Published in final edited form as:

Immunol Lett. 2015 February ; 163(2): 199–205. doi:10.1016/j.imlet.2014.10.002.

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.

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

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The authors have no conflict of interest.

Appendix A. Supplementary data

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 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 180–200mg/dl were considered indicative of prediabetic hyperglycemia. Two consecutive readings $\geq$ 300mg/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 300µg 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 0.25µg per 10^6 cells in a volume of 100µl to deplete endogenous Tregs during the separation. Purified CD4⁺ T cells were cultured in 6-well plates coated with anti-CD3 (145-2C11, 5µg/ml) and anti-CD28 (37.51, 5µg/ml) purchased from BioXcell in complete RPMI-1640 medium for 5 days. The cultures were supplemented with 10µg/ml anti-IFN-γ (XMG1.2 or R46A2), 200units/ml rIL-2 (NCI Biological Resource Branch, Frederick), and 10ng/ml rTGF-β1 (BioLegend) as we previously described [8,9]. These conditions elicit Tregs 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 suspensions 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 (1µg/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-β_{1,2,3} (1D11), IL-10 (JES-2A5), and IFN-γ (XMG1.2). The cells were restimulated with 50ng/ml PMA (Sigma-Aldrich) and 1µg/ml ionomycin (Sigma-Aldrich) with 10µg/ml Brefeldin A (Sigma-Aldrich) for 4 hours. The cells were first stained for surface markers, and after fixation and permeabilization with Cytotfix/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 (FJK-16S, eBioscience). 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 hematoxylin and eosin. Insulitis indices were performed by scoring islet infiltrates as described in Fig. 2B. Between 10 and 30 islets were analyzed in each of six levels of sections that differed by 30µm for each individual animal.

2.6 Statistical Analyses

Groups of mice treated with control or anti-CD44 antibodies were compared using an unpaired *t*-test, since the variances were equivalent, using 95% confidence intervals. The data were analyzed using the Prism program (GraphPad Software, Inc.).

3. Results

3.1 Effect of anti-CD44 treatment on the spontaneous diabetes

To investigate whether anti-CD44 treatment alters spontaneous T1D, prediabetic hyperglycemic NOD mice with blood glucose levels of 180–200mg/dl were treated with anti-CD44 mAb or control IgG, 2×/week for 2 weeks. Progression to diabetes was then monitored for 80 days. As shown in Fig. 1A, this brief course of antibody treatment was sufficient to delay diabetes onset in 40% of the animals. The data demonstrate that even at the immediate prediabetic stage, blocking CD44 had efficacy in altering disease progression, supporting the conclusion of a previous study that examined T1D development in an induced model where the antibody was administered at the time of transferring spleen cells from diabetic mice [3].

3.2 Effect of anti-CD44 treatment on pancreatic infiltrates

To address the impact of anti-CD44 treatment on T cell localization in the pancreas and pancreatic LN (PLN), we initiated anti-CD44 or control antibody treatment by *i.p.* injection, of 300µg anti-CD44 immediately prior to *i.v.* adoptive transfer of total spleen cells containing 4×10^6 CD3⁺ cells from diabetic NOD.Thy1.1 mice into NOD.*Scid* mice. With this model, 100% of the mice become diabetic between 4–6 weeks after cell transfer. On Day 3 after treatment, the recovery of Thy1.1⁺ donor cells from the pancreas and PLN was greatly reduced (Fig. 2A) without diminishing recovery from the spleen or non-draining LN. This result indicates that diabetogenic T cells failed to accumulate in the target organ. Therefore, anti-CD44 treatment could block either homing of activated T cells to, or their retention in the pancreas as an immediate effect that reduces the local autoimmune response. In parallel, we also observed significant reduction in the levels of insulitis after anti-CD44 treatment (Fig. 2B).

