Published in final edited form as:

Autoimmunity. 2016 August ; 49(5): 298–311. doi:10.1080/08916934.2016.1183657.

Age-dependent divergent effects of OX40L treatment on the development of diabetes in NOD mice

Christine S. Haddad¹, Palash Bhattacharya¹, Khaled Alharshaw¹, Alejandra Marinelarena¹, Prabhakaran Kumar¹, Osama El-Sayed¹, Hatem A. Elshabrawy¹, Alan L. Epstein², and Bellur S. Prabhakar¹

¹Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, Illinois

²Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, California

Abstract

Earlier, we have shown that GM-CSF derived bone marrow dendritic cells (G-BMDCs) can expand Foxp3⁺ regulatory T-cells (Tregs) through a TCR-independent, but IL-2 dependent mechanism that required OX40L/OX40 interaction. While some reports have shown suppression of autoimmunity upon treatment with an OX40 agonist, others have shown exacerbation of autoimmune disease instead. To better understand the basis for these differing outcomes, we compared the effects of OX40L treatment in 6-week-old pre-diabetic and 12-week-old near diabetic NOD mice. Upon treatment with OX40L, 6-week-old NOD mice remained normoglycemic and showed a significant increase in Tregs in their spleen and lymph nodes, while 12-week-old NOD mice very rapidly developed hyperglycemia and failed to show Treg increase in spleen or LN. Interestingly, OX40L treatment increased Tregs in the thymus of both age groups. However, it induced Foxp3⁺CD103⁺CD38⁻ stable-phenotype Tregs in the thymus and reduced the frequency of autoreactive Teff cells in 6-week-old mice; while it induced Foxp3⁺CD103⁻CD38⁺ labile-phenotype Tregs in the thymus and increased autoreactive CD4⁺ T cells in the periphery of 12-week-old mice. This increase in autoreactive CD4⁺ T cells was likely due to either a poor suppressive function or conversion of labile Tregs into Teff cells. Using *ex vivo* cultures, we found that the reduction in Treg numbers in 12-week-old mice was likely due to IL-2 deficit, and their numbers could be increased upon addition of exogenous IL-2. The observed divergent effects of OX40L treatment were likely due to differences in the ability of 6- and 12-week-old NOD mice to produce IL-2.

Keywords

regulatory T-cells; Foxp3; stable Tregs; labile Tregs; IL-2

Corresponding Author: Bellur S. Prabhakar, MSc. PhD, Professor, Departments of Microbiology and Immunology, and Ophthalmology, Associate Dean for Technological Innovation and Training, University of Illinois College of Medicine, Room E-705, (M/C 790), 835 S. Wolcott Ave, Chicago, IL 60612, USA, Tel: +1-312-996-4945, FAX: +1-312-996-6415, bprabhak@uic.edu.

Declaration of interest

The authors have no competing financial interests.

Introduction

OX40L and its unique receptor OX40 belong to the TNF-super family (1), and their interaction can provide co-stimulatory signal during TCR-mediated T-helper activation (2–4). OX40⁺ T lymphocytes have been detected in the tumor-microenvironment (5), and treatment with an OX40 agonistic antibody has been shown to enhance antitumor responses in tumor models of melanoma, breast, lung and colon cancers (5–8). Based on these promising pre-clinical findings, OX40 agonist is currently being tested in clinical trials as an immune-enhancer in cancer therapy (9, 10). Furthermore, OX40 mediated signaling has been reported to be antagonistic to regulatory T-cell (Treg) generation and function, and can even contribute to the development of autoimmunity (11, 12). In addition, blocking OX40L/OX40 signaling has been shown to diminish Experimental Autoimmune Encephalomyelitis (EAE) (13), Inflammatory Bowel Disease (IBD) (14), and Type-1 diabetes (T1D) (11).

In contrast to its co-stimulatory function in TCR mediated T-cell activation, OX40 is constitutively expressed on regulatory T cells (Tregs) (15), and OX40L-OX40 interaction has been shown to contribute to Treg survival, proliferation and suppressive function in a mouse model of colitis (16). Interestingly, while OX40^{-/-} mice show a reduction in the number of Foxp3⁺ Tregs compared to wild type controls (17), transgenic mice over expressing OX40L show increased Treg numbers in both the spleen and the thymus (17). Moreover, administration of an OX40 agonist can lead to *in vivo* Treg expansion and protection of mice against developing EAE or T1D (18–20). However, OX40 agonist has been shown to exert divergent effects *in vivo* depending upon the presence or absence of pro-inflammatory cytokines (18). An OX40 agonist could increase Tregs *in vivo* and protect mice from developing EAE only when administered prior to the disease onset. In contrast, when administered after the disease onset, it failed to increase Tregs and exacerbated the disease (18).

In our earlier studies we showed that treatment of mice with low-dose GM-CSF prevented the development of T1D in NOD mice (21). Similarly, beneficial effects of GM-CSF treatment was observed in CBA mice with experimental autoimmune thyroiditis (EAT) (21–23) and C57Bl/6 mice with experimental autoimmune myasthenia gravis (EAMG) (24–26). In subsequent studies, we showed that GM-CSF-treatment induced tolerogenic dendritic cells (DCs) which expressed matured levels of MHC class-I/II and B7 molecules with little or no expression of pro-inflammatory cytokines. Antigen presentation by these tolerogenic DCs resulted in Treg proliferation, which suppressed antigen specific effector T cell responses through enhanced production of IL-10 (21, 23).

Recently, we reported that bone marrow (BM) precursor cells differentiated in the presence of GM-CSF (G-BMDCs), when co-cultured with naïve CD4⁺ T cells, were able to selectively expand Tregs, but not T effectors (Teffs), while spleen derived DCs (SpDCs) exposed to GM-CSF failed to do so (27). Further, using MHC class-II^{-/-} G-BMDCs, we showed that Treg expansion occurred in the absence of canonical TCR engagement, but required G-BMDC-T cell contact and exogenous IL-2. G-BMDCs induced Treg expansion was dependent upon higher levels of OX40L expression on their surface and the Treg expansion could be blocked by an anti-OX40L antibody, which could be lifted upon addition

of an OX40 agonist. These results showed the importance of OX40L/OX40 interaction in TCR-independent Treg expansion (27), and suggested a critical role in Treg development/homeostasis.

While administration of soluble OX40L for selective expansion of either Tregs or augmenting Teffs function is a promising therapeutic strategy for treating autoimmune diseases or cancer respectively, it is critically important to better understand the basis for the above mentioned differing outcomes of OX40L/OX40 interaction prior to its clinical use. In an effort to do that we chose NOD mice because, unlike the mouse model of EAE, they spontaneously develop T1D and the pro-inflammatory responses in these mice are naturally exacerbated as they get older. Therefore, we wanted to determine the immunological consequence of treating NOD mice at different ages with a soluble OX40L.

We found that treatment of 6-week-old pre-diabetic NOD mice with soluble OX40L led to a significant increase in Tregs in the spleen and pancreatic lymph nodes (LNs). However, in 12-week-old NOD mice, when they typically begin to show overt autoimmunity, OX40L administration caused acute onset of hyperglycemia with no increase in Tregs in the spleen or pancreatic LNs. Upon further investigation, although we noted an increase in thymic Tregs in both age groups of mice, the Tregs from 12-week-old mice exhibited a labile phenotype while the Tregs from 6-week old mice showed a more stable phenotype (28). Furthermore, our results suggested that although OX40L administration can cause Treg proliferation *in vivo*, a combination of other factors including reduction in IL-2 and increase in TH17 family of pro-inflammatory cytokines likely led to a loss of Treg phenotype and function in the periphery, which probably contributed to the acute onset of hyperglycemia in 12-week-old mice. Thus, sustenance of Treg suppressor phenotype and function in an inflammatory setting may require signaling in addition to that mediated through OX40.

Materials and methods

Animals

6- to 8-week-old NOD mice, thymectomized NOD mice and twelve-week-old Balb/c were purchased from the Jackson laboratory. Thymectomy was performed on 4-week-old NOD mice by the vendor (Surgical Services, Jackson Lab). Mice were housed in the Biological Resources laboratory facility at the University of Illinois (Chicago, IL) and provided food and water ad libitum. All animal experiments were approved by the University of Illinois at Chicago animal care and use committee. 6- or 12-week-old non-diabetic NOD mice were treated intraperitoneally with OX40L once a week over a period of 3 weeks (200µg/mouse/treatment). Glucose was measured in blood samples collected from tail vein using Contour glucometer (Bayer) and a blood glucose level above 200 mg/dl was considered hyperglycemic (29–31).

Cytokines and antibodies

Recombinant mouse GM-CSF (PMC2013) and CellTrace Violet (C34557) were purchased from Invitrogen (Carlsbad, USA). Recombinant mouse TGF-β (7666-MB-005) was purchased from R&D Systems (Minneapolis, MN). Production of soluble Fc-muOX40L has

been described earlier (8). Carboxyfluorescein Succinimidyl Ester (CFSE) (65-0850); PE-conjugated anti-OX40L (12-5905), anti-OX40 (12-1341), anti-CD25 (12-0251), anti-IFN- γ (12-7311-81), anti-IL-4 (12-704-81); APC-conjugated anti-OX40L (17-5905), anti-Foxp3 (17-5773), anti-CD8 α (17-0081), anti-CD11c (17-0114), anti-IL-17 (17-7177-81); eFlour-450 conjugated anti-CD11c (48-0114); FITC conjugated anti-CD8 α (11-0081), anti-CD4 (11-0041); anti-mouse CD3 (16-0032), and mouse recombinant IL-2 (34-8021) were purchased from eBiosciences (San Diego, CA). Pacific Blue-conjugated anti-CD4 (100428) was purchased from Biolegend (San Diego, CA).

Isolation of DC and T-cell populations

Bone marrow cells were cultured in complete RPMI medium containing 10% heat-inactivated FBS in the presence of 20ng/ml GM-CSF. Fresh medium containing 20ng/mL GM-CSF was added on days 4 and 6. On the 8th day, non-adherent CD11c⁺ DCs (G-BMDCs) or specific sub populations of G-BMDCs (i.e. OX40L⁺ or OX40L⁻) were sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with appropriate antibodies. CD4⁺ cells were isolated from the spleens by using either the Mouse CD4⁺ T Cell Isolation Kit II (130-095-248) from MiltenyiBiotec (San Diego, CA), or sorted using a MoFlo flow cytometer (Beckman/Coulter) following staining with anti-CD4 antibodies.

In vitro co-cultures of DCs and T-cells

G-BMDCs (5×10^4) and CD11c⁺SpDCs were cultured with CD4⁺ T-cells at a ratio of 1:2 for 5 days. For proliferation assays, total CD4⁺ T-cells were labeled with CFSE at 10uM or CellTrace Violet according to manufacturer's instruction prior to co-culturing them with DCs. After 5 days, cells were fixed, permeabilized, stained with anti-CD4 and anti-Foxp3 antibodies and analyzed using a Cyan ADP Flow Cytometer. Each co-culture experiment was set up in triplicates for each sample.

Induction of Tregs ex vivo

Splenocytes from NOD mice were stained with anti-CD4 and anti-CD25 antibodies, and then sorted using a MoFlow flow cytometer (Beckman.Coulter). CD4⁺CD25⁻ (5×10^5 cells) were then cultured in the presence of anti-CD3 (2 μ g/mL) and TGF- β (3 ng/mL) or anti-CD3 alone (control) for 48 hrs. Subsequently, the cells were washed thoroughly to remove anti-CD3/TGF- β , labeled with CFSE and then co-cultured for 5 days with G-BMDCs or SpDCs at a ratio of 2:1 (1×10^5 T cells: 5×10^4 DCs). Cells were then stained for CD4, Foxp3 and analyzed for Treg proliferation using Cyan ADP flow cytometer (Beckman/Coulter).

Isolation of in-vitro generated Tregs (G-BMDCs-Tregs)

Total CD4⁺ T cells were co-cultured with G-BMDCs for 5 days, as described above. Cells from G-BMDC-CD4⁺ T cell co-cultures were stained with anti-CD4 and anti-CD25 antibodies, and CD4⁺CD25⁺ T cells were sorted. Purified Tregs were then co-cultured with CFSE-CD4⁺CD25⁻ T cells in the suppression assay as described below.

Suppression assay

CD4⁺CD25⁻ T cells were isolated from the spleens of NOD mice by FACS following staining with anti-CD4 and anti-CD25 antibodies. Cells were then labeled with CFSE and plated in a U-bottom 96-well plate (5×10^4 cells/well) in the presence of anti-CD3 (2 μ g/mL) and splenic APCs. Freshly isolated or G-BMDC-expanded Tregs were sorted and co-cultured with CFSE-CD4⁺CD25⁻ T effectors at different ratios. After 3 days, cells were fixed & permeabilized, stained with anti-CD4 and anti-Foxp3 antibodies and analyzed using a Cyan Flow Cytometer (Beckman/Coulter).

In-vitro Treg stimulation assay

CD4⁺CD25⁺Tregs were isolated from the spleens and PLN of 6- and 12-week-old NOD mice by FACS sorting. Purified Tregs were then labeled with CFSE. Labeled cells from each age group were mixed back with splenocytes or PLN. Cells were cultured in the presence or absence of Cell-Stimulation Cocktail (PMA/Ionomycin) (eBiosciences) with or without IL-2 (10ng/mL) for 24 hrs. After 24 hrs, cells were stained with anti-CD4 and anti-Foxp3 antibodies, according to the staining protocol described below. Cells were analyzed for conversion of Foxp3⁺ T cells by FACS.

Intracellular staining

Briefly, at the end of co-culture experiments, T-cells were first stained with Pacific blue labeled anti-mouse CD4 antibody. Then, for intracellular staining, surface stained cells were fixed and permeabilized using a Foxp3/Transcription Factor Staining Buffer Set (eBiosciences, San Diego, CA) and incubated with specified antibodies.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-2 were measured using Mouse IL-2 ELISA Ready-SET-Go kit (eBioscience). Splenocytes from 6- and 12-week-old mice were cultured in the presence of cell stimulating cocktail (eBioscience) for 24 hrs; supernatants were collected and used to measure the levels of IL-2 according to the manufacturer's instructions.

Tetramer staining

Mouse InsB9-23 MHCII tetramer (peptide sequence:HLVERLYLVAGEEG) was obtained from the NIH Tetramer Core Facility. Single-cell suspensions were prepared from the spleens or pancreatic lymph nodes of 6- or 12-week-old NOD mice either treated with OX40L or left untreated. Cells were incubated with the MHCII tetramer in 0.5% BSA at room temperature for 1 hr. Tetramer labeled cells were stained with a CD4 antibody, and then analyzed by FACS.

Flow Cytometry

Freshly isolated and *ex vivo* cultured cells were washed with PBS containing 0.5%BSA-EDTA. For surface staining, the cells were labeled with specified FITC, PE, APC conjugated antibodies for 30 min. For cell proliferation assays, the cells were labeled with CFSE, fixed, permeabilized and incubated with fluorescent coupled antibodies for intracellular staining. Stained cells were washed three times and analyzed by FACS.

H&E staining

Pancreatic tissues were collected from sacrificed mice and fixed in formalin, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E) and subjected to microscopic examination to determine the degree of infiltration.

Adoptive transfer

Three groups of 3 mice each were adoptively transferred, via i.v. injection, with either i) PBS, ii) 1×10^6 purified CD11c⁺ DCs from untreated control NOD mice or iii) or 1×10^6 CD11c⁺ G-BMDC sorted from BM cultures in GM-CSF. Seven days after the adoptive transfer, mice were sacrificed, and the spleens and lymph nodes were isolated and analyzed for percentage of Foxp3⁺ Tregs.

Cell stimulation and cytokine expression analysis by qRT-PCR

Splenocytes from the control and OX40L treated 6- and 12-week-old NOD mice were stimulated with 500X cell stimulation cocktail containing phorbol 12-myristate 13-acetate (PMA) and ionomycin for 24h. Total RNA was isolated from cells using RNAeasy columns (Qiagen, Valencia, CA). cDNA synthesis from RNA was done using RevertAid cDNA synthesis kit (Thermo Scientific). Expression of mRNA encoding cytokines such as IFN- γ , IL-12 α , IL-12 β , TNF- α , IL-4, IL-5, IL-6, IL-17 and IL-21 was analyzed by RT-qPCR using primers shown in Table-1. RT-qPCR was carried out with Fast SYBR green master mix (Applied Biosystems) and gene specific primers (IDT Technologies) by using AB ViiA7 real time PCR instrument (Applied Biosystems). Gene expression values were calculated by comparative Ct method after normalization to GAPDH internal control and expressed as fold change over respective controls.

Statistical analysis

Mean, standard deviation, and statistical significance were calculated using the MS-Excel application software. Statistical significance was determined using the one tailed Students t-test. A *p-value* of 0.05 was considered significant. Log-rank test for statistical significance was conducted through the online program at <http://bioinf.wehi.edu.au/software/russell/logrank/>.

Results

NOD derived G-BMDCs can expand autologous Tregs both *ex vivo* and *in vivo* through OX40L/OX40 interaction

In our earlier studies, we have shown that G-BMDCs-mediated TCR-independent Treg expansion is dependent on OX40/OX40L signaling in CBA mice (32). To determine if this is also the case in NOD mice, which have several inherent immunological defects, we cultured bone marrow precursors from NOD mice with GM-CSF to determine the expression of OX40L on GM-CSF-differentiated G-BMDCs. After seven days in culture, about 35% of the CD11c⁺ BMDCs were OX40L⁺ (Fig. 1A). We then co-cultured G-BMDCs with splenic CD4⁺ T cells labeled with CFSE, in the absence of any exogenous antigen. Only G-BMDCs, and not splenic DCs (SpDCs; negative control) could induce the proliferation of Foxp3⁺

Tregs (Fig. 1B). Next, we sorted G-BMDCs into OX40L⁺ and OX40L⁻ subsets and co-cultured each subset with CFSE-labeled naïve CD4⁺ T cells for 5 days, in the absence of any exogenous antigen. As indicated by the extent of CFSE dilution, significant proliferation of Foxp3⁺ Tregs occurred only when co-cultured with OX40L⁺ G-BMDCs but not with OX40L⁻ G-BMDCs (Fig. 1C). Collectively, these data suggested that OX40L/OX40 interaction is critical for G-BMDC induced Treg expansion *ex vivo*.

To test the capacity of G-BMDCs to expand Tregs *in vivo* in NOD mice, we adoptively transferred G-BMDCs or SpDCs (as a control) (1×10⁶ cells/mouse) to 10-week-old NOD mice. Mice were sacrificed 7 days after adoptive transfer and analyzed for the percentage of Foxp3⁺ Tregs in the spleen and PLNs (PLN) (Fig. 1D). Mice adoptively transferred with G-BMDCs showed increased percentages of Foxp3⁺ Tregs in both the spleen and the PLNs compared to the adoptive control. In contrast, adoptive transfer of SpDCs did not increase Tregs in either the spleen or the PLNs.

In addition to expanding naturally occurring Foxp3⁺ Tregs, we wanted to further determine the capacity of G-BMDCs to expand *ex vivo* generated adaptive Tregs (iTregs). iTregs were generated by culturing CD4⁺CD25⁻ T cells from NOD mice in the presence of anti-CD3 and TGF-β as previously described (33, 34). After 48 hrs, a fraction of these cells were stained with CD4 and Foxp3 antibodies to confirm the presence of Foxp3⁺ T cell population. About 50% of the cells had undergone conversion as determined by Foxp3 expression (Supplementary Fig 1A, **upper panel**). Cells were then labeled with CFSE and co-cultured with either G-BMDCs or SpDCs for 5 days. After 5 days, iTregs showed marked proliferation only when co-cultured with G-BMDCs, but not with SpDCs (Supplementary Fig1A, **lower panel**). These data confirm that G-BMDCs are capable of expanding nTregs, isolated from the spleens of NOD mice, as well as *ex-vivo* generated iTregs.

Previous studies have demonstrated that *in vitro* expansion of Tregs leads to loss of Foxp3 expression and their suppressive function (35, 36). Therefore, we set-up an *in vitro* suppression assay to determine whether *ex vivo* expanded Tregs, using G-BMDCs, retain their capacity to suppress Teff proliferation. CD4⁺CD25⁻ Teff were isolated from the spleens of NOD mice, labeled with CFSE, and stimulated with anti-CD3 and splenic APCs. *Ex vivo* expanded CD4⁺CD25⁺ (G-BMDC Tregs) sorted from 5-day old G-BMDC-CD4⁺ T cell co-culture were added to the CFSE labeled-CD4⁺CD25⁻ Teff at different ratios (Supplementary Fig. 1B). Tregs sorted from freshly obtained splenocytes, cultured at 1:1 ratio with Teff cells, were used as a positive control. After 3 days, cells were analyzed for T effector cell proliferation. T effector cells proliferated robustly when stimulated with anti-CD3 in the absence of Tregs (~80%). In contrast, proliferation was markedly reduced in the presence of *ex vivo* expanded G-BMDC Tregs. As expected, the extent of suppression by *ex vivo* expanded G-BMDC Tregs was dependent on the Tregs:Teff ratio in the co-culture (reduced to 36%-58% in the presence of 1:1 to 1:6 Treg:Teff ratio). Furthermore, the suppressive function of *ex vivo* expanded Tregs was comparable to that of freshly isolated splenic Tregs (Supplementary Fig. 1B). Taken together, these results strongly suggested that G-BMDCs can induce the proliferation of Tregs *ex vivo* and *in vivo*, and that expanded Tregs maintain their suppressive capacity.

OX40L treatment increases Tregs in the spleen and lymph nodes of 6-week old but not 12-week-old NOD mice

We treated 6-week and 12-week-old NOD mice with OX40L (200ug/mouse weekly for 3 weeks). Interestingly, OX40L treatment induced a rapid onset of hyperglycemia in the 12-week-old NOD mice, with 80% and 100% of mice becoming diabetic within the first and second weeks of treatment respectively, while 80% of the untreated control mice remained disease free at 15 weeks of age (Fig. 2A). However, treatment of 6-week-old NOD mice did not induce rapid onset of T1D, and like control mice they remained diabetes-free following three treatments with OX40L (Fig. 2A). The rapid onset of hyperglycemia in 12-week-old mice was accompanied by extensive destruction of islet cells as evidenced in H&E stained pancreatic tissue sections of experimental mice (Fig 2B). In contrast, untreated 12-week-old mice showed intact islets with either periinsulinitis or more advanced stages of lymphocytic infiltrations. Interestingly, islets from both treated and untreated 6-week-old mice showed normal morphology (Fig 2B). These data suggested that the rapid onset of hyperglycemia in 12-week-old NOD mice is not the result of direct β -cell damage by OX40L but rather due to their accelerated destruction by the immune system.

To elucidate the immunomodulatory effects of OX40L, particularly on Treg phenotype and function, spleen and PLNs from 12- and 6-week-old treated mice were analyzed for Foxp3 expression (Fig. 2C, Supplementary Fig. 2A). The percentage of Foxp3⁺ Tregs in 12-week-old mice treated with OX40L was only modestly increased in the spleen and the PLNs compared to 12-week-old untreated controls. Interestingly, 6-week-old NOD mice treated with OX40L showed a marked increase in the percentage of Foxp3⁺ Tregs in both the spleen and the PLNs compared to 6-week-old controls. Intriguingly, and in contrast to our findings in the peripheral lymphoid tissues, 12-week-old NOD mice showed a significant increase in the percentage of thymic Foxp3⁺ Tregs upon OX40L treatment compared to the control (Fig. 2D, Supplementary Fig 2B). Similarly, thymic Foxp3⁺ Tregs were also increased in 6-week-old NOD mice treated with OX40L compared to the untreated controls. These data showed that the effect of OX40L on Treg proliferation was divergent at two levels; a) between thymus and periphery within 12-week-old mice, and b) in the spleen and lymph node Tregs between 12-week and 6-week-old mice.

OX40L primarily acts on the thymus and the age-dependent divergent effects are specific to NOD mice

To determine if the effect of OX40L treatment was primarily on the thymus or the peripheral lymphoid organs, we used NOD mice thymectomized at 4-weeks of age. Starting at 6 or 12 weeks of age these mice were treated weekly with OX40L (200 μ g/mouse). As expected, thymectomized mice treated at 12 weeks of age did not show increased Tregs in either spleen or LN (data not shown). Thymectomized 6-week-old NOD mice also failed to show an increase in Tregs in the spleen and LN, suggesting that the OX40L mediated increase in Tregs primarily occurred in the thymus and their migration into the periphery likely accounted for their increase in spleen and LN seen in wild type NOD mice (Fig 3A and 3B).

To determine if the discrepancy between Treg phenotype of 6-week and 12-week-old NOD mice after OX40L treatment was due to a general phenomenon of aging (e.g. decreased

migration of Tregs from the thymus to the periphery) or was unique to NOD mice, we treated 12-week-old Balb/c mice with OX40L (Fig. 3C and 3D). Interestingly, upon OX40L treatment, these mice showed a significant increase in Foxp3⁺Tregs in the thymus, spleen, and PLN, compared to the untreated control group. Therefore, OX40L is capable of inducing Tregs in the thymus and consequently in the periphery of 12-week-old WT Balb/c mice, and suggested that the absence of Tregs expansion in the periphery of 12-week-old NOD mice was not due to a universal effect of aging, but rather due to a specific defect in NOD mice that occurs around 12 weeks of age.

Autoreactive T cells are increased in 12-week-old mice and decreased in 6-week-old NOD mice upon OX40L treatment

It has been suggested that autoimmunity occurs due to autoreactive T cells escaping thymic negative selection (37, 38). MHC class II tetramers are important tools to identify and enumerate antigen-specific CD4⁺ T cells (39). Autoreactivity towards the insulin peptide has been strongly implicated in the pathogenesis of T1D (40, 41). T cells with receptor specificity for Ins1/2B9–23 peptide have been linked to the development of diabetes in NOD mice (41–43). Therefore, to determine if OX40L treatment caused an increase in autoreactive CD4⁺ T cells in 12-week-old mice, we enumerated the frequency of InsB9–23 pMHC-II tetramer binding T cells. Interestingly, 6-week-old mice treated with OX40L showed a marked reduction in the frequency of InsB9–23-specific CD4⁺ T cells in both the spleen and the PLNs compared to 6-week-old untreated controls (Fig. 4A and 4B). In contrast, OX40L-treated 12-week-old mice showed a significant increase in InsB9–23-specific CD4⁺ T cells in the PLNs compared to 12-week-old controls, while there was no significant difference in the frequency of InsB9–23-specific CD4⁺ T cells in spleens of OX40L-treated and untreated mice.

The rapid onset of hyperglycemia in 12-week-old NOD mice upon OX40L treatment is not due to direct effect of OX40L on OX40⁺ effector T cells

The increase in InsB9–23-specific CD4⁺ T cells in the PLNs in OX40L treated 12-week-old mice could be either due to a direct effect of OX40L on T-effector cells, or due to conversion of Tregs. Effector T cells are known to express OX40 transiently upon TCR activation (15). Thus, we tested whether CD4⁺ T cells from 12-week-old NOD mice had higher expression of OX40 to which OX40L could bind and cause their proliferation. Thus, the thymus, spleen and PLNs from 6- and 12-week-old NOD mice were analyzed for the expression of OX40 on total CD4⁺ and for Foxp3⁺ T cells. Interestingly, frequencies of CD4⁺ OX40⁺T cells were similar in the thymus, spleen and PLNs between 12-week and 6-week old NOD mice (Fig 5A). While OX40L treatment caused a general increase in the frequency of OX40 expressing cells in the thymus, spleen, and PLN in both 6 and 12-week-old NOD mice it was increased significantly more in the spleen of 6-week-old mice and thymus in the 12-week-old mice ($p < 0.01$ for spleen between OX40L treated and control and $p < 0.01$ for thymus between OX40L treated and control) (Fig 5B; **left panel**). In all cases, the noted increase in OX40⁺ T-cells was predominantly in the Foxp3⁺Tregs subpopulation (Fig 5B; **right panel**). Therefore, it is unlikely that over expression of OX40 or increased frequency of OX40⁺ Teff cells was responsible for rapid proliferation of effector T cells induced by OX40L in 12-week-old NOD mice.

OX40L treatment increases labile Tregs in 12-week-old, but not in 6-week-old, NOD mice

A recent study by Sharma et al. identified a new subset of “labile” Foxp3⁺Tregs that have the capacity to reprogram and gain helper-like function by promoting the differentiation of naïve CD8⁺ T cells into effector cells under certain pro-inflammatory conditions (28). These labile Tregs are characterized as Foxp3⁺CD103⁻CD38⁺. In contrast, stable Tregs, characterized as Foxp3⁺CD103⁺CD38⁻, sustain their phenotypic and functional properties under pro-inflammatory conditions (28). Since OX40L treatment increased Foxp3⁺Tregs in the thymus but not in the periphery of 12-week-old NOD mice, we tested whether these mice have an altered Treg phenotype (i.e. increase in labile Tregs) which upon OX40L treatment can increase in the thymus and are converted into Teff in the peripheral lymphoid organs. Thus, the thymus, spleens and PLNs of 12- and 6-week-old, OX40L treated and untreated, mice were stained with CD38 and CD103 to determine the relative distribution of stable vs. labile Tregs (Fig. 5C). Interestingly, we noted that the OX40L-induced Treg expansion in 6-week-old NOD mice occurred mainly in the stable Foxp3⁺CD103⁺CD38⁻Treg subset in the thymus, spleen, and PLNs, compared to untreated controls. Moreover, OX40L-treated 6-week-old mice had a concomitant decrease in the Foxp3⁺CD103⁻CD38⁺ labile population in the thymus, spleen, and PLNs (Fig. 5D; **left panel**). In contrast, the increase seen in thymic Tregs in 12-week-old NOD mice upon OX40L treatment was mainly due to an increase in the labile Foxp3⁺CD103⁻CD38⁺Treg subset compared to untreated controls. The frequency of labile Tregs in the spleen and PLN of OX40L-treated 12-week-old mice was similar to that noted in untreated 12-week-old controls (Fig. 5D; **right panel**). Additionally, the frequency of stable Tregs was also similar in the treated and untreated control 12-week-old mice in the thymus, spleen, and PLNs. Given that the expanded thymic Tregs in OX40L treated 12-week-old mice were mostly labile Tregs, it is likely that these cells had converted to become non-Tregs and thus could not be detected in the periphery. It is also important to note that 12-week-old mice showed an increase in the Foxp3⁺CD103⁻CD38⁺labile Tregs compared to the 6 week old mice in the thymus, spleen, and PLNs. (Fig. 5C, -D). This altered Treg phenotype may be an important underlying defect that might have contributed to the acute pathogenesis of T1D in 12-week-old NOD mice.

Conversion of labile Tregs in 12-week-old NOD mice likely occurs due to IL-2 deficiency

Labile Tregs have been shown to undergo conversion into “helper-like” cells under inflammatory setting (28). To confirm if labile Tregs from 12-week-old NOD mice were more likely to convert into Foxp3⁻T cells, we tested for conversion of Foxp3⁺Tregs into Foxp3⁻ T cells. Briefly, we sorted for CD4⁺CD25⁺ Tregs from the freshly isolated spleens and PLNs. The purity of the sort was confirmed by staining each purified sample for Foxp3 (~80% Foxp3⁺ in 4 different samples). Tregs were then labeled with CFSE and co-cultured back with their respective total splenocytes (Treg depleted) in the presence of cell stimulation cocktail (PMA/Ionomycin). After 24 hours, cells were stained with CD4/Foxp3 antibodies and analyzed for the percentages of CFSE⁺Foxp3⁺ vs. CFSE⁺Foxp3⁻ cells (Fig 6A). In stimulated cultures, the majority of CFSE labeled cells from 6-week-old NOD mice remained Foxp3⁺ in both the spleen and PLNs. However, in 12-week-old NOD mice a higher percentage of Foxp3⁺ T cells converted into CFSE⁺Foxp3⁻ T cells in the spleen as

evidenced by the reduction in the percentage of Foxp3⁺ and in the PLNs. In the unstimulated cultures, cells from both 12- and 6-week-old mice showed marked conversion in the spleen and PLNs (Fig 6A).

To see if the cytokine environment drives *ex vivo* conversion of Tregs in 12-week-old NOD mice, CD4⁺CD25⁺Tregs were sorted from freshly isolated spleens, labeled with CFSE and co-cultured CFSE-labeled Tregs from 12-week-old mice with total splenocytes from 6-week-old mice, and Tregs from 6-week-old mice with splenocytes from 12-week-old mice (Fig 6B). Interestingly, co-culturing Tregs from 12-week-old-mice with splenocytes from 6-week-old mice prevented the conversion as most of CFSE⁺Tregs expressed Foxp3, compared to Tregs from 12-week-old mice co-cultured with splenocytes from 12-week-old mice. In contrast, culturing Tregs from 6-week-old mice with splenocytes from 12-week-old mice resulted in higher conversion of CFSE⁺ Foxp3⁺Tregs compared to the co-culture of Tregs and splenocytes both of which were from 6-week-old mice (Fig 6B).

Instability of Treg phenotype in 12-week-old NOD mice is correlated with reduced IL-2 secretion and increased production of pro-inflammatory cytokines

We noted that in the absence of cell stimulation (PMA/ionomycin), Tregs from both 12- and 6-week-old NOD mice were converting to Foxp3⁻ T cells. IL-2 is known to be required for Treg cell survival and maintenance. Deficiencies in IL-2 production and IL-2 signaling have been implicated in the pathogenesis of T1D in NOD mice (44, 45). In contrast, We analyzed the expression of CD25 (IL-2R α) and Foxp3 within CD4⁺ T-cell populations in the thymus and spleen of 12 and 6-week old NOD mice to verify if there was a dissociation of their expression with age. We found a small increase in the thymic Foxp3⁺CD25⁺ Tregs in 12-week old mice compared to 6-week old mice; other populations were not significantly different. These data suggested that the phenotypic instability of Tregs in older mice was not due to a defect in CD25 expression (Supplementary Fig 3).

To determine if splenocytes from 12 week old mice had reduced capacity of IL-2 secretion, we first isolated CD4⁺CD25⁺ cells (~80% purity) from both 6- and 12-week-old NOD mice, labeled them with CellTrace violet and co-cultured back with their respective total splenocytes in the presence of cell stimulation cocktail (PMA/Ionomycin). Twenty four hours post stimulation, splenocytes from 12-week-old NOD mice showed a higher loss of Foxp3 expression than splenocytes from 6-week-old NOD mice as determined by FACs (Figure 7A). We used the supernatant from both co-cultures to determine IL-2 levels. We found about 2-fold higher IL-2 levels in the supernatant from co-cultures of splenocytes from 6-week-old mice compared to splenocytes from 12-week-old mice (p=0.04) (Fig 7B). These data suggested that IL-2 deficiency in splenocyte cultures from 12-week-old mice may be responsible for loss of Foxp3 expression.

Next, we tested whether addition of IL-2 to the co-culture could prevent the loss of Foxp3 expression. Interestingly, addition of IL-2 even in the absence of PMA/Ionomycin stimulation maintained Foxp3⁺Treg population in cultures from both 12- and 6-week-old mice as indicated by the high percentage of CFSE⁺Foxp3⁺ T cells, compared to cells cultured in the absence of IL-2 and PMA/Ionomycin (Fig 7C). Further, addition of IL-2 to co-cultures stimulated with PMA/Ionomycin significantly reduced conversion of Foxp3⁺

Tregs in cultures from 12-week-old mice, compared to PMA/Ionomycin stimulation without IL-2. These data suggested that the increased conversion of Foxp3⁺Tregs from 12-week-old NOD mice *ex vivo* upon PMA/Ionomycin is most likely due to IL-2 deficiency in these cultures (Fig 7C).

We further addressed whether this defect in IL-2 production was associated with an increase in the secretion of inflammatory cytokines in 12-week-old mice. Thus we cultured splenocytes from 6- and 12-week-old mice in the presence of PMA/Ionomycin and compared the expression of intracellular IFN- γ (Th1), IL-4 (Th2) and IL-17 (Th17) by FACS (Fig 7D). While the levels of IFN- γ and IL-4 were comparable between the cultures, we observed a significantly elevated expression of IL-17 in 12-week-old splenocytes compared to 6-week-old splenocytes. To explore this issue further, we extracted total RNA from these cultures and compared the relative levels of mRNA transcripts for TH17-associated and other relevant cytokines by qRT-PCR (Figure 7E). We found significantly increased levels of IL-6, IL-17a and IL-21 transcripts in splenocytes from 12-week-old mice.

Discussion

First, we demonstrated the critical requirement for OX40L/OX40 interaction in the *ex vivo* proliferation of Tregs from NOD mice. To determine the relevance of this signaling on Treg expansion *in vivo*, we treated NOD mice with OX40L at two different ages. Unlike 6-week old mice which remained normoglycemic, treatment of 12-week-old (near diabetes onset) NOD mice with OX40L resulted in a very rapid onset of hyperglycemia with concomitant lymphocytic infiltration and destruction of islets. This divergent effect of OX40L treatment was accompanied by a robust expansion of Tregs in the peripheral lymphoid organs of 6-week-old mice, while it failed to occur in 12-week-old NOD mice.

In contrast, OX40L treatment increased thymic Foxp3⁺ Tregs in both age groups of NOD mice. Interestingly, it failed to increase Tregs in the periphery of 6-week-old thymectomized NOD mice, which indicated that the primary effect of OX40L was on the thymus. Additionally, treatment of 12-week-old Balb/c mice with OX40L increased Tregs in both the thymus and in the peripheral lymphoid organs, ruling out the possibility that the lack of Tregs in the periphery of 12-week-old NOD mice was due to a generalized age-related phenomenon. These findings suggested that the failure of OX40L to increase Tregs in the periphery of 12-week-old NOD mice is likely a consequence of loss of Foxp3 expression either due to the peripheral microenvironment and/or is reflective of an inherent property of the induced Tregs in these mice.

Interestingly, concomitant with an increase in Foxp3⁺ Tregs, the frequency of InsB₉₋₂₃-specific CD4⁺ T cells were decreased in the peripheral lymphoid organs of OX40L treated 6-week-old NOD mice. In contrast, we found no increase in Foxp3⁺ Tregs in the periphery, but found a significant increase in InsB₉₋₂₃-specific CD4⁺ T cells in the PLN 12-week-old NOD mice treated with OX40L. These later findings are consistent with the rapid onset of hyperglycemia in 12-week-old NOD mice.

Next, we investigated to see if the increase in $\text{InsB}_{9-23}^+\text{CD4}^+$ T cells in 12-week-old NOD mice could be due to a direct effect of OX40L on OX40^+ Teff cells leading to their expansion or on OX40^+ Tregs leading to their conversion to become Foxp3^- Teff cells in the periphery. Since OX40 expression on Teff cells was comparable in both groups of mice and OX40L failed to increase $\text{CD4}^+\text{OX40}^+$ Teff cells in either group, it is unlikely that OX40L acted directly on Teff cells. The above observations led us to investigate if OX40L treatment increased different subsets of Tregs in the thymus of 6- and 12-week-old mice that could undergo differential re-programming in the periphery (28). Interestingly, while OX40L increased labile Tregs in the thymus but not in the peripheral lymphoid organs of 12-week-old NOD mice, it induced stable Treg expansion in the thymus as well as in the periphery of 6-week-old mice. These results strongly suggested that OX40L acted primarily on the thymus where it induced marked expansion of Tregs in both age groups. Interestingly, while the Tregs that migrated from the thymus were sustained in the periphery in 6-week-old mice, they were rapidly converted into Foxp3^- T cells in 12-week-old NOD mice. Although not proven, it is possible that some of these converted Tregs may have been responsible for the noted increase in autoreactive T cells and contributed to early disease onset in 12-week-old NOD mice.

In trying to understand the reasons behind the Treg instability in 12-week-old NOD mice, we found that while splenocytes from 6-week-old mice could sustain Foxp3 expression in Tregs from either 6- or 12-week-old NOD mice (69% and 71% respectively; Fig 6B) in *ex vivo* cultures stimulated with PMA/Ionomycin, splenocytes from 12-week-old mice were less effective in sustaining Foxp3 expression in Tregs from either 12- or 6-week-old NOD mice (55% and 61% respectively). Deficiencies in IL-2 production and IL-2 signaling have been implicated in the pathogenesis of T1D in NOD mice (46). Therefore, we investigated the role of IL-2 in Treg survival and function. We found lower concentrations of IL-2 and higher levels of Th17 related cytokines in splenocyte cultures from 12-week-old NOD mice when compared to splenocyte cultures from 6-week-old NOD mice. Interestingly, 6-week-old splenocyte Treg co-cultures produced a significantly higher amount of IL-2 relative to 12-week-old splenocyte Treg co-cultures (Fig 7B). In addition, the reduced capacity of 12-week-old splenocytes to sustain Foxp3 expression in Tregs in PMA/Ionomycin stimulated *ex vivo* cultures could be rectified upon addition of exogenous IL-2 (Fig 7C). Collectively, these data suggested that a deficit in IL-2 production by the splenocytes from 12-week-old NOD mice was likely responsible for their reduced capacity to sustain Foxp3 expression in Tregs. Thus, it is possible that the rapid development of diabetes in 12-week-old NOD mice may be a result of Treg instability.

The phenomenon of Treg cell instability and/or plasticity has been implicated in the pathogenesis of autoimmune diseases in both mice and humans (47). One study on NOD mice showed that loss of Foxp3 expression in Tregs during autoimmune diabetes led to conversion of suppressor Tregs into a highly auto-aggressive Foxp3^- population (i.e. exFoxp3 cells) expressing IL-17 (48). These NOD mice had higher percentages of Foxp3^- T cells in pancreatic infiltrates. Moreover, BDC2.5 TCR-transgenic exFoxp3 cells were capable of inducing severe insulinitis and diabetes upon adoptive transfer into NOD/SCID mice (48). It has been suggested that this Treg instability may be related to a deficiency in IL-2 signaling in Tregs, thus leading to the loss of Foxp3 expression and conversion into

diabetogenic Teff cells (48) Polymorphism in IL-2R α (CD25) gene has been previously linked to type 1 diabetes (49–51). Although not statistically significant, we found higher mean percentages of Foxp3⁺CD25⁻ Tregs in the spleen of 12-week old compared to 6-week old NOD mice (Supplementary Fig 3). Interestingly, increased percentages of this subpopulation in the pancreatic infiltrate has been found to correlate with late stages of inflammation (52). Further, increase in the percentages of a similar subpopulation was also found in T1DM patients (53). This could potentially be a response to lower IL-2 availability concomitant with age. Additionally, a progressive breakdown in T cell tolerance leading to the development of diabetes in NOD mice has been associated with a defect in IL-2 production (44). Our results showed expansion of labile Tregs with concomitant increase in autoantigen specific Teff cells in 12-week-old NOD mice, while stable Tregs were expanded with associated reduction in autoantigen specific Teff cells in 6-week-old mice. Interestingly, addition of exogenous IL-2 into Treg cultures from 12-week-old mice prevented their conversion and thus sustained Treg numbers in cultures stimulated with PMA/ionomycin. These findings are consistent with findings from other studies, which have shown that IL-2 treatment alone was sufficient to increase Tregs in NOD mice and confer protection from the development of diabetes (44, 54). Most interestingly, low dose IL-2 is being tested in clinical trials to determine its efficacy in the treatment of diabetes (55, 56).

In summary, while several studies have indicated a role for OX40 agonist in enhancing Teff responses (5–11, 13, 14), some others have shown that it can suppress the development of diabetes in NOD mice (20). OX40 agonists have been shown to increase Tregs in naïve mice in a dose dependent manner (18). Thus, OX40L (or an OX40 agonist) administration can become a viable option for treating autoimmune disorders in addition to its current use in cancer therapy. An earlier study has shown that if an OX40 agonist is given before the onset of the disease, it can increase Tregs and delay the onset of the disease. Our results show, however, that if OX40L treatment is given near-onset of an autoimmune disease, it can accelerate and/or exacerbate the disease. Thus it is prudent to fully understand the potential benefits and limitations of treatment with OX40 agonists prior to their routine clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the grants # 1R41AI085677-01A1 and R01 AI107516-01A1 to Dr. Prabhakar from the National Institutes of Health. Christine Haddad was supported by pre-doctoral fellowship #13PRE14470009 from American Heart Association. Prabhakaran Kumar was supported by post-doctoral fellowship #15POST25090228 from American Heart Association.

Abbreviations

Tregs	regulatory T-cells
nTregs	natural Tregs
iTregs	inducible Tregs

NOD	Non-Obese Diabetic mouse
BM	bone marrow
DCs	dendritic cells
BMDCs	bone marrow dendritic cells
G-BMDCs	GM-CSF-induced bone marrow derived dendritic cells
LN	lymph nodes
SpDC	spleen derived dendritic cells

References

1. Bossen C, Ingold K, Tardivel A, Bodmer JL, Gaide O, Hertig S, Ambrose C, Tschopp J, Schneider P. Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human. *J Biol Chem*. 2006; 281:13964–13971. [PubMed: 16547002]
2. Hoshino A, Tanaka Y, Akiba H, Asakura Y, Mita Y, Sakurai T, Takaoka A, Nakaike S, Ishii N, Sugamura K, Yagita H, Okumura K. Critical role for OX40 ligand in the development of pathogenic Th2 cells in a murine model of asthma. *Eur J Immunol*. 2003; 33:861–869. [PubMed: 12672051]
3. Ito T, Amakawa R, Inaba M, Hori T, Ota M, Nakamura K, Takebayashi M, Miyaji M, Yoshimura T, Inaba K, Fukuhara S. Plasmacytoid dendritic cells regulate Th cell responses through OX40 ligand and type I IFNs. *J Immunol*. 2004; 172:4253–4259. [PubMed: 15034038]
4. Akiba H, Oshima H, Takeda K, Atsuta M, Nakano H, Nakajima A, Nohara C, Yagita H, Okumura K. CD28-independent costimulation of T cells by OX40 ligand and CD70 on activated B cells. *J Immunol*. 1999; 162:7058–7066. [PubMed: 10358148]
5. Vetto JT, Lum S, Morris A, Sicotte M, Davis J, Lemon M, Weinberg A. Presence of the T-cell activation marker OX-40 on tumor infiltrating lymphocytes and draining lymph node cells from patients with melanoma and head and neck cancers. *Am J Surg*. 1997; 174:258–265. [PubMed: 9324133]
6. Weinberg AD, Rivera MM, Prell R, Morris A, Ramstad T, Vetto JT, Urba WJ, Alvord G, Bunce C, Shields J. Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J Immunol*. 2000; 164:2160–2169. [PubMed: 10657670]
7. Morris A, Vetto JT, Ramstad T, Funatake CJ, Choolun E, Entwisle C, Weinberg AD. Induction of anti-mammary cancer immunity by engaging the OX-40 receptor in vivo. *Breast Cancer Res Treat*. 2001; 67:71–80. [PubMed: 11518468]
8. Sadun RE, Hsu WE, Zhang N, Nien YC, Bergfeld SA, Sabzevari H, Lutsiak ME, Khawli L, Hu P, Epstein AL. Fc-mOX40L fusion protein produces complete remission and enhanced survival in 2 murine tumor models. *J Immunother*. 2008; 31:235–245. [PubMed: 18317364]
9. Redmond WL, Ruby CE, Weinberg AD. The role of OX40-mediated co-stimulation in T-cell activation and survival. *Crit Rev Immunol*. 2009; 29:187–201. [PubMed: 19538134]
10. Weinberg AD, Morris NP, Kovacsovics-Bankowski M, Urba WJ, Curti BD. Science gone translational: the OX40 agonist story. *Immunol Rev*. 2011; 244:218–231. [PubMed: 22017441]
11. Pakala SV, Bansal-Pakala P, Halteman BS, Croft M. Prevention of diabetes in NOD mice at a late stage by targeting OX40/OX40 ligand interactions. *Eur J Immunol*. 2004; 34:3039–3046. [PubMed: 15368274]
12. Nohara C, Akiba H, Nakajima A, Inoue A, Koh CS, Ohshima H, Yagita H, Mizuno Y, Okumura K. Amelioration of experimental autoimmune encephalomyelitis with anti-OX40 ligand monoclonal antibody: a critical role for OX40 ligand in migration, but not development, of pathogenic T cells. *J Immunol*. 2001; 166:2108–2115. [PubMed: 11160262]
13. Weinberg AD, Bourdette DN, Sullivan TJ, Lemon M, Wallin JJ, Maziarz R, Davey M, Palida F, Godfrey W, Engleman E, Fulton RJ, Offner H, Vandenbark AA. Selective depletion of myelin-

- reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis. *Nat Med.* 1996; 2:183–189. [PubMed: 8574963]
14. Higgins LM, McDonald SA, Whittle N, Crockett N, Shields JG, MacDonald TT. Regulation of T cell activation in vitro and in vivo by targeting the OX40-OX40 ligand interaction: amelioration of ongoing inflammatory bowel disease with an OX40-IgG fusion protein, but not with an OX40 ligand-IgG fusion protein. *J Immunol.* 1999; 162:486–493. [PubMed: 9886424]
 15. Vu MD, Xiao X, Gao W, Degauque N, Chen M, Kroemer A, Killeen N, Ishii N, Li XC. OX40 costimulation turns off Foxp3+ Tregs. *Blood.* 2007; 110:2501–2510. [PubMed: 17575071]
 16. Griseri T, Asquith M, Thompson C, Powrie F. OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med.* 2010; 207:699–709. [PubMed: 20368580]
 17. Takeda I, Ine S, Killeen N, Ndhlovu LC, Murata K, Satomi S, Sugamura K, Ishii N. Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. *J Immunol.* 2004; 172:3580–3589. [PubMed: 15004159]
 18. Ruby CE, Yates MA, Hirschhorn-Cymerman D, Chlebeck P, Wolchok JD, Houghton AN, Offner H, Weinberg AD. Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *Journal of immunology.* 2009; 183:4853–4857.
 19. Griseri T, Asquith M, Thompson C, Powrie F. OX40 is required for regulatory T cell-mediated control of colitis. *The Journal of experimental medicine.* 2010; 207:699–709. [PubMed: 20368580]
 20. Bresson D, Foustieri G, Manenkova Y, Croft M, von Herrath M. Antigen-specific prevention of type 1 diabetes in NOD mice is ameliorated by OX40 agonist treatment. *J Autoimmun.* 2011; 37:342–351. [PubMed: 22063316]
 21. Cheatem D, Ganesh BB, Gangi E, Vasu C, Prabhakar BS. Modulation of dendritic cells using granulocyte-macrophage colony-stimulating factor (GM-CSF) delays type 1 diabetes by enhancing CD4+CD25+ regulatory T cell function. *Clin Immunol.* 2009; 131:260–270. [PubMed: 19171501]
 22. Vasu C, Dogan RN, Holterman MJ, Prabhakar BS. Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4+/CD25+ T cells and suppresses experimental autoimmune thyroiditis. *J Immunol.* 2003; 170:5511–5522. [PubMed: 12759428]
 23. Gangi E, Vasu C, Cheatem D, Prabhakar BS. IL-10-producing CD4+CD25+ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis. *J Immunol.* 2005; 174:7006–7013. [PubMed: 15905543]
 24. Sheng JR, Li L, Ganesh BB, Vasu C, Prabhakar BS, Meriggioli MN. Suppression of experimental autoimmune myasthenia gravis by granulocyte-macrophage colony-stimulating factor is associated with an expansion of FoxP3+ regulatory T cells. *J Immunol.* 2006; 177:5296–5306. [PubMed: 17015715]
 25. Sheng JR, Li LC, Ganesh BB, Prabhakar BS, Meriggioli MN. Regulatory T cells induced by GM-CSF suppress ongoing experimental myasthenia gravis. *Clin Immunol.* 2008; 128:172–180. [PubMed: 18502693]
 26. Meriggioli MN, Sheng JR, Li L, Prabhakar BS. Strategies for treating autoimmunity: novel insights from experimental myasthenia gravis. *Ann N Y Acad Sci.* 2008; 1132:276–282. [PubMed: 18567878]
 27. Bhattacharya P, Gopisetty A, Ganesh BB, Sheng JR, Prabhakar BS. GM-CSF-induced, bone-marrow-derived dendritic cells can expand natural Tregs and induce adaptive Tregs by different mechanisms. *Journal of leukocyte biology.* 2011; 89:235–249. [PubMed: 21048215]
 28. Sharma MD, Huang L, Choi JH, Lee EJ, Wilson JM, Lemos H, Pan F, Blazar BR, Pardoll DM, Mellor AL, Shi H, Munn DH. An inherently bifunctional subset of Foxp3+ T helper cells is controlled by the transcription factor eos. *Immunity.* 2013; 38:998–1012. [PubMed: 23684987]
 29. Waldron-Lynch F, von Herrath M, Herold KC. Towards a curative therapy in type 1 diabetes: remission of autoimmunity, maintenance and augmentation of beta cell mass. *Novartis Found Symp.* 2008; 292:146–155. discussion 55-8, 202-3. [PubMed: 19203097]
 30. Waid DM, Vaitaitis GM, Pennock ND, Wagner DH Jr. Disruption of the homeostatic balance between autoaggressive (CD4+CD40+) and regulatory (CD4+CD25+FoxP3+) T cells promotes diabetes. *J Leukoc Biol.* 2008; 84:431–439. [PubMed: 18469093]

31. Akirav EM, Lebastchi J, Galvan EM, Henegariu O, Akirav M, Ablamunits V, Lizardi PM, Herold KC. Detection of beta cell death in diabetes using differentially methylated circulating DNA. *Proc Natl Acad Sci U S A*. 2011; 108:19018–19023. [PubMed: 22074781]
32. Gopisetty A, Bhattacharya P, Haddad C, Bruno JC Jr, Vasu C, Miele L, Prabhakar BS. OX40L/Jagged1 cosignaling by GM-CSF-induced bone marrow-derived dendritic cells is required for the expansion of functional regulatory T cells. *J Immunol*. 2013; 190:5516–5525. [PubMed: 23630352]
33. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*. 2003; 198:1875–1886. [PubMed: 14676299]
34. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol*. 2004; 172:5149–5153. [PubMed: 15100250]
35. Hoffmann P, Boeld TJ, Eder R, Huehn J, Floess S, Wieczorek G, Olek S, Dietmaier W, Andreesen R, Edinger M. Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. *Eur J Immunol*. 2009; 39:1088–1097. [PubMed: 19283780]
36. Tran DQ, Andersson J, Hardwick D, Bebris L, Illei GG, Shevach EM. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. *Blood*. 2009; 113:5125–5133. [PubMed: 19299332]
37. Kishimoto H, Sprent J. Negative selection in the thymus includes semimature T cells. *J Exp Med*. 1997; 185:263–271. [PubMed: 9016875]
38. Sprent J, Kishimoto H. The thymus and central tolerance. *Philos Trans R Soc Lond B Biol Sci*. 2001; 356:609–616. [PubMed: 11375064]
39. Nepom GT. MHC class II tetramers. *J Immunol*. 2012; 188:2477–2482. [PubMed: 22389204]
40. Zhang L, Nakayama M, Eisenbarth GS. Insulin as an autoantigen in NOD/human diabetes. *Curr Opin Immunol*. 2008; 20:111–118. [PubMed: 18178393]
41. Chaparro RJ, Dileonzo TP. An update on the use of NOD mice to study autoimmune (Type 1) diabetes. *Expert Rev Clin Immunol*. 2010; 6:939–955. [PubMed: 20979558]
42. Daniel D, Gill RG, Schloot N, Wegmann D. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur J Immunol*. 1995; 25:1056–1062. [PubMed: 7537670]
43. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature*. 2005; 435:220–223. [PubMed: 15889095]
44. Tang Q, Adams JY, Penaranda C, Melli K, Piaggio E, Sgouroudis E, Piccirillo CA, Salomon BL, Bluestone JA. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity*. 2008; 28:687–697. [PubMed: 18468463]
45. Hulme MA, Wasserfall CH, Atkinson MA, Brusko TM. Central role for interleukin-2 in type 1 diabetes. *Diabetes*. 2012; 61:14–22. [PubMed: 22187370]
46. Turka LA, Walsh PT. IL-2 signaling and CD4+ CD25+ Foxp3+ regulatory T cells. *Front Biosci*. 2008; 13:1440–1446. [PubMed: 17981641]
47. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, Tanaka S, Bluestone JA, Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nat Med*. 2014; 20:62–68. [PubMed: 24362934]
48. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*. 2009; 10:1000–1007. [PubMed: 19633673]
49. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, Bailey R, Nejentsev S, Field SF, Payne F, Lowe CE, Szeszeko JS, Hafler JP, Zeitels L, Yang JH, Vella A, Nutland S, Stevens HE, Schuilburg H, Coleman G, Maisuria M, Meadows W, Smink LJ, Healy B, Burren OS, Lam AA, Ovington NR, Allen J, Adlem E, Leung HT, Wallace C, Howson JM, Guja C, Ionescu-Tirgoviste C, Genetics of Type 1 Diabetes in F, Simmonds MJ, Heward JM, Gough SC, Wellcome Trust Case Control C, Dunger DB, Wicker LS, Clayton DG. Robust associations of four new chromosome

- regions from genome-wide analyses of type 1 diabetes. *Nat Genet.* 2007; 39:857–864. [PubMed: 17554260]
50. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, Bourget K, Plagnol V, Field S, Atkinson M, Clayton DG, Wicker LS, Todd JA. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. *Nat Genet.* 2007; 39:1074–1082. [PubMed: 17676041]
51. Qu HQ, Montpetit A, Ge B, Hudson TJ, Polychronakos C. Toward further mapping of the association between the IL2RA locus and type 1 diabetes. *Diabetes.* 2007; 56:1174–1176. [PubMed: 17395754]
52. Kaminitz A, Mizrahi K, Askenasy N. Surge in regulatory T cells does not prevent onset of hyperglycemia in NOD mice: immune profiles do not correlate with disease severity. *Autoimmunity.* 2014; 47:105–112. [PubMed: 24328490]
53. Zoka A, Barna G, Somogyi A, Muzes G, Olah A, Al-Aissa Z, Hadarits O, Kiss K, Firneisz G. Extension of the CD4(+)Foxp3(+)CD25(-/low) regulatory T-cell subpopulation in type 1 diabetes mellitus. *Autoimmunity.* 2015; 48:289–297. [PubMed: 25523632]
54. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, Cagnard N, Carpentier W, Tang Q, Bluestone J, Chatenoud L, Klatzmann D, Salomon BL, Piaggio E. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. *J Exp Med.* 2010; 207:1871–1878. [PubMed: 20679400]
55. Rosenzweig M, Churlaud G, Mallone R, Six A, Derian N, Chaara W, Lorenzon R, Long SA, Buckner JH, Afonso G, Pham HP, Hartemann A, Yu A, Pugliese A, Malek TR, Klatzmann D. Low-dose interleukin-2 fosters a dose-dependent regulatory T cell tuned milieu in T1D patients. *J Autoimmun.* 2015; 58:48–58. [PubMed: 25634360]
56. Yu A, Snowwhite I, Vendrame F, Rosenzweig M, Klatzmann D, