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Generation of Regulatory T Cells Against Islet Neoantigen

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ABSTRACT

Type 1 diabetes (T1D) results from T cell-mediated destruction of insulin-producing pancreatic beta-cells. Recently, the immune-provoking role of stress-related neoantigens has become evident. Neoantigens unlikely contribute to central tolerance and therefore hold strong immunogenic potential, but their role in peripheral immune regulation is unknown. Here, we sought proof of concept that Tregs can be generated against islet neoantigen INS-DRiP that results from stress-induced ribosomal misreads of insulin mRNA. Tregs were induced from naïve CD4 T cells isolated from a healthy donor and co-cultured with monocyte-derived tolerogenic DCs either pulsed with neoantigen INS-DRiP or native autoantigen (proinsulin-peptide C19A3) that can induce Tregs in T1D patients. Their phenotypes, cytokine profiles and suppressive capacity were compared. Tregs induced against neoantigen completely inhibited proliferation of naïve T cells upon cognate antigen-pulsed DC stimulation, which was indistinguishable from Tregs induced against C19A3. Phenotype and cytokine profiling showed co-clustering of native autoantigen- and neoantigen-specific Tregs, and distinction from T cells generated with antigen-pulsed proinflammatory instead of tolerogenic DCs. Naïve T cells exist against islet neoantigen that can be primed to become Tregs despite the high immunogenic potential of neoantigen. Induction of immune regulation to neoantigens may be useful as immune intervention or prevention of T1D.

1 | Introduction

The pathogenesis of type 1 diabetes (T1D) is characterized by beta-cell distress and dysfunction, leading to the generation of islet neoantigens and infiltration of islet autoreactive T cells, which results in the selective destruction of beta-cells [1]. Environmental risk factors can trigger a stress response in concert with genetic risk factors affecting the stress-coping mechanism of beta-cells [2–4]. The defective ribosomal protein (INS-DRiP) is a neoantigen that is produced by translational misreading of insulin mRNA by ribosomes during stress [5]. Importantly, CD8 T cells against INS-DRiP are detectable in the insulitic lesions of T1D patients, implying a role in beta-cell destruction [6]. INS-DRiP and other neoantigens possess high binding affinity for HLA favoring their presentation over native peptides and weakly binding autoantigenic peptides, and thus more potent provocateurs of immune cells [6, 7]. Interestingly, naïve autoreactive T cells against ZnT8 and other islet epitopes are also present in healthy individuals, but exhibit a regulatory phenotype, implying that the existence of autoreactive T cells to native autoantigens need not cause disease [8, 9]. Instead, we propose that neoantigens play a key role in T1D pathogenesis when both central tolerance and peripheral regulation are absent.

Abbreviations: C19A3, proinsulin peptide epitope; inflamDC, inflammatory dendritic cell; INS-DRiP, defective ribosomal product of insulin; mDC, mature dendritic cell; tolDC, tolerogenic dendritic cell; T1D, type 1 diabetes; Treg, regulatory T cell; Teff, effector T cell.

Nicoline H. M. Den Hollander and Chelsea Gootjes contributed equally to this work.

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Recently, an insulin gene variant was identified that protects against T1D by accelerated decay of insulin mRNA by IRE1a that becomes activated during ER stress, alleviating beta-cell stress and reducing the generation of INS-DRiP, rendering beta-cells less immunogenic [2]. Patients developing T1D despite carrying this protective variant lacked autoimmunity to INS-DRiP, insulin and proinsulin, and presented more insulin-specific Tregs compared to insulin-specific effector T cells (Teffs) in response to oral insulin treatment, whereas this balance was opposite in patients not carrying the protective variant, further underscoring the importance of beta-cell stress and neoantigens in both development of T1D and protection thereof [10]. We recently showed that therapeutic blockade of Tregs by immune checkpoint inhibition (ICI, pembrolizumab; anti-PD-L1) as immunotherapy in cancer unleashed strong autoimmunity to INS-DRiP and primed naïve T cells reactive to native islet autoantigens, followed by acute development of T1D [11]. This observation points to the importance of inducing or restoring peripheral immune tolerance against islet neoantigen.

