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Targeting of a T Cell Agonist Peptide to Lysosomes by DNA Vaccination Induces Tolerance in the Nonobese Diabetic Mouse

Elisa I. Rivas,* John P. Driver,† Nahir Garabatost,* Maximiliano Presa,* Conchi Mora,‡ Fernando Rodriguez,* David V. Serreze, † and Thomas Stratmann*

CD4 T cells are crucial effectors in the pathology of type 1 diabetes (T1D). Successful therapeutic interventions for prevention and cure of T1D in humans are still elusive. Recent research efforts have focused on the manipulation of T cells by treatment with DNA. In this paper, we studied the effects of a DNA treatment strategy designed to target antigenic peptides to the lysosomal compartment on a monospecific T cell population termed 2.5mi+ T cells that shares reactivity with the diabetogenic T cell clone BDC-2.5 in the NOD mouse. MIC class II tetramer analysis showed that repeated administrations were necessary to expand 2.5mi+ T cells in vivo. This expansion was independent of Ag presentation by B cells. A single peptide epitope was sufficient to induce protection against T1D, which was not due to Ag-specific T cell anergy. Typical Th2 cytokines such as IL-10 or IL-4 were undetectable in vivo. This expansion was independent of natural regulatory T cell (Treg). Although Ag-specific Foxp3+ Treg did not expand in response to treatment, diabetes onset was delayed in Treg-reconstituted and DNA-treated NOD.SCID mice. These observations provide evidence for a Treg-mediated protective mechanism that is independent of the expansion or de novo generation of Ag-specific Treg.

Type 1 diabetes (T1D) is caused by the destruction of the insulin-producing β cells in pancreatic islets (1). Numerous studies have underlined the crucial role of autoreactive T cells as the main effectors in β cell destruction (2). In the NOD mouse model system for T1D, CD4+ as well as CD8+ T cells contribute to tissue destruction in the pancreas. This has been demonstrated in several TCR transgenic (Tg) mouse models that express TCRs originating in diabetogenic T cell clones of either cell subset (3, 4). These studies have conclusively demonstrated that some autoreactive T cells not only escape negative thymic selection, but also resist mechanisms normally leading to peripheral tolerance. T1D in the NOD mouse is closely linked to the expression of a peculiar MHC class II β-chain allele, A^B7, that pairs with the α-chain of I-A^d (5). Polymorphisms with biochemical characteristic similar to A^B7 are also found in DQ8 and DQ2, two MHC class II alleles that are considered major risk factors for T1D in humans (6, 7). Therefore, the NOD mouse is particularly well suited as a natural model to study diabetogenic CD4 T cells.

Successful therapeutic interventions in human patients for the prevention and the cure for T1D are still elusive. An attractive approach to manipulate T cells in vivo is the treatment with DNA. Some of the advantages of such a strategy are the relative low production cost of DNA and, most importantly, the bypass of recombinant protein production and purification, which for some proteins can be labor intensive, costly, and not always successful. An additional advantage is the option to include specific signal sequences into the coding sequence, allowing one to direct recombinant proteins to the desired intracellular compartment of choice or to force their secretion. To date, a series of different DNA vaccine strategies have been used in the NOD mouse to induce Ag-specific tolerance, but with variable outcomes. Routes of administration (i.e., intradermal, i.m., or oral), choice of Ag, as well as coadministration of Ag-coding and IL-coding plasmids determine protection versus acceleration of T1D in this model (8–13). In most cases, these experiments have been carried out without a detailed analysis of the Ag-specific T cell response due to the lack of suitable reagents. A well-characterized model is BDC-2.5, a NOD-derived diabetogenic CD4 T cell clone isolated based on its responsiveness to pancreatic β cells (14). Transfer of the T cell clone into young NOD mice accelerates T1D (15). Although T cell clones as well as TCR Tg mice offer the advantage of a plethora of cells with a given Ag specificity, neither Tg animals nor T cell transfer models adequately mimic physiological con-
ditions in wild-type (wt) NOD mice in which low frequencies of polyclonal T cell populations respond to more than one Ag simultaneously. This drawback becomes even more evident when strategies for T1D prevention or treatment are explored that may be tested conceptually in these models (13) but need further evaluation in a complex natural setting. Therefore, we recently approached this problem by the generation of Aβ7 tetramers complexed to a strong peptide agonist for BDC-2.5 (16). Tetramer analysis of NOD mice facilitated the identification of a natural complexed to a strong peptide agonist for BDC-2.5 (16). Tetramer analysis of Foxp3+ T cells, animals received three injections of plasmid DNA at weekly intervals, starting at week 4 of age. Blood glucose levels were monitored using the EndoFree plasmid mega kit from Qiagen (Hilden, Germany).

In this study, we used the 2.5mi+ T cell system in wt NOD mice to examine the response to a DNA treatment approach designed to target peptides to the lysosomal MHC class II Ag-loading site. We found that Ag-specific T cells respond to the treatment by a brief expansion phase of IFN-γ-producing Th1 T cells. Our results provide evidence that long-term protection is achieved possibly in part in a regulatory T cell (Treg)-dependent fashion.

Materials and Methods
Reagents
All Abs were purchased from eBioscience (La Jolla, CA) or BD Biosciences (Franklin Lakes, NJ), respectively. Cell-culture media were obtained from Lonza (Barcelona, Spain). Streptavidin-PE was obtained from Columbia Bioscience (Columbia, MD). Unless otherwise mentioned, all other reagents were obtained from Fluka or Sigma-Aldrich (Madrid, Spain).

Mouse strains and treatment
NOD/LtJ, BDC-2.5/N TCR Tg, NOD.SCID, and B6.

