Editing T cell repertoire by thymic epithelial cell-directed gene transfer abrogates risk of type 1 diabetes development

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Insulin is the primary autoantigen (Ag) targeted by T cells in type 1 diabetes (T1D). Although biomarkers precisely identifying subjects at high risk of T1D are available, successful prophylaxis is still an unmet need. Leaky central tolerance to insulin may be partially ascribed to the instability of the MHC-InsB9-23 complex, which lowers TCR avidity, thus resulting in defective negative selection of autoreactive clones and inadequate insulin-specific T regulatory cell (Treg) induction. We developed a lentiviral vector (LV)-based strategy to engineer thymic epithelial cells (TECs) to correct diabetogenic T cell repertoire. Intrathymic (it) LV injection established stable transgene expression in EpCAM⁺ TECs, by virtue of transduction of TEC precursors. It-LV-driven presentation of the immunodominant portion of ovalbumin allowed persistent and complete negative selection of responsive T cells in OT-II chimeric mice. We successfully applied this strategy to correct the diabetogenic repertoire of young non-obese diabetic mice, imposing the presentation by TECs of the stronger agonist InsulinB9-23R22E and partially depleting the existing T cell compartment. We further circumscribed LV-driven presentation of InsulinB9-23R22E by micro-RNA regulation to CD45⁻ TECs without loss of efficacy in protection from diabetes, associated with expanded insulin-specific Tregs. Overall, our gene transfer-based prophylaxis fine-tuned the central tolerance processes of negative selection and Treg induction, correcting an autoimmune prone T cell repertoire.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease mediated by T cells that infiltrate and destroy insulin-producing β cells within the pancreatic islets, often starting in childhood or adolescence. The incidence of T1D has increased significantly in recent decades, particularly in young children. Serocconversion to a detectable titer of more than one autoantibody (autoAb) specific for islet-associated antigens (Ags: insulin, Ins; glutamic acid decarboxylase 65-kDa isofrom, GAD65; insulinoma-associated-2, IA-2; zinc transporter 8, ZnT8) characterizes the pre-symptomatic stage of T1D, which in most cases evolves into overt autoimmune diabetes within a decade. Although multiple islet autoAbs are biomarkers of high-risk T1D development, the disease cannot currently be prevented. Thus, an effective prophylactic intervention remains an urgent unmet medical need.

Autoimmune responses to insulin are associated with T1D development, especially in younger patients harboring HLA DR4-DQ8. In this patient population insulin may be the initial autoantigen. The disease observed in non-obese diabetic (NOD) mice is also driven by autoimmunity to insulin. Therefore, insulin is the ideal target for testing Ag-specific tolerogenic approaches for disease prevention in NOD mice, which may have translational relevance for a subset of individuals.

Human and rodent thymi are fully active in shaping the T cell repertoire only for a limited time window before undergoing an irreversible involution. Thymic epithelial cells (TECs) play a pivotal role in thymocyte selection by presenting self-Ag-derived epitopes. The rate of autoimmune regulator (AIRE)-driven transcription of prionsulin in medullary (m)TECs is genetically determined and impacts susceptibility to T1D, affecting thymic selection of the T cell repertoire. Thus, a weak expression of proinsulin by mTECs is associated with suboptimal negative selection of insulin-reactive T cell clones. Several strategies for Ag-specific tolerance induction have been tested to achieve Ag expression in the thymus and drive negative selection of Ag-reactive thymocytes and/or induction of regulatory T cells (Tregs). However, none of them employed a selective cell-targeting approach to impose the presentation of a specific epitope only by
TECs, failing to fulfill safety requirements. In addition, none of them showed the capacity to achieve a profound and stable negative selection of Ag-reactive clones and drive the selection of additional Treg clonotypes to correct an autoimmune prone T cell repertoire.

