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Targeting CD44 augments the efficacy of Tregs in autoimmune diabetes

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ABSTRACT

Curing type 1 diabetes (T1D) will require lasting control of the autoimmune response that destroys insulin-producing islet β-cells. Re-establishing tolerance by restoring/replacing Tregs has significant potential for treatment of T1D but will require strategies to augment and maintain their efficacy. We previously showed that polyclonal *in vitro*-induced Tregs can reverse recent onset of T1D in ~50% of NOD mice. Here we report that treatment of newly hyperglycemic animals with a short course of anti-CD44 at the time of Treg transfer improved diabetes reversal to >90%. Anti-CD44 treatment alone delayed diabetes onset and increased the frequencies of pancreatic $CD4^+$ T cells producing IL-2 or TGF-β, cytokines that support Treg function and survival, without altering production of IFN-γ. These anti-CD44 effects on endogenous T cells were also observed in the context of polyclonal Treg transfer, and the combination treatment also reduced pancreatic infiltrates. The results provide compelling evidence that approaches to modulate the pancreatic milieu to support Treg function and counteract inflammation in the pancreas can greatly enhance the efficacy of adoptively transferred Tregs, and suggest that approaches achieving these outcomes hold promise for long-term control of autoimmunity in T1D.

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1. Introduction

The ultimate goal for treatment of T1D is to reestablish long-term glucose regulation in patients by maintaining and/or restoring the necessary β-cell mass. This goal will remain elusive until a safe and effective approach to protect β-cells from autoimmune destruction can be developed. Although many studies taking advantage of the NOD mouse model of T1D reveal that it is in principle possible to restore tolerance by various treatments, the clinical translation has remained problematic, in part because of the genetic diversity of the human population susceptible to T1D, the prolonged period of disease development, and the complexity and changing nature of the autoimmune response over time. There is increasing recognition that more than one approach will be needed to successfully treat T1D. Although transfer of polyclonal Tregs has clinical potential for controlling T1D, it is now clear that treatments to promote Treg function and persistence will be an important component of a successful therapy [1].

Inhibiting localization of effector T cells in the pancreas has long been considered an approach to controlling pathogenesis in T1D, but the need for a continuous blockade renders this strategy clinically unrealistic. However, our recent studies suggest that CD44, a widely expressed adhesion receptor for hyaluronic acid (HA) that is upregulated on activated T cells can regulate the responses of effector T cells [2]. Other studies indicate that antibody blockade of CD44-HA interactions can delay T1D development in an adoptive transfer model by reducing effector T cell migration into the islets [3]. Blocking CD44 can also contribute to the loss of effector T cell motility and function within tissues [4] or, as shown by our studies, eliciting Th1 effector cell apoptosis [2]. Importantly, CD44 can also have a critical role in the regulation of Tregs. On Tregs, high expression of CD44 together with HA-binding activity marks cells with the most potent inhibitory function [5,6], and Tregs from *Cd44*$^{-/-}$ mice exhibit impaired suppressive capacity and persistence [7]. Furthermore, ligation of CD44 via immobilized anti-CD44 can enhance Treg function and FoxP3 expression *in vitro* [7], suggesting that engagement of CD44 could provide a means to enhance Treg activity. Thus, we hypothesized that targeting CD44 might improve control of autoimmunity through effects on Treg function after emergence of hyperglycemia in T1D and potentially alter effector T cell migration and function. To address these possibilities, we used a combination treatment with a non-depleting anti-CD44 antibody and adoptive transfer of polyclonal induced Tregs to reverse spontaneous

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hyperglycemia in NOD mice. We observed that although a short course of anti-CD44 treatment delayed diabetes onset in hyperglycemic animals, when combined with administration of polyclonal induced Tregs, diabetes was prevented in >90% of the recipients. Greater Treg efficacy was associated with higher frequencies of $CD4^+$ T cells producing IL-2 and TGF-β in the draining LN and pancreas as well as more limited pancreatic infiltrates, demonstrating that counteracting the inflammatory milieu associated with the autoimmune response to one that can support Treg function can dramatically improve Treg control of autoimmunity.

2. Materials and methods

2.1. Mice

Age-matched female NOD mice were purchased from the Jackson Laboratory. NOD.*Scid* mice and NOD.Thy1.1 mice were bred in house from breeders obtained from Jackson. Only female mice were used. The animals were maintained in a specific pathogen free vivarium at Sanford-Burnham Medical Research Institute (SBMRI). Hyperglycemic NOD mice were identified by weekly blood glucose testing using Bayer's Countour meters. Blood glucose levels of 180–200 mg/dl were considered indicative of prediabetic hyperglycemia. Two consecutive readings ≥300 mg/dl were considered indicative of diabetes. All experiments were approved by the Institutional Animal Care and Use Committee of SBMRI.

2.2. Antibody treatments

Anti-CD44 (clone IM7) was purchased from BioXcell and administered in a dose of 300 μg by *i.p.* injection 2×/week for 2 weeks to NOD or NOD.*Scid* mice. The control was polyclonal rat IgG (Jackson ImmunoResearch Laboratories), which was administered in the same amount and dosing schedule.

