ mice were injected with 2 × 10^7 total splenocytes from a pool of hyperglycemic NOD mice alone, or co-injected with equivalent numbers of splenocytes from pools of untreated or anti-DEC-PIns-treated normoglycemic NOD mice. Prior to injection, splenocytes were prepared by erythrocyte lysis (Qiagen). Blood glucose levels were measured using whole blood from the tail vein and Accu-Chek® Aviva (Roche). Blood glucose levels of NOD mice in experimental groups were routinely determined once a week, whereas NOD-Rag1$^{-/-}$ recipients in the adoptive cell transfer model of diabetes were tested at least twice a week. Mice were considered diabetic at blood glucose levels above 200 mg/dl on at least two consecutive measurements or with blood glucose levels once above 400 mg/dl.

Results

Generation of recombinant anti-DEC-205 antibodies to selectively deliver pancreatic β-cell antigen to DCs

As an approach towards manipulation of β-cell-reactive CD4$^+$ T cells by DC targeting of β-cell Ag in NOD mice, we took advantage of a well-established TCR transgenic model system that consists of mice expressing the BDC2.5 TCR as a transgene [30]. BDC2.5 CD4$^+$ T cells that are highly diabetogenic in NOD mice are reactive to islet β-cells in the context of the major histocompatibility complex class II (MHC-II) molecule A^{g7}. While agonistic mimotope peptides that stimulate BDC2.5 T cells at nanomolar concentrations had been described some years ago [29], the islet cell Ag responsible for pathogenicity has only recently been identified as chromogranin A [31].

To activate BDC2.5 T cells through DC-targeted Ag we followed a protocol described previously [23]. We generated IgH chain-encoding eukaryotic expression vectors of the agonistic mi-

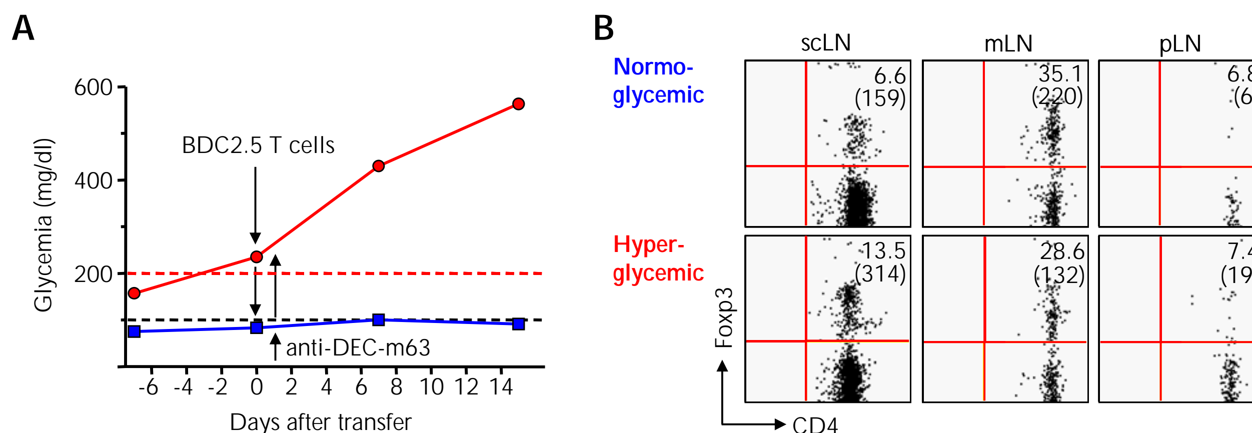

Figure 4. Efficacy of anti-DEC-205-mediated Foxp3 induction in transferred BDC2.5 T cells is independent of the glycemic status of NOD recipient mice. A: Blood glucose levels (in mg/dl) in NOD-Thy1.2 mice with normoglycemia (solid blue line) or early diabetes onset (solid red line) that were adoptively transferred with FACS-purified Thy1.1$^+$ BDC2.5 T cells (~1.7 × 10^6) and subsequently injected with 500 ng anti-DEC-m63 antibody (~10 ng m63 peptide), as indicated. Dashed lines in black and red indicate normal (100 mg/dl) and high (200 mg/dl) blood glucose levels, respectively. **B**: Flow cytometry of induced Foxp3 expression among gated CD4$^+$Thy-1.1$^+$ BDC2.5 T cells recovered from indicated LNs of normoglycemic (top panels) and hyperglycemic (bottom panels) NOD recipient mice at day 14 after cell transfer and anti-DEC-m63 antibody administration. Numbers in dot plots indicate percentages and numbers in brackets absolute numbers of Foxp3$^+$ BDC2.5 T cells. Data are representative of 2 mice per experimental condition.

motope peptide 1040-63 (RTRPLWVRME, hereafter referred to as m63) added in frame to the C terminus of cloned anti-DEC-205 (anti-DEC-m63), or III/10 isotype control antibody (III/10-m63). To ensure the specificity of antigen targeting the rat IgG2a constant regions of the original NLDC-145 and isotype control antibodies were replaced with mouse IgG1 constant regions, which carry point mutations interfering with Fc receptor binding [32]. The integrity of produced recombinant IgH chains was confirmed by SDS-PAGE (data not shown).

