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A multi-epitope DNA vaccine enables a broad engagement of diabetogenic T cells for tolerance in Type 1 diabetes

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A B S T R A C T

Type 1 diabetes (T1D) is caused by diabetogenic T cells that evaded tolerance mechanisms and react against multiple β-cell antigens. Antigen-specific therapy to reinstate tolerance (typically using a single β-cell antigen) has so far proved unsuccessful in T1D patients. Plasmid DNA (pDNA)-mediated expression of proinsulin has demonstrated transient protection in clinical trials, but long-lasting tolerance is yet to be achieved. We aimed to address whether pDNA delivery of multiple epitopes/mimotopes from several β-cell antigens efficiently presented to $CD4^+$ and $CD8^+$ T cells could also induce tolerance. This approach significantly delayed T1D development, while co-delivery of pDNA vectors expressing four full antigens protected more mice. Delivery of multiple epitopes resulted in a broad engagement of specific T cells, eliciting a response distinct from endogenous epitopes draining from islets. T-cell phenotypes also varied with antigen specificity. Unexpectedly, the repertoire of T cells reactive to the same epitope was highly polyclonal. Despite induction of some $CD25^+$ $Foxp3^+$ regulatory T cells, protection from disease did not persist after treatment discontinuation. These data demonstrate that epitope-based tolerogenic DNA vaccines constitute effective precision medicine tools to target a broad range of specific $CD4^+$ and $CD8^+$ diabetogenic T-cell populations for prevention or treatment of T1D.

1. Introduction

Type 1 diabetes (T1D) results from a destruction of insulin-producing β-cells in the pancreas. This autoimmune response is mediated by autoreactive $CD4^+$ and $CD8^+$ T cells that recognize multiple epitopes derived from β-cell antigens in both non-obese diabetic (NOD) mouse and T1D patients [1,2]. These diabetogenic T cells that have escaped deletion or inactivation due to a breakdown in central and/or peripheral tolerance play a critical role in disease onset and progression. Targeting these T cells specifically to be deleted, inactivated or to adopt a protective phenotype, without affecting the rest of the immune system, has been the goal of antigen-specific therapies for T1D [3]. Particularly, tolerogenic DNA vaccines, which rely on self-antigens endogenously encoded by plasmid DNA (pDNA), have been investigated for many years with the goal to induce antigen-specific tolerance. Antigen-specific therapies in general have not been successful so far in inducing durable protection from T1D, possibly because the antigens selected may have been insufficient and/or inadequate. The preferred use of proinsulin as antigen mainly stems from the fact that insulin B:9–23 is a dominant MHC class II-restricted epitope that is necessary for the initiation of disease in NOD mice [4], the insulin gene is associated with T1D in humans [5,6], and insulin-reactive T cells are commonly found in human islets [7,8]. But as disease progresses, epitope spreading occurs in both NOD mice and humans [9,10], such that other autoreactive T-cell populations specific to a variety of epitopes across different β-cell antigens become also involved.

DNA-based antigen-specific therapies present several unique advantages, as pDNA vectors are easy and cheap to produce, and can achieve prolonged expression of encoded antigens compared to other forms of antigen and delivery methods. Like other antigen-specific therapies, tolerogenic DNA vaccines have primarily focused on delivering pDNA-encoded proinsulin or GAD65, showing reduced diabetes development in preventive or reversal settings in NOD mice [11–13]; however therapeutic efficacy was found to be transient in clinical studies [14]. One of the limitations affecting the efficacy of current DNA vaccines in T1D might be the limited fraction of diabetogenic T cells engaged due to the use of a single antigen, encoded in native form by the vaccine, despite ample evidence of epitope spreading and existence of neoepitopes (post-transcriptional peptide modifications [15,16] and hybrid peptides [10,17]).

We recently demonstrated the ability of a new DNA construct, that express epitopes derived from multiple β-cell antigens, to optimally and simultaneously engage both $CD8^+$ and $CD4^+$ T cells of different antigen specificities by different types of antigen-presenting cells (APCs) [18]. This more efficient presentation of epitopes to $CD4^+$ and $CD8^+$ T cells was made possible by differential MHC targeting within the cell, and via the use of mimotopes, which have been selected to better engage antigen-specific T cells when compared to native sequences [17,19,20]. After validating these multi-epitope constructs in vitro [18], we developed two variants (epitopes confined to the expressing

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cells or secreted) to test in vivo as DNA vaccine.

In the work presented here, we investigated whether delivery of major epitopes from multiple β-cell antigens confers any potential advantage over delivery of a single or multiple proteins as source of antigens (no mimotopes present in whole proteins). To this end, we assessed the efficacy of this new DNA vaccine for autoimmune diabetes and the characteristics of the antigen-specific T-cell responses elicited in vivo. This unique approach allowed us to evaluate the antigen-specific T-cell response to at least four different epitopes from the DNA constructs and demonstrate that different clones specific to different epitopes or even to the same epitopes behave differently.

2. Materials and methods

2.1. Plasmid constructs

We designed two epitope-expressing constructs, both expressing the same major epitopes from several β-cell antigens, including native epitopes and mimotopes for higher affinity recognition (Fig. S1A). The first construct (AI, intracellular) with segregated polypeptides allows each epitope to be efficiently targeted to its appropriate MHC pathway (ie. all $CD4^+$ T-cell epitopes targeted to the endosome while $CD8^+$ T-cell epitopes are processed via the proteasome) [18]. The second construct (BS, secreted) allows all epitopes to be efficiently secreted as a single polypeptide, allowing dissemination and uptake by additional APCs. All constructs and genes were cloned into pBHT568, gifted by Dr. Peggy Ho (Stanford University), in place of the original proinsulin gene [13]. This vector has CpG motifs substituted by GpG immunomodulatory sequences to minimize immune stimulatory activity [21,22]. The AI and BS constructs, and the glutamic acid decarboxylase (GAD65) and mCherry genes were codon-optimized and synthesized by GeneArt (ThermoFisher). The chromogranin A (ChgA) and islet-specific glucose-6-phosphatase-related protein (IGRP) genes were subcloned after PCR amplification from MIN6 pancreatic β-cell line, while the Luciferase (Luc) gene was subcloned from pGL4.10[luc2] (Promega) (Fig. S2A). All vectors were propagated in *E. coli* DH5α, purified with the GenElute Endotoxin free pDNA purification kit (Sigma), and concentrated under sterile conditions in PBS, at a final concentration of 0.5 mg/ml. DNA quality and quantity were determined by Nanodrop and agarose gel electrophoresis. The expression of all pDNA-encoded products was validated by qPCR, flow cytometry (FCM) or Luc assay (Figs. S1 and S2B).

2.2. Mice

All mouse strains were purchased from the Jackson Laboratory and bred in our barrier facility: NOD (#001976), NOD.SCID (#001303), NOD.Thy1.1 (#004483), NOD.CD45.2 (#14149) and T-cell receptor transgenic (TCR-Tg) mice: BDC2.5 (#004460), BDC12–4.1 (#006303/006304) and NY8.3 (#005868). TCR-Tg T cells from these mice respectively recognize the p79/2.5 mimotope (2.5mi) [23], $InsB_{9\text{-}23}$ epitopes and mimotopes [24], and $IGRP_{206\text{-}214}$ epitope, all encoded by our constructs AI and BS. BDC12–4.1.TCRαKO mice were produced as previously described [18]. Male and female mice donors and recipients were used at 8–16 weeks of age for studies of T-cell response and female NOD mice were used at 10 weeks of age for preclinical experiment. All studies were approved by Columbia University's Institutional Animal Care and Use Committee.

2.3. Biodistribution and uptake of pDNA

Animals received an intramuscular (i.m.) or intradermal (i.d.) injection of 50 μg of pDNA (Luc or mCherry) split between the two quadriceps muscles or the two flanks of the abdominal area, respectively. Luc expression in different tissues was measured in live animals at different time points using the IVIS Spectrum (Perkin Elmer). Prior to in vivo imaging, mice were anesthetized by isoflurane and injected intraperitoneally with 200 μl of Luciferin (0.15 mg/ml). Luc signal was also measured in excised tissues (e.g. draining LNs) ex vivo afterwards. The presence of transfected $mCherry^+$ cells was assessed in LNs and skin after i.d. pDNA administration. Skin cells were released after digestion of treated abdominal skin with collagenase IV (Worthington, 1 mg/ml) and dispase (Invitrogen, 1 mg/ml). Cell suspensions obtained were analyzed by FCM 24 and 48 h after DNA injection.

