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Antigen-specific prevention of type 1 diabetes in NOD mice is ameliorated by OX40 agonist treatment

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Abstract

Antigen-specific therapies are possibly the safest approach to prevent type 1 diabetes (T1D). However their clinical translation has yielded poor results and greater efforts need to be put into the development of novel strategies to ameliorate their clinical outcome. OX40 is a costimulatory molecule expressed by T cells after antigen recognition and has been implicated in the control effector but also regulatory T cells (Tregs) function *in vivo*. The activity of OX40 signal on Tregs function has been controversial. In this context we investigated whether an anti-OX40 agonist antibody treatment can ameliorate antigen-specific immune intervention for the prevention of T1D. We show that treatment of non-obese diabetic (NOD) mice with an OX40 agonistic antibody (OX86) reduced type 1 diabetes (T1D) incidence by inducing both CD4⁺CD25⁺Foxp3⁺ Tregs and CD4⁺Foxp3⁻ T cells expressing the latency-associated peptide (LAP). These OX86-induced CD4⁺Foxp3⁻LAP⁺ T cells also demonstrated suppressive activity *in vitro*. A significant increase in protection was observed when OX86 was combined with insulin B9:23 (insB9:23) peptide immunizations. Synergy resulted from an expansion of IL-10-expressing insB9:23-reactive Tregs which augmented the proportion of CD4⁺ T cells with *in vivo* suppressive activity. Consequently, CD4⁺ T cells purified from OX86/insB9:23 combination treatment prevented T1D development when adoptively transferred into recipient mice. These findings suggest that the requirement for OX40 signaling by antigen-induced Tregs can be dominant over its well-documented need for effector memory cell function and may have potentially important implications for improving the clinical translation of antigen-specific prevention of T1D and possibly other autoimmune disorders.

Keywords

Type 1 diabetes (T1D); Immunotherapy; Tolerance; Regulatory T cells (Tregs); OX40; Insulin B9-23 peptide

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Author contributions

D.B. researched data, contributed to discussion and wrote the manuscript. G.F. researched data and contributed to discussion. Y.M. researched data. M.C. contributed to discussion and reviewed/edited manuscript. M.v.H. contributed to discussion and reviewed/edited manuscript.

1. Introduction

Autoimmune diabetes, also known as juvenile or type 1 diabetes (T1D), results from the destruction of insulin-producing pancreatic beta-cells by auto-reactive immune cells. Despite the improvements in our understanding of the physiopathology of the disease, the incidence of T1D has been continuously rising over the past years, especially in young children [1,2]. The development of immune interventions for the treatment of T1D has been the focus of intense research over the past decades but still remains to be improved [3,4]. Ultimately a safe and potent approach to prevent the destruction of pancreatic beta-cells before the clinical symptoms of overt T1D (i.e. hyperglycemia) will be highly desirable.

One of the most attractive approach to prevent T1D is the deployment of islet (auto)antigen (aAg)-specific regulatory T cells (Tregs) [5]. These cells can be induced through a plethora of immunization methods using aAg-expressing DNA vaccines [6], peptides derived from islet antigens [7] or with full-length islet proteins [8]. When administered in a tolerogenic fashion during the prediabetic phase in animal models, these vaccines mediate protection by expanding islet-specific CD4⁺ Tregs. Mechanistically, there is a growing body of evidence suggesting that islet-specific Tregs operate *in vivo* by migrating and selectively proliferating in the pancreatic lymph nodes (PLN) where islet antigens are presented to the immune system during diabetes development [9,10]. When activated at the right time (early in diabetes development) and at the right place (in the pancreatic lymph nodes), Tregs can suppress expansion and activation of polyclonal autoaggressive T cells located in their vicinity thanks to a mechanism called bystander suppression and most likely via modulation of antigen-presenting cells [11]. The appealing aspect about this type of intervention is the generation of islet aAg-specific Tregs that will locally and permanently dampen multiple autoaggressive effector T cells, then circumventing the need to identify all aAg targets and avoiding systemic side-effects.