Adoptive T cell transfer, cell preparation, cell staining, and flow cytometry analysis
Ex vivo T cell analysis was carried out as previously described (16). Briefly, single-cell suspensions were blocked with avidin (Sigma-Aldrich) in FACS buffer (PBS containing 2% FCS and 0.04% NaN3) and stained with PE-labeled MHC/peptide tetramers on ice. For costing of surface markers, FITC–anti-CD4, PE cyanine 5–anti-B220, and PE cyanine 5–anti-CD8 from eBioscience were used; dead cells were excluded by addition of propidium iodide (PI). Flow cytometry was performed using an FACScan, FACSCalibur, or FACSVantage instrument (BD Immunocytometry Systems, Mountain View, CA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

For analysis of Foxp3 expression by intracellular staining with Ab, NOD. NON-Thy1a (Thy1b+) recipient female mice were sublethally irradiated with 600 rad before transfer via tail vein of 5 × 106 total splenocytes extracted from BDC-2.5/N TCR (Thy1b+) Tg female mice. DNA treatment was carried out starting at 4 wk posttransfusion. Splenocytes were prepared as described above and intracellular staining for Foxp3 was performed using the eBioscience kit under the conditions recommended by the manufacturer. FITC–anti-Foxp3 and PE cyanine 5–anti-CD8 were used (eBioscience).

Alternatively, Treg were isolated from naive BDC-2.5/N TCR Tg mice using a CD4+CD25+ Regulatory T Cell isolation kit (Miltenyi Biotec). For in vivo transfer experiments, 3 × 107 CD4+CD25+ T cells were transferred to 4-wk-old NOD.SCID mice by injection into the tail vein. Four weeks later, mice were treated in weekly intervals three times with 100 μg DNA (pCMV-2.5mi-LIMPII or pCMV-GPI-LIMPII as control) and rested for an additional 4 wk. To assess function of Treg, total splenocytes were isolated from BDC-2.5/N TCR Tg mice, activated in vitro for 48 h with 5 μM synthetic 2.5mi peptide, and 2.5 × 106 of activated cells were transferred vial tail vein into the reconstituted and treated NOD.SCID mice. Diabetes onset was assessed as mentioned above.

For the analysis of B6.Foxp3EGFP × NOD F1 mice, surface marker Abs allophycocyanin–anti-CD4, PE-cyanine 5–anti-B220, and PE-cyanine 5–anti-CD8 were used (eBioscience).

Nested RT-PCR
Nested RT-PCR was carried out as published (18). Briefly, stained T cells were sorted using a FACS Vantage sort dot (BD Biosciences) directly into 25 μl (2.5mi+CD4+ cells) or 100 μl (total 2.5mi+CD4+) of cDNA reaction mixture containing 15 U/μl MLV-RT (Fermentas) with the buffer provided by the manufacturer, 0.5 nM spemidine (Sigma-Aldrich), 100 μg/ml BSA, 10 ng/ml oligo(dT), 200 μM each 2′-deoxynucleoside 5′-triphosphate (Fermentas), 1 mM DTT, 1 μl (Sigma-Aldrich), 220 U/ml RNasin (Promega), 100 μg/ml Escherichia coli RNA (Boehringer Ingelheim), and 1% Triton X-100. The reaction mixture was incubated at 42°C for cDNA synthesis and stored at −80°C. The PCR reactions were carried out using nested primer pairs and the amount of cDNA corresponding to 40 cells under the exact conditions as published (18).

Immunohistochemistry
In situ detection of 2.5mi+ T cells has been published (16). Briefly, pancreata were embedded in Tissue-Tek OCT compound (Sakura Fine Tek, Torrance, CA) and immediately frozen at −80°C. Six-micrometer sections were cut using a microtome at −16°C, transferred onto Super frosted slides (Fisherbrand), and incubated overnight at 4°C with 200 μl solution containing allophycocyanin–tetramer and rat anti-mouse CD4 Ab in PBS containing 2% FCS. Slides were washed three times with PBS and fixed for 30 min at room temperature with PBS-buffered 2% formaldehyde. After washing three times in PBS, slides were incubated for 3 h at 4°C with rabbit anti-allophycocyanin (Biomedica, Foster City, CA) diluted in PBS containing 2% FCS, washed three times in PBS and incubated with rhodamine red X-conjugated goat anti-rabbit Ab (both from Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 2% FCS. Slides were washed three times as before and mounted using SlowFade (Molecular Probes, Eugene, OR). Sections were analyzed using a laser scanning confocal microscope (Olympus) as published.

MHC class II tetramers
MHC–peptide tetramer complexes were prepared as previously described in detail (16) using Drosophila melanogaster-derived SC2 cells transfected with DNA plasmids coding for the Aα chain containing a biotinylation sequence [W83 (19)] and the Aβ chain β-chain connected N-terminally to the 2.5mi or the GPI peptide, respectively. Molecules purified from culture supernatants were biotinylated using the BirA enzyme, and tetramers were generated by incubation of Aβ7 monomers with PE-labeled streptavidin in a 5:1 ratio.
ELISPOT

ELISPOT assays were carried out using a mouse IFN-γ ELISPOT reagent set (BD Biosciences, Franklin Lakes, NJ) as indicated by the manufacturer. Total splenocytes from treated (three immunizations with pCMV-2.5mi-LIMPII or pCMV-2.5mi) or naive control female NOD mice (three mice per group) were prepared 4 d after the last immunization as described above, and 2.5 × 10^5 cells/well were plated in RPMI plus 10% FBS in the absence or presence of 1 or 10 μM 2.5mi peptide. After 4 d of incubation at 37°C, ELISPOT assays were performed as indicated by the manufacturer.

Statistical analysis

Cumulative incidence of diabetes was determined by Kaplan-Meier estimates using the GraphPad Prism software version 5.0 (GraphPad), and statistical analysis of difference was determined by log-rank test. A p value < 0.05 was considered significant. Bar diagrams were analyzed using the GraphPad Prism software (GraphPad) and the Bonferroni test for selected pairs of columns or unpaired t test.

Results

Treatment with pCMV-2.5mi-LIMPII leads to expansion of 2.5mi+ T cells

The LIMPII tail targets fused proteins or peptides to the lysosomal compartment (20). We decided to analyze the efficacy of a DNA vaccine that targets a T cell peptide agonist to this compartment. To do so, we generated a DNA plasmid that codes for 2.5mi N-terminally fused to the last 20 aa of the LIMPII tail (pCMV-2.5mi-LIMPII). We also generated a control plasmid (pCMV-GPI-LIMPII) in which the 2.5mi was replaced by GPI282–292, a naturally processed peptide derived from GPI that binds to A^B (21) (Supplemental Fig. 1).