BDC2.5 CD4$^+$ T cell activation and induction of Foxp3 expression by DC-targeted pancreatic β-cell Ag in vitro

To determine whether m63 antigen fused to recombinant anti-DEC-205 antibody can be processed by DCs for MHC class II presentation to BDC2.5 T cells, CD11c$^+$ DCs were purified from spleens of normoglycemic NOD mice. They were briefly incubated with titrating amounts of anti-DEC-m63 or III/10-m63 isotype control antibody unable to bind DEC-205, and subsequently rigorously washed to remove unbound antibodies. Throughout this study, naïve BDC2.5 T cells were FACS-purified from pools of spleen and lymph nodes (LNs) of BDC2.5 transgenic NOD mice based on CD4$^+$Vβ4$^+$CD62L^{high}CD25$^-$ expression (Figure 1A). Highly pure populations of naïve BDC2.5 T cells containing ≤1% Foxp3$^+$ cells were labeled with CFSE and co-cultured with purified DCs as antigen-presenting cells (APCs) that had been pulsed with recombinant antibodies.

The analysis of T cell activation marker expression and cell division at day 3 of cultures revealed that DCs pulsed with minute amounts of anti-DEC-m63 (100 ng/ml or 1 μg/ml) antibody (corresponding to ~2 or 20 ng m63 peptide, respectively) efficiently induced strong activation and proliferation of BDC2.5 T cells (Figure 1B, top panels). The activation level was comparable to that of control cultures, to which synthetic m63 peptide was added without removal of unbound peptide (Figure 1C, left panel). The III/10-m63 isotype control antibody was ineffective in mediating T cell activation and proliferation (Figure 1B, bottom panels), unless excessive amounts of III/10-m63 were added without removal of unbound antibody (Figure 1C, right panel). This confirmed that the produced III/10-m63 isotype control antibody was functional. Thus, DEC-205-mediated delivery of β-cell-derived Ag to splenic DCs *in vitro* results in efficient processing and MHC-II presentation of m63 peptide to BDC2.5 T cells.

Cytokines (such as IL-2 or IFN-γ) that are locally produced during immune responses and/or enhanced TCR/co-stimulatory receptor signaling have been shown to abrogate efficient upregulation of Foxp3 expression in *in vitro* and *in vivo* settings of Treg conversion ([22, 33, 34] and references therein). This and several reported defects in DC populations of NOD mice [35-40] encouraged us to extend our functional *in vitro* analysis to DEC-205$^+$ DCs from pLNs and other peripheral lymphoid organs (spleen, mLNs and scLNs) from NOD mice with normoglycemia or early diabetes onset. These experiments revealed no appreciable differences between synthetic m63 peptide-loaded APCs and anti-DEC-m63-targeted DCs from single cell suspensions of the various lymphoid organs from normo- or hyperglycemic NOD mice, with regard to their capacity to induce Ag-specific activation and proliferation of co-cultured BDC2.5 T cells (data not shown, and Figure 1D). Notably, when exogenous TGF-β was added to the stimulation cultures to induce Foxp3 expression in initially naïve Foxp3$^-$CD25$^-$ BDC2.5 T cells [41, 42], both percentages (Figure 1E, left panels) and numbers (Figure 1E, right panels) of Foxp3$^+$ cells were comparable between the different lymphoid organs of normo- or hyperglycemic NOD mice.

Tracking self-reactive CD4$^+$ T cells during DC targeting of pancreatic β-cell antigen in vivo

To determine the relative contribution of endogenous self-antigen presentation on pancreatic β-cell-reactive T cell proliferation, we injected CFSE-labeled congenic Thy1.1$^+$ BDC2.5 T cells into Thy1.2$^+$ NOD recipient mice. Kinetic studies revealed some proliferation and accumulation of adoptively transferred BDC2.5 T cells in all peripheral lymphoid organs analyzed (Figure 2A). Most prominently, the cells were found in pLNs. This is most likely due to increased endogenous presentation of BDC2.5's natural β-cell Ag at this site. Proliferation of BDC2.5 T cells could be further increased by single-dose administration of as little as 50 ng recombinant anti-DEC-m63 antibody, corresponding to ~1 ng m63 peptide (Figure 2B).