So far, the clinical outcomes of islet aAg-specific vaccination for the prevention [12] or the reversion [13,14] of human autoimmune diabetes have been disappointing probably because treatment efficacy has been hindered by a weak *in vivo* expansion of aAg-specific Tregs [15]. Therefore, an improvement of this procedure is mandatory to reach clinical efficacy and might require the use of *in silico* modeling to optimize different immunization parameters (such as the impact of dose, frequency of administration and age at treatment) [16], but maybe also a combination with other immune interventions which will support the expansion of aAg-specific Tregs *in vivo* [4,17],

OX40 (CD134, TNFRSF4) is a member of the TNF receptor superfamily expressed on a variety of immune cells including activated T cells, NKT cells, NK cells and neutrophils [18]. Naturally occurring CD4⁺ Tregs (nTregs) constitutively express OX40 in mice, while human nTregs up-regulate OX40 on their surface upon TCR cross-linking. Conflicting data from the literature have raised the issue that OX40 agonist signals might transiently inhibit nTreg suppressive function [19–23], but also at the same time, or under particular inflammatory conditions, promote nTreg persistence and survival [24]. Similar to data with nTregs, both *in vitro* and *in vivo* studies on antigen-specific adaptive (aTregs) led to contrasting results [20,21,25–29] revealing that OX40 signaling has a more complex physiology on Tregs than previously thought.

In this context we decided to evaluate whether OX40 agonist treatment would be beneficial to increase the anti-diabetogenic potential of aAg-specific aTregs *in vivo*. Here, we show that treatment with a non-depleting anti-OX40 agonist antibody can control T1D in non-obese diabetic (NOD) mice by expanding simultaneously CD4⁺Foxp3⁺ and CD4⁺Foxp3⁺LAP⁺ Tregs. We also show that anti-OX40 can synergize with insulin B9:23

(insB9:23) peptide immunotherapy to expand *in vivo* IL-10-secreting insB9:23-specific aTregs. This study points for the first time towards a therapeutic utility of OX40 agonist signal in conjunction with antigen-specific induction of aTregs to protect from T1D.

2. Results

2.1. Antigen-specific prevention of type 1 diabetes in NOD mice is ameliorated in presence of OX40 agonist antibody

To evaluate the ability of the non-depleting anti-OX40 agonist antibody (OX86) to affect Treg function in an autoimmune environment we treated non-obese diabetic (NOD) mice early during type 1 diabetes (T1D) pathogenesis and followed disease development. T1D was significantly delayed and the incidence was reduced after anti-OX40 treatment (31% protection versus 12% for the control group at 30 weeks post-treatment; $p = 0.0275$) (Fig. 1A). Since nasal insulin therapy did not significantly delay T1D in humans [30,31], in order to mimic this situation in NOD mice we employed a sub-optimal vaccination schedule consisting of high-frequency nasal insB9:23 peptide immunizations as we previously described [16]. At this dose intranasal insB9:23 peptide mono-therapy did not significantly delayed T1D (Fig. 1A) although a lower incidence was observed at 30 weeks post-treatment as compared to the control group (33% versus 12% protection). Efficacy of both mono-therapies and in particular insB9:23 peptide therapy was significantly augmented when combined with OX40 agonist antibody (Fig. 1A) leading to 54% protection (B9:23 alone versus B9:23+OX86; $p = 0.0139$ and OX86 versus B9:23+OX86; $p = 0.036$).

The efficacy of OX40 agonist therapy was accompanied by a reduction of potentially pathogenic intra-pancreatic T cells secreting pro-inflammatory cytokines. As observed Fig. 1B, the frequency of TNF-secreting CD4⁺ T cells was significantly diminished in the pancreas of treated NOD mice ($19.49 \pm 4.18\%$ versus $9.27 \pm 5.58\%$ for controls, $**p < 0.01$). Collectively these data suggest that antigen-specific therapy of prediabetic NOD mice is ameliorated in presence of OX40 agonist signal.

2.2. OX40 agonist stimulation in combination with antigen-specific therapy prevents type 1 diabetes in NOD mice by expanding Tregs

To determine whether efficacy of anti-OX40 and insB9:23 treatments were associated with an expansion of Tregs, we first enumerated CD4⁺CTLA-4⁺Foxp3⁺ Tregs in the spleen and pancreatic lymph nodes (PLN) two weeks after treatment. The percentage of Tregs was augmented in the PLN after both anti-OX40 and insB9:23 mono-therapies as compared to control NOD mice receiving an isotype antibody and intranasal PBS1X immunization (Fig. 2A and B). Synergy between OX40 agonist stimulation (OX86) and insB9:23 peptide immunization correlated with an additional expansion of CD4⁺CTLA-4⁺Foxp3⁺ Tregs in the PLN after anti-OX40/insB9:23 combination treatment and correlates with the improvement of efficacy seen Fig. 1A.