We designed a lentiviral vector (LV) platform capable of establishing presentation of specific Ags in a renewable population of TECs to correct thymic selection in a model resembling young subjects at risk for T1D development. We used NOD female mice at age 5 weeks as a model of young subjects at high risk for T1D development. At this age, mice have a mature T cell compartment comprising diabetogenic T cells, and primitive InsB9-23 responses have already started, while the β cell mass is still intact.12,13

Here, we show that intrathymic (it) administration of an LV encoding for invariant-chain (Ii) fused to a portion of the Ag of interest, including immunodominant T cell epitope, constitutes a successful strategy to generate a stable subset of engineered TECs, by transducing the common EpCAM+/CD49F+/Sca-1+ TEC precursor.14 This results in persistent and robust presentation of autoAg by TECs to developing thymocytes. In addition, microRNAi42-negative post transcriptional regulation of Ii expression was exploited to target LV-driven epitope presentation to TECs. Upon transduction, TECs are permissive for transgene expression since they do not belong to a hematopoietic lineage and do not express microRNAi42.15 By enabling presentation of the strong agonist variant of insulin InsB9-23,16 reshaping the T cell repertoire and preventing T1D development.

RESULTS

LV-mediated transduction of the TEC progenitors enables stable transgene expression in cortical and medullary TECs

Aiming to perturb the T cell selection process, we tested whether the injection of LV into the thymus could result in a durable genetic modification of TECs. To determine distribution of transgene expression in the body and success rate of the injection procedure, NOD mice (n = 10) were intrathymically injected with luciferase-encoding LV under the control of phosphoglycerate kinase (PGK) promoter (LV.Luc, 2 × 10⁷ transduction unit [TU]/mouse). Ten days post injection, 90% of LV-treated mice displayed a thymus-localized luciferase activity (Figure S1). To determine whether TECs can be transduced via it-LV administration, 5-week-old C57Bl6 mice (n = 6) were intrathymically injected with a GFP-encoding vector (LV.GFP, 2 × 10⁷ TU/mouse). At 1 and 9 weeks (n = 3/time point) post LV.GFP treatment, thymus were harvested and CD45⁻ TEC-enriched cells were isolated to determine the percentage of GFP expressing TECs (EpCAM⁺), including cortical (c) (CD80low/int Ly51⁺) and medullary (m)TECs (CD80°Ly51low/int) and their common precursors (CD49F°Sca-1⁻) (Figure 1A). Results indicate that an average of 2.8% of TECs (CD45° EpCAM⁺) expressed GFP at 1 week post treatment. The expression was stably maintained up to 9 weeks post it-LV by virtue of the transduction of 1.4% of TEC precursors (Figures 1B and 1C). A comparable fraction of GFP⁺ was observed in cTECs and mTECs at 1 week post it-LV (Figure 1B). Conversely, at 9 weeks post injection GFP expression was higher in cTECs, possibly due to progressive tissue disorganization (Figure 1C). Therefore, a single it-LV treatment leads to vector integration into TEC precursors, resulting in a stable modification of mature TEC compartment (Figure 1D), which persists beyond their 2-week turn-over time.19 This opens the possibility of expressing genes to modulate thymocyte selection for the entire window of activity of the primary lymphoid organ.

TEC-mediated Ag presentation imposed by it-LV gene transfer alters thymocytes selection overtime

To prove the potential of it-LV gene transfer in redirecting thymocyte selection, CD45.1.C57Bl6 mice (n = 9) were lethally irradiated, while lead-shielding the thymus to preserve tissue architecture and function; and bone marrow cell transplantation (BMT) from CD45.2.OT-II T cell receptor (TCR) transgenic mice was performed to achieve mixed chimerism (Figures 2A–2C). This model ensured the migration of CD45.2.OT-II T cell precursors with a known Ag-specificity for Ovalbumin323-339 (OVA323-339) from bone marrow (BM) to the thymus, where immature T cells will complete maturation and undergo thymic selection. As soon as CD45.2.OT-II CD4⁺ T cells were detectable in the peripheral blood of transplanted mice (week 5 post BMT), they received it-LV encoding for invariant-chain (Ii) fused to chicken Ovalbumin315-353 (Ii.OVA315-353) fragment (it-LV.OVA, n = 6), to achieve OVA323-339 presentation by TECs, or it-LV encoding for Ii fused to GAD65500-585 (it-LV.GAD65, n = 3), as unrelated Ag control. The frequency of CD45.2.OT-II CD4⁺ T cells was determined by cyt fluorimetric analysis of circulating cells and the ratio [CD45.2.OT-II CD4⁺ T cells/CD45.2 CD11b°GR1° myeloid cells] was determined at the indicated time points (Figures 2B–2D). Results showed that it-LV.OVA led to TEC transduction and consequent OVA323-339 presentation in the context of major histocompatibility complex class II (MHC class II) to developing thymocytes, as confirmed by the complete negative selection of CD45.2.OT-II CD4⁺ T cells in it-LV.OVA mice. Conversely, CD45.2.OT-II CD4⁺ T cells egressed from the thymus in it-LV.GAD65-treated control mice. Of note, 2 × 10⁷ TU/mouse dose resulted in ~3% of transgene-expressing TECs (Figure 1B); thus, in chimeric mice we expect that a similar percentage of TECs were presenting cognate Ag (OVA323-339) to OT-II T cells. Since levels of engraftment mirror the composition of T cell precursors, we can estimate that up to 35% of them, originating from OT-II hematopoietic stem and progenitor cells, were negatively selected by a few LV-engineered TECs, demonstrating the potency and precision of this strategy.

Intrathymic LV gene transfer alters thymocyte selection and abrogates T1D development in NOD mice

To evaluate whether perturbing the selection of thymocytes responsive to insulin immunodominant epitope (InsB23) may reduce T1D development, 5-week-old NOD female mice (n = 15) received it-LV encoding for II.Insulin B chain4-23R22E (it-LV.InsB.R22E, moleculartherapy.org
n = 8) or Li.OVA315-353 (it-LV.OVA, n = 7) as control (Figure 3A). R22E aminoacidic substitution improves stability of [InsB9-23-IAg7] complex (IAg7 is MHC class II in NOD mice). To reduce the frequency of autoreactive diabetogenic cells already in circulation at the time of treatment, and to allow the reconstitution of the T cell repertoire conditioned by it-LV-driven insulin epitope presentation, the day after it-LV injection mice were treated with an Ab cocktail to deplete CD4+ and CD8+ T cells \((\text{D{T}})\) in vivo. After 5 weeks, the T cell compartment was almost completely reconstituted by T cells selected by the it-LV-conditioned thymus and by homeostatic expansion of T cells spared by the depletion regimen (Figure 3B). LV-mediated genetic modification was comparable in the two groups of it-LV-treated mice, as indicated by quantification of vector copy number (VCN) in the thymus (Figure 3C). Blood glucose levels (bgl) were monitored up to age 33 weeks in it-LV-treated mice. Results indicate that insulin in it-LV-treated mice was compatible with that of normoglycemic NOD mice (Figure S2). Percentages of CD4+ T cells, CD8+ T cells, and Foxp3+ Tregs were determined in the pancreatic lymph nodes (PLNs) and spleen, revealing a tendency to a lymphocyte reduction in it-LV.InsB.R22E-treated mice (Figures S3A and S3B). Notably, there were significantly more Foxp3+ Tregs in the islets of it-LV.InsB.R22E-treated mice than in the islets of diabetic NOD mice (Figures S3C–S3E).

Histological analysis of the thymi did not show differences between it-LV-treated mice and untreated controls. The architecture of the thymic tissue at age ~6 months showed a typical slight decrease of the cortical layer\(^20\) (Figure S4).

Based on these results, we postulate that T1D protection is due to improved processes of central tolerance to insulin by optimized induction of insulin-specific Tregs and/or negative selection of insulin-reactive T cell clones. This alteration in thymic output modulated T cell responses against insulin presented by antigen-presenting cells (APCs) in secondary lymphoid organs, overall preserving \(\beta\) cell mass and glucose homeostasis.

**TEC-specific LV.142T gene transfer enables editing of T cell selection in the thymus**

To limit off-target transgene expression in hematopoietic cells following it-LV administration, without the loss of TEC transduction, negative regulation mediated by hematopoietic-specific micro-RNAs was exploited. To determine whether TECs can be transduced by administering it.LV comprising four repeated target sequences for...
hematopoietic-specific micro-RNA142 at 3′ untranslated region, 5-week-old C57Bl6 mice received intrathymically micro-RNA regulated GFP-encoding vector (it-LV.GFP.142T, 2 × 10^7 TU/mouse, n = 4). One week post it-LV.GFP.142T treatment, thymi were harvested and CD45+ TEC-enriched cells were isolated to determine the percentage of GFP-expressing TECs (EpCAM+), including cTECs, mTECs, and their common precursors. Results indicate that an average of 3.6% of CD45+ EpCAM+ cells express GFP, equally represented in cTECs and mTECs, and ~1.5% of TEC precursors were GFP+ (Figures 4A and 4B). Therefore, it-LV.142T targeted TECs at levels comparable with those achieved by it-LV. Moreover, comparative studies confirmed that it-LV.142T targeted TECs and showed a significant reduction of transgene expression in hematopoietic cells, such as thymic and peripheral APC subsets (Figures 4C, 4D, and S5) and thymocytes (Figure S5). Thus, it-LV.142T provides a safer pattern of transgene expression, excluding cell subsets potentially able to trigger an autoimmune reaction upon presentation of a relevant autoAg in secondary lymphoid organs.

**TEC-mediated InsB9-23R22E overexpression is effective in reshaping the T cell repertoire to achieve prevention of T1D development**

To further confirm that targeting LV-driven Ag presentation to TECs represents a strategy to prevent the risk of autoimmunity, 5-week-old female NOD mice (n = 48) received intrathymically micro-RNA142-regulated LV encoding for Ii.B4-29R22E (it-LV.InsB.R22E.142T, n = 19), with Ii.OVA315-353 (it-LV.OVA, n = 8) as unrelated control Ag. To elucidate the contribution of a T cell-depleting regimen, a fraction of it-LV.142T-treated mice, as well as untreated controls, received an Abs cocktail to deplete in vivo CD4+ and CD8+ T cells (ΔT) at day +1 (Figure 5A). Results indicate that the T cell-depleting regimen did not reduce the risk of T1D development per se, resulting in only minimally delaying disease onset. Conversely, a depleting regimen was required to achieve complete protection from T1D development in mice treated with it-LV.InsB.R22E.142T (Figures 5B and S7). LV-driven presentation of wt.InsB9-23 did not protect from T1D development, even in association with the T cell-depleting regimen (ΔT) (Figures S6 and S7). The absence of any protection from T1D development by it-LV.OVA further demonstrates the precision of the Ag-specific activity of this strategy (Figures 5B, S6, and S7).

At the end of the observation period, frequencies of insulin-specific T cells were investigated using IAg7-InsB9-23R22E tetramers. Although the frequency of insulin-specific conventional T cells (Tconv) was not altered by any treatment (Figures 5C and 5D), insulin-specific Tregs (IAg7-InsB9-23R22E tetramer+CD25+GR1+ myeloid cells within CD45+ - and OT-II-derived CD45+2.2 cells (B)), Ratio between [%CD4+,%CD11b+GR1+] was determined over time within CD45+2.2 cells to normalize differences in engraftment (C). Single values, mean ± SEM are reported. Representative dot plots of the study are reported (D).

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**Figure 2. LV-driven presentation of an immunodominant T cell epitope by TECs results in profound and durable negative selection of Ag-reactive T cell clones**

(A–D) CD45.1-C57BL6 mice (n = 9) were conditioned by lethal irradiation, protecting the thymus using a lead shield, and transplanted with CD45.2.OT-II bone marrow cells. Five weeks post BMT CD45.2.OT-II T cells became detectable in circulation. Chimeric mice were treated intrathymically (it) with LV.PGK.Ii.OVA315-353 (it-LV.OVA, n = 6) or LV.PGK.Ii.GAD65500-585 (it-LV.GAD, n = 3) as control. Post transplant, T cell compartment reconstitution was monitored for ~100 days after it-LV treatment (A), determining the frequency of CD4+ T cells and CD11b+GR1+ myeloid cells within CD45+ - and OT-II-derived CD45+2.2 cells (B), Ratio between [%CD4+,%CD11b+GR1+] was determined over time within CD45+2.2 cells to normalize differences in engraftment (C). Single values, mean ± SEM are reported. Representative dot plots of the study are reported (D).
PLN Tregs of it-LV.InsB.R22E.142T+ΔT mice, which are protected from T1D, we found sharing of several clonotypes (Figure 6A) and, among the 23 TCR α complementarity-determining region 3 (CDR3) Treg sequences shared, 17 were found exclusively in mice treated with it-LV.InsB.R22E.142T, with or without T cell depletion (Figure 6B, in yellow). Among the 17 it-LV.InsB.R22E.142T treatment-associated clones, only 6 were ascribable to the Treg compartment (Figure 6C, in red) since they were not shared with PLN Tconv sequences. One clonotype was shared by CD4+ Tconv from untreated NOD mice, suggesting that it-LV treatment possibly promoted its conversion into Tregs.