To test the efficacy of pCMV-2.5mi-LIMPII, we carried out a series of dose-response experiments. Five- to 6-wk-old NOD females were injected up to three times with 100 μg plasmid into the muscles tibialis, and the expansion of 2.5mi+ T cells in the spleen and in a collection of LN was analyzed 4 d later by staining with the A^B/2.5mi tetramer. No expansion of 2.5mi+ T cells was detected after one or two injections. However, 4 d after the third injection (day 18 of the entire treatment protocol), an averaged 6-fold increase of 2.5mi+ T cells was found in spleen and draining iliac LN, but not in any other LN analyzed (inguinal LN, popliteal LN, mesenteric LN; Fig. 1A, 1B). Control staining with the A^B/GPI tetramer revealed no T cell expansion (Fig. 1A). These data suggested that proliferation of 2.5mi+ T cells was a result of an accumulative effect of three successive injections with DNA. To assess whether pCMV-2.5mi-LIMPII augmented the 2.5mi+ T cell response in comparison with the same vector coding for the 2.5mi peptide lacking the LIMPII tail, we generated additional plasmids in which a stop codon was introduced after the 2.5mi and the GPI-coding sequences (pCMV-2.5mi and pCMV-GPI). Six- to 7-wk-old NOD females treated three times in weekly intervals with pCMV-2.5mi-LIMPII showed a significantly higher response (over 2-fold on average) in comparison with pCMV-2.5mi at day 4 after the last dose of DNA. Treatment with the pCMV-GPI-LIMPII or pCMV-GPI control plasmids did not lead to any significant 2.5mi+ T cell expansion (Fig. 1C). These experiments confirmed that fusion to LIMPII is a valid approach to augment CD4+ T cell stimulation in vivo.

DNA immunization leads to a mild inflammation at the point of injection (22). Repeated immunization at the same site is likely to increase local inflammation and recruitment and activation of a large number of APCs, such as macrophages and dendritic cells. It was therefore possible that the first two injections were merely necessary to recruit sufficient APCs to the site of injection that were transfected in vivo in sufficient quantities by the third injection to allow expansion of Ag-specific T cells. In this scenario, the Ag encoded by the DNA used during the first two injections was likely to be irrelevant. To test this hypothesis, NOD females were injected three times with naked DNA as above; however, pCMV-GPI-LIMPII was used for the first and second injection, whereas pCMV-2.5mi-LIMPII was only used for the last injection. No expansion of 2.5mi+ T cells could be detected 4 d after the last injection (Supplemental Fig. 2), indicating that even if additional APCs were attracted as a result of multiple injections, specific Ag-coding DNA needed to be applied more than once.

A time-course experiment revealed that maximal percentage of 2.5mi+ T cells within the CD4+ T cell subset was found in the spleen at day 3 after the third injection and remained elevated at day 10. By week 4, cell numbers were essentially indistinguishable from age-matched naive littersmates (Fig. 1D). In conclusion, only after the third dose of DNA, a fast expansion of Ag-specific CD4 T cells was observed, which is typical for a memory response (18).

B cells are required for in vivo expansion of 2.5mi+ T cells in naive NOD mice but dispensable for Ag-specific response upon DNA treatment

Previously, it has been shown that NOD mice devoid of the B cell compartment (NOD.Ig^null mice) are protected from insulitis and diabetes (23). Reintroduction of a BCR specific for a xenogeneic antigen (hen egg lysozyme) into the NOD.Ig^null background does not restore diabetes in these animals (24), indicating that the Ag specificity of B cells in the NOD background is relevant for pathogenesis. Therefore, we investigated how the absence of B cells affected the 2.5mi+ T cells population upon DNA treatment. As recently published (25), 2.5mi+ T cells in naive NOD. Ig^null mice were almost undetectable, whereas the size of the total CD4+ T cell compartment remained unaffected (Fig. 2). Treatment with three doses of pCMV-2.5mi-LIMPII revealed that the absence of B cells did not prevent expansion of 2.5mi+ T cells. However, their numbers were significantly lower (p < 0.05) compared with treated wt NOD mice, possibly mirroring the increased frequency of 2.5mi+ T cell precursors in naive wt NOD mice compared with naive NOD.Ig^null animals (Fig. 2).

These data indicated a requirement of B cells for the natural expansion and possibly for the maintenance of 2.5mi+ T cells in naive animals in vivo. However, B cells are dispensable for Ag-specific 2.5mi+ T cell expansion upon in vivo challenge with DNA.

In vivo delivery of LIMPII-2.5mi prevents diabetes

Next, we explored whether protection against T1D was afforded by treatment with pCMV-2.5mi-LIMPII. To this end, starting at 4 wk of age, NOD females were injected on a weekly basis three times consecutively with pCMV-2.5mi-LIMPII or with pCMV-GPI-LIMPII. Disease onset and prevalence in NOD females treated with the control plasmid was similar to the one observed in naive NOD female littersmates (Fig. 3). In contrast, ∼50% of animals treated with the pCMV-2.5mi-LIMPII were protected from diabetes. We concluded that diabetes prevention was due to the presentation of the 2.5mi peptide and not to the in vivo delivery of the pCMV-LIMPII plasmid per se.

Histological analysis of pancreata of surviving animals at the endpoint of the experiments revealed islet infiltration of a similar degree in both treatment groups, indicating that insulitis was not completely prevented in animals that were protected by treatment with pCMV-2.5mi-LIMPII (not shown). Further analysis of infiltrated islets by immunohistochemistry using anti-CD4 Abs and the A^B/2.5mi tetramer revealed only very few 2.5mi+ T cells in pCMV-2.5mi-LIMPII DNA-treated animals, similar to what we
had previously observed in naive NOD females (Fig. 4A) (16). In a control experiment, almost all CD4 T cells of a BDC/N TCR Tg mouse were stained by the Ag7/2.5mi tetramer but not by the Ag7/GPI tetramer in the spleen, demonstrating the specificity of the reagent (Fig. 4B). These results indicate that 2.5mi+ T cells are neither massively recruited to the pancreas after treatment nor is their migration to the pancreas completely prevented at this late stage of insulitis.