Next we asked whether efficient DC-targeted induction of Foxp3 expression can be observed in self-reactive CD4$^+$ T cells, or whether this process is abrogated by chronic activation of those T cells

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by endogenous self-Ag. For this, we analyzed the expression of Foxp3 in initially naïve, congenically marked $CD4^+CD25^-Foxp3^-$ BDC2.5 T cells at various time-points after adoptive transfer into normoglycemic Thy1.2$^+$ NOD recipient mice and anti-DEC-m63 antibody administration (Figure 3). As expected, BDC2.5 T cells in III/10-m63 isotype control-injected or untreated recipients showed marginal Foxp3 expression (Figure 3A, middle and right panels) and limited proliferation (Figure 3E). In contrast, BDC2.5 T cells weakly activated through DC-targeted m63 peptide and which underwent only a few cell divisions, efficiently upregulated Foxp3 expression. Whereas, cells that had proliferated most extensively did not (Figure 3E, top left panel). Thus, the inverse relationship of cell division and induced Foxp3 expression in TCR transgenic $CD4^+$ T cells recognizing non-self model antigens [22] is also true for self-reactive $CD4^+$ T cells.

Overall, compared to controls, anti-DEC-m63 targeting consistently resulted in substantially increased frequencies and numbers of Foxp3$^+$ cells in various lymphoid organs as early as day 7 after recombinant antibody administration (Figure 3, A-D). As an example, at day 18 after initiation of the experiment, up to 35% of BDC2.5 T cells in mLNs of NOD recipients that received 50 ng recombinant anti-DEC-m63 antibody (~1 ng m63 peptide) expressed Foxp3 (Figure 3B). This corresponds to an approximate 10-fold increase of the Foxp3$^+$ BDC2.5 T cell number (Figure 3D). This observation held true for all lymphoid organs analyzed, with the exception of pLNs that exhibited accumulation of only few Foxp3$^+$ BDC2.5 T cells at any time point analyzed after adoptive transfer and