2.4. NOD disease prevention with DNA vaccines

Ten week-old NOD females were injected i.m. weekly for a total of 8 weeks. Five groups of 12 mice received one or multiple pDNAs encoding full proteins Luc (a), proinsulin only (b), proinsulin, GAD65, ChgA and IGRP (c), AI and BS constructs (encoding epitopes and mimotopes) (d) or were left untreated (e) (Fig. S2C). Because of the varying size of those vectors (Fig. S2A), all mice received the same molar amount of pDNA (24 pmol, based on 50 μg of the proinsulin-encoding vector), which was split between the two quadriceps. Mice from different groups were mixed in cages. Blood glucose was monitored weekly (up to 35 weeks) using Prodigy glucometer and test strips. Mice were diagnosed as diabetic after two consecutive blood glucose levels greater than 250 mg/dl and sacrificed upon diagnosis or at the end of the observation period if normoglycemic.

2.5. Adoptive transfer of TCR-Tg T cells

T cells were isolated by negative selection from spleen and pooled lymph nodes (LNs) of donor TCR-Tg mice: $CD4^+$ $CD25^-$ T cells from BDC2.5 or BDC12–4.1.TCRαKO using the Mojosort mouse $CD4^+$ T-cell isolation kit (Biolegend) supplemented with biotinylated anti-CD25 (PC61); $CD8^+$ T cells from NY8.3 mice using the Mojosort mouse $CD8^+$ T-cell isolation kit. T-cell purities were around 94–98% as confirmed by FCM. Purified T cells were labeled with a green (CFSE) or violet cell proliferation dye (VCPD) (eBioscience) prior to intravenous transfer into the tail vein of congenic NOD.Thy1.1 or NOD.CD45.2 recipients ($0.5–1x10^6$/mouse).

2.6. Flow cytometric analysis of T-cell responses

T-cell responses were analyzed 3 days after a single or multiple treatments with pDNA (3 doses every 2–3 days). LNs draining the site of injection (inguinal LNs (ILNs) for i.d. or para-aortic LNs (PALNs) for i.m.) were collected. Cervical LNs (CLNs) and pancreatic LNs (PLNs) were used as control. In adoptive transfer systems, PLNs were used as positive control, as TCR-Tg T cells proliferate to endogenous islet-derived antigens in the PLNs. When treated mice were NOD mice (no adoptive transfer), LN cells were first stained with MHC tetramers to identify endogenous antigen-specific T-cell populations by FCM. Premade $I\text{-}A^{g7}$ MHC-II tetramers with 2.5mi/p79 (AAAAVRPLWVRMEAA), $GAD65_{286\text{-}300}$ (KKGAAALGIGTDSVI) and $InsB_{9\text{-}23}$ R22E (HLVERLYLVCGEEG) peptides, and made-to-order $H\text{-}2K^d$ MHC-I tetramer with $IGRP_{206\text{-}214}$ mimotope (KYNKANVFL) were obtained from the NIH Tetramer Core Facility. Cells were incubated with tetramers for 1 h in the dark (room temperature for $I\text{-}A^{g7}$ MHC-II tetramers or 4 °C for $H\text{-}2K^d$ MHC-I tetramer), then washed twice. LN cells from all treated mice (with or without adoptive transfer) were stained for extracellular and/or intracellular markers for 30 min at 4 °C. The following antibodies were used to phenotype T cells: CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD25 (PC61), CD44 (IM7), CD45 (30-F11), CD45.1 (A20), CD62L (MEL-14), CD73 (TY/11.8), CD90.2 (30-H12), CD103 (2E7), CD279 (RMP1-30), CD326 (G8.8), FR4 (12A5), Ly-6C (HK1.4), $I\text{-}A^k$ (10-3.6, cross-reactive with $I\text{-}A^{g7}$), Ki67 (16A8), IFNγ (XMG1.2), IL-2 (JES6-5H4), TNFα (MP6-XT22), IL-10 (JES5-16E3) were from Biolegend and Foxp3 (FJK-16s) was from eBioscience. The True-Nuclear Factor (Biolegend) and CytoFix/CytoPerm (BD

Bioscience) kits were used for intracellular staining of Foxp3 and cytokines respectively, following manufacturer's instructions. After wash, cells were resuspended in buffer with viability dye and analyzed on BD Fortessa or LSRII. Data analysis was performed using FlowJo 9 or FCS Express 6.06.

2.7. Adoptive cell transfer into NOD.SCID mice

Eight-week old NOD mice were injected i.d. and i.m. with mCherry pDNA or a 1:1 mix of AI and BS pDNA every two days for a total of three doses. Two days after the last injection, spleens, ILNs, PALNs and PLNs were harvested. Cells from pooled LNs (1×10^6) and from spleens (4×10^6) were mixed with splenocytes (2×10^6) from 16-week old diabetic NOD mice and injected intravenously into 10-week old NOD.SCID mice. Blood glucose was monitored weekly until mice were diagnosed as diabetic (as above).

2.8. Single cell sorting and TCR & cytokines mRNA sequencing

Following 3 doses (i.d.) of mCherry pDNA or a 1:1 mix of AI and BS pDNA, ILNs and PLNs were collected, and cells were restimulated with PMA (150 ng/ml) and ionomycin (750 ng/ml) for 3 h in vitro and stained as above. 2.5mi-tetramer (tetr)$^+$ CD4$^+$ T cells were single-cell sorted (with indexing) using the BD Influx into 96-well plates containing RT-PCR buffer (Qiagen). Single-cell mRNA sequencing of paired TCRα/β and selected T-cell cytokines and transcription factors were achieved by a series of three nested PCR reactions, barcoding and sequencing on MiSeq (Illumina) as described [25]. For the TCR sequencing reaction, multiple internally nested TCRVα, TCRVβ, TCRCα and Cβ primers were used.

2.9. Statistical analysis

All statistical testing was performed using GraphPad Prism 5.0. Unless otherwise indicated, values were expressed as mean ± SEM. All experiments were repeated two to three times with reproducible results. Statistical significance of differences between the groups were analyzed by a two-tailed Student's t-test or the Mann–Whitney *U* test, and the exact P values are reported in the figures. Kaplan-Meier curve statistical analysis was done using the log-rank test and Chi-square test for comparisons. The p-values were compensated for multiple comparison (Bonferroni) of survival curves. *P < 0.05, **P < 0.01 and ***P < 0.001.

3. Results

3.1. In vivo uptake, transport and expression of DNA vectors

We first determined the biodistribution and duration of vector expression following i.d. or i.m. delivery of naked pDNA, in order to better understand where antigens are produced and for how long. The signal from a standard dose of Luc-pDNA (50 μg) was measured over time by IVIS imaging of the injected area in NOD mice. A similar kinetic profile was achieved with both routes of delivery: expression at the sites of injection (skin or muscle) was detected as early as 4 h after administration, reached maximum level after 2–3 days and remained substantial for at least 7 days (Fig. 1A). Signals from i.d.-injected Luc pDNA tended to be slightly higher, possibly due to more superficial location and reduced tissue absorption. However, no Luc expression above background was detected in excised draining ILNs or PALNs or more distal LNs using this technique (Fig. S1C). No differences in Luc signal were found 3 days after a single dose or after three consecutive doses (data not shown).

Using mCherry pDNA delivered i.d., we determined more precisely which cells were capable of taking up and expressing the transgene by FCM analysis. Expression of mCherry was found in few immune (CD45$^+$) and non-hematopoietic (CD45$^-$) cells at the site of injection (skin) and in few CD45$^+$ cells within the draining ILNs (Fig. 1B). Skin CD11c$^+$ CD11b$^+$ DCs were the main CD45$^+$ population expressing mCherry; they also expressed Ly6C, but not Epcam or CD103, markers which define other DC populations (Fig. 1C–E).