Interestingly, five of the seven latter clonotypes were also found in pancreas-infiltrating T cells of it-LV.InsB.R22E.142T-treated mice, but not in it-LV.InsB.R22E.142T+ΔT mice (Figure 6D, in white), suggesting a different Treg dynamic and localization within the two groups. Therefore, these seven CDR3 sequences were regarded as bona fide Treg clonotypes induced by it-LV.InsB.R22E.142T treatment, either in PLNs (in mice receiving T cell depletion and achieving full protection) or in pancreatic infiltrates (in the absence of T cell depletion and with only partial protection from T1D) (Figures 6E and 6F).

In conclusion, these data demonstrate that selective targeting presentation of hyper-agonist T cell epitopes to TECs in combination with a T cell-depleting regimen is an effective preventive strategy to dampen the diabetogenic potential of T cell repertoire, resulting in protection from autoimmune T1D.

**DISCUSSION**

This study shows that the T cell repertoire that predisposes NOD mice to autoimmune T1D can be modified by the intrathymic administration of LV for TEC-directed gene transfer to achieve central tolerance to insulin and preventing autoimmunity. The concept of modulation of Ag-specific central tolerance has been explored in the past in different models of autoimmune diseases by vector-mediated expression or direct injection of the protein of interest into the thymus. We have substantially improved several aspects of this.
strategy, defining a specific and efficacious platform, potentially applicable to subjects at risk of T1D. We show that a single LV dose is sufficient to engineer less than 5% of TEC that, despite low frequency, successfully promotes negative selection of highly represented clones (e.g., TCR transgenic OT-II thymocytes) and achieves an almost complete protection from T1D development in NOD mice, predominantly through the selection of a new family of thymic-derived Tregs.

Previous attempts based on transgenic LV-mediated expression of proinsulin have shown only a partial prevention of T1D in NOD mice,22 and their potential for clinical translation remains elusive. Bettini and colleagues elegantly showed that forced presentation of wt-InsB immunodominant epitope by professional APCs in the thymus of specific retro-genic mice promoted induction of Ag-specific Tregs, while presentation of the modified epitope (InsB9-23.R22E) led to negative selection of a specific insulin-reactive T clone.23 Although the use of retro-genic mice represents a precise approach to study mechanisms of thymic selection, results were limited to a few insulin-specific clones, losing the complexity that results from the selection of multiple clones potentially reactive to the it-LV-encoded epitope. We show that InsB9-23.R22E presentation is able to promote expansion of insulin-specific Tregs in fully immunocompetent NOD mice. Unfortunately, in our experimental settings we were unable to definitely define whether negative selection of InsB9-23-specific clones occurred. However, altering the strength of TCR signaling of InsB9-23 by introducing R22E aminoacidic substitution we are likely altering the fate of several clonotypes; therefore, correcting the T cell repertoire of naive T cells and Tregs. We can envisage that, among billions of clonotypes, those recognizing InsB9-23, destined to be naive T cells in untreated NOD, will be selected to be Tregs, or deleted after encountering InsB9-23.R22E-presenting mTECs.

Alterations of central tolerance mechanisms in autoimmunity are still not completely understood. The immunodominant autoAg InsB9-23, while restricted to T1D-predisposing MHC class II IAg7 molecule in NOD mice, as well as the homologous HLA-DQ8 in humans, generates an unstable MHC-peptide complex,17 which seems to lower TCR T cell avidity, leading to inadequate T cell selection.24 To optimize
thymocytes, we propose an innovative LV-mediated gene transfer approach specifically targeting TECs in vivo to generate presentation of MHC class I and II-restricted epitopes, persisting beyond their typical 2-week turnover time, as a consequence of TEC precursor transduction. AutoAg-derived epitopes may be modified to increase the strength of the TEC-thymocyte interaction, as for InsB9-23R22E. This may be exploited to overcome the suboptimal selection of insulin-reactive clones by the wt-InsB9-23 epitope and promote differentiation of insulin-specific Tregs. Moreover, our LV platform may potentially be applied to generate central tolerance to neo-Ags described both in humans with T1D and NOD mice (namely, hybrid insulin peptides [HIPs]), generated by post-translational modification within secretory granules of β cells upon covalent binding of (pro)insulin peptides and other peptides derived from β cell-related Ags. In murine models, HIP-specific T cells transfer disease, as shown for InsB9-23-specific T cells in NOD mice. Furthermore, in recent studies conducted in humans genetically at risk for T1D, T cell responses to HIPs were more prevalent than responses to insulin peptides, and were strongly associated with formation of anti-insulin autoAbs and to T1D progression.