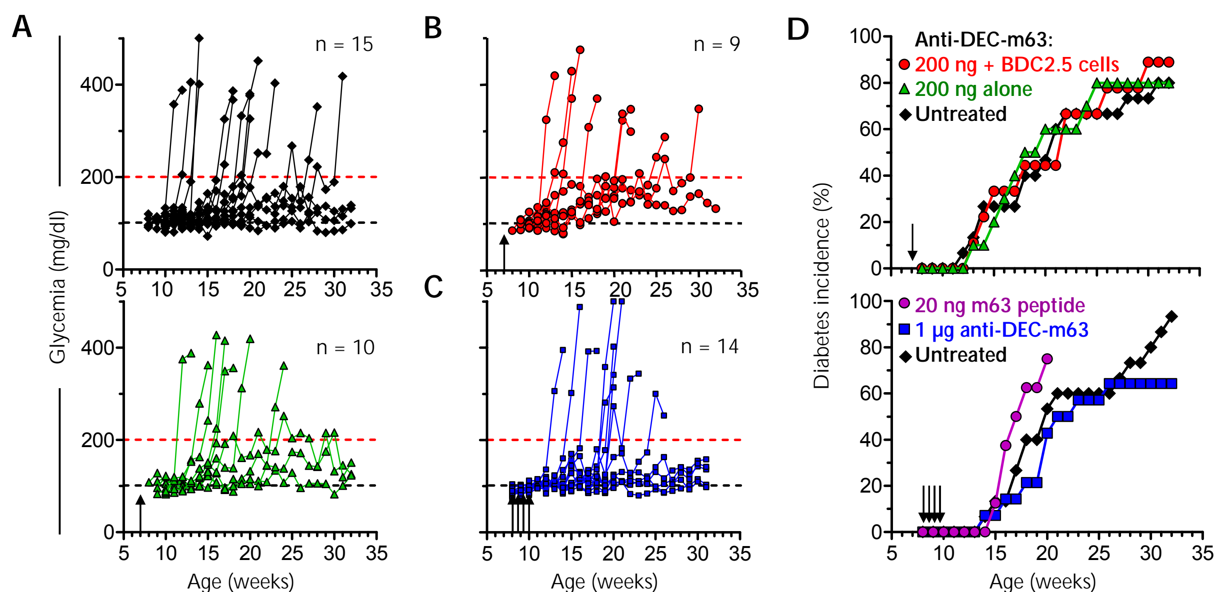

Figure 5. Anti-DEC-m63 treatment of prediabetic NOD mice and BDC2.5 Treg cell conversion has limited impact on progression towards diabetes. Cohorts of NOD mice at 7 weeks of age were either left untreated as controls (**A**, top), or treated with a single-dose injection of 200 ng anti-DEC-m63 antibody (~4 ng m63 peptide) without (**A**, bottom) or with prior adoptive transfer of 2 x 10^6 FACS-purified BDC2.5 T cells (**B**), or 4 consecutive injections of 1 µg anti-DEC-m63 antibody (~20 ng m63 peptide) within 2 weeks (**C**). Blood glucose concentrations of individual mice in the various experimental groups were determined weekly and plotted against age of mice in weeks. The experiment was terminated when the mice reached 32 weeks of age. Arrowheads indicate time-points of recombinant anti-DEC-m63 antibody administration. Dashed lines in black and red indicate normal (100 mg/dl) and high (200 mg/dl) blood glucose levels, respectively. **D**: Incidence of diabetes among experimental groups. Black diamonds: untreated controls; green triangles: single-dose of 200 ng anti-DEC-m63 antibody only; red circles: BDC2.5 T cell transfer prior to single-dose of 200 ng anti-DEC-m63 antibody; blue squares: 4 consecutive injections of 1 µg anti-DEC-m63 antibody; pink circles: 4 consecutive injections of 20 ng synthetic m63 mimotope peptide (n = 8). Statistical analysis (Log-rank) revealed no significant differences between the respective untreated control group and the groups that received 200 ng anti-DEC-m63 alone ($p = 0.8461$), 200 ng anti-DEC-m63/BDC2.5 ($p = 0.7828$) (upper panel), or four consecutive injections of 20 ng synthetic m63 peptide ($p = 0.1242$) or of 1 µg anti-DEC-m63 ($p = 0.1661$, bottom panel).

anti-DEC-m63 antibody treatment. The ineffective accumulation of Foxp3^{+} BDC2.5 T cells selectively in pLNs is consistent with the notion that pro-inflammatory conditions that enhance T cells proliferation during Treg cell *de novo* generation (Figure 3E) abrogate efficient induction of Foxp3 expression [22]. Of note, efficiency of Foxp3 induction and *de novo* Treg cell generation in non-pancreas-draining lymphoid organs was independent of the recipients' glycemic status. This was revealed by the comparative analysis of initially naïve BDC2.5 T cells 14 days after adoptive transfer and anti-DEC-m63 antibody treatment of normo- or hyperglycemic recipients (Figure 4, A and B). In conclusion, selective delivery of minute amounts of m63 peptide to DCs *in vivo* efficiently induced upregulation of Foxp3 expression in initially naïve CD4^{+}CD25^{-}Foxp3^{-} BDC2.5 T cells.

Impact of anti-DEC-m63 antibody treatment on disease progression

The above described experiments formally demonstrate that pancreatic β-cell-reactive CD4^{+} T cells can be encouraged to upregulate Foxp3 expression upon activation by DC-targeted self-Ag. This prompted us to investigate whether anti-DEC-m63 antibody treatment to promote BDC2.5