3.2. Multiple-epitope vaccination delayed the onset of diabetes in NOD mice

We postulated that diversifying the number of antigens delivered or only providing selected epitopes (including mimotopes) from several major antigens may enable a broader tolerance induction over using a single antigen. In order to test this hypothesis, we evaluated the in vivo efficacy of different DNA constructs in preventing T1D in NOD mice (Fig. S2C): a) control pDNA (reporter gene), b) proinsulin pDNA alone, c) a combination of four pDNA expressing the full proteins proinsulin, GAD65, ChgA and IGRP proteins, and d) a combination of two pDNA expressing epitopes or mimotopes from those four proteins that are produced intracellularly or secreted (AI and BS). We performed this study with i.m. injections of pDNA for better comparison with historical preclinical and clinical data with this vector [13,14]. We initiated the treatment at late stage diabetes (10 weeks of age) for 8 consecutive weeks. Delivery of multiple epitopes/mimotopes specifically targeted to CD4$^+$ and CD8$^+$ T cells (AI/BS) significantly delayed diabetes development compared to control NOD mice at this late stage of disease, but did not induce greater protection than proinsulin or the mix of antigens (Fig. 2). Disease was delayed for the duration of the treatment, and mice started to develop diabetes after the treatment was discontinued. Delivery of four full antigens (including proinsulin) resulted in greater protection from diabetes (% of diabetes-free animals) than all other treatments (Fig. 2). A marginal and non-significant effect of the vector backbone (expressing Luc as an irrelevant protein) was consistent with previous studies [13,26].

3.3. Validation and location of epitope-specific T-cell responses

We next set out to confirm that the different pDNA-encoded epitopes were being presented in vivo and assess how the resulting local T-cell responses compared with the response to native epitopes in islet-draining PLNs. Using adoptive transfer of TCR-Tg T cells, we evaluated the initial response of antigen-specific T cells (CD4$^+$ and CD8$^+$) to three of the pDNA-encoded epitopes in AI and BS constructs (2.5mi, $InsB_{9\text{-}23}$ mimotope and $IGRP_{206\text{-}214}$, recognized by BDC2.5 CD4$^+$, BDC12–4.1 CD4$^+$ and NY8.3 CD8$^+$ T cells respectively) in various LNs after i.m. and i.d. vaccination (Fig. 3A). As expected, these T cells extensively proliferated in the PLNs, regardless of pDNA treatment, upon encountering islet-derived antigens, but not in other LNs (CLN, ILN, and PALN) in absence of pDNA vaccination (Fig. 3B–E). All three antigen-specific populations were stimulated in the draining LNs after a single treatment (in PALNs with i.m. route (Fig. 3B and C) and in ILNs with i.d. route (Fig. 3D and E). In contrast to PLNs, many of these responding T cells expressed CD25 and their proliferation was more limited. The secreted polypeptide encoded by BS induced a more pronounced CD8$^+$ T-cell response in proliferation and CD25 expression compared to AI, with both i.d and i.m. routes (Fig. 3C,E, lower panels), reflecting the fact that those epitopes are more widely disseminated. In contrast, the level of CD25 induction and proliferation in CD4$^+$ T cells was similar between AI and BS constructs (Fig. 3C,E, upper and middle panels). TCR-Tg T cells possess an activated phenotype (CD62L$^-$ CD44$^+$) in PLNs and a naïve phenotype (CD62L$^+$ CD44$^-$) in other LNs (ILNs, PALNs) until after pDNA treatment when these cells become CD62L$^{-/low}$ CD44$^+$ similar to PLNs (Fig. S3).

3.4. Frequency of endogenous antigen-specific T cells after pDNA treatment

With the sites of T-cell engagement established with the more sensitive adoptive transfer model, we then moved on to evaluate the

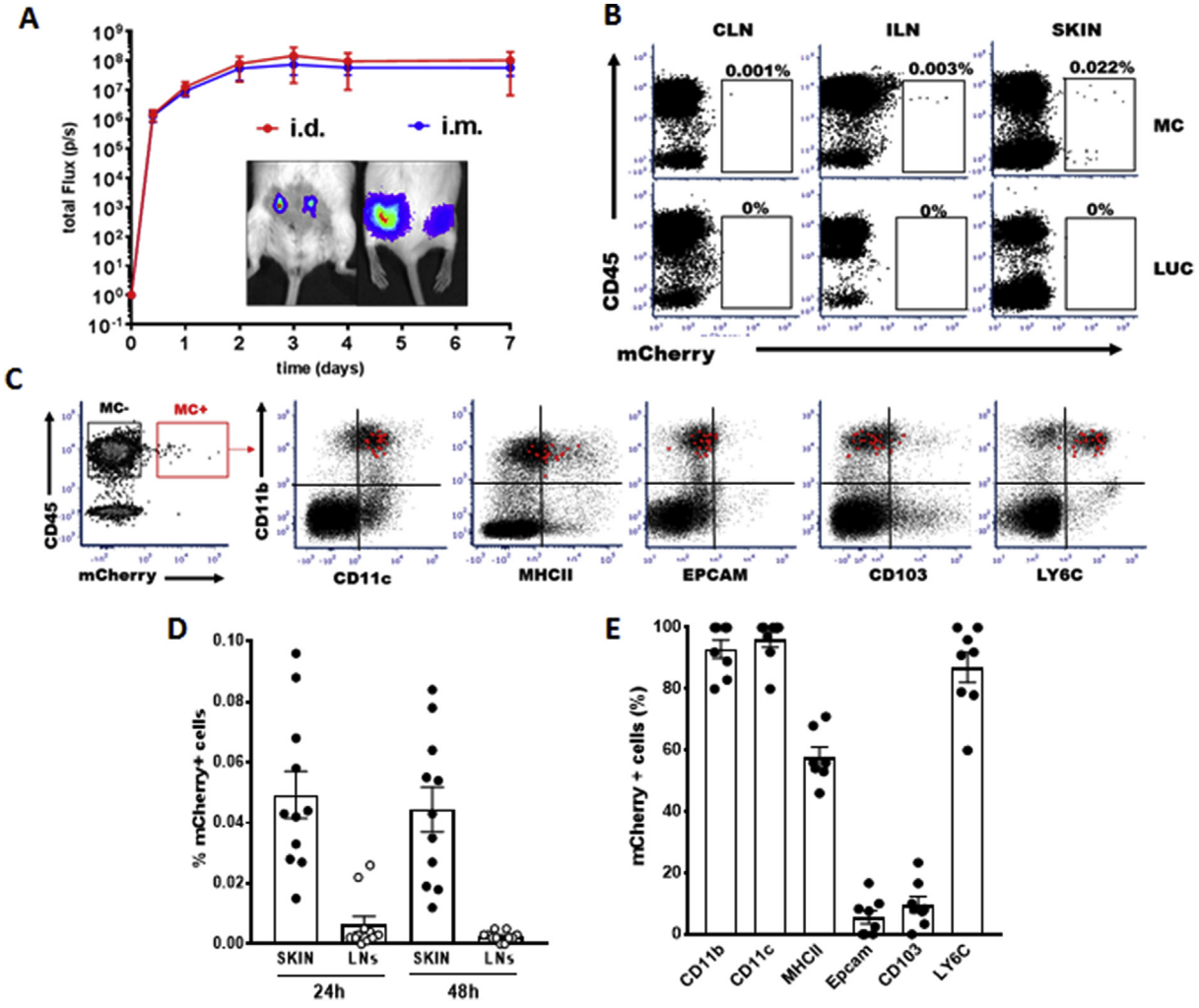

Fig. 1. DNA expression and uptake in vivo. (**A**) Kinetics of Luc expression after i.d. or i.m. administration of 50 μg of Luc pDNA. Insert: representative visualization of Luc signal using IVIS Spectrum Optical Imaging System. (**B**) FCM plots of cells prepared from skin, CLNs and ILNs, 24 h after i.d. injection of mCherry (MC) or Luc-encoding pDNA. (**C**) FCM plots on skin cells showing the phenotype of CD45$^+$ mCherry$^+$ cells (red) relative to the overall population. (**D**) Percentage of mCherry$^+$ cells in skin and ILNs 24 h and 48 h after i.d. injection. (**E**) Percentage of cells expressing specific markers among CD45$^+$ mCherry$^+$ cells in the skin. All bar graphs show mean ± SEM (n = 5 per group in **A**; n as indicated in scatter dot plot in **D-E**).