3.3 Anti-CD44 treatment enhances the efficacy of Treg therapy

We showed previously that induced polyclonal FoxP3⁺ Tregs can persist and function as memory cells *in vivo* with the capacity to reverse hyperglycemia and protect against diabetes development [8]. However, their efficacy, like that of naturally occurring *in vitro* expanded FoxP3⁺ Tregs, is typically ~50–60% (Fig. 1B). We now addressed whether anti-CD44 treatment could be used as an adjunct therapy to enhance their efficacy. Anti-CD44 or

control antibodies were injected immediately prior to the Tregs, and at 3 1/2 day intervals for 2 weeks. As shown in Fig. 1B, the combination of anti-CD44 and polyclonal Tregs dramatically improved the rate of hyperglycemic reversal to >90%.

3.4 Anti-CD44 treatment downmodulates the proinflammatory T cell response in the pancreas

To address the *in vivo* mechanisms of action of anti-CD44, we examined the effects of treatment on T cell infiltration in the pancreas and PLN of prediabetic mice. In the absence of Treg transfer, at 2 weeks after antibody administration to prediabetic mice, anti-CD44 did not significantly affect the recovery of mononuclear cells, CD4⁺ T cells, or FoxP3⁺ Tregs in the pancreas (Fig. S1A) or PLN (Fig. S1B). CD8⁺ T cells, B cells, and $\gamma\delta$ T cells were also not significantly altered (data not shown). This result supports a conclusion that although anti-CD44 treatment may have the capacity to limit the recruitment of cells that contribute to T1D pathogenesis, it does not alter their retention in the pancreas. We did not observe upregulation of FoxP3 in either the pancreas or PLN (Fig. S1A, S1B far right panels), which was reported with CD44 ligation by immobilized but not soluble anti-CD44 *in vitro* [7]. We also did not observe impaired immune responses against influenza infection after anti-CD44 treatment (Fig. S4), suggesting that generalized inhibitory effects on immune responses were not occurring.

We next examined the functions of T cells from the pancreas and PLN as measured by cytokine production. We observed marked increases in the frequencies of CD4⁺FoxP3⁻ T cells that produced IL-2 (Fig. 3A) and TGF- β (Fig. 3B). The results from individual animals are shown in Fig. 3C (pancreas) and Fig. 3D (PLN), and indicate that no differences in IL-10 or IFN- γ were observed. We did not detect differences in the frequencies of IL-2 and TGF- β producing CD4⁺FoxP3⁻ T cells from the spleens. We also did not detect changes in cytokine production (IL-10, TGF- β) by FoxP3⁺ Tregs themselves from any tissue (data not shown). However, greater levels of IL-2 and TGF- β , cytokines that have established effects on Treg survival and function, could alter the proinflammatory milieu of the pancreas and draining LN to favor greater control of the autoimmune response. However, the effects were not sustained, implying that the response to anti-CD44 wanes as antibody levels decay.

3.5 Effects of anti-CD44 treatment on T cell functions in the presence of Tregs

We next addressed changes that occurred with the combination treatment of anti-CD44 and polyclonal Tregs in prediabetic mice. As shown in Fig. 4A, at 2 weeks after treatment we observed marked increases in the frequencies of IL-2 and TGF- β producing FoxP3⁻CD4⁺ T cells in the pancreas that were more significant than those found with anti-CD44 alone, without changes in IL-10 or IFN- γ producers (data not shown). There was also a reduction in pancreatic infiltrating cells (Fig. 4A, far right panel), without changes in the proportions of host FoxP3⁻CD4⁺ cells or FoxP3⁺ cells, or of donor Tregs (Fig. S3). These changes were not observed in the PLN (Fig. 4B and data not shown). The results demonstrate that anti-CD44 does not have a selective effect on the distribution of effector and regulatory CD4⁺ T cells when combined with exogenous Treg transfer. We did not observe consistent changes in cytokine production by either host or donor Tregs (data not shown), suggesting that with

respect to cytokine production, effector CD4⁺ T cells were the major population to be impacted by anti-CD44.