### Functionality of 2.5mi+ T cells

Several mechanisms could explain the protective effect of the treatment with pCMV-2.5mi-LIMPII. Some of these mechanisms include the induction of anergy in the Ag-specific T cell population or the conversion of Th1 to Th2 cells, a reported mechanism by which autoimmunity may be prevented (26). To test the first possibility, starting at 4 wk of age, NOD females were injected three times with pCMV-2.5mi-LIMPII or with pCMV-GPI-LIMPII. Naive mice (n = 7) as well as mice treated with pCMV-GPI-LIMPII or pCMV-GPI served as controls. Expansion of 2.5mi+ T cells was analyzed at day 4 after the last injection by tetramer analysis.

### FIGURE 1

**Ag-specific T cell response to pCMV-2.5mi-LIMPII challenge in NOD mice.** A. 2.5mi+ T cell expansion is detectable by tetramer staining in spleen and iliac (ILN), but not in pancreatic (PLN), popliteal (PopLN), or inguinal LN (ILN) after three doses of pCMV-2.5mi-LIMPII. Left group of panels, Staining with the Aβ7/2.5mi tetramer; right group of panels, control staining with the Aβ7/GPI tetramer. Top panels, Naive NOD mice; bottom panels, treated NOD mice. T cells are gated on CD8+ B220−, (PI)−, and CD4+. T cells were analyzed at day 4 after the last injection. B. Five- to 6-wk-old NOD females were injected with pCMV-2.5mi-LIMPII i.m., up to three times in weekly intervals, and the percentage of 2.5mi+ T cells within the CD4+ T cells compartment was analyzed by staining with the Aβ7/2.5mi tetramer in spleen and iliac LN (ILN). Cells were gated as in A. T cell expansion is statistically significant after three injections compared with zero, one, and two injections, respectively (∗p values all <0.05). T cells were analyzed at day 4 after the last injection. C. Differential expansion of 2.5mi+ T cells after injection of pCMV-2.5mi-LIMPII or pCMV-2.5mi. Six- to 7-wk-old NOD females (n = 12 per plasmid) were injected three times in weekly intervals with 100 µg pCMV-2.5mi-LIMPII or pCMV-2.5mi. Naive mice (n = 7) as well as mice treated with pCMV-GPI-LIMPII or pCMV-GPI served as controls. Expansion of 2.5mi+ T cells was analyzed at day 4 after the last injection by tetramer analysis. D. Time-course analysis of 2.5mi+ T cell response to treatment with pCMV-2.5mi-LIMPII. NOD mice received three doses of DNA, and the Ag-specific T cell response in spleen was determined by staining with Aβ7/2.5mi tetramers at the indicated time points after the last challenge. T cell expansion is statistically significant at day 4 (∗∗∗p < 0.01) and day 5 (∗p < 0.05) as compared with day 0. Cells were gated as in B. ∗∗∗p < 0.0001.

### FIGURE 2

**Expansion of 2.5mi+ T cells in B cell deficient NOD mice upon challenge with DNA.** NOD and NOD.Igα−/− mice (n = 5/group) were analyzed for the presence of 2.5mi+ T cells before and after three injections with pCMV-2.5mi-LIMPII. Numeric differences of tetramer-positive T cells are significant (∗∗p < 0.01).

### FIGURE 3

**Cumulative diabetes incidence in NOD mice treated with pCMV-2.5mi-LIMPII or the control pCMV-GPI-LIMPII.** Starting at 4 wk of age, NOD females (n = 30/group) were treated with three injections of DNA in weekly intervals. Animals with blood glucose levels exceeding 200 mg/dl were considered diabetic. The graph represents a compilation of three independent experiments. The diabetes incidence of the naive colony (n = 20) is shown for comparison. ∗p < 0.05.
LIMPII, and then challenged 4 or 40 wk after the last injection with r2.5mi. In either case, a strong expansion of the 2.5mi+ T cell population was detected in the spleen after 4 d, indicating that these cells remained fully responsive in vivo (Fig. 5A,5B).

To test conversion of Th1 to Th2 cells, we analyzed the cytokine profile from sorted splenic 2.5mi+ T cells originating from naive and DNA-treated animals by RT-PCR. Contrary to total CD4 T cells, sorted 2.5mi+ T cells from either naive or treated mice produced detectable amounts of IFN-γ just like the BDC-2.5 T cell clone (27) (Fig. 6A). To the contrary, 2.5mi+ T cells did not produce traceable amounts of IL-10, and none produced IL-4 or IL-17 (not shown), indicating that 2.5mi+ T cells of naive and treated NOD mice were Th1 and neither Th2 nor Th17 cells. Strong production of TNF-α was detected exclusively in 2.5mi+ T cells, indicating that these cells had been activated. Finally, animals that had received the pCMV-2.5mi-LIMPII DNA developed more IFN-γ–producing cells in the spleen than naive littermates, as demonstrated by ELISPOT assay using the 2.5mi peptide for in vitro stimulation (Fig. 6A, 6B). Mice treated with the control vaccine pCMV-2.5mi generated only marginal amounts of IFN-γ–producing cells, further confirming the enhanced T cell stimulation in vivo achieved by the fusion approach to the LIMPII tail. Taken together, these experiments clearly show that treatment with pCMV-2.5mi-LIMPII led to expansion of Ag-specific, IFN-γ–producing T cells.