engagement of endogenous antigen-specific T cells in response to several pDNA-encoded epitopes, using four different MHC tetramers corresponding to three CD4 epitopes and one CD8 epitope. A significant increase in the frequency of T cells reactive to the 2.5mi and insulin mimotopes was already evident in the draining PALNs after a single i.m. injection of AI/BS pDNA (Fig. 4). As seen with transferred T cells, the secreted construct BS led to a greater increase of the 2.5mi-reactive T cells after i.d injection. (Fig. S4). The BS construct also seemed to induce an increase on GAD65-tetr$^+$ cells in ILNs after a single i.d. dose but it was only significant in PLNs after three doses (Fig. S4). Three doses further increased the frequency of 2.5mi-tetr$^+$ CD4$^+$ T cells in the ILNs but not in PLNs (Fig. S4). In contrast, CD4$^+$ T cells reactive to insulin and GAD65 epitopes accumulated in the PLNs after 3 doses, suggesting a kinetic of redistribution distinct from 2.5mi-reactive CD4$^+$ T cells (Fig. S4). IGRP-reactive CD8$^+$ T cells were also increased in PALNs and PLNs after a single i.m. injection (Fig. 4) and in ILNs after three doses of BS (Fig. S4), similar to what we found with NY8.3 TCR-Tg CD8$^+$ T cells (Fig. 3).

3.5. Endogenous T-cell responses are highly polyclonal with limited clonal expansion

As 2.5mi-reactive CD4$^+$ T cells exhibited the greatest increase in frequency relative to other antigen-specific T cells (from 30% increase with i.m. route to up to 5-fold increase with i.d. route), we interrogated the TCR repertoire of this population using single-cell targeted RNA sequencing in order to assess its clonality. We performed a sampling of 2.5mi-tetr$^+$ CD4$^+$ T cells from ILNs and PLNs following three i.d. doses of pDNA encoding either mCherry (control) or antigens (AI/BS). Analysis of the mRNA sequencing of TCRαβ chains revealed a similar usage of Vβ and Vα families between samples, with a preferential usage of TRAV3 and TRBV13 in almost half of the clones sorted (Fig. S5A). Within each Vα and Vβ family, the VJα and VJβ usage was very diverse, indicating that the responding T cells were highly polyclonal in all sites (Table S1). In fact, our sampling of 48 cells per group and tissue revealed no replicated clone, except in the ILN of antigen-treated mice where only 5 clones (~10%) were replicated (identical VJα and VJβ) (Fig. S5B), suggesting minimal clonal expansion despite significant increase (3–5 fold) in the frequency of these T cells in draining ILNs (Fig. 5A). To further assess the extent of clonal expansion of these endogenous T cells, we performed Ki67 staining and observed that around 60% of 2.5mi-tetr$^+$ CD4$^+$ T cells were cycling after pDNA vaccination (3 doses) (Fig. 5B). This proliferation was accompanied by CD25 upregulation after one or three doses in ILNs and PLNs respectively (Fig. 5C), and other epitope-reactive T cells also upregulated CD25 to a limited extent (Fig. 4, Fig. S5C). Altogether, these data indicate that a

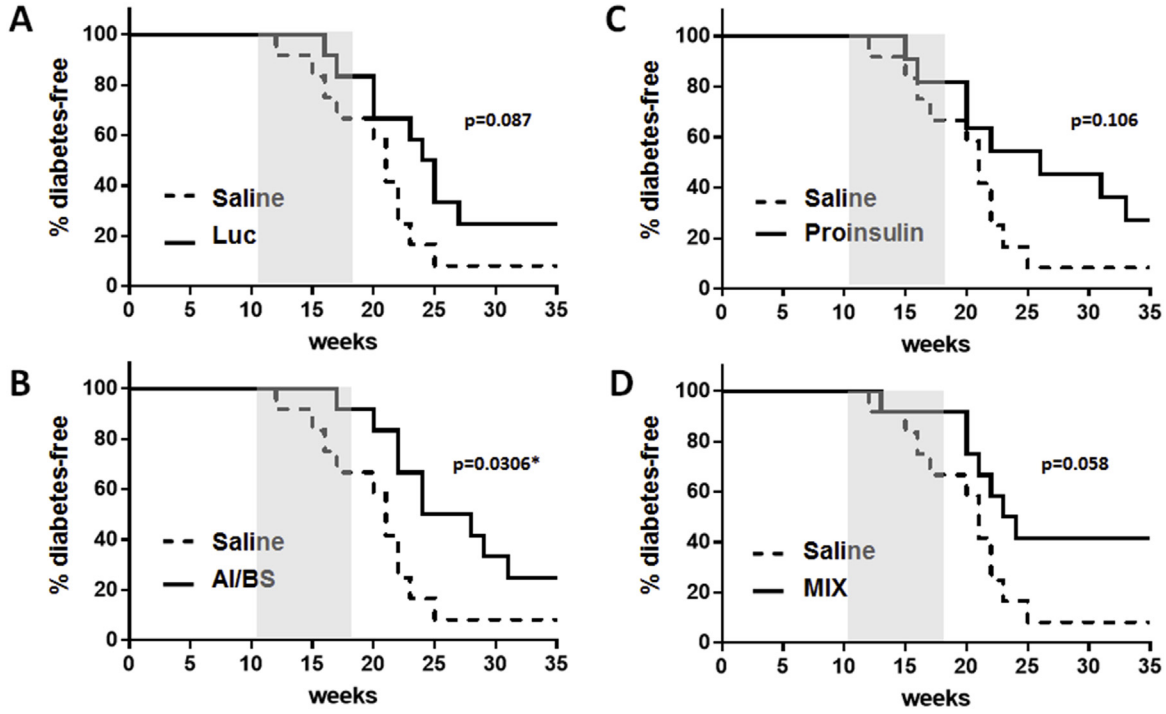

Fig. 2. Multi-epitope pDNA delays T1D. Disease progression with pDNA treatments encoding Luc (**A**), proinsulin, (**B**), a 1:1 mixture of non-secreted (AI) and secreted (BS) constructs (**C**), a mixture of proinsulin, ChgA, GAD65 and IGRP (MIX) (**D**) versus saline controls. The treatment was performed over 8 weeks (gray shading) with weekly i.m. injections of 24 pmol pDNA (50 μg proinsulin pDNA) (n = 12/group); p value by Log Rank test.

large and clonally heterogeneous population of antigen-specific T cells preexists and are consistent with a moderate expansion of a large number of clones rather than an extensive expansion of a few.

3.6. Phenotype of antigen-specific T cells after pDNA treatment

In an attempt to infer the mechanism of tolerance elicited (e.g. induction of Tregs or anergy), we analyzed the response of both adoptively transferred and endogenous antigen-specific T cells. The percentage of Foxp3$^+$ among adoptively transferred BDC2.5 CD4$^+$ T cells increased from ~1% to 4–6% in draining ILNs (Fig. 6A). In contrast, the Foxp3$^+$ T cells among endogenous 2.5mi-specific CD4$^+$ T cells increased from ~5% to 10–15% but in the PLNs and only after 3 doses (AI or BS) (Fig. 6B, Fig. S6A), and their presence was also evident in our single-cell analysis (AI/BS, Fig. 6C). The frequency of endogenous polyclonal Foxp3$^+$ T cells was also increased with AI (Fig. S6A). To determine if these induced Tregs were sufficient to mediate protection, we transferred cells from draining LNs and spleen of NOD mice treated 3x with AI/BS, proinsulin or mCherry (control) into NOD.SCID mice. While recipient mice receiving cells from treated (AI/BS or ProIns) mice had a delayed incidence of disease, this did not reach significance ($p = 0.09$; Fig. 6D, Fig. S6B).

