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*J Immunol* published online 23 February 2011
http://www.jimmunol.org/content/early/2011/02/23/jimmunol.0902395

Supplementary Material  http://www.jimmunol.org/content/suppl/2011/02/23/jimmunol.0902395

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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 Garabatos,* 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. MHC 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 Ag presentation by B cells. A single peptide epitope was sufficient to induce protection against T1D by DNA treatment was completely lost in NOD.CD28−/− mice which are largely deficient of natural regulatory T cells (Treg). Although Ag-specific Foxp3+ Treg did not expand in response to DNA 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.

The Journal of Immunology, 2011, 186: 000–000.

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, Aβ7, that pairs with the α-chain of I-Aα (5). Polymorphisms with biochemical characteristic similar to Aβ7 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 mice 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 Ag7 tetramers complexed to a strong peptide agonist for BDC-2.5 (16). Tetramer analysis of NOD mice facilitated the identification of a natural CD4+ T cell population that shared the same reactivity as the BDC-2.5 T cell clone. This polyclonal T cell population, termed 2.5mi+, displays signature behavior of autoreactive T cells: the cells escape like BDC-2.5 thymic negative selection, expand in pancreatic lymph node (LN), and home to pancreatic islets (16).

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.foxp3<sup>GFP</sup> mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and further bred in our special pathogen-free animal facility (Parc Científic and Hospital Clinic, Barcelona, Spain). For separate sets of experiments, NOD/LtJ, BDC-2.5/N TCR Tg (Thy1b<sup>+</sup>), NOD.Non- Thy1a (Thy1b<sup>-</sup>), NOD.CD28<sup>-/-</sup>, and NOD.Jger<sup>−/−</sup> mice were bred at The Jackson Laboratory and used in place.

For short-term in vivo T cell response studies, animals of indicated age were treated by injection into both tibialis muscles with plasmid DNA reconstituted in saline solution, with 100 μg DNA per animal and immunization, unless outlined differently for the individual experiments in the text. Animals were sacrificed at different time points after the last injection, as indicated in every experiment. For long-term experiments or analysis of Foxp3<sup>+</sup> T cells, animals received three injections of plasmid DNA at weekly intervals, starting at week 4 of age. Blood glucose levels were measured weekly (using blood glucose strips; Roche) starting at week 13 of age. Animals with blood glucose levels above 200 mg/dl were considered diabetic.

Generation of DNA plasmids

The plasmid pCMV-LIMPII coding for the last 20 aa of the lysosomal targeting sequence of the lysosomal integral membrane protein II (LIMPII) has been published (17). pCMV-LIMPII was further modified by insertion of annealed oligonucleotides into a BglII site coding for the start codon, followed by the 2.5mi peptide (AHHPIW ARMDA; pCMV-2.5mi-LIMPII) or the glucose-6-phosphate isomerase-derived peptide (GPI)<sub>282–292</sub> (LSIAL- HVGFDH; pCMV-GPI-LIMPII) in frame and N-terminally of the LIMPII tail. pCMV-2.5mi and pCMV-GPI were generated similar to 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 × 10<sup>6</sup> of activated cells were transferred via tail vein into the reconstituted and treated NOD.SCID mice. Diabetes onset was assessed as mentioned above.

For the analysis of NOD.foxp3<sup>GFP</sup> × NOD F1 mice, surface marker Abs allophycocyanin–anti-CD4, PE-cyanine 5–anti-B220, and PE-cyanine 5–anti-CD8 were used (eBioscience).