FIGURE 4. 2.5mi+ T cells are not prevented from migrating to pancreatic islets after treatment with pCMV-2.5mi-LIMPIII. A, Surviving animals from long-term treatment studies were analyzed for islet-infiltrating 2.5mi+ T cells by immunohistochemistry. An infiltrated islet stained with an anti-CD4 Ab, and the Aβ/2.5mi tetramer is shown. a and c, Overlay of anti-CD4 Ab and Aβ/2.5mi tetramer staining; b and d, Aβ/2.5mi tetramer staining only of the same section; c and d, same as a and b, enlarged. B, Control staining of a spleen section from a BDC/N TCR Tg animal using the same reagents as in A. Top row left and right, Aβ/2.5mi tetramer; bottom row left and right, Aβ/GPI tetramer. Left panels, Tetramer and anti-CD4 overlaid; center panels, anti-CD4 only; right panels, tetramer only. Original magnification ×60.

No pCMV-2.5mi-LIMPII–mediated T1D protection in NOD. CD28−/− mice

NOD.CD28−/− mice are largely devoid of natural Treg (nTreg) (28), leading to an accelerated onset of diabetes in these animals. We decided to explore the efficacy of pCMV-2.5mi-LIMPII in a side-by-side analysis of NOD.CD28−/− and NOD mice. Animals were treated three times with the vaccine in weekly intervals, starting at 4 wk of age. Treatment led to a similar ~5-fold increase of this population in both strains. However, either due to the higher frequency of Ag-specific T cell precursors or the lack of Treg that control expansion of these cells in NOD.CD28−/− animals, approximately three times more 2.5mi+ T cells were detected in these animals compared with treated wt NOD mice after DNA injection (Fig. 1A, 7A). We next monitored T1D progression in NOD.CD28−/− animals. As previously reported (29), diabetes onset in these animals was accelerated in comparison with wt NOD mice. Disease onset and prevalence was essentially indistinguishable in pCMV-2.5mi-LIMPII–treated NOD.CD28−/− animals compared with naive littermates or treated with the control pCMV-GPI-LIMPII DNA (Fig. 7B). These results suggested that in a more aggressive disease model, pCMV-2.5mi-LIMPII was not effective, CD28 was necessary for protection, or nTreg were involved in disease protection mediated by the DNA treatment.
2.5mi+ Foxp3+ T cells exist in NOD mice but do not expand upon DNA treatment

The failure of the pCMV-2.5mi-LIMPII to induce protection in an nTreg-impaired model prompted us to investigate the role of Foxp3+ Treg further. We decided to explore whether DNA treatment led to de novo generation of Ag-specific Tregs or to expansion of a pre-existing Ag-specific Treg population. First, we transferred splenocytes from BDC-2.5/N TCR animals into Thy1-mismatched, sublethally irradiated NOD females and thereby generated mice that contained between 20 and 25% of clonotypic CD4 T cells (Supplemental Fig. 3). Intracellular staining revealed ∼3% of Foxp3+ BDC-2.5 T cells in the spleen in both DNA-treated and naive animals (Fig. 8A), similar to previously published frequencies of Foxp3+ T cells found in the BDC/N TCR Tg strain (30). This indicated that under these experimental conditions, DNA treatment did not lead to either substantial expansion or de novo generation of clonotypic Foxp3+ T cells.

Next, we analyzed whether and to what extent Foxp3+ T cells formed part of the natural 2.5mi+ T cell population. A combined analysis of tetramer and intracellular Foxp3 staining by Ab led to a high background staining. To circumvent this technical difficulty, we crossed hemizygous B6 males that carry a bicistronic Foxp3 locus coexpressing enhanced GFP (EGFP) under the endogenous Foxp3 promoter [hereafter referred to as B6.Foxp3EGFP mice (31)] with NOD wt females. The resulting heterozygous B6.Foxp3EGFP ∞ NOD F1 females were analyzed for the presence of CD4+EGFP+ T cells in the spleen. Approximately 10% of total CD4 T cells were EGFP+. B6.Foxp3EGFP ∞ NOD F1 females contained a distinct 2.5mi+ T cell population, 8% of which also expressed Foxp3. Triple immunization with pCMV-2.5mi-LIMPII leads to a 5-fold expansion of 2.5mi+ T cells by day 4; however, as in the BDC-2.5 system, 2.5mi+Foxp3+ did not expand (Fig. 8B, Table I). A further analysis of the percentage of CD4+Foxp3+ T cells within the CD4 T cell compartment of naive and treated NOD mice did not reveal any difference between both groups (Fig. 8A). Together, these experiments suggest that treatment with pCMV-2.5mi-LIMPII does not lead to the expansion or de novo generation of 2.5mi+Foxp3+ T cells.

Delayed diabetes onset in Treg-reconstituted and DNA-treated NOD.SCID mice

To further analyze the function of Ag-specific Treg after immunization, we isolated CD25+CD4+ T cell from naive BDC-2.5/N TCR mice and transferred them to 4-wk-old NOD.SCID mice. Four weeks later, the reconstituted mice were treated with either...
Splenocytes from BDC/N TCR Tg Thy1b+ mice transferred to sublethally irradiated Thy1b+
(Thy1b+, P I F1 generation female of B6.
splenocytes is shown throughout the entire figure. LIMPII DNA (control, analyzed for Foxp3 expression as deduced from presence or absence of EGFP. Total events are gated on PI
population was assessed by intracellular staining using an anti-Foxp3 Ab (2.5mi+ T cells by tetramer analysis. Total events are gated on PI
no expansion of 2.5mi+Foxp3+ T cells following treatment with pCMV-2.5mi-LIMPII.
FIGURE 8. No expansion of 2.5miFoxp3+ T cells following treatment with pCMV-2.5mi-LIMPII. A. Foxp3 expression within the CD4 T cell pop-
ulation was assessed by intracellular staining using an anti-Foxp3 Ab (left and center panels) or expression of EGFP (right panels). Animals were left naive (top left panel) or treated with pCMV-GPI-LIMPII DNA (control, top center and right panels) or pCMV-2.5mi-LIMPII DNA (bottom panels). Left panels, Splenocytes from BDC/N TCR Tg Thy1b+ mice transferred to sublethally irradiated Thy1b+ NOD females. FACS analysis shows CD4+ T cells gated on Thy1b+, P I , B220+, CD8+, and CD4+ cells. Center panels, NOD wt animals. FACS analysis shows CD4+ T cells gated on P I , B220+, and CD8+. Right panels, An F1 generation female of B6.Foxp3GFP males crossed with NOD wt females is shown. FACS analysis as for center panels. B, Foxp3 expression within the 2.5mi+ T cell population. CD4 T cells from F1 generation females of B6. Foxp3GFP males crossed with NOD wt females were analyzed for coexpression of Foxp3 and 2.5mi-specific TCRs. Left panels, Analysis of 2.5mi+ T cells by staining with the A7/2.5mi tetramer of animals treated with pCMV-GPI-
LIMPII DNA (control, top panels) or with pCMV-2.5mi-LIMPII DNA (bottom panels). Right panels: Gated 2.5mi+ T cells from left panels were further analyzed for Foxp3 expression as deduced from presence or absence of EGFP. Total events are gated on P I , B220+, CD8+, and CD4+ cells. Analysis of splenocytes is shown throughout the entire figure.