Most 2.5mi-tetr$^+$ T cells, like other CD4$^+$ T cells, expressed TNF-α after ex vivo polyclonal restimulation, but only cells from treated mice expressed IFNγ and/or IL-10 (Fig. 6C, Fig. S6C-E). Other markers associated with peripheral tolerance were measured. PD-1 was specifically upregulated in 2.5mi-tetr$^+$ and IGRP-tetr$^+$ T cells in ILNs and PLNs after i.d. pDNA injection of AI or BS pDNA (3 doses) (Fig. 7A, Fig. S7A,B). Moreover, anergy markers CD73 and FR4 were increased in insulin-reactive BDC12–4.1 and 2.5mi-tetr$^+$ T cells, but relatively unchanged in transferred BDC2.5 T cells and NY8.3 T cells (Fig. 7B, Fig. S7C). Overall, there were more PD-1$^+$ and CD73$^+$ FR4^{hi} cells in PLNs that in ILNs (Fig. S7).

4. Discussion

Antigen-specific therapies have been pursued to downregulate diabetogenic autoimmune responses in T1D [27,28]. Therapies targeting antigen-specific T cells were deemed among the safest approaches and a variety of antigen delivery methods compatible with tolerance induction have been explored. Earliest attempts at translating antigen-specific therapies to the clinic have been met with disappointment and optimizations are required to overcome possible T1D-associated defective or excessive functions of T cells [29] and APCs [30]. Furthermore, questions remain as to whether the breadth of antigens or epitopes administered is sufficient, and whether particular antigens would be more appropriate for certain endotypes of T1D patients (requirement for a precision medicine approach). DNA vaccines have many advantages over delivering soluble peptides or recombinant proteins: 1) they are cheaper and easier to produce, 2) they offer a more sustained antigen presence after treatment, 3) endogenously expressed antigens may acquire unique post-translational modifications that recombinant antigens lack, and 4) they facilitate expression of complete antigens (e.g. proinsulin, as opposed to insulin used as recombinant protein to induce tolerance but lacking the important epitopes from the C-peptide [31,32]). Importantly, not only DNA vaccines are safe, but they can also delay the loss of C-peptide expression (i.e. β-cell function) in T1D patients [14]. Further delaying or halting the loss of C-peptide will require further optimization of this platform [13,33] and a better understanding of its mechanism of action.

We have recently reported on the validation of DNA/RNA constructs encoding multiple epitopes from β-cell antigens targeted in T1D [18]. When endogenously expressed, epitopes and mimotopes can be targeted to the adequate MHC molecules for optimal engagement of the target T cells. Because mimotopes are generally stronger agonists [23,24], one concern is whether these could overstimulate diabetogenic T cells and exacerbate disease. Administration of mimotopes of the InsB9-23 epitope at low dose in NOD mice have been shown to block disease by some [34] or worse disease by others [35]. Mimotopes or

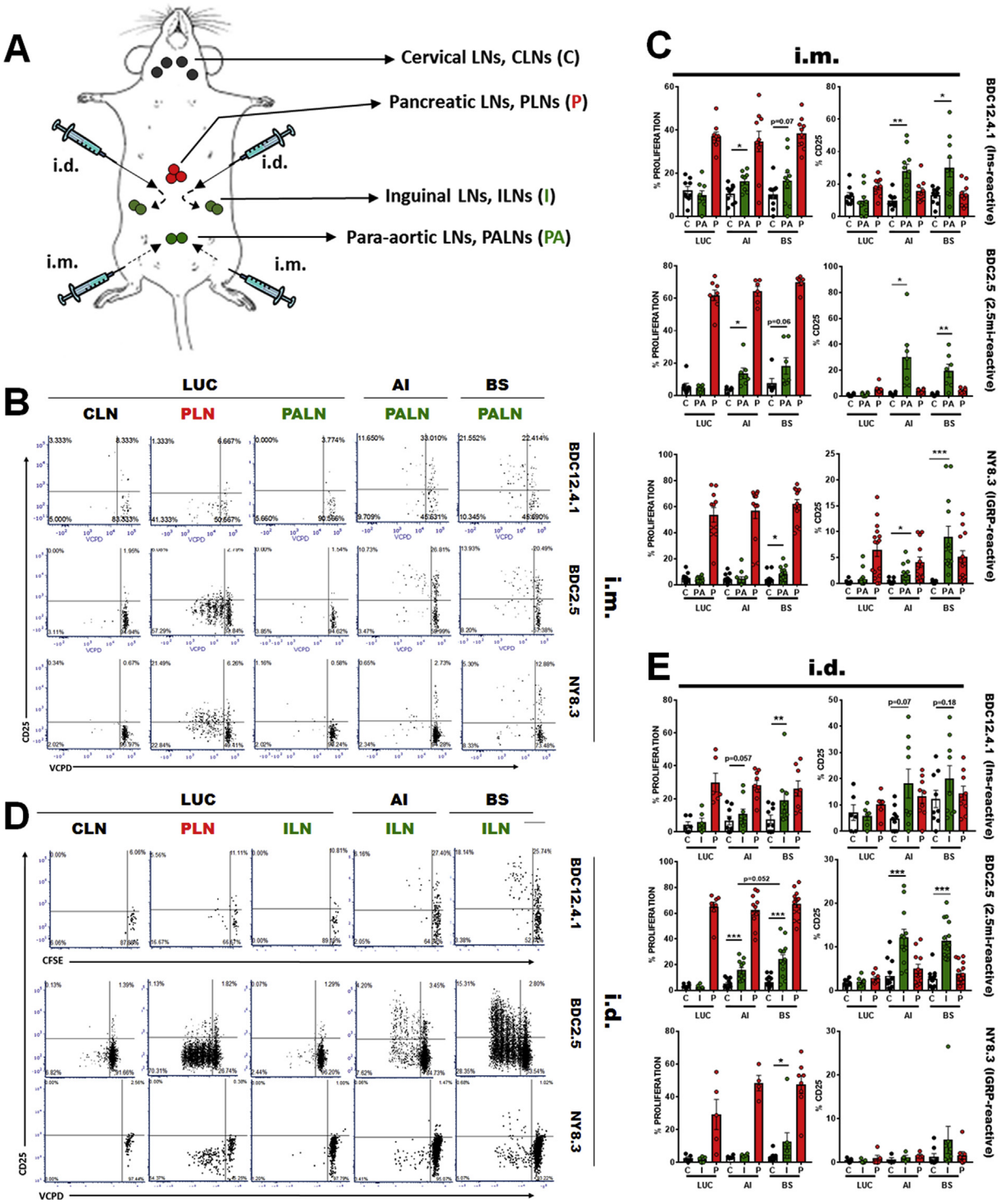

Fig. 3. Engagement of TCR-Tg T cells by pDNA-encoded epitopes in vivo. (**A**) Schematic diagram of pDNA treatment sites in NOD mice and the LNs collected to assessed TCR-Tg T-cell responses. (**B-C**) Representative FCM plots (**B**) and bar graphs (**C**) of proliferation and CD25 expression on transferred BDC2.5, BDC12.41 and NY8.3 after i.m. delivery of pDNA. (**D-E**) Representative FCM plots (**D**) and bar graphs (**E**) of proliferation and CD25 expression on transferred BDC2.5, BDC12.41 and NY8.3 after i.d. delivery of pDNA. T-cell responses were measured 3 days after a single dose of Luc, AI or BS-encoding pDNA. LNs analyzed: CLNs (C) (non-draining LN, negative control), ILNs (I) (draining LN for i.d. delivery), PALNs (PA) (draining LN for i.m. delivery), PLNs (P) (positive control, with natural islet-derived antigens). The baseline is indicated by two controls: (1) response in CLNs with all treatments, and (2) response to LUC pDNA in CLNs and ILNs/PALNs. Experiments included both male and females NOD mice. All bar graphs show mean ± SEM.