Expansion of *de novo* Tregs upon anti-OX40 agonist antibody (OX86) treatment in prediabetic NOD mice was observed in the spleen and PLN (Fig. 3A), as well as in the pancreas (data not shown), and subsequently confirmed by an increase in the number of CD4⁺Foxp3⁺ Tregs expressing the proliferation marker Ki67 (Fig. 3B). A higher percentage of Tregs stained positive for Ki67 after OX86 than isotype control treatment in the spleen ($50.58 \pm 3.21\%$ vs $35.05 \pm 3.21\%$, $**p < 0.01$) and pancreatic lymph nodes ($42.9 \pm 3.4\%$ vs $33.05 \pm 5.46\%$, $*p < 0.05$) but not in the blood ($54.65 \pm 4.38\%$ vs $60.87 \pm 4.02\%$). Only a small increase in CD4⁺Foxp3⁻Ki67⁺ cells was observed in each group, presumably as a result of homeostatic proliferation of CD4⁺ effector T cells (Teffs) (Fig. 3B).

2.3. OX40 agonistic antibody treatment expands LAP-expressing CD4⁺ Tregs in the pancreas of NOD mice

Previous studies have identified TGF β -dependent Tregs characterized by surface expression of latency-associated peptide (LAP; [32]). We next determined whether OX40 agonist antibody treatment promotes the generation of CD4⁺LAP⁺ T cells. The percentage of total CD4⁺LAP⁺ T cells induced upon OX40 agonist treatment gradually expanded (10% expansion two weeks post-treatment) in the pancreas of treated NOD mice (Fig. 4A). The expansion seen in the spleen and PLN at 1 week after OX40 agonist therapy (~6%) was not sustained at 2 weeks. In addition to anti-OX40 therapy, insB9-23 peptide immunization given alone also significantly expanded the percentage of CD4⁺LAP⁺ T cells in the pancreas of treated NOD mice (Fig. 4B). However, the percentage of total CD4⁺LAP⁺ T cells was not affected after anti-OX40 (OX86)/insB9-23 combination treatment (CT) as compared to the control group. This observation can be explained by the fact that the percentage of CD4⁺Foxp3⁺LAP⁻ Tregs parallely increased in the CT group (Fig. 5A). Consequently, the percentage of LAP⁺ T cells remained constant within the CD4⁺ population. We next assessed whether anti-OX40 and insB9-23 therapies given alone or in combination expanded insB9-23-reactive CD4⁺LAP⁺ T cells. As depicted Fig. 4C and D, both mono-therapies significantly expanded insB9-23-reactive CD4⁺LAP⁺ T cells in the pancreas when insB9-23 peptide stimulation was used as compared to unstimulated cells (OX86: 57.1 $\pm$ 2.6% vs 51.4 $\pm$ 3.4%, * p = 0.019 and insB9-23: 62.0 $\pm$ 1.4% vs 55.2 $\pm$ 3.1%, ** p = 0.0079. Although the percentage of total CD4⁺LAP⁺ T cells diminished upon CT in the pancreas (Fig. 4D) the percentage of insB9-23-specific CD4⁺LAP⁺ T cells was significantly augmented when compared to unstimulated cells (OX86/insB9-23: 52.6 $\pm$ 2.3% vs 44.4 $\pm$ 3.1%, * p = 0.008).

In addition even if CD4⁺CD25⁺Foxp3⁺LAP⁺ Tregs expanded upon OX40 antibody treatment (Fig. 5A) only a small proportion of OX40-induced CD4⁺LAP⁺ T cells were Foxp3⁺ (Fig. 5B). In line with these data, previous studies showed that TGF β -mediated conversion of CD4⁺CD25⁻ into CD4⁺CD25⁺Foxp3⁺ Tregs induced LAP not only on T cells that converted to Foxp3⁺ but also on Foxp3⁻ T cells [33].

We next investigated whether CD4⁺LAP⁺ T cells generated upon OX40 agonist treatment exhibit suppressive function. We compared the suppressive capacity of OX86 treated or non-treated CD4⁺LAP⁺ T cells in co-culture assays using CFSE-labeled CD4⁺CD25⁻LAP⁻ responder T cells. CD4⁺LAP⁺ T cells purified from OX86 treated mice resulted in ~50% suppression of responder T cells (Fig. 6). Therefore OX40-specific antibody treatment expands CD4⁺LAP⁺ T cells with suppressive ability.