4. Discussion

In this study, we show that combining immunotherapy with anti-CD44 at the time of treatment with polyclonal Tregs greatly improves reversal of hyperglycemia in the context of spontaneous development of T1D. Although the effects of a brief course of anti-CD44 treatment are transient, producing only a relatively short delay in the onset of overt diabetes in the majority of mice, the dramatic effects on diabetes control by polyclonal Tregs reinforces the concept that treatments that support Treg function have striking clinical potential for treatment of T1D. Our results show that although anti-CD44 can prevent the development of *de novo* pancreatic infiltrates, established infiltrates are not greatly affected, suggesting that blocking CD44 does not impact the retention of cells that have previously migrated into the pancreas. In addition, our earlier studies showed that induced Tregs can limit entry of diabetogenic T cells into the pancreas [10], and our results here indicate that anti-CD44 can amplify this effect.

The most significant effects of anti-CD44 treatment were the increased frequencies of CD4⁺ effector cells that produce IL-2 and TGF- β in the local environment in the pancreas and in the draining LN. These cytokines regulate Treg function and homeostasis, although we did not observe changes in the frequencies of endogenous FoxP3⁺ Tregs. However, our data show that to achieve lasting control of diabetes, it is critical that exogenous Tregs be used to optimally counteract the autoimmune response in the context of modulating effector CD4⁺ T cell function by CD44. Although we did not find increased numbers or frequencies donor Tregs following anti-CD44 treatment, ligation of CD44 *in vivo* could enhance the stability of induced Tregs by mimicking the engagement of CD44 by galectin-9 and acting together with TGF- β 1 as recently reported [11]. Based on our previous studies, better access to TGF- β is likely to be a contributing factor in the improved diabetes control after Treg transfer [10].

The induction of IL-2 could also contribute to stabilizing host Tregs and donor Tregs as well as limiting their conversion to pathogenic effectors. Inflammation has detrimental effects on the stability of Tregs in NOD mice [12,13], and plasticity in terms of proinflammatory cytokine production is associated with Tregs from T1D patients [14]. IL-2 was shown to be essential for stabilizing FoxP3⁺ Tregs when they localize in a proinflammatory milieu [15]. Furthermore, systematic administration of low doses of IL-2 could reverse new onset of diabetes in NOD mice, an effect associated with increased numbers of FoxP3⁺ Tregs in the pancreas [16,17]. IL-2 therapy, in combination with rapamycin was shown to transiently increase Treg numbers in T1D patients, and provide long lasting improvement in IL-2 responsiveness by Tregs [18]. However, adverse events associated with systemic IL-2 treatment as well as with rapamycin currently limit clinical translation. Our finding that anti-CD44 induces increased frequencies of IL-2 producing CD4⁺ T cells selectively in the PLN and pancreas, the sites of the autoimmune response, suggests that approaches that achieve localized IL-2 production will have greater translational potential. Although increased local availability of IL-2 could potentially also support the responses of pathogenic effector T

cells, it is possible that the enhanced availability of TGF- β after anti-CD44 treatment inhibits their responses [19].

Previous studies have shown that anti-CD44 treatment of T cells can enhance TCR signals [20] and can costimulate CD4⁺ T cell production of IL-2 *in vitro* in the context of low levels of TCR stimulation [21]. Our data suggest that anti-CD44 treatment *in vivo* may also costimulate CD4⁺ T cell production of TGF- β . However, the effects we observe on cytokine production could also be indirect, with anti-CD44 acting on APCs. For example, blocking CD44 could prevent engagement of DCs by effector CD4⁺ T cells and thereby limiting their production of inflammatory cytokines *in situ* [22]. Since we did not detect consistent anti-CD44-dependent changes in cytokine production or levels of FoxP3 expression by Tregs themselves, it is possible that targeting CD44 on Tregs could impact other facets of their function that include modulating the immunogenicity of DCs or the access of effector T cells to them [23]. From our results, we propose that enhanced frequencies of IL-2 and TGF- β producing FoxP3⁺CD4⁺ T cells in the PLN and pancreas support Treg function in part by alleviating the proinflammatory milieu. It is important to note that the anti-CD44 clone we used does not activate CD44 nor cause apoptosis of activated CD4⁺ T cells as was shown in our previous study with a CD44 agonist antibody [2].