In this study we sought proof of concept that regulatory T cells can be generated against INS-DRiP (DRIP) by culturing naïve T cells with tolerogenic DRIP-pulsed DCs.

2 | Methods

2.1 | Generation of Monocyte-Derived DCs and Antigen-Specific T Cell Lines

Tolerogenic DCs (tolDCs) and inflammatory DCs (inflamDCs) were generated from a HLA-DR3/4-DQ2/8 donor (Sanquin) as described previously [12]. DCs were either loaded with 10 μg/mL INS-DRiP whole protein (LUMC) to allow natural antigen-processing at start of maturation, and 7.5 μg/mL C19A3 peptide (LUMC) during the last 4 h of maturation, or without islet antigen to account for potential antigen presentation in the culture. To generate antigen-specific Treg and Teff lines, freshly isolated naïve CD4 T cells were cultured for 14 days with mature antigen-loaded tolDCs or inflamDCs as described before [13]. In total six T cell lines were generated; T cells cultured with tolDCs that were either non-pulsed (Treg), C19A3 peptide-pulsed ($Treg^{PI}$), or DRIP-pulsed ($Treg^{DRIP}$), and T cell cultured with inflamDCs that were either non-pulsed (Teff), C19A3 peptide-pulsed ($Teff^{PI}$), or DRIP-pulsed ($Teff^{DRIP}$).

2.2 | Treg Suppressive Capacity

Autologous naïve CD4 T cells were labelled with 1 μM CFSE (Invitrogen) and cultured with inflamDCs loaded with C19A3 peptide or INS-DRiP or no islet antigen in a 10:1 ratio in a 96-wells round bottom plate coated with suboptimal concentration of 0.3 μg/mL anti-CD3 (Biolegend) that requires additional DC stimulation to induce T cell proliferation, as described previously [14]. $Treg^{DRIP}$ or $Treg^{PI}$ were added in a 1:1 ratio to the CFSE-labelled responder cells. Each condition was tested in triplicate. On Day 4, the cells were harvested and acquired by 5-laser Aurora flow cytometer (Cytek Biosciences). Data were analyzed using FlowJo Software.

2.3 | Phenotype Analysis of T Cell Lines

Naïve CD4 T cells (Day 0) and T cells (Day 14) were stained with surface staining mix (Table S1), washed, stained with LIVE/DEAD Blue (Invitrogen), and acquired using the SpectroFlo Software v2.2.0.2. Equal numbers of CD4+ T cells (Figure S1) from each line were subsampled and analyzed by heatmap and opt-SNE clustering using OMIQ Software (Dotmatics). Furthermore, UMAP dimension reduction was performed, followed by Wanderlust trajectory analysis using Day 0 T cells as starting population.

2.4 | Cytokine Analysis of T Cell Lines

Treg and Teff cells were re-stimulated on Day 14 with mature cognate antigen-pulsed inflamDCs or non-pulsed inflamDCs at a 10:1 ratio for 24 h in duplicate. Supernatant was collected and cytokines were measured using the Bio-plex Human Cytokine Th1/Th2 Assay according to the manufacturer's protocol (Bio-Rad). GM-CSF was not produced by T cells, while IL-13 and TNF exceeded the detection limit and were therefore excluded from comparison between lines. Cytokine levels from all six lines were analyzed by heatmap and opt-SNE clustering using OMIQ Software.