2.4. IL-10-expressing insB9-23-specific T cells expand upon anti-OX40/insB9-23 combination treatment

We previously showed that insB9-23 immunization increases IL10-secreting T cells in the NOD mice [16]. We next examined whether OX40 agonist antibody therapy in conjunction with insB9-23 immunization generates IL-10-expressing insB9-23-specific T cells. Although insB9-23 immunizations induced both IL-4- and IL-10-producing insB9-23 reactive CD4⁺ T cells in the PLN, addition of anti-OX40 agonist antibody promoted the expansion of insB9-23-specific CD4⁺ T cells expressing IL-10 but not IL-4 (Fig. 7). All together, these data clearly demonstrate that OX40 agonist signal can promote expansion of fully functional adaptive Tregs when combined with antigen-specific immunization.

2.5. Antigen-specific regulatory T cells induced upon anti-OX40/insB9-23 combination treatment can transfer tolerance

To further test the ability of the Tregs induced upon anti-OX40 (OX86)/insB9-23 CT to prevent autoimmune diabetes, we performed adoptive transfer experiments into NOD.SCID

mice. Splenocytes from overtly diabetic NOD mice containing diabetogenic cells (Diab) were adoptively transferred alone into NOD.SCID recipient mice or in conjunction with purified CD4⁺ T cells from NOD mice treated with OX86, insB9-23 (B:9-23) or CT (Fig. 8). NOD.SCID mice receiving diabetogenic cells rapidly developed T1D (within 5–7 weeks post transfer). Co-administration of CD4⁺ T cells obtained from NOD mice treated with OX86 in combination with insB9-23 peptide (CT) significantly reduced T1D incidence in NOD.SCID recipients. We conclude from these experiments that anti-OX40/insB9-23 CT of prediabetic NOD mice augments the proportion of CD4⁺ T cells with *in vivo* suppressive T1D activity.

3. Discussion

In vivo and *ex vivo*-expanded Tregs are currently under clinical evaluation to restore long-term tolerance in type 1 diabetes (T1D) [4,14,34–36]. This approach comes however with potential side-effects as Tregs have been shown to be involved in dampening the immune response to tumors and infectious agents. For that reason one has to develop therapeutic strategies enhancing anti-autoimmunity without affecting anti-tumor immunity. One option is to expand antigen-specific aTregs to avoid deleterious effects of transferring large numbers of multi-specific Tregs. However, the agents capable of inducing such an expansion *in vivo* and currently under clinical evaluations like anti-CD3 or anti-thymoglobulin antibodies are associated with side-effects mainly due to short-term lympho-depletion [9,37–41]. Our data here describe a novel approach using a combination of insB9-23 peptide immunization with a non-depleting OX40 agonist antibody to expand islet-specific Treg populations that can prevent autoimmune diabetes.

OX40 has been implicated in several mechanisms controlling the development of effector and memory CD4 T cells as well as the generation and maintenance of memory CD8 T cells (reviewed in [18]). OX40 is expressed on activated CD4⁺ and CD8⁺ T cells and consequently can be found at the surface of both Tregs and Teffs. Although anti-OX40 agonist treatment induced long-term tolerance (for at least 30 weeks) by favoring selective expansion of Tregs in our T1D model, and short-term tolerance (for up to 15 days) in another model of autoimmune disease [28], the same anti-OX40 antibody is able to promote anti-tumor immunity by blocking Tregs function in tumor rejection models [18,22]. It is worth noting that treatment with an OX40 agonist antibody did not result in accelerated T1D when injected early in the prediabetic phase of NOD mice. In contrast OX40 agonist therapy slowed down T1D progression (Fig. 1).

Although not formally demonstrated here, the tumor micro-environment may provide a milieu favoring the immunogenic rather than tolerogenic activation status of tumor-infiltrating T cells, thus neutralizing Tregs while activating Teffs through a rescue of tumor-infiltrating dendritic cell (DC) function. This unique ability of OX40 agonist agents to exert differential activities on Tregs depending on the pathologic context will be highly beneficial to avoid potential side-effects, such as lympho-depletion and anti-tumor immunity [42], if used in the clinic to restore tolerance in autoimmune settings.