altered peptides of human insulin binding to HLA-DQ8 or HLA-A2 induced regulatory $CD4^+$ and $CD8^+$ T cells in humanized mice [36,37]. Moreover, one of the BDC2.5 mimotopes ("p31") delivered by DNA vaccine (i.m.) reduced the incidence of disease in NOD mice [38]. Our constructs contained two InsB9-23 mimotopes (p8E and p8G) [24] and another BDC2.5 mimotope ("p79") [23], and delivery of those constructs on DNA vaccine delayed rather than exacerbated disease. However, late stage prevention (starting at 10 weeks of age) seemed to require continuous treatment as protection was transient, similar to what was observed in clinical trials [3,14]. In contrast, earlier stage

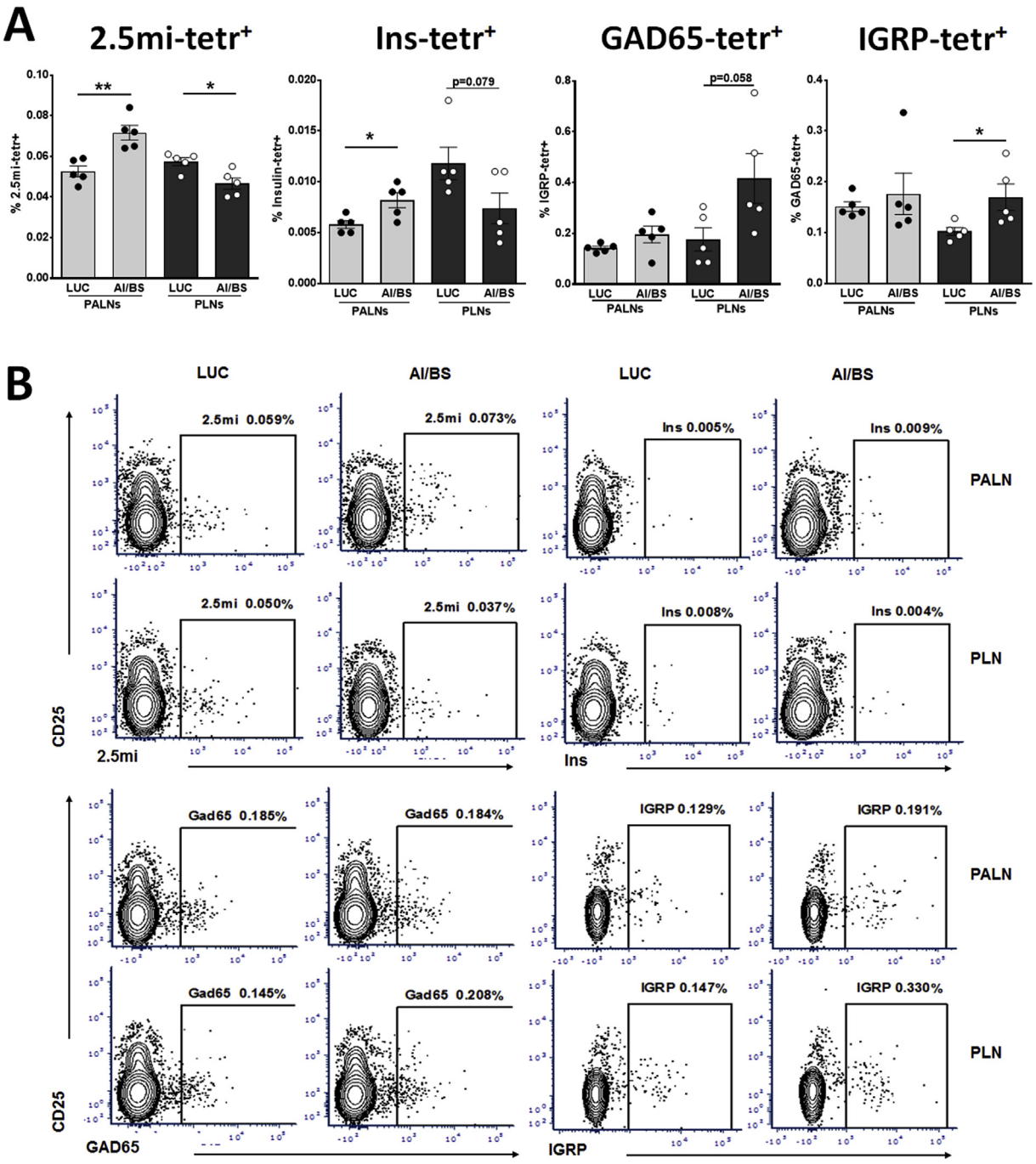

Fig. 4. Response of endogenous antigen-specific T cells in vivo. (**A**) Identification and quantification of antigen-specific T cells by tetramer staining in PALNs and PLNs from NOD males after a single i.m. dose of AI and BS pDNA (50 μg) compared to Luc pDNA-treated controls. (**B**) Representative FCM plots of the staining of four different MHC tetramers in PALNs and PLNs from NOD mice after AI/BS treatment compared to Luc controls. All bar graphs show mean ± SEM.

prevention (treatment at 4–6 weeks of age) with a single mimotope had a more sustained effect [38]. Administration of multiple pDNA vectors encoding the full protein of four antigens resulted in the highest proportion of mice protected from disease, however there was no significant difference between administering proinsulin alone, in conjunction with three other protein antigens or only selected epitopes. The incidence profile achieved with proinsulin alone was very similar to that of another study [39]. Longer treatments are needed to assess whether delivering multiple epitopes or antigens has any benefit over proinsulin alone, and protection might be achieved with less frequent administrations depending on the duration of antigen expression in vivo (at least one week in our hands).

The mechanism of action of tolerogenic DNA vaccines remains elusive. Antigen-specific T-cell responses (in absence of any adjuvant) had been characterized more often by an increase in IFNγ expression (± IL-10 or IL-4) in response to antigens [13,38,40] than not [41]. We have leveraged TCR-tg T cells, MHC tetramers and single-cell targeted RNA sequencing as powerful tools to provide a more comprehensive analysis of antigen-specific T-cell responses to pDNA-encoded epitopes from multiple antigens in vivo. Interestingly, engagement of antigen-specific T cells in draining LNs of pDNA inoculation sites markedly induced CD25 expression in TCR-tg T cells (and to a lesser extent in endogenous antigen-specific T cells) while the same TCR-tg T cells proliferated more vigorously in the PLNs without CD25 expression. Half