Discussion
A number of different approaches have been used in the NOD mouse to prevent T1D or ameliorate disease at the early-onset phase. However, the lack of suitable reagents has presented a major drawback to systematically analyze therapeutic effects on Ag-specific CD4 T cells in vivo. In this study, we used a targeted DNA treatment approach in combination with A7 tetramers to study its effect on the 2.5mi+ CD4 T cell population that shares its reactivity with the well-characterized diabetogenic BDC-2.5 T cell clone.

In naive NOD mice, a fraction of 2.5mi+ T cells are activated. Yet, contrary to our expectations, three successive injections were necessary to induce measurable Ag-specific T cell expansion (at day 18). These results are in contrast to a typical secondary-type expansion in which proliferation is detected at day 3 or 4 after challenge, similar to what we detect after injection of 2.5mi peptide and what has been observed in other CD4 T cell model systems (32). We experimentally excluded that this was a delayed response, as two injections alone did not lead to any measurable expansion at day 18 (not shown). Instead, the response was a result of cumulative administrations of the same Ag. Replacement of the 2.5mi peptide coding DNA with the control plasmid coding for the GPI peptide during the first two injections did not lead to T cell expansion upon the third dose of DNA. Therefore, the two first DNA injections did not simply favor a better DNA uptake by mechanical force, either by attraction of APCs or by damage of

FIGURE 7. T1D onset is not prevented in DNA-treated NOD.CD28−/− mice. A, WT NOD mice or NOD.CD28−/− mice were treated three times with pCMV-2.5mi-LIMPII or left naive and analyzed for the presence of 2.5mi+ T cells by tetramer analysis. Total events are gated on P I , B220+, CD8+, and CD4+ cells. B, Cumulative diabetes incidence of NOD.CD28−/− mice treated three times with pCMV-2.5mi-LIMPII, pCMV-GPI-LIMPII, or left naive (n = 6 for each group). Mice were checked for blood glucose levels every 3 d after the last dose of DNA. Animals with blood glucose levels exceeding 200 mg/dl were considered diabetic. The graph represents a compilation of two independent experiments.

FIGURE 8. No expansion of 2.5miFoxp3+ T cells following treatment with pCMV-2.5mi-LIMPII. A. Foxp3 expression within the CD4 T cell pop-
ulation was assessed by intracellular staining using an anti-Foxp3 Ab (left and center panels) or expression of EGFP (right panels). Animals were left naive (top left panel) or treated with pCMV-GPI-LIMPII DNA (control, top center and right panels) or pCMV-2.5mi-LIMPII DNA (bottom panels). Left panels, Splenocytes from BDC/N TCR Tg Thy1b+ mice transferred to sublethally irradiated Thy1b+ NOD females. FACS analysis shows CD4+ T cells gated on Thy1b+, P I , B220+, and CD8+. Center panels, NOD wt animals. FACS analysis shows CD4+ T cells gated on P I , B220+, and CD8+. Right panels, An F1 generation female of B6.Foxp3GFP males crossed with NOD wt females is shown. FACS analysis as for center panels. B, Foxp3 expression within the 2.5mi+ T cell population. CD4 T cells from F1 generation females of B6. Foxp3GFP males crossed with NOD wt females were analyzed for coexpression of Foxp3 and 2.5mi-specific TCRs. Left panels, Analysis of 2.5mi+ T cells by staining with the A7/2.5mi tetramer of animals treated with pCMV-GPI-
LIMPII DNA (control, top panels) or with pCMV-2.5mi-LIMPII DNA (bottom panels). Right panels: Gated 2.5mi+ T cells from left panels were further analyzed for Foxp3 expression as deduced from presence or absence of EGFP. Total events are gated on P I , B220+, CD8+, and CD4+ cells. Analysis of splenocytes is shown throughout the entire figure.
myocytes. DNA uptake by myocytes as well as by local APCs has been demonstrated (33). As pCMV-LIMPII has been designed to target peptides to lysosomes, direct transfection of APCs would be an efficient way to present peptides to 2.5mi+ T cells. However, murine as well as human myocytes have been shown to express MHC class II molecules upon DNA uptake and to process Ag, which is dependent on IFN-γ (33–35). Accumulation of myocytes transfected with the 2.5mi coding plasmid together with locally transfected APCs may be responsible for presenting the peptide to primed 2.5mi+ T cells. In addition, by means of receptor export from the lysosomes to the cell surface, processed but unbound peptide is likely to leak out into the intracellular space where it could be picked up by resident or circulating dendritic cells and macrophages, allowing priming of naive 2.5mi+ T cells.

Although B cells may aid in the process of peptide presentation, B cell-deficient mice still responded to treatment with DNA. Expansion of 2.5mi+ T cells was of similar magnitude in B cell-deficient mice (∼6–8-fold) as in wt animals; however, the percentage in B cell-deficient mice was lower, both in naive as well as in treated animals. Therefore, similar to recently published data (25), we found that B cells are implicated in the expansion and/or maintenance of the 2.5mi+ T cell population in naive NOD mice, although they are not absolutely required in the expansion phase of DNA treatment with the LIMPII plasmid.