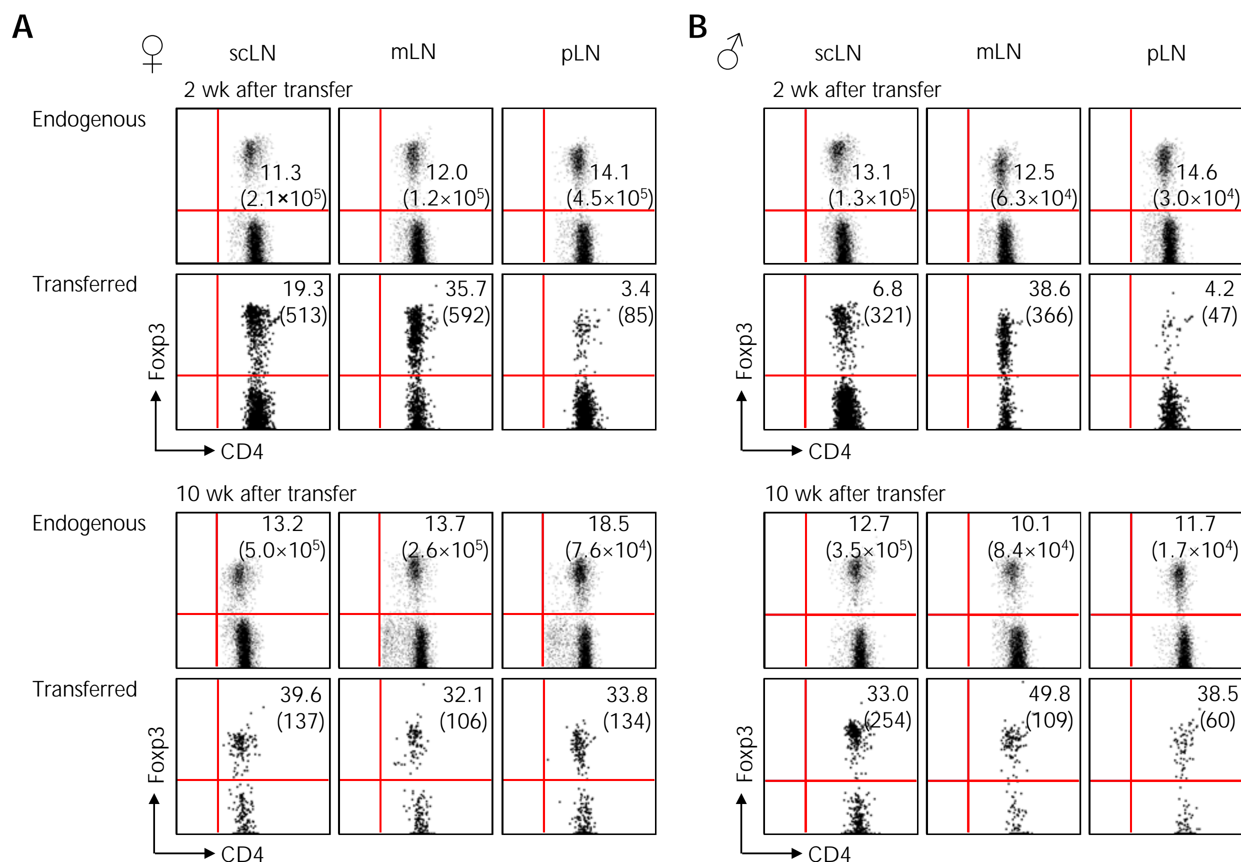

Figure 6. Stability of *in vivo* induced Foxp3^{+} BDC2.5 Treg cells. Normoglycemic female (**A**) or male (**B**) NOD-Thy1.2 recipient mice were adoptively transferred with Thy1.1[+] BDC2.5 T cells as described in the legend of Figure 2. Recipients were then injected with 200 ng anti-DEC-m63 (~4 ng m63 peptide). Induced Foxp3 expression among gated CD4^{+}Thy-1.1^{-} endogenous T cells (top) or CD4^{+}Thy-1.1^{+} BDC2.5 T cells recovered from indicated LNs were analyzed by flow cytometry at 2 and 10 weeks after cell transfer and recombinant antibody administration. NOD females analyzed at 10 weeks after cell transfer and antibody injection exhibited high blood glucose levels. Numbers in dot plots indicate percentages of Foxp3^{+} T cells among gated CD4^{+}Thy1.1^{-} or CD4^{+}Thy1.1^{+} T cells. Numbers in brackets indicate absolute numbers of CD4^{+}Foxp3^{+} T cells recovered from indicated lymph nodes. Data are representative of two independent experiments with one mouse per condition.

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Treg cells *in vivo* is suitable to interfere with disease progression in prediabetic NOD mice. Diabetes incidence in cohorts of 7 week-old mice was comparable to that of non-manipulated control animals (Figure 5, A and D, top). The cohort mice were either treated with a single-dose injection of 50 ng (data not shown), or 200 ng anti-DEC-m63 antibody alone (Figure 5A), or in conjunction with adoptively transferred naïve BDC2.5 T cells to increase the relationship of antigen-specific precursor cells to antigen-specific Foxp3+ Treg (Figure 5B).

Four consecutive injections of 1 μg anti-DEC-m63 antibody (~20 ng m63 mimotope peptide each) within 2 weeks resulted in an initial delay in progression towards hyperglycemia and a marginal reduction in overall disease incidence compared with untreated control NOD mice (Figure 5, C and D, bottom). Interestingly, administration of equivalent amounts of free synthetic mimotope peptide (20 ng) following the same treatment regimen appeared to accelerate progression towards overt diabetes (Figure 5D, bottom).

Next, we asked whether the limited impact on progression towards overt diabetes that we observed when recombinant anti-DEC-m63 antibodies were administered to prediabetic NOD mice, could be attributed to a decreased survival of *de novo*-generated m63-reactive Foxp3+ BDC2.5 Treg cells. High frequencies of Foxp3+ BDC2.5 T cells could be readily detected in pLNs and other peripheral lymphoid organs of NOD females 10