Micro-RNA142-regulated vector design avoids triggering of autoimmunity by activation of peripheral mature T cells and is mandatory for safety. Indeed, our study shows that a T cell-depleting regimen applied immediately after LV-mediated conditioning of thymus, although not effective per se, contributes to achieving a complete prevention of T1D, likely by removal of diabetogenic peripheral mature T cells at the time of treatment. However, we cannot exclude a role for thymic resident APCs in transgenic Ag presentation, which may still be translated in the case of leakiness of micro-RNA142 regulation or acquired from thymic fibroblast. Moreover, upon Ag-loaded-MHC class II exposure on the cell surface LV-modified TECs themselves may release an excess of transgenic Ag copies stored in the endosome, thus leading to a potential contribution in Ag presentation by professional APCs in the thymus.

Our results further highlight how strategies to temporarily deplete and inactivate peripheral T cells are insufficient in disarming diabetogenic T cell repertoire in NOD mice and in subjects at risk for T1D. Indeed, Herold et al. recently showed clinical results of a 14-day treatment with Teplizumab (anti-CD3 mAb) in a large cohort of genetically predisposed high-risk subjects. This T cell-directed regimen led to a significant delay of T1D onset, but it was unable to protect patients from disease progression.
context, strategies aimed at the correction of the central tolerance machinery may represent a concrete option for prevention of autoimmune diabetes.

Expanding the clonality of insulin-specific Tregs by naturally occurring mechanisms may, in the long term, strengthen the regulatory arm of the immune system to regulate autoreactive cells spared by T cell-directed regimen. Although ethical concerns may arise from LV-based preventive treatment in pediatric at-risk subjects, our study suggests that an early intervention with intrathyMIC gene therapy associated with a T cell-depletion regimen may result in suppression of relevant autoimmune reaction prevalently occurring in lymphoid organs (PLN), as suggested by the localization of Treg clones in it-LV.InsB₄₋₂₉R₂₂E.1₄₂T+ΔT-treated mice.

Discrepancies in maturation of T cell compartment and primary lymphoid organs in humans and mice have been considered. In humans, T cell compartment and lymphoid tissues are almost completely established at birth, and the thymus is fully active until puberty, when it starts to involute, turning into fatty tissue with residual activity of T cell selection. Conversely, mice are lymphopenic at birth with a small thymus that grows during the first weeks of life, while changes in the size and cortico-medullary structure become visible starting from 2.5 months. Involution of the thymus progressively evolves with age, preserving a basal activity to refill life-long naive T cell compartment.

Limitations of our strategy may derive from in vivo administration of integration-competent LV and from the choice of the autoAg. Several years of follow-up of patients enrolled in hematopoietic stem cell ex vivo gene therapy clinical trials22,23 did not show signs of genotoxicity in highly replicating cells, such as hematopoietic stem cells, with a pattern of integration in active genes, typical of the LV.34 This also occurred in non-human primates undergoing LV in vivo gene transfer directed to hepatocytes.

Although T cell epitopes in T1D are well characterized based on the human HLA,36 we envisage that a personalized it-LV therapy for T1D could be designed according to pre-screening T cell reactivity, with the possibility of including multiple target epitopes. Overall, our study may contribute to the definition of a strategy for prevention, not only for T1D, but generally applicable to T cell-mediated autoimmune diseases.

MATERIALS AND METHODS

Study design
To investigate whether the correction of T cell repertoire selection could abrogate susceptibility to T1D, NOD mice received an integration competent LV dose in the thymus to impose specific Ag presentation to developing thymocytes, followed by a T cell-depleting regimen. Mice were treated at the age of 5 weeks, largely before the onset of T1D but when insulin tolerance has already been broken in vivo. Experimental groups were dimensioned to allow statistical analysis. Mice were randomly assigned to each group, but the experimenter was not blinded to group identity.