Mechanistically, both the activation status of Tregs and the cytokine milieu [28] seem to play a key role in this dual effect. Valzasina et al. [20] showed that OX40 triggering does not suppress Tregs function when they are formerly activated *in vitro* [20] an effect not seen with the glucocorticoid-induced tumor necrosis factor receptor (GITR). Based on our data, one would argue that prediabetic NOD mice display an immune status (both at the cytokine and Treg activation levels) favoring expansion of functional Tregs upon OX40 stimulation. Consistent with this notion we observed a mild but significant increase of insB:9-23-specific CD4⁺LAP⁺ T cells in the pancreas of OX86 treated animals (Fig. 4D). As previously

observed pancreas-resident antigen-presenting cells (APCs) can capture apoptotic beta-cells and present islet-derived autoantigens (aAgs) to the immune system [43]. Then one can hypothesize that these aAgs-loaded APCs prime and expand insB:9-23-reactive T cells during OX86-mediated expansion of CD4⁺LAP⁺ T cells.

One attractive feature of anti-OX40 agonist antibody treatment consists in the fact that both CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻LAP⁺ T cells were expanded. Proliferation of CD4⁺Foxp3⁺ is probably resulting from the expansion of de novo-induced adaptive Tregs from pre-existing natural Tregs as previously described [28]. Indeed in our model, combination of OX86 with insB9-23 peptide immunization resulted in an increased percentage of insB9-23-reactive CD4⁺Foxp3⁺ (Fig. 4). In addition the percentage of insB9-23-reactive CD4⁺LAP⁺ T cells was also increased in the pancreas and spleen of NOD mice treated with OX86/insB9-23 CT (Fig. 4D). Such a simultaneous expansion of antigen-specific T cells might explain the superior *in vivo* suppressive capacity observed when total CD4⁺ T cells from OX86/insB9-23 treated NOD mice are used in an adoptive co-transfer experiment (Fig. 8). In this situation the recipient mice received spleen-derived CD4⁺ T cells containing a significant number of insB9-23-specific CD4⁺LAP⁺ T cells (Fig. 4D) as well as PLN-derived insB9-23-specific CD4⁺ T cells expressing Foxp3⁺ (Fig. 1) and/or IL-10 (Fig. 7) known as one of the most potent suppressor cytokine. An important aspect to be addressed in future studies is whether all the different populations generated upon OX86/insB9-23 CT possess equivalent suppressive activity in prediabetic NOD mice.

Several therapeutic settings can be foreseen for the treatment of autoimmunity and transplant rejection. Among them the use of aTregs might be the safest for clinical application. They can be either expanded *ex vivo* followed by re-implantation into patients or *in vivo* using antigen vaccination alone or in conjunction with antibodies targeting key receptors to promote synergy [9]. We showed here *in vivo* that OX40 signals can have a beneficial effect on aTregs to suppress autoimmune diabetes. Our data highlighted that short-term treatment with a non-depleting anti-OX40 agonist antibody expands CD4⁺ T cells with suppressive activity in prediabetic NOD mice. Since antigen-based immune interventions have been relatively inefficient in humans to prevent autoimmune diabetes [3], the synergy we observed here between anti-OX40 antibody and insB9:23 peptide provides a novel option for efficiently translating antigen-specific treatment into the clinic. Ultimately, a better knowledge of the action of OX40/OX40L pathway on aTregs function might provide new attractive targets to harness these signals and generate more potent Tregs for clinical use.

4. Materials and methods

4.1. Mice

NOD/LtJ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Diabetes was defined as two consecutive blood glucose values superior to 250 mg/dl. This study was approved by the La Jolla Institute for Allergy and Immunology Animal Care Committee.

4.2. Treatments

Six-week old female NOD mice were immunized intranasally with insulin B:9-23 (insB9:23) peptide at 40 µg/immunization (three times a week for the first two weeks; once a week for five more weeks and once every two weeks until the mice reached 25 weeks of age). Some mice also received three intraperitoneal injections (200 µg/injection) of anti-OX40 stimulating antibody (clone OX86; BioXcell, West Lebanon, NH) on days 0, 2 and 4.