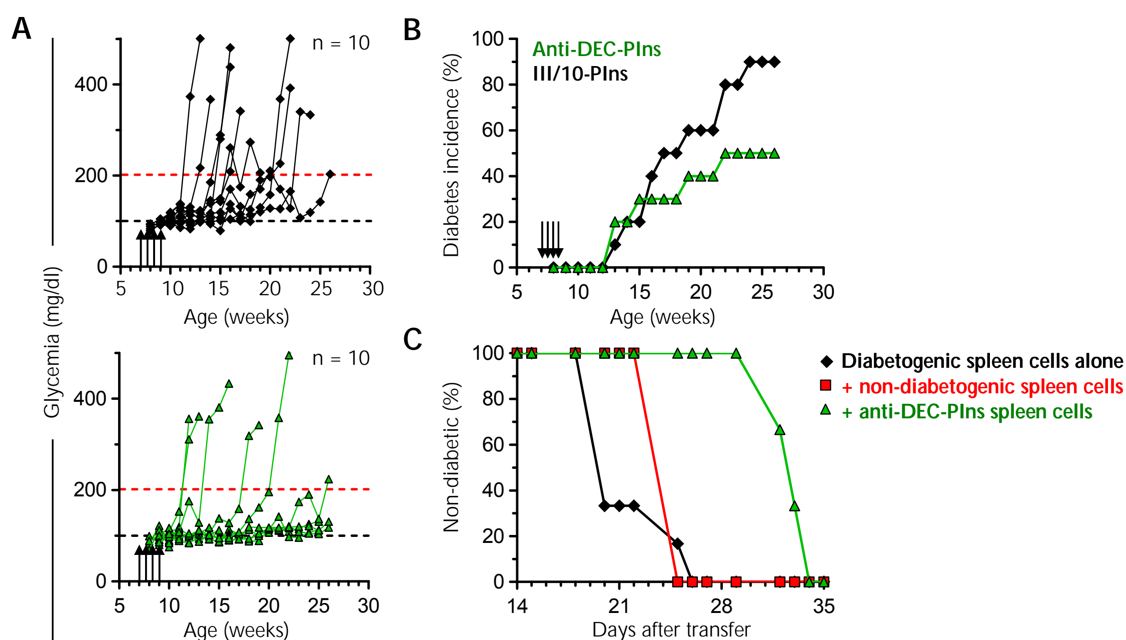

Figure 7. Selective delivery of proinsulin to DCs in prediabetic NOD mice limits progression towards diabetes. Cohorts of NOD mice received 4 consecutive injections of either 1 μg III/10-PIns isotype control antibody (top) or 1 μg anti-DEC-PIns antibody (bottom) within 2 weeks beginning at 7 weeks of age. Blood glucose concentrations (in mg/dl) of individual mice were determined weekly and plotted against age of mice in weeks (**A**). 1 μg recombinant antibody corresponds to ~100 ng proinsulin peptide. Arrowheads indicate time-points of recombinant antibody administration. Dashed lines in black and red indicate normal (100 mg/dl) and high (200 mg/dl) blood glucose levels, respectively. **B**: Incidence of diabetes among experimental groups. Black diamonds: III/10-PIns isotype control; green triangles: anti-DEC-PIns at 7 weeks of age (p = 0.1345, Log-rank test). The experiment was terminated when the mice reached 26 weeks of age (see below). **C**: Blood glucose levels of NOD-Rag1-/- recipient mice that received splenocytes harvested from diabetic NOD donor mice only (black diamonds, n = 6, average diabetes development at day 21.8 ± 2.6), or that were co-injected with equivalent numbers of splenocytes from either normoglycemic NOD donors (red squares, n = 2, 25.0 ± 0.0), or splenocytes from NOD donors depicted in **A** and **B** that maintained normoglycemia until 26 weeks of age after treatment with anti-DEC-PIns antibody beginning at 7 weeks of age (green triangles, n = 3, 33.0 ± 0.8). Log-rank test comparing NOD-Rag1-/- recipient mice that received splenocytes from diabetic donor mice alone or were co-injected with splenocytes from anti-DEC-PIns-treated NOD mice revealed a p value of 0.0068.

weeks after adoptive transfer of BDC2.5 T cells, and anti-DEC-m63 antibody treatment of normoglycemic NOD recipients that, at the time of analysis, had progressed to hyperglycemia (Figure 6A, bottom panels). In addition, accumulation of Foxp3$^+$ BDC2.5 T cells in female recipients was comparable to that observed in age-matched male recipients (Figure 6B). The latter are known to exhibit less invasive and destructive insulitis, and a much lower incidence of diabetes compared to females. m63-reactive Foxp3$^+$ Treg cells that had been generated by DC targeting *in vivo* survived for several months, without any appreciable adverse impact on hyperglycemia.