2.3. Adoptive transfer of T cells from diabetic mice and of Tregs

Total spleen cells from diabetic NOD.Thy1.1 mice were injected *i.v.* into NOD.*Scid* mice in a dose containing 4×10^6 $CD3^+$ cells. Antibody treatments were initiated immediately prior to cell transfer. Lymphoid tissues and pancreata were analyzed on Day 14. To generate Tregs, $CD4^+$ T cells were isolated from the lymphoid tissues of 6–8-week old NOD.Thy1.1 mice using EasySep kits (StemCell Technologies) according to the manufacturer's instructions, except that biotin-conjugated anti-CD25 antibody (clone PC61, BioLegend, San Diego) was included in a dose of 0.25 μg per 10^6 cells in a volume of 100 μl to deplete endogenous Tregs during the separation. Purified $CD4^+$ T cells were cultured in 6-well plates coated with anti-CD3 (145-2C11, 5 μg/ml) and anti-CD28 (37.51, 5 μg/ml) purchased from BioXcell in complete RPMI-1640 medium for 5 days. The cultures were supplemented with 10 μg/ml anti-IFN-γ (XMG1.2 or R46A2), 200 units/ml rIL-2 (NCI Biological Resource Branch, Frederick), and 10 ng/ml rTGF-β1 (BioLegend) as we previously described [8,9]. These conditions elicit Tregs that uniformly express FoxP3. Tregs were transferred into NOD recipient mice by *i.v.* injection in a dose of 2×10^6 cells.

2.4. Flow cytometry

Single cell supensions of lymphoid tissues were prepared by mechanical disruption. Pancreata were finely minced, digested with collagenase P (Roche), and mechanically disrupted to release infiltrating cells. The viable mononuclear cell recovery was determined by flow cytometry using FITC-labeled particles for standardization (Spherotech) according to the manufacturer's formula and using propidium iodide (1 μg/ml) to distinguish dead cells. Fluorochrome-conjugated antibodies for FACS analysis were purchased from BioLegend, with the exception that PE-conjugated anti-mouse FoxP3 (clone FJK-16S) was purchased from eBioscience (San Diego, CA). For cell-surface staining, we used antibodies specific for CD4 (GK1.5), CD8α (53–6.7), CD19 (6D5), γδ TCR (GL3), and Thy1.1 (OX7). For intracellular cytokine staining, antibodies specific for the following were used: IL-2 (JES6-5H4), TGF-$\beta_{1,2,3}$ (1D11), IL-10 (JES-2A5), and IFN-γ (XMG1.2). The cells were restimulated with 50 ng/ml PMA (Sigma–Aldrich) and 1 μg/ml ionomycin (Sigma–Aldrich) with 10 μg/ml Brefeldin A (Sigma–Aldrich) for 4 h. The cells were first stained for surface markers, and after fixation and permeabilization with Cytofix/Cytoperm buffer (BD Biosciences, San Diego, CA), were then stained with anti-cytokine antibodies as previously described [10]. For FoxP3 staining, the cells were stained for surface markers first; after fixation and permeabilization, the cells were stained with PE-conjugated anti-mouse FoxP3. The gating strategies to identify endogenous $CD4^+$ T cells and donor $Thy1.1^+$ cells are shown in Fig. S2A, and to distinguish $FoxP3^+$ and $FoxP3^-$ endogenous $CD4^+$ T cells are shown Fig. S2B. The flow cytometry data were acquired using an LSR-Fortessa instrument (BD) and were analyzed using FlowJo software (Tree Star).

2.5. Histology

Pancreata were fixed in 10% buffered Formalin and embedded in paraffin. Four-micrometer-thick sections were stained with

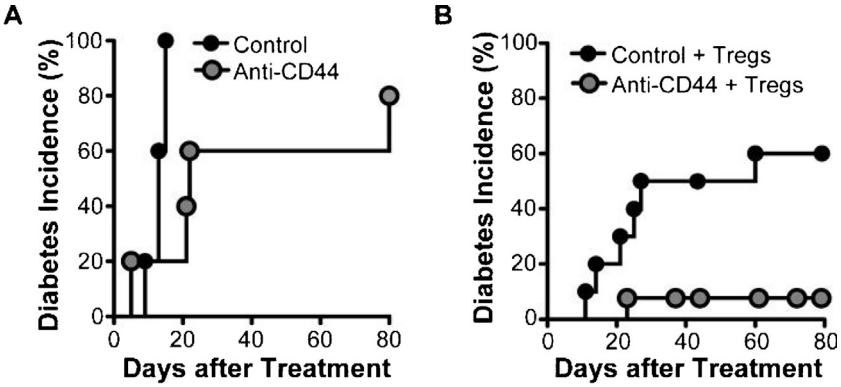

Fig. 1. Anti-CD44 treatment delays progression from hyperglycemia to diabetes and improves glycemic control with Treg transfer. Adult female NOD mice with blood glucose levels of 180–200 mg/dl were given anti-CD44 (IM7) or control polyclonal rat IgG (300 μg, *i.p.*, 2×/week) for 2 weeks without (A) (n = 8/group) or immediately prior to *i.v.* injection with 2×10^6 *in vitro* induced polyclonal Tregs (B) (n = 10/group). Diabetes incidence was then followed until 80 days. The experiment shown in (A) is representative of 3 additional repeats with up to 6/group. The experiment shown in (B) is representative of 2 additional repeats (n = 6–7/group).

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