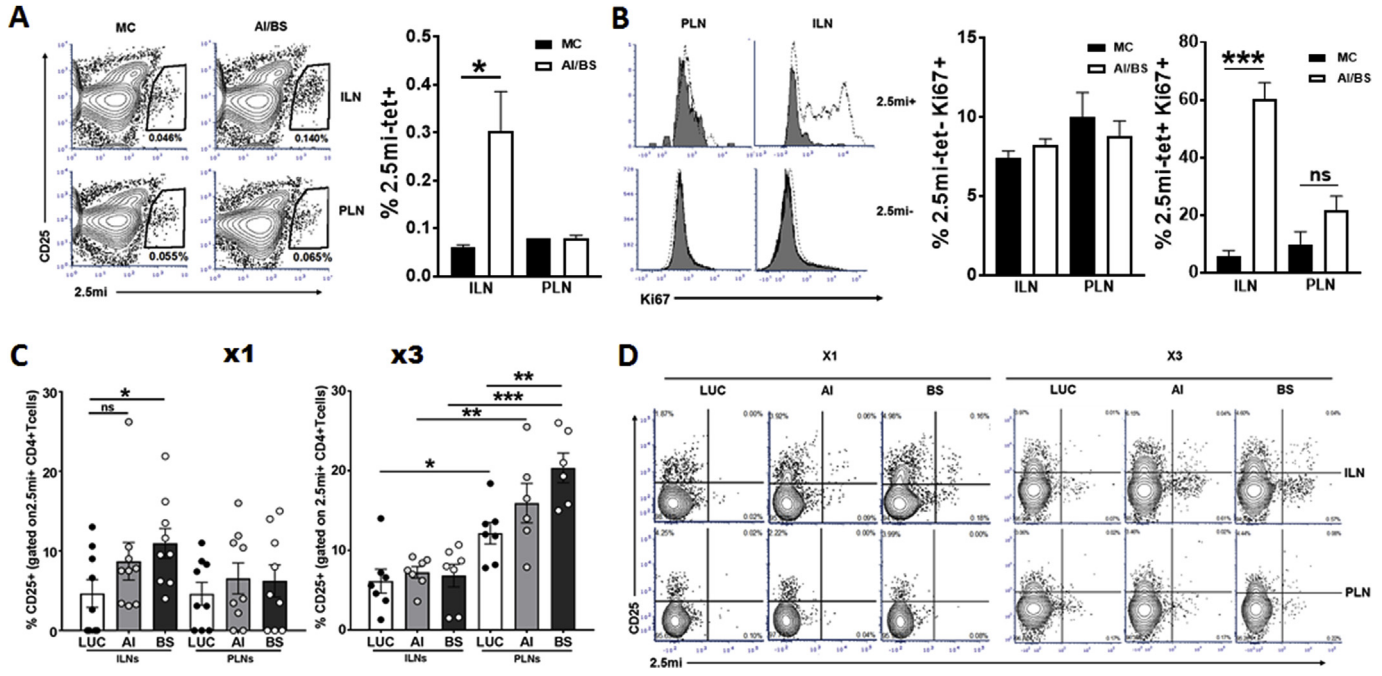

Fig. 5. Proliferation and CD25 expression in endogenous antigen-specific T cells in vivo. (**A**) Dot plots and bar graphs for average frequency of 2.5mi-specific CD4$^+$ T cells used for single-cell sorting after i.d. administration (3 doses) of mCherry (MC) or 1:1 mix of AI and BS (AI/BS) pDNAs (n = 3/group). Cells were first gated on live singlets, CD3$^+$ and CD4$^+$ cells. (**B**) Histograms and bar graphs of Ki67$^+$ cells within 2.5mi-tetr$^+$ and polyclonal (2.5mi-tetr-) CD4$^+$ T cells in ILNs and PLNs after the same pDNA treatment (n = 3/group, separate experiment). (**C**) CD25 expression on 2.5mi-tetr$^+$ CD4$^+$ T cells after 1 dose (x1) or 3 doses (x3) of Luc, AI and BS pDNA treatment. (**D**) Representative plots (gated on total CD4$^+$ T cells). All bar graphs show mean ± SEM.

of these CD25$^+$ TCR-tg T cells were Foxp3$^+$, but among endogenous antigen-specific T cells, Foxp3$^+$ cells were not detected until after several doses and accumulated in the PLNs rather than the draining LNs, while IL-10$^+$ T cells appeared in draining LNs. Unequal redistribution of different antigen-specific T-cell populations over time may be dictated by the relative abundance of the different antigens between the two sites, due to unequal retention and accumulation of circulating T cells. Unfortunately, these Tregs did not appear to exert sufficient suppression at this late stage of disease, as suggested by a lack of protection from transfer of both LN and spleen cells into NOD.SCID mice in our studies and others [13], and by the lack of persistent protection after treatment cessation in NOD mice. However, we cannot exclude the possibility of functional Tregs at other sites not used for those transfers. It is also possible that the induced Tregs were unstable and lost their suppressive function [42]. In contrast, disease transfer was delayed when using cells from early stage prevention experiments, although in this case, the Tregs did not appear to be antigen-specific [38]. While PD-1 upregulation may better predispose T cells to tolerance, we have not found evidence of clonal deletion after multiple injections, and anergy induction, based on CD73 and FR4 co-expression, appeared to be epitope-dependent. While the PLN environment did not support CD25 upregulation, it was generally more conducive of induction of FR4^{hi} CD73$^+$ anergic T cells compared to ILNs, consistent with previous studies [43]. The contrasting T-cell responses between draining LNs and PLNs may reflect differences in their microenvironment and/or in the nature of APCs involved, leading to different outcomes.

The clonal composition of T cells responding to a specific epitope was not addressed in prior DNA vaccine studies. We show that T cells reactive to the 2.5mi are highly polyclonal even though most broadly fall into the TRVB13 and TRAV3 families. Although a majority of 2.5mi-specific T cells were Ki67$^+$ in the draining LNs, there were few identical clones amplified, suggestive of a limited proliferation of many different clones rather than preferential and extensive expansion of a few specific clones. The increased frequency of 2.5mi-tetr$^+$ in the draining LNs may also reflect both proliferation and accumulation of circulating T cells that are retained upon antigen engagement with or without subsequent proliferation. It is also possible that many of these clones were 'silent' low affinity T cells that were oblivious to regulatory mechanisms, but more reactive to the mimotope than their putative natural antigen. Surprisingly, the well-studied BDC2.5 clone (Vβ4 Vα1; TRBV2 TRAV7), which is very reactive to the 2.5mi, does not belong to the most represented TRBV/TRAV families and was never picked up in our sampling of 2.5mi-specific T cells. It is possible that the monoclonal response measured with transferred BDC2.5 is also atypical and is not representative of the whole 2.5mi-specific polyclonal T-cell population. Comparison of the different TCR families indicate that the most represented clonal families (TRBV13 TRAV3) decreased in relative proportion, while previously underrepresented families emerged upon treatment. Interestingly, we found that most IL-10-expressing cells (83%) were found among those emerging clones.

T-cell responses to pDNA-encoded antigens may be influenced by the route of injection. While the majority of pDNA encoding β-cell antigens have been tested via the i.m. route, alternative routes have been explored, including i.d., oral, and intranasal [40,41,44]. Both i.d. injection and oral delivery of a GAD65-encoding pDNA appeared to be superior to i.m. injection for suppression of T1D [41], and gene-gun administration into the skin skewed the T-cell response more toward Th2 than i.m. delivery [40]. We did not observe notable differences in T-cell responses between i.d. and i.m. antigen delivery. Keratinocytes and myocytes are the main parenchymal cells expressing the transgene after i.d. and i.m. injection respectively [45,46], however, DCs are central to the induction of immune responses by DNA vaccines administered via either route. Immune responses are promoted by direct priming (antigens expressed by directly transfected DCs) or by antigen transfer from non-hematopoietic parenchymal cells. Here we confirmed that both hematopoietic and non-hematopoietic cells are able to express DNA-encoded products at the site of injection, with only the former capable of migrating to local LNs. Parenchymal transfected with conventional DNA vaccine may not release antigens unless dying. In contrast, a secreted polypeptide containing all epitopes can be picked up