Selective delivery of proinsulin to DCs ameliorates autoimmune diabetes

BDC2.5's natural Ag that has recently been identified as chromogranin A [31] showed widespread expression in various tissues. Whereas, insulin and its precursor proinsulin are uniquely secreted by pancreatic β-cells. There is increasing evidence that autoimmunity to (pro)insulin may be central to disease pathogenesis [43-45]. This, and the view that (pro)insulin might be the primary and initiating self-Ag encouraged us to investigate the influence of DC-targeted proinsulin on disease progression in prediabetic NOD mice. To this end, we generated fusion proteins of proinsulin 2 (A-chain, C-peptide and B-chain), added inframe to the C terminus of anti-DEC-205 (anti-DEC-PIns) or III/10 isotype control (III/10-PIns) antibody. When 7 week-old NOD mice were treated with four consecutive injections of 1 μg anti-DEC-PIns antibody over a period of 2 weeks, 50% of mice maintained normoglycemia until the end of the observation period at 26 weeks of age (Figure 7, A and B). Whereas, 90% of littermate control NOD mice injected with equivalent amounts of III/10-PIns isotype control antibody, or left untreated, had progressed to overt diabetes (Figure 7, A and B, and data not shown).

We attempted to identify the mechanisms by which DC-targeted PIns ameliorates autoimmune diabetes in NOD mice. We asked whether cotransfer of spleen cells, from anti-DEC-PIns-treated NOD mice, can ameliorate diabetes development in immunocompromised NOD-Rag1$^{-/-}$ recipients of spleen cells from diabetic NOD donor mice. In these experiments, injection of spleen cells from a pool of hyperglycemic NOD donor mice resulted in diabetes development in all NOD-Rag1$^{-/-}$ recipients at day 21.8 ± 2.6. Co-transfer of spleen cells from non-manipulated normoglycemic NOD donor mice had little, if any, effect on diabetes development (Figure 7C). Whereas, diabetes development in NOD-Rag1$^{-/-}$ recipients occurred at day 33.0 ± 0.8 after co-transfer of spleen cells from diabetic NOD mice with a pool of spleen cells from anti-DEC-PIns-treated NOD mice that had normal blood glucose levels at 26 weeks of age (Figure 7C).

Discussion

We have reported on experiments to evaluate DC targeting of pancreatic β-cell antigen *in vivo*. These were designed as an approach to induce antigen-specific Foxp3$^+$ Treg cells and immunological tolerance in autoimmune diabetes. Previous studies employing the non-self model antigens hemagglutinin [22] and ovalbumin [24] indicated that, in certain experimental settings, selective delivery of agonist ligands to DEC-205$^+$ DCs can induce Foxp3-expressing Treg cells from initially naïve CD4$^+$CD25$^-$Foxp3$^-$ T cells. We should consider whether this pathway of artificial Treg cell generation also applies to self-antigens and self-reactive T cells. It now appears to be a relevant question in the context of novel therapy concepts in T1D prevention and β-cell replacement. The latter may be possible, either by transplantation or stimulation of β-cell regeneration. At present, both of which are hampered by reoccurring autoimmune-mediated β-cell destruction.

For several reasons, it was unclear whether previously described experimental protocols for the generation of antigen-specific Foxp3$^+$ Treg *de novo* by confronting CD4$^+$ T cells with antigen *in vivo* could be extended to pancreatic β-cell-reactive T cells in NOD mice. Several reported DC [35-40] and T cell [6-8] defects contribute to the failure to establish and maintain T cell tolerance in NOD mice. Considering these defects, it was unclear whether extrathymic DC-targeted *de novo* Treg cell generation could be achieved in autoimmune-prone animals, and T cells that are chronically exposed to self-antigen. Specifically, previous studies concerned with modalities of DC-targeted peripheral Treg cell conversion [22], established that efficient induction of Foxp3 expression required subimmunogenic conditions (i.e. low antigen dose, immature DC maturation stage, reduced IL-2R, and enhanced TGF-βR signaling). Recent studies suggested that limited proliferation of converting T cells [22] might be necessary to oppose cell cycle-dependent epigenetic silencing of the gene locus

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encoding Foxp3 [46]. Since these studies employed TCR transgenic CD4$^+$ T cells recognizing non-self model antigens, T cell proliferation was attributable to exogenous DC-targeted Ag. Furthermore, Treg cell conversion has been shown to work effectively with naïve T cells, while fully differentiated memory-type T cells are relatively resistant to the conversion process [21]. These observations suggested that efficient *de novo* Treg cell generation is hampered by endogenous self-Ag-mediated preactivation of β-cell-reactive CD4$^+$ T cells. The latter are unlikely to exhibit a truly naïve phenotype, even when selected for high expression of CD62L, as done in the present study. Lastly, continuous autoimmune inflammation and dysregulated glucose metabolism in NOD mice may negatively impact Treg cell conversion efficiency and survival of *de novo* generated Treg cells.