3 | Results and Discussion

3.1 | Inhibition of T Cell Proliferation by Tregs Induced Against INS-DRiP

Given the involvement of neoantigens in the development of T1D and INS-DRiP in specific, we tested whether Tregs can be generated against neoantigen INS-DRiP. Previously, we successfully induced regulatory T cells against native proinsulin C19-A3 peptide in vitro and in vivo in T1D patients in a clinical trial assessing C19-A3-pulsed tolDCs as inverse vaccine to reverse islet autoimmunity. These iTregs could modulate antigen-presenting mDCs in vitro by changing their phenotype to anti-inflammatory DCs—thus inducing infectious tolerance. We demonstrated that the mechanism of inhibition by iTregs is "linked suppression," implying that inhibition is extended to T cells recognizing antigens that are presented by the same modified APC, but only triggered by antigen-specific activation of the iTregs [13–16]. Given the absence of central tolerance and immunogenic character of DRIP, we speculated that induction of peripheral tolerance against this neoantigen might prove more difficult to achieve, yet important in reversing or preventing islet-autoimmunity [11].

We generated Treg lines against INS-DRiP and C19A3, using tolDCs from a healthy donor carrying the T1D predisposing HLA-DR3/4-DQ2/8 genotype and tested their suppressive function (Figure 1). As expected, CFSE-labeled responder T cells did not proliferate with sub-optimal concentration of anti-CD3 in absence of inflammatory DCs, but showed strong proliferation in presence of either C19A3-loaded or DRIP-loaded inflammatory DCs (Figure 1). Addition of $Treg^{PI}$ or $Treg^{DRIP}$ cells in absence of cognate antigen-pulsed DCs reduced proliferation of responder T cells, whereas complete inhibition of proliferation was observed in presence of cognate antigen-pulsed DCs (Figure 1). This indi-

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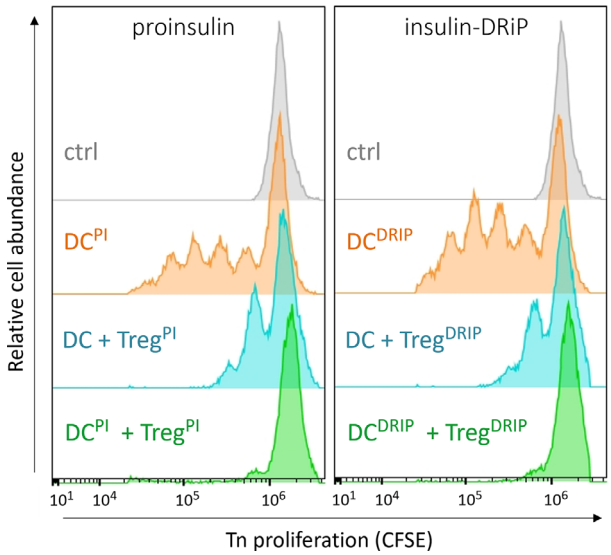

FIGURE 1 | Antigen-specific Tregs inhibit naïve T-cell proliferation. CFSE staining shows proliferation of naïve CD4 T cells upon suboptimal anti-CD3 stimulation (grey; negative control) combined with antigen-pulsed inflamDCs (orange), non-pulsed inflamDCs with Tregs (blue), and antigen-pulsed inflamDCs with Tregs (green). Proliferation is assessed by the number of peaks from right to left (divisions per cell) and amplitude (relative cell abundance per division).

cates that Tregs can be generated against neoantigen (INS-DRiP) that possess adaptive and suppressive capacity indistinguishable from Tregs against native islet antigen (C19A3), despite the immunogenic nature of neoantigens.