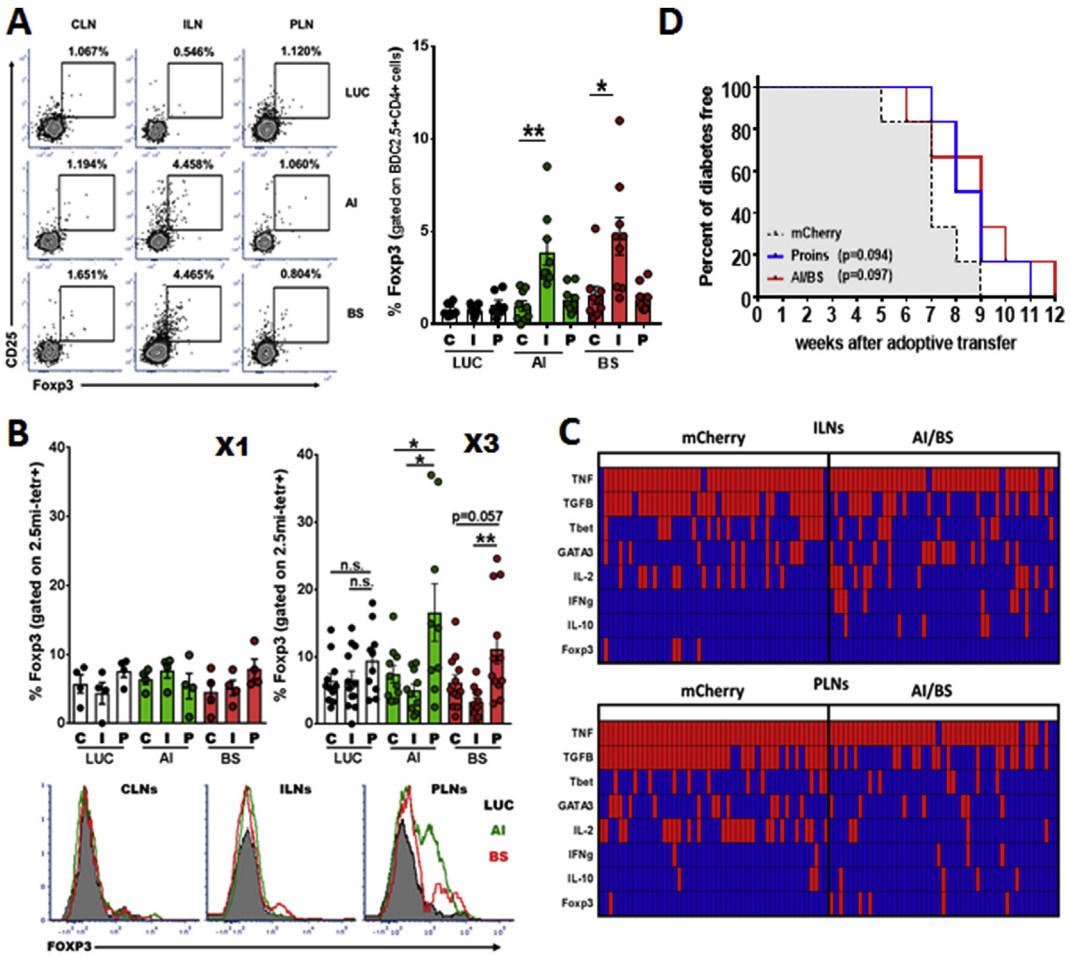

Fig. 6. Regulatory T cells and other Th subsets induced by pDNA in vivo. (**A**) Foxp3 induction in BDC2.5 TCR-Tg CD4$^+$ T cells in different LNs after i.d. pDNA treatment (one dose). (**B**) Foxp3 induction in 2.5mi-tetr$^+$ CD4$^+$ T cells in different LNs after i.d. pDNA treatment (one dose X1 and 3 doses X3). LNs tested: CLN (C), ILN (I) and PLN (P). (**C**) Detection of several T helper cytokines and transcription factors in single-cell sorted 2.5mi-tetr$^+$ CD4$^+$ T cells from ILNs and PLNs after DNA treatment (3 doses i.d.). Red: detected; blue: not detected. (**D**) Percentage of diabetes-free NOD.SCID mice after adoptive cell transfer of LN and spleen cells from NOD mice treated with either mCherry, proinsulin or AI/BS pDNAs (3 doses i.d.). All bar graphs show mean ± SEM.

and transported by non-transfected DCs and/or flow directly to draining LNs to be acquired and presented by resident DCs. Indeed, the secreted polypeptide (from BS) resulted in a more efficient T-cell engagement than non-secreted polypeptides (from AI), although both induced similar T-cell phenotypes. The increased CD8$^+$ T-cell response obtained with the secreted epitopes would suggest that antigen cross-presenting DCs substantially contribute to the response and directly transfected DCs may not mediate CD8$^+$ T-cell responses as much as previously thought [46]. While assessing the contribution of these different APCs is challenging, it is worth investigating, because each APC subset may have different tolerogenic potential and may also be differentially defective in T1D-prone individuals [30].

5. Conclusions

The responsiveness of T1D patients to insulin or GAD65 delivery appears to be linked to their endotypes characterized by distinct underlying autoimmune responses reflected by autoantibodies [47], and as a consequence, antigen-specific therapies will likely move towards more personalized approaches. By eliciting antigen-specific CD4$^+$ and CD8$^+$ T-cell responses to multiple epitopes across different β-cell antigens using a single easily customizable construct, our platform is poised to greatly facilitate this transition. It allows inclusion of mimotopes that more efficiently engage diabetogenic T cells for tolerance without disease exacerbation. Additional neoepitopes such as the newly discovered hybrid insulin peptides [17,48] can easily be incorporated in this platform as opposed to approaches using recombinant proteins. We propose that this versatile approach will be most effective when combined with immunomodulatory elements that help overcome defective mechanisms of tolerance induction and more efficiently promote regulatory antigen-specific T cells that sustain therapeutic benefits long-term.

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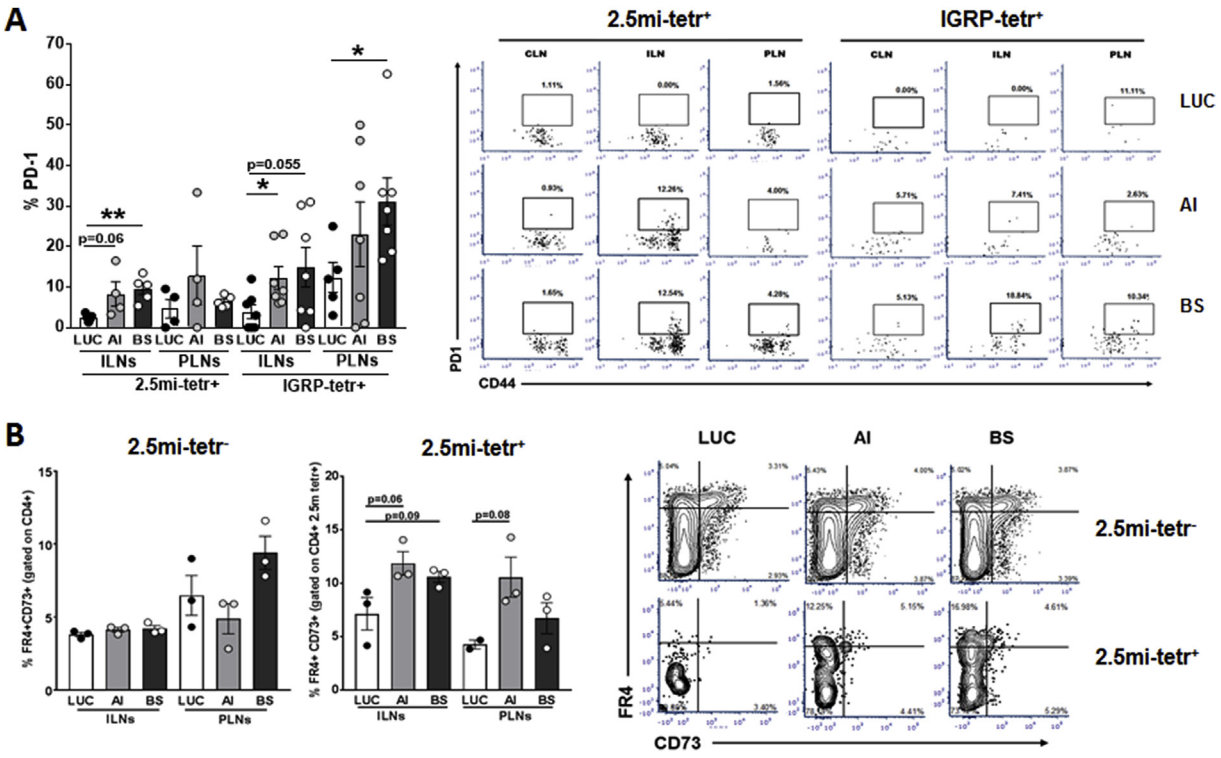

Fig. 7. Expression of PD-1 and anergy markers FR4/CD73. (**A**) PD-1 induction in 2.5mi-tetr$^+$ CD4$^+$ T and IGRP-tetr$^+$ CD8$^+$ T cells in ILNs and PLNs after pDNA treatment (3 doses i.d.). Scatter bar graphs of % PD-1 among Tetr$^+$ CD4$^+$ T cells and representative dot plots of PD-1 versus CD44, showing that PD-1$^+$ cells are antigen-experienced CD44$^+$ T cells. (**B**) FR4 and CD73 expression in 2.5mi-tetr$^+$ and 2.5mi-tetr$^-$ CD4$^+$ T cells after 3 doses (i.d.) of AI or BS pDNA. All bar graphs show mean ± SEM.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.11.003.

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