Treatment with pCMV-2.5mi-LIMPII had a clear therapeutic effect and reduced T1D in ∼50% of animals compared with control-treated mice. It is remarkable that manipulation of a single T cell specificity had an effect in such a complex system as the NOD mouse. Possibly, the target Ag of BDC-2.5 has a similar key function as insulin in the development of T1D in the NOD mouse (36). As the target Ag of BDC-2.5, chromogranin A, has only very recently been identified (37), studies to clarify this question have not yet been reported. However, it remains an open question to which extent key Ags are relevant in humans. During the course of T1D, multiple autoantigens are targets of T cell responses (2). Thus, prevention of or treatment at early-onset diabetes by elimination or silencing of a single T cell specificity is likely to be difficult. An alternative, more realistic possibility is the expansion or activation of T cells that have some regulatory properties. In our experimental conditions, DNA treatment clearly did not lead to the elimination of 2.5mi+ T cells. In fact, quite the opposite occurred. At least for a brief period, these T cells increased in number. These cells were not anergic either during a short-term experiment or at the endpoint of a long-term treatment study, as they expanded vigorously after in vivo administration of r2.5mi peptide. Treatment did not completely prevent 2.5mi+ T cells from penetrating the pancreas, but neither did we observe a massive infiltration of these cells upon treatment.

If percentages of 2.5mi+ T cells augment after DNA treatment, why then was disease not accelerated in these animals? A possible explanation would be the deviation of a Th1 to a Th2 profile that has been reported for Ag-specific treatment in NOD mice (26). However, our results demonstrate that this was not the case as 2.5mi+ T cells from treated mice had a Th1 profile, just like the ones from naive mice or BDC-2.5 T cells. The absence of detectable amounts of IL-10 formally excludes the generation of Tr1 cells (38). ELISPOT analysis by Solvason and colleagues (39) suggested that an insulin-based tolerizing DNA vaccine led to the initial decrease, followed by an increment of Ag-specific IFN-γ T cells during a long-term response. Our ELISPOT experiments suggest an initial increment of IFN-γ-producing T cells. However, the tetramer analysis clearly demonstrated that the boost of Ag-specific T cells is short lived. Therefore, the DNA treatment likely generated a memory T cell population that readily expanded upon Ag encounter and possibly secreted larger amounts of IFN-γ compared with T cell from naive animals.

Treatment of NOD.CD28−/− mice with pCMV-2.5mi-LIMPII did not protect these animals from T1D, indicating that functional CD28 is a requirement for DNA-mediated protection. Possible explanations for the failure of the DNA treatment to induce protection in this aggressive model include that insulitis is likely to be more advanced compared with NOD mice at the initiation of treatment or that CD28 expression on effector cells is required for disease prevention. Yet one of the hallmarks of CD28−/− animals is the severe reduction of Foxp3-expressing nTreg, leading to enhanced T1D onset in the NOD background. As nTreg are known to be crucially involved in the prevention of systemic autoimmunity (40), we thus analyzed whether DNA treatment expanded nTreg or generated Treg de novo (inducible Treg). Little is known about the Ags recognized by these T cells (41) or whether there is a natural pendant of Foxp3-positive CD4 T cells to each effector CD4 T cell specificity. Our adoptive transfer experiments of BDC-2.5 T cells into the NOD strain suggested that a part of BDC-2.5

Table I. Expansion of 2.5mi+Foxp3− T cells versus 2.5mi+Foxp3+ T cells in NODwt × B6.Foxp3EGFP F1 animals after DNA treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Aβ/2.5mi+ CD4+</th>
<th>Number of Foxp3+ Aβ/2.5mi+ CD4+</th>
<th>Percent Foxp3+ Aβ/2.5mi+ CD4+</th>
<th>Percent Foxp3+ Aβ/2.5mi+ CD4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-2.5mi-LIMPII</td>
<td>361 ± 164</td>
<td>13 ± 5</td>
<td>4.1 ± 2.2</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>pCMV-2.GPI-LIMPII</td>
<td>91 ± 7</td>
<td>9 ± 2</td>
<td>10.1 ± 1.7</td>
<td>0.03 ± 0.005</td>
</tr>
</tbody>
</table>

Values are number ± SD.

a Each group included four animals.

b Numbers are normalized to 2.5 × 10⁵ CD4 T cells.

FIGURE 9. Delayed onset of diabetes in Treg-reconstituted and DNA-treated NOD-SCID mice. CD4+CD25+ T cells isolated from BDC-2.5/N TCR Tg mice were transferred to 4-wk-old NOD-SCID (n = 3 per plasmid treatment) and treated in weekly intervals three times with pCMV-2.5mi-LIMPII or pCMV-GPI-LIMPII. Four weeks after the last injection, in vitro-stimulated total splenocytes from BDC-2.5/N TCR Tg mice were transferred via tail vein into the reconstituted and treated NOD-SCID mice. Diabetes onset was assessed by weekly measurements. The cumulative incidence of diabetes (blood glucose levels above 200 mg/dl) is shown.

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T cells express Foxp3. According to previous studies, these Foxp3+ Treg are BDC-2.5 clonotypic cells that express a second, rearranged α-chain (30). Prevention of α-chain rearrangement by backcrossing BDC-2.5/N TCR Tg animals into a RAG1-deficient or into the NOD.SCID background prevents the generation of BDC-2.5 clonotype Treg and leads to aggressive and accelerated diabetes onset (42). Compared to the percentage of Foxp3+ T cells within the CD4+ T cell population in naive NOD mice, the percentage of Foxp3+ cells in the BDC-2.5 population was reduced to one fourth. These percentages were similar to published frequencies of Foxp3+ T cells in the BDC-2.5/N TCR Tg mouse (30), indicating that, if any, homeostatic expansion did not have a major effect on the ratio of effector to Foxp3+ T cells. In this experimental setup, DNA treatment did not lead to a significant, measurable expansion of Foxp3+ BDC-2.5 T cells.