3.2 | Tregs Against Neo- and Native Autoantigen Are Phenotypically Comparable

Phenotype analyses of T cell lines at the end of culture period (Day 14) showed that all Treg lines clustered together and separately from Teff lines, sharing higher expression of PD-1, CD27, CD103, CD45RA, TIGIT, LAP, and CCR4 compared to Teff lines (Figure 2A). In line with our previous findings [13, 14], (neo)antigen-specific Tregs induced ex vivo from conventional T cells exhibited a phenotype signature that is in concord with a broad spectrum of induced Treg phenotypes and differs from thymic-derived Tregs that are $CD25^{hi}$, $CD127^{lo}$ and express FOXP3 [17]. Our induced neo- or autoantigenic Tregs expressed low CD25 and CD127 (Figure S2)—presumably due to the standard use of low dose IL-7 in culture—but also high PD-1, LAP, CCR4, CD103, and TIGIT, which play important roles in T cell suppression, and are often impaired in autoimmune disease but increased in cancer [18–22]. CD25 expression is usually relatively low on iTregs compared to Teff lines that are activated by inflamDCs, and in contrast to nTregs. Furthermore, opt-SNE analysis clustered Treg and Teff lines separately (Figure 2B). Comparison of the three different Treg lines showed no difference in clusters, only a difference in cell numbers within a cluster, indicating that $Treg^{DRIP}$ and $Treg^{PI}$ are phenotypically comparable (Figure 2C). Furthermore, trajectory analysis showed both Treg and Teff lines diverging from the starting population at Day 0 (Figure 2D). However, cells from all three Treg lines were placed earlier in the differentiation trajectory compared to Teff lines, and the trajectory of Treg lines was the same irrespective of the target antigen. These findings highlight that tolDCs can induce Tregs against neo- or native auto-antigen, which are indistinguishable in their suppressive capacity and phenotype, but distinct from Teff lines.

3.3 | Tregs Against Neo or Native Autoantigen Exhibit Similar Cytokine Profiles

Cytokine release by the Treg and Teff lines was determined after restimulation of the lines for 24 h with inflammatory DCs loaded with their cognate neo or native autoantigen or without antigen. Based on their cytokine production profile all Treg lines clustered together, and separately from the Teff lines (Figure 2E). The Treg lines to either neo or native autoantigen produced higher levels of IL-2 (30-fold), IL-10 (5-fold), and IFN-γ (2-fold) compared to Teff lines. Opt-SNE analysis clearly showed distinct clustering of the Tregs versus Teff lines (Figure 2F). Of note, while TNF was excluded from the comparisons, this cytokine was above detection limit in all Treg lines but in none of the Teff lines. Although IL-2 and TNF are generally considered to mark acute inflammation, we have demonstrated an indispensable role for mTNF in the induction of autoantigen-specific Tregs, with prolonged exposure to TNF enhancing differentiation and suppressive capacity in Tregs and inducing TCR downregulation and apoptosis in autoreactive T cells [23]. Furthermore, TNFR2 co-stimulation increased Treg proliferation, whereas no effect was found in conventional T cells [24]. Indeed, TNF inhibitors can precipitate new autoimmune diseases (e.g., MS, psoriasis, autoimmune hepatitis, or T1D), indicating that TNF also possesses regulatory properties that can differ between inflammatory diseases [25–27]. IL-2 is critical for survival and suppressive function of Tregs [28]. IL-10 also promotes differentiation, survival and function of Tregs [16]. Importantly, islet-specific Tregs secreting both IL-10 and IFN-γ have been shown to suppress cognate islet antigen-presenting mDC [29]. Therefore, the combined secretion of IFN-γ with IL-10 by our iTregs, which is absent in Teff lines, accords with their suppressive nature. We cannot reconcile why IL-2 is higher in supernatant of Treg lines compared to Teff lines, but we speculate that higher CD25 expression in Teff lines and their stronger proliferation rate, may have led to more IL-2 consumption [14]. Thus, our results fit with previous studies showing that autoantigen-recognizing naïve T cells exist that can be primed to produce TNF, IL-2, and IL-10 upon antigen recognition and suppress activation of naïve T cells.