In an initial attempt to address the above described issues, we have tracked the fate of adoptively transferred β-cell-reactive congenic BDC2.5 CD4$^+$ T cells that had been confronted with minute amounts of DC-targeted mimotope peptide in immunocompetent NOD recipient mice. Based on these experiments, several important conclusions can be drawn regarding DC-targeted *de novo* generation of β-cell-reactive Treg cells in autoimmune diabetes. Firstly, DEC-205$^+$ DCs of NOD mice have the capacity to efficiently process and present targeted antigen to BDC2.5 CD4$^+$ T cells, *in vitro* and *in vivo*. Secondly, consistent with studies on TCR transgenic CD4$^+$ T cells recognizing the model antigens influenza hemagglutinin [22] or ovalbumin [24], selective delivery of a BDC2.5 mimotope peptide to immature DCs resulted in long-term survival of DC-primed Ag-specific CD4$^+$ T cells. Induction of efficient deletion can be observed for hen's egg lysozyme-specific 3A9 [27], or ovalbumin-specific OT2 [34] TCR transgenic CD4$^+$ T cells. However, this was not observed in our experiments. This result appears particularly important because promoting dominant tolerance pathways are likely to have superior efficacy in both preventive and therapeutic interventions in T1D, compared to deletional tolerance. Thirdly, our experiments indicate that selective delivery of minute amounts of a BDC2.5 mimotope peptide to DCs can induce Foxp3 expression and a Treg cell phenotype in a significant proportion of β-cell-reactive BDC2.5 CD4$^+$ T cells that initially exhibited a CD62L^{high}CD25$^-$Foxp3$^-$ phenotype. Tracking the proliferation of converting BDC2.5 T cells by CFSE dilution, indicated that the inverse relationship of cell division and induced Foxp3 expression, which had been described for TCR transgenic CD4$^+$ T cells recognizing non-self model antigens [22, 24], may also apply to self-reactive BDC2.5 CD4$^+$ T cells. Of note, additional experiments suggested that the efficiency of *de novo* Treg cell generation, accumulation, and long-term survival of induced BDC2.5 Treg cells appeared to be independent of the glycemic status of NOD mice. Further studies, involving large-sized cohorts and *de novo* generated Treg cells recognizing different β-cell-derived self-Ag, are required to substantiate this observation.

Additional studies that involved selective delivery of a major pancreatic β-cell-derived self-Ag to DCs in prediabetic NOD mice revealed a beneficial effect of DC-targeted proinsulin on progression towards overt diabetes. This was corroborated by the observation that co-transfer of spleen cells from anti-DEC-PIns-treated NOD mice delayed diabetes development in a NOD-Rag1$^{-/-}$ adoptive transfer model of autoimmune diabetes. Encouraged by this observation our current efforts concentrate on evaluating the relative contributions of recessive and dominant tolerance mechanisms that operate to protect β-cells from autoimmune destruction in anti-DEC-PIns-treated NOD mice. This may include deletion and induction of an anergic state in proinsulin-reactive T cells, as well as *de novo* generation and expansion of preexisting proinsulin-reactive Treg cells. Although consistent with the concept that proinsulin is a major auto-Ag and primary target during the early stages of T1D development, at this point, we can only speculate as to why DC-targeted BDC2.5 mimotope peptide was less effective than whole proinsulin in ameliorating autoimmune diabetes progression in prediabetic NOD mice. The higher complexity of proinsulin with regard to the number of contained MHC-I and MHC-II T cell epitopes, compared with the MHC-II mimotope peptide, may be part of the explanation. To reveal the full story, further experiments are required, involving individual peptide epitopes of proinsulin and additional major β-cell self-antigens.

Acknowledgements: We thank M. Nussenzweig (The Rockefeller University, New York, USA) for providing plasmid vectors of cloned anti-DEC-205 NLDC-145 and III/10 isotype control antibodies; E. Bonifacio (CRTD / DFG-Center for Regenerative Therapies Dresden, Germany) for critically reading the manuscript and helpful advice; C. Friebel for excellent technical assistance; and A. I. Garbe for critically reading the manuscript. This work was supported by the Kompetenznetz Diabetes mellitus (Competence Network for Diabetes mellitus) funded by the Federal Ministry of Education and Re-

search (BMBF, FKZ 01GI0805-07) and by a grant from the BMBF to the German Center for Diabetes Research (DZD e.V., FKZ01GI0924).

Disclosures (conflict of interests statement): The authors report no conflict of interests.

Author contributions: C.P. and J.R. designed and performed the experiments and C.P. contributed to the writing of the manuscript. T.K. and S.S. helped with some experiments. K.K. conceived the research, guided its design, analysis and interpretation and wrote the manuscript.

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