Several reports have claimed the generation of Foxp3+ T cells following immunization with DNA (43–45). However, none of these studies have directly enumerated Ag-specific Treg in vivo. In our studies, we found that a Foxp3+2.5mi+ T cell population does exist in mice expressing Aβ7 (e.g., B6.Foxp3EGFP × NOD F1 animals), for which the proportion within the 2.5mi+ T cell population was comparable to total Foxp3+ T cells within the CD4 T cell compartment. However, expansion upon DNA treatment was essentially confined to 2.5mi Foxp3+ T cells. We were unable to detect a change of the total nTreg population. Yet, pCMV-2.5mi-LMIIIP treatment of Treg-reconstituted NOD.SCID mice led to a delay in diabetes onset once BDC-2.5mi effector cells were transferred in comparison with control-treated mice. These combined observations suggest that DNA treatment alters the in vivo function of nTreg rather than affecting their expansion or de novo generation. To our knowledge, this is the first report describing a combined analysis with Foxp3 and MHC class II tetramers in the NOD mouse.

An additional mechanism of how disease could be affected by DNA treatment is a change of the composition of the 2.5mi+ T cells population at a clonal level. Our previous studies have demonstrated that the 2.5mi+ T cell population is polyclonal and that not all clones provide a response of similar magnitude to islets in vitro (16). Possibly, repeated stimulation with the 2.5 mimotope leads to preferential expansion of 2.5mi+ T cells that are less islet reactive. Whether these cells carry out some type of regulatory function remains to be established. This might be combined with IFN-γ produced by these cells. IFN-γ−/− deficient NOD mice were largely resistant to treatment protocols using CFA or bacillus Calmette-Guérin vaccine, both of which lead to T1D prevention in wt NOD animals (46), indicating that IFN-γ is necessary for tolerance induction. Experimental autoimmune encephalomyelitis has been reported to be exacerbated in IFN-γ−/− or IFN-γ receptor knockout mice (47–49). It was shown that one consequence of IFN-γ removal was to augment IL-17–producing cells (50). On the contrary, in IL−17−/− mice, a higher frequency of IFN-γ–producing cells was observed (50). Therefore, a further possibility how DNA treatment may have prevented diabetes was a skewing away from a Th17 response by incrementing IFN-γ–producing T cells.

Protection afforded by the LMIIIP-based DNA treatment was not transferable, at least not in the conditions tested in which either total splenocytes from diabetic animals or purified BDC-2.5 T cells or total splenocytes from BDC-2.5/N TCR Tg mice were cotransferred with purified CD4 T cells from DNA-treated NOD mice into NOD.SCID animals (not shown). However, these conditions lead to aggressive T1D onset, unlike in wt animals, as either experimental setup introduces large numbers of diabetogenic T cells that may overcome the regulatory capacity of transferred T cells. In addition, standard conditions were used in these experiments, in which generally 106 CD4 T cells of treated NOD mice were used for transfer. Because generally only 0.2–0.3% of CD4 T cells are 2.5mi+ T cells in treated animals, not more that 3000 clonotypic T cells were transferred in these experiments. This is likely to be insufficient to produce a protective T cell population when confronted with a large amount of fully developed diabetogenic mono- or polyclonal T cell populations. An alternative approach would be to enrich 2.5mi+ T cells by tetramer staining and then transfer them to NOD.SCID animals. However, injection of MHC class II tetramers or dimers alone has been reported to prevent autoimmunity (51–54). Therefore, pretreatment of clonotypic T cells by means of tetramer staining may affect these cells, making it difficult to dissect whether disease prevention originates from DNA treatment or tetramer manipulation.

Altogether, our results demonstrate that targeting a monospecific CD4 T cell population by DNA vaccination leads to prevention of T1D. Mechanistically, we show that the DNA-mediated disease protection is dependent on the presence of CD28. Our study also indicates that, in part, a functional change rather than the expansion of 2.5mi Foxp3+ T cells is likely responsible for the long-term protection against the disease. Our study paves the road for a rational design and treatment protocol using Ag-fusion vaccines designed to target the MHC class II loading compartment.

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Disclosures
The authors have no financial interests of conflict.

References


Supplemental Figure Legends

**Supplemental Fig. S1** Fusion Proteins encoded by pCMV-LIMPII.
The pCMV promotor based vector codes for the last 20 amino acids of LIMP II. Peptides (2.5mi and GPI_{282-293}, respectively) are inserted into a Bg/II site 5’ of the LIMPII tail coding sequence. The resulting duplicated Bg/II site 3’ of the peptide-coding sequence is translated (RS) while the 5’ cloning site remains untranslated. The start codon is introduced *de novo* into each peptide-coding sequence during cloning.

**Supplemental Fig. S2** No expansion of 2.5mi⁺ T cells after 2 injections with pCMV-GPI-LIMPII, followed by 1 injection of pCMV-2.5mi-LIMPII.
Six week-old NOD females (n = 3) were injected at day 0 and at day 7 with pCMV-GPI-LIMPII, followed by 1 injection with pCMV-2.5mi-LIMPII at day 14 (treated). Expansion of 2.5mi⁺ T cells was analyzed at day 18 in spleen and iliac LN (IILN). Naïve NOD females are shown for comparison. Differences between groups are not statistically significant.

**Supplemental Fig. S3** Percentage of BDC-2.5 CD4 T cells after adoptive transfer of BDC2.5/N TCR tg splenocytes into NOD mice.
Total splenocytes from BDC/N TCR tg mice (Thy1b⁺) were adoptively transferred into sublethally irradiated NOD Thy1b⁻ females (n = 4). After a 4-week recovery period, animals received 3 doses of pCMV-2.5mi-LIMPII DNA (bottom panels) or were left untreated (control, top panels). At day 4 after the last DNA treatment, animals were analyzed for recovery of BDC-2.5 T cells by staining with the Aβ7/2.5mi tetramer.
Irradiated Host: NOD Thy1b⁻

Transfer of Splenocytes

Donor: BDC2.5/N TCRtg Thy1b⁺

4-Week Recovery

Comparison of A₉/2.5 mi tetramer expression in CD4⁺ T cells:
- Control: 23.0 ± 2.7 %
- pCMV-2.5 mi-LIMPII: 22.0 ± 2.6 %