3.4 | Limitations

This proof-of-concept study demonstrates the possibility to induce Tregs against neoantigen, despite lack of central or peripheral tolerance and strong immunogenicity. This study does not attempt to compare different donors (biological replicates), assess statistical significance, or exhibit reproducibility. The tolDC yield, extensive culturing protocol, challenges to propagate immunosuppressive lymphocytes and necessity to test Treg function freshly restricted the range of suppression assays, for instance assessing DCs pulsed with irrelevant peptide. Nevertheless, DCs are loaded with endogenous and exogenous

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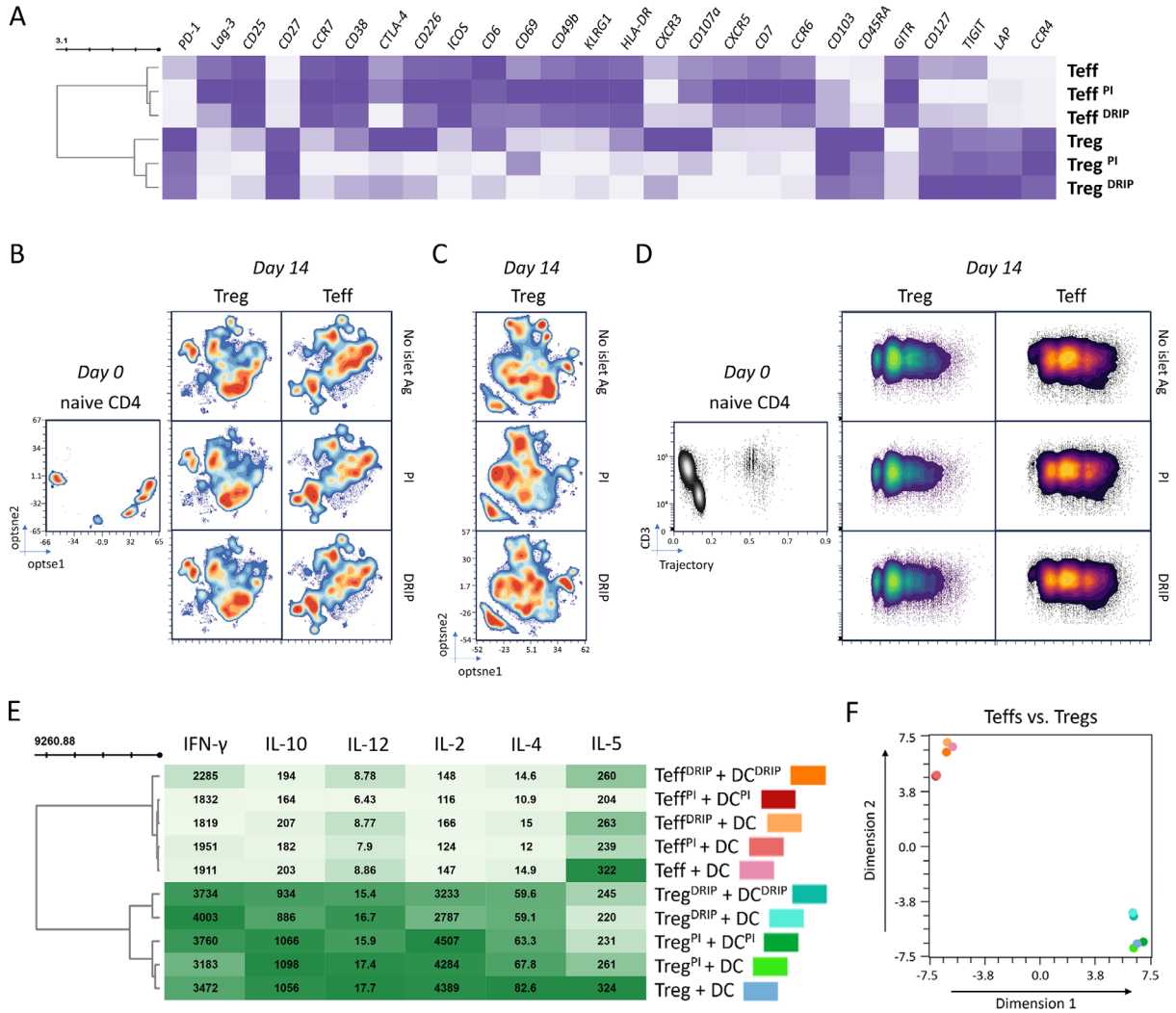