Lentiviral vectors
InsulinB₄₋₂₉ (QHLGCGSHVEALYVCGERGFYTM) and Insulin B₄₋₂₉R₂₂E (QHLGCGSHVEALYVCGERGFYTM) or control Ovalbumin315-353 (CGISSAESLKISQAVHAAHAEINEAGREVVGSAEAG) were cloned into linearized BamHI-SalI vectors for transgene expression: pCCLsin.PGK.wpre (LV.PGK), and pCCLsin.PGK.wpre142-3pT (LV.PGK.142T), including the ubiquitously active template gDNA using a primers/probe set designed on the primer binding site region of LV, as described above for LV titration. The amount of endogenous murine DNA was quantified by a Kozak sequence upstream of the transcription start site and a stop codon at the terminus. Therefore, sequences were cloned into in PCR amplification Kit (Promega). VCN in murine DNA was determined by ddPCR, starting from 5–20 ng of template gDNA using a primers/probe set designed on the primer binding site region of LV, as described above for LV titration.

Integrated vector quantification of copies
For mice experiments, DNA was extracted from thymus samples using Maxwell 16 Tissue DNA Purification Kit (Promega). VCN in murine DNA was determined by ddPCR, starting from 5–20 ng of template gDNA using a primers/probe set designed on the primer binding site region of LV, as described above for LV titration. The amount of endogenous murine DNA was quantified by a primers/probe set designed on the murine sema3a gene (Sema3A fw: 5′-AC CGATTCCAGATGATTGGC-3′; Sema3A rv: 5′-TCCATATTAAT GCAGTGCTTGC-3′; Sema3A probe: HEX 5′-AGAGGCGTGTC CGAAGCTCATGG-3′). The PCR reaction was performed with each primer (900 nM) and the probe (250 nM) following the manufacturer’s instructions (Bio-Rad), read with a QX200 reader.
and analyzed with QuantaSoft software (Bio-Rad). Transduced CEM (lymphoblastic cells line) clones carrying a known number of copies were used as standard and internal control. Results are expressed as VCN/diploid genome.

Mice and treatments
Female non-obese diabetic (NOD/LtJ), C57Bl6 (C57BL/6NCrl), C57Bl6 congenic CD45.1 (B6.SJL-PtprcaPepcb/JBoyCrl), and OT- ILC57Bl6 (C57BL/6-Tg(TcraTcrb)425Cbn/Grl) mice were purchased (Charles River, Calco, Italy) and housed in specific pathogen-free conditions. Diabetes was determined by two consecutive measures of glycemia ≥ 250 mg/dL. Blood glucose measurements were determined by a Bayer Breeze blood glucose monitoring system (Bayer). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at San Raffaele Institute, Milan (no. 1062).

Intrathymic LV injection in the thymus was performed as described previously. In brief, mice were anesthetized and placed supine to make a small (4–5 mm) transverse incision of the skin in coincidence to a line joining the anterior legs to identify intercostal spaces. Two injections were performed by Hamilton syringe (Neuros, 33 gauge) through the second intercostal space on either side of the sternum with an ~30°–40° angle to access both the thymic lobes. An LV dose of 2 × 10^7 TU/mouse was typically injected in a volume of 10–15 μL/lobe. The skin was closed by stitches.

A mix containing anti-CD4 (100 μg/dose, clone GK1.5) and anti-CD8 (100 μg/dose, clone YTS169.4) depleting Abs (Bio X Cell) was administered intravenously. Transgene expression following it-LV.PGK luciferase treatment was evaluated in NOD mice at the indicated time points by a small-animal bioluminescence imaging procedure using the IVIS Spectrum CT System (PerkinElmer).

OT-II chimeric mice conditioning and BM transplantation
BM from 6-week-old OT-II mice was harvested by flushing femurs. CD45.1 mice received total body irradiation (935 cGy split into two doses performed at least 2 h apart) protecting the thymus with a lead shield; afterward OT-II BM cells (7.5 × 10^6 cells/mouse) were injected intravenously. Mice were bled, and the engraftment of donor cells was determined by flow cytometry at weekly intervals.

Lymphocyte isolation and immunophenotyping
Spleens and PLNs were homogenized using frosted glass slides and filtered after RBC lyses (where needed) to obtain a single-cell suspension. Single-cell suspensions were stained with the following Abs: anti-CD4 (RM4-5), anti-CD8 (53–67) anti-CD62L (Mel14), anti-CD25 (PC61), anti-CD3 (17A2), anti-CD45 (30F11), anti-CD44 (IM7), anti-GR1 (RB6-8C5), anti-CD11b (M170), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD19 (1D3), anti-CD45RB/B220 (RA3-6B2), (BD Pharmingen), anti-CD11c (N418), and anti-Foxp3 (FJK-16s) (eBioscience). Intracellular Foxp3 staining was performed according to the manufacturer’s instructions. Labeled cells were analyzed using a BD Symphony cytometer (BD) and analyzed using Flowjo-10 software. Class II MHC tetramer IAg InsB15-23R22E MHC class II (HLVLYLVCGERG) and MHC class I H2-K^d InsB15-23 (LYLVCGERG) were provided by the NIH Tetramer Core (Emory University, Atlanta, GA).

TEC isolation and characterization
Murine TEC isolation was performed at the indicated time points following it-LV treatment. Murine thymus was cleaned of fat and stromal tissue, and then digested at 37°C with an enzymatic solution containing Liberase TL and DNAse I. Digested tissues were collected in DMEM with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin. Single thymic cell suspensions were then incubated with anti-CD45 micro-beads (Miltenyi Biotec) and processed with the AutoMACS Pro Separator (Miltenyi Biotec). The CD45 negative fraction was retrieved and then tested by multiparametric cytometry analysis for the expression of TEC markers.

To check TEC enrichment after isolation, cells were immune-stained by anti-CD45 (clone 30-F11, BioLegend), anti-EP Cam (clone G8.8, BioLegend) and pan major MHC class II (IA/IE MHC-II) (clone M5/114.15.2), anti-CD49F (GoH3) (BioLegend), anti-Ly5.1 (clone 6C3) (Miltenyi), and anti-CD80 (16-10A1) (BD Pharmingen). Exclusion of dead cells was done by adding 200 μg/mL of DAPI solution just before acquisition.

Immunohistochemistry and islets scoring
Pancreata and thymi were harvested, fixed in 10% buffered formalin (Bio Optica) and embedded in paraffin. Serial sections (4 μm) were stained with hematoxylin and eosin for morphological analysis and insulitis scoring. To determine levels of insulitis, we performed an unbiased software quantification analysis based on the Aperio Scan Software System. Thus, pancreatic islets were assigned as healthy islets with no lesions/infiltraion: 1%–25%, 26%–50%, 51%–75%, and 76%–100% of infiltration/islet destruction.

To assess immunolocalization of Foxp3, rabbit anti-Foxp3 (FjK-16s) antibody was used after antigen retrieval with Tris-EDTA (pH 9) in a warm bath and revealed by rat on a rodent HRP-polymer and rabbit on a rodent HRP-polymer (Biocare Medical), using 3,3-diaminobenzidine as chromogen (BioGenex). Slides were counterstained with hematoxylin. To normalize Foxp3+Treg in the islet was measured using an unbiased software quantification analysis tool based on Aperio Scan Software System.

TCR α/β-sequencing
TCR α and β chains were sequenced using a modified rapid amplification of cDNA end PCR protocol, independent of multiplex PCR. Total RNA extracted from FACs-sorted subpopulations was reverse transcribed and amplified using primers specific to murine α/β chain constant regions and for the template-switching sequence added during cDNA synthesis. PCR amplicons were purified with AMPure beads (Beckman Coulter) and sequenced on a MiSeq platform (Illu- mina). Data were analyzed using MiXCR software. Non-functional
CDR3 amino acid sequences were excluded from the analysis using the VDJtools package.47

Statistical analyses
Statistical analyses were performed using GraphPad Prism software. Incidence of diabetes was compared by Mantel-Cox test. Fisher’s exact test (chi-square test) was used to compare levels of infiltration between experimental groups. ANOVA was used to determine statistical differences between multiple experimental groups, following verification of normal distribution of measurements in each group by Shapiro-Wilk test, while Mann-Whitney U test was used to compare means between two independent groups. Findings were considered significant with values for $p \leq 0.05$.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.04.017.

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AUTHOR CONTRIBUTIONS
F.R., R.C., L.P., and I.B. designed and performed experiments and analyzed data. E.R. preformed TCR sequencing. F.S. coordinated histological analyses. S.G. and A.V. interpreted data and edited the manuscript. A.A. designed and coordinated the project, interpreted data, and wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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