In light of our findings that induced Tregs can persist and function as protective memory cells after diabetes reversal [8], it is possible that increases IL-2 and TGF- β 1 promote their ability to form and maintain memory. The results highlighted here imply that treatment strategies to support Treg functions at the time of initiating Treg therapy have the potential to greatly improve the likelihood of achieving durable control of autoimmunity in T1D. Another example of a dual functioning treatment is anti-CD154, which promotes Treg expansion/function while inhibiting CD4⁺ and CD8⁺ effector functions in murine T1D models [24,25]. Importantly, FoxP3⁺ Tregs can be induced from the CD4⁺ T cells of T1D patients [26]. Furthermore, humanized anti-CD44 antibodies are currently under preclinical evaluation for cancer treatment [27,28]. Our current study provides a proof of concept that the efficacy of induced Tregs in controlling T1D can be dramatically improved by modulating the inflammatory milieu of the pancreas and draining LN to facilitate their function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from NIH (AI081238) and from JDRF (32-2008-353) to LMB. The authors would like to thank Jennifer Nguyen (Sanford-Burnham Medical Research Institute) for overseeing the maintenance and blood glucose monitoring of the animals used in these studies.

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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 180–200mg/dl were given anti-CD44 (IM7) or control polyclonal rat IgG (300µg, *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).

Fig. 2. Anti-CD44 treatment decreases T cell homing into the pancreas and islet infiltration
(A) NOD.*Scid* mice were injected with anti-CD44 or control IgG in a dose of 300 μ g *i.p.* just prior to *i.v.* injection of total splenocytes containing 4×10^6 CD3⁺ cells from diabetic NOD.Thy1.1 mice. Recovery of donor T cells on Day 3 after cell transfer were calculated based on total cell counts and percentage of the population (n=5/group). **(B)** NOD.*Scid* mice treated in (A) were given additional doses of the antibodies on Days 3, 6, 9, and 12 after cell transfer. Islet infiltration was analyzed at Day 14. Individual animals are shown. * p < 0.05 and ** p < 0.01 represent statistically different outcomes. These experiments are representative of 2 additional repeats.

Fig. 3. Anti-CD44 treatment does not alter pancreatic infiltrates, but enhances the frequencies of IL-2 and TGF- β producing non-Tregs

Ten-week old NOD female mice were treated with anti-CD44 or control IgG for 2 weeks (n=5/group) as in Figure 1A. Representative plots showing CD4⁺ cells producing IL-2 (A) or TGF- β (B) in the pancreas and pancreatic lymph nodes (PLN). Frequencies of CD4⁺ cells producing IL-2, IL-10, TGF- β , and IFN- γ in the pancreas (C) and PLN (D) from the individual animals in the experiment. Where shown, * represents statistically different results (p 0.05). Shown is representative of a total of 3 repeats with n= 4-5/group.

Fig. 4. Anti-CD44 treatment together with Tregs enhances the frequencies of IL-2 and TGF-β producing host CD4⁺ non-Tregs and reduces pancreatic infiltrates

Ten-week old female NOD mice were treated with anti-CD44 or control IgG and administered *in vitro* generated Tregs that were trackable by Thy1.1 (Fig. S2) immediately afterwards (n=4/group) as for Fig. 1B. Frequencies of cytokine producing host non-Treg CD4⁺ T cells in (A) the pancreas and (B) the pancreatic lymph node (* p 0.05, ** p 0.01, *** p<0.001 represent statistically different outcomes.) Data shown are representative of 3 repeats with n=4–5/group.