4.3. Antibodies and reagents for flow cytometry

We purchased the following antibodies from BD Biosciences: Alexa Fluor 488-conjugated Ki67-specific (B56; cross-reactive with mouse Ki67), PE-conjugated CTLA-4-specific (UC10-4F10-11), PercPCy5.5- and APC-conjugated CD4-specific (RM4-5), FITC-conjugated CD25-specific (7D4), APC-conjugated CD62L-specific (MEL-14), PE-conjugated IFN γ (XMG1.2), FITC-conjugated TNF (MP6-XT22) and CD16/CD32-specific (FcBlock). We purchased the following antibodies from Biolegend: Pacific Blue-conjugated CD25-specific (PC61) and PE-conjugated CD44-specific (IM7). We purchased Pacific Orange-conjugated CD8-specific antibody (Ly-2) and Alexa Fluor 488-conjugated streptavidin from Invitrogen/Life Technologies. We purchased PE-Cy7-conjugated Foxp3-specific antibody (FJK-16S) and the Foxp3 staining buffer set from eBio-science. Biotinylated anti-LAP antibody (BAF246) was purchased from R&D systems (Minneapolis, MN).

4.4. Intracellular cytokine staining (ICCS)

Single-cell suspensions were prepared from intra-pancreatic lymphocytes of age-matched NOD mice either treated with OX86 antibody alone (2 weeks post-treatment) or non-treated. A fraction of the cells were cultured and stimulated in-vitro with a mixture of Phorbol myristate acetate (PMA) at 15 ng/ml and ionomycin at 500 ng/ml for 6 h in presence of Golgi-stop (Cytofix/cytoperm kit, Becton Dickinson) or remained unstimulated as negative controls. Then, cells were stained in FACS buffer (PBS-1X supplemented with 1% FCS) with the conjugated-antibodies specific for cell surface markers (20min at 4 °C). After permeabilisation with the cytofix/cytoperm kit, cells were incubated with a series of cytokine-specific conjugated antibodies (30min at 4 °C) and fixed with a 4% para-formaldehyde solution (15min at room temperature). Cells were washed in FACS buffer, acquired and analyzed on a FACSCalibur or LSRII flow cytometer (BD Biosciences).

4.5. Isolation of pancreas-infiltrated lymphocytes

Freshly collected pancreata were cut into small pieces with a scissor and digested for 30min at 37 °C in 3 ml/pancreas of Hank's Balanced Salt Solution (HBSS) 1X with Ca^{2+} and Mg^{2+} complemented with collagenase P at 2 mg/ml. The digestion is optimized by frequent pipetting using a P1000 pipettor. The digestion was stopped by adding 3 volumes of RPMI-1640 buffer supplemented with 10% FCS (complete RPMI) and the big clumps of undigested pancreas were removed by filtration through a 500 μm mesh. After centrifugation (1,350 rpm, 5min) the cellular pellet was resuspended in a 40% Percoll solution (8 ml/pancreas) and transferred into a 15-ml conical tube. Intra-pancreatic lymphocytes were purified at the interphase of a 40%/75% Percoll density gradient after centrifugation (2,200rpm, 20min at RT). Purified lymphocytes are rapidly washed in complete RPMI and store on ice until further analysis.

4.6. Detection of Ki67⁺ cells

The staining was performed as previously described [44]. Briefly, single cell suspensions were stained for the surface marker CD4 and subsequently fixed and permeabilized using the Foxp3 staining kit from eBioscience according to the manufacturer's instructions. Then, Foxp3- and Ki67-specific antibodies (or their isotype controls) were incubated for 30min at 4 °C. After washings the cells were acquired using an LSRII flow cytometer.

4.7. Detection of cytokines and latency-associated peptide (LAP)

IL-4 and IL-10 cytokines were detected by enzyme-linked immunosorbent spot assay (ELISpot) as previously described [45]. Briefly, CD4⁺ T cells were purified by negative magnetic Dynabead[®] selection (114-16D, Invitrogen/Life Technologies) from the

splenocytes or pancreatic lymph nodes (PLN) cells of treated NOD mice (2 weeks after treatment). Serial dilutions of purified (>95% purity) CD4⁺ T cells (from 10⁴ to 10⁵ cells/well) were resuspended in HL-1 medium (BioWhittaker) in presence of T cell-depleted splenocytes from non-diabetic 6-week old NOD female mice as antigen-presenting cells (APCs) and cultured in 96-well ELISPOT plates (Millipore) coated with anti-IL-4 or anti-IL-10 Abs (mouse ELISPOT antibody pairs, BD Biosciences) for 72 h at 37 °C. Insulin B9-23 peptide or anti-CD3 antibody (clone 145 2C11) were added at a final concentration of 10 or 0.5 µg/ml respectively. Plates were washed, incubated with the appropriate biotinylated anti-mouse cytokine Abs and streptavidin-HRP (P0397, DakoCytomation), and spot forming units developed with 100 mM sodium acetate buffer containing 0.3 mg/ml 3-amino-9-ethylcarbazole (Sigma–Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader was used to count spot forming unit per well.

The number of LAP⁺ T cells were measured as previously described [46]. Briefly, single cell suspensions were incubated with biotinylated anti-LAP polyclonal antibody at 0.25 µg/ml for 1 h at 4 °C. After washing the cells were incubated with AlexaFluor 488-labeled streptavidin at 1/1000 for 20min at 4 °C. The number of LAP⁺ cells was enumerated by flow cytometry.

4.8. Adoptive cell transfers

To assess the presence and function of regulatory T cells after treatment with OX86 alone insulin B9-23 alone or both in combination, splenocytes and PLN cells were purified from treated animals. Total CD4⁺ were purified by negative selection (114-16D, Invitrogen/Life Technologies). Six- to eight-week old female NOD-SCID recipient mice were injected intravenously with 3 × 10⁶ diabetogenic cells (splenocytes from overtly diabetic NOD females) alone or a mixture of diabetogenic cells and purified CD4⁺ T cells (10⁶ cells/mouse) containing putative regulatory T cells.

4.9. Statistical analysis

Data analysis was performed using GraphPad Prism version 4.00. For survival analysis, survival curves were computed using the method of Kaplan–Meier. For other *in vivo* data the significance of the differences observed between the number diabetic and non-diabetic mice was evaluated using a two-way ANOVA test. Statistical significance for other data was measured using an unpaired two-tailed Mann–Whitney *U* test. All values of *p* < 0.05 were considered significant. *, *P* < 0.05; **, *P* < 0.01.

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Fig. 1.

Antigen-specific prevention of T1D is augmented by using an agonistic OX40-specific antibody. (A) Six-week old female NOD mice were immunized intranasally with the insulin B-chain 9-23 (B:9-23) peptide ($n = 12$ mice). Two groups of mice were treated intraperitoneally with the anti-OX40 agonistic mAb OX86 (days 1, 3 and 5; 200 μ g/injection) alone (group OX86; $n = 16$ mice) or in conjunction with B:9-23 peptide (group B:9-23 + OX86; $n = 15$ mice). In the control group, B:9-23 and OX86 treatments were substituted for PBS and isotype control treatments respectively ($n = 12$ mice). NS, not significant (B) Seven-week old NOD females were treated with anti-OX40 agonistic mAb (OX86). Two weeks later pancreas-infiltrating leukocytes were purified and the percentage of IFN- γ and TNF- α expressing CD4 $^{+}$ and CD8 $^{+}$ T cells infiltrated in the pancreas was enumerated by intracellular cytokine staining after *in vitro* stimulation with PMA (25 ng/ml) and Ionomycin (500 ng/ml) (two independent experiments; $n = 8$ mice per group). **, $p < 0.01$ as compared to non-treated mice.

Fig. 2. Anti-OX40 treatment in prediabetic NOD mice expands CD4⁺CTLA4⁺Foxp3⁺ regulatory T cells. (A–B) Six-week old female NOD mice were immunized intranasally with the insulin B-chain 9-23 (InsB:9-23) peptide or with PBS alone as control. During the first week of treatment, some mice received three injections of anti-OX40 agonistic (OX86) or isotype control antibody (on days 1, 3 and 5; 200 µg/injection). Two weeks later the percentage of CD4⁺CTLA4⁺Foxp3⁺ regulatory T cells (Tregs) was measured in the spleens and pancreatic lymph nodes (PLN). *, $p < 0.05$ and **, $p < 0.01$ as compared to control mice. Two independent experiments (5 pooled mice/experiment).

Fig. 3. OX86 treatment induces CD4⁺Foxp3⁺ Tregs proliferation in prediabetic NOD mice. (A) Six-week old NOD mice were treated with OX86 or an isotype control antibody (days 1, 3 and 5; 200 μ g/injection). Day 7 post-treatment the percentage of CD4⁺Foxp3⁺ Tregs was calculated in the spleen, pancreatic lymph nodes (PLN) and blood of each individual mouse ($n = 5$ mice per group). (B) Representative histograms showing the Ki67 profiles of spleen-, PLN- and blood-derived CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cells from OX86 or isotype treatment groups (percentages of CD4⁺Foxp3⁺Ki67⁺ cells are shown). NS; not significant ($p > 0.05$).

Fig. 4. OX40 agonist antibody treatment in prediabetic NOD mice augments the proportion of CD4⁺LAP⁺ T cells. (A) The expansion of CD4⁺LAP⁺ T cells in the spleen, pancreatic lymph nodes (LN) and pancreas was evaluated at 1 and 2 weeks post-treatment with OX40 agonist Ab (200 µg on days 1, 3 and 5). (B–D) Six-week old female NOD mice were immunized intranasally with the insulin B-chain 9-23 (InsB:9-23) peptide or with PBS alone as control ($n = 5$ mice per group). During the first week of treatment, some mice received three injections of anti-OX40 agonistic (OX86) or isotype control antibody (on days 1, 3 and 5; 200 µg/injection). Two weeks later the percentage of CD4⁺LAP⁺ T cells was measured in the spleen, PLN and pancreas of treated NOD mice with or without prior *in vitro* stimulation with insB9-23 peptide. The data were reproduced in two independent experiments with 4–5 mice/group. *, $p < 0.05$ and **, $p < 0.01$.

Fig. 5. OX40 agonist treatment increases the number of both CD4⁺CD25⁺Fxp3⁺CTLA4⁺LAP⁺ and CD4⁺Fxp3⁻LAP⁺ T cells. (A) Six-week old NOD mice were treated with (i) OX40 agonist antibody (OX86), (ii) insulin B(9-23) peptide or (iii) both in combination. Control mice received a combination of intranasal PBS and isotype antibody control. Day 10 post-treatment the percentage of CD4⁺CD25⁺Fxp3⁺LAP⁺ T cells Tregs was calculated in the pancreatic lymph nodes (PLN). (B) Six-week old NOD mice were treated with OX86 (days 1, 3 and 5; 200 µg/injection). Day 10 post-treatment the percentage of CD4⁺Fxp3⁺ Tregs within the CD4⁺LAP⁺ and CD4⁺LAP⁻ T cells was calculated in the spleen, pancreatic lymph nodes (PLN) and pancreas.

Fig. 6.

CD4⁺LAP⁺ T cells expanded after OX40 agonist treatment display suppressive activity. CD4⁺LAP⁺ T cells were purified from non-treated (NT) or OX40 agonist Ab (OX86) treated NOD mice. Proliferation of responder CD4⁺CD25⁻ cells (5×10^4) was assessed 72h after stimulation with anti-CD3 Ab (1 μ g/ml) and mitomycin C treated T cell-depleted antigen-presenting cells (2×10^5) in the presence or not of CD4⁺LAP⁺ cells (5×10^4) from non-treated (NT) or OX86 treated mice. Data are representative of two independent experiments. *, $p < 0.05$.

Fig. 7. Anti-OX40/insB9-23 combination treatment expands insB9-23-specific IL-10-producing T cells. The number of insB:9-23-reactive T cells producing IL-4 and IL-10 in the spleen and PLN was enumerated by ELISPOT assay following insB:9-23 *in vitro* stimulation. In parallel, total IL-4- and IL-10-producing T cells were revealed after anti-CD3 stimulation. ELISPOT was performed using CD8-depleted splenocytes stimulated for 3 days in the presence of 10 µg/ml insB:9-23 or 0.5 µg/ml anti-CD3. The data represent the average ± SEM after subtracting the background (without stimulation). Data are representative of 5 mice per group and two independent experiments.

Fig. 8.

Anti-OX40/insB9-23 combination treatment in prediabetic NOD mice augments the proportion of CD4⁺ T cells with in vivo suppressive activity. Six-week old NOD mice were treated with (i) OX40 agonist antibody (OX86), (ii) insulin B(9-23) peptide (B:9-23) or (iii) both in combination (CT). Diabetogenic cells (Diab) from fully diabetic NOD mice were injected alone into NOD-SCID recipient mice or co-transferred with CD4⁺ T cells purified from NOD mice 2 weeks after treatment with OX86, B:9-23 or CT. Autoimmune diabetes development and kinetics were followed in each group ($n = 7$ or 8 mice per group). *, $p < 0.05$.