Nestled RT-PCR

Nestled RT-PCR was carried out as published (18). Briefly, stained T cells were sorted using a FACS Vantage sorter (BD Biosciences) directly into 25 μl (2.5mi+CD4<sup>+</sup> cells) or 100 μl (total 2.5mi+CD4<sup>+</sup>) of cDNA mixture containing 15 U/ml MLV RT (Fermentas) with the buffer provided by the manufacturer, 0.5 mM spermidine (Sigma-Aldrich), 100 μg/ml BSA, 10 ng/ml oligo(dT), 200 μM each 2′-deoxynucleoside 5′-triphosphate (Fermentas), 1 mM DTT (Sigma-Aldrich), 220 U/ml RNasin (Promega), 100 μg/ml E. coli-derived cDNA (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<sup>+</sup> 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 micrometre at −16°C, transferred onto Super- frost 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 (Biodera, Foster City, CA) diluted in PBS containing 2% FCS, then washed three times in PBS and incubated for 3 h at room temperature with cyanine 5-conjugated goat anti-rabbit Ab and rhodamine red X-conjugated goat anti-rat Ab (both from Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS containing 2% FBS. 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 A<sup>b</sup> α-chain containing a biotinylation sequence [85 (19)] and the A<sup>b</sup> 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 A<sup>b</sup><sup>+</sup> 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 × 10^6 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 Ag7 (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 musculus 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^β/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^β/ 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 sequence (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 translocated 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 littermates (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) are protected from insulinitis and diabetes (23). Reproduction of a BCR specific for a xenointigen (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 littermates (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^β/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

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**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 A67/2.5mi tetramer; **right group of panels,** control staining with the A67/GPI tetramer. **Top panels,** Naive NOD mice; **bottom panels,** treated NOD mice. T cells are gated on CD8-2, B220-2, (PI)-2, 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 A67/2.5mi 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 A67/2.5mi 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.Ignull 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. 6B, 6C). 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 LIMP II 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.

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, approximate 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, 2.5miFoxp3+ 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
the pCMV-2.5mi-LIMPII or the pCMV-GPI-LIMPII control vaccine three times in weekly intervals and rested for an additional 4 wk after the last injection. Next, we transferred into these animals total splenocytes from BDC-2.5/N TCR Tg mice that had been activated in vitro in presence of 2.5mi peptide and monitored diabetes onset. Although all animals eventually became diabetic, this occurred with a significant delay ($p < 0.05$) in pCMV-2.5mi-LIMPII-treated mice (Fig. 9). Because at the time of DNA treatment presumably few, if any, BDC-2.5 effector cells were present, and 4 wk after the last DNA injection few, if any, effector cells will encounter sufficient peptide to proliferate substantially as suggested by our time-course experiments (Fig. 1), these data suggest a functional change of Treg suppressor activity.

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 A^E^7 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...
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 control-treated mice. It is remarkable that manipulation of a single T cell specificity had an effect in such a complex system as the control-treated mice. It is remarkable that manipulation of a single T cell specificity had an effect in such a complex system as the control-treated mice. It is remarkable that manipulation of a single T cell specificity had an effect in such a complex system as the control-treated mice. It is remarkable that manipulation of a single T cell specificity had an effect in such a complex system as the control-treated mice.

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 ones from naive mice or BDC-2.5 T cells. The absence of detectable amounts of IL-10 formally excludes the generation of Tr1 ones from naive mice or BDC-2.5 T cells. The absence of detectable amounts of IL-10 formally excludes the generation of Tr1 ones from naive mice or BDC-2.5 T cells. The absence of detectable amounts of IL-10 formally excludes the generation of Tr1 ones from naive mice or BDC-2.5 T cells. The absence of detectable amounts of IL-10 formally excludes the generation of Tr1 ones from naive mice or BDC-2.5 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.
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 AβF (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-LIMPII 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 cell 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−/− 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 LIMPII-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 105 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 than 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 monoclonal 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.

Acknowledgments

We thank P. Mias for critical reading of the manuscript, L. Teyton for the DNA constructs and cell lines to generate AβF/2.5mi and AβF/GPI tetramers, and L. Mandrile for the setup of photographs of the confocal microscopy.

Disclosures

The authors have no financial conflicts of interest.

References