FIGURE 2 | Regulatory T cells are phenotypically comparable and have similar cytokine profiles, but are distinct from effector T-cell lines. Phenotype analysis of Treg and Teff lines generated by antigen-loaded tolDCs and inflamDCs. (A) Heatmap showing the relative expression level per marker among the T-cell lines on Day 14 of culture (low expression = white, high expression = purple). (B) Opt-SNE analysis of Day 0 (naïve) and Day 14 Treg/Teff lines. (C) Opt-SNE analysis of the different Treg lines on Day 14. (D) Trajectory analysis of Day 0 (naïve) and Day 14 Treg/Teff lines. (E) Heatmap showing clusters based on the production of selected cytokines by Treg and Teff lines after stimulation with cognate antigen-pulsed or non-pulsed inflamDCs for 24 h. Values show absolute cytokine levels (pg/mL) for each condition and the color depicts relative levels per cytokine among Teff and Treg lines (low = white, high = green). (F) Two-dimensional opt-SNE plot showing clustering of Treg and Teff lines based on the cytokine production.

peptides from the culture media, sera and buffy coat, potentially contributing to suppression by Tregs when DCs were not pulsed. Yet, complete suppression in the presence of relevant neo- or autoantigen underscores their antigen-specific nature and action.

3.5 | Future Perspectives

The finding that naïve T cells can be primed to become neoantigen-specific Tregs with suppressive capacity, indistinguishable from Tregs induced against native autoantigen, opens avenues to explore neoantigen-targeting tolerance induction therapy for the prevention and treatment of autoimmune diseases with known disease-driving targets [30]. We previously found that PPI-specific iTregs could directly inhibit PPI-reactive CD8 T cells, implying that these neoantigen-specific iTregs have potential to protect islets against CD8 killing [14]. Since generation of neoantigen is likely driven by perturbations limited to the disease lesion and distressed tissue, the potential risk of increasing inflammation against healthy tissue and aggravate disease is considerably lower for neoantigen-driven therapy than for native autoantigen-driven therapy. Therefore, neoantigen-specific Tregs may also prevent autoimmune diseases following checkpoint blockade, provided that the neoantigen of choice is exclusively expressed on tumor cells.

4 | Concluding Remarks

To our knowledge, we provide the first evidence for the possibility to induce Tregs against neoantigen. These Tregs are

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indistinguishable from those induced against native autoantigen in terms of their suppressive capacity, phenotype, and cytokine profile, despite the strong immunogenic nature and inflammatory potential of neoantigens, opening novel avenues to explore therapeutic strategies to induce or restore immune tolerance against neoantigens.

Author Contributions

Conceptualization: Diahann T. S. L. Jansen and Bart O. Roep. Formal analysis: Nicoline H. M. Den Hollander, Chelsea Gootjes, and Diahann T. S. L. Jansen. Investigation: Diahann T. S. L. Jansen, Nicoline H. M. Den Hollander, Chelsea Gootjes, and Maurits G. Staal. Writing – original draft: Nicoline H. M. Den Hollander, Diahann T. S. L. Jansen, and Chelsea Gootjes. Writing – review and editing: Bart O. Roep and Tatjana Nikolic. Visualization: Nicoline H. M. Den Hollander, Chelsea Gootjes, Diahann T. S. L. Jansen, and Bart O. Roep. Resources: Antoinette M. Joosten. Supervision: Diahann T. S. L. Jansen and Bart O. Roep. Funding acquisition: Diahann T. S. L. Jansen and Bart O. Roep.

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Ethics Statement

Informed consent and Ethical Committee approval was obtained via Sanquin (Amsterdam, the Netherlands) allowing the use of anonymized buffy coat in accordance with the Declaration of Helsinki.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The experimental data is available upon request.

Peer Review

For transparency, the peer review documents associated with this article are available at https://doi.org/10.1002/eji.70229.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: eji70229-sup-0001-SuppMat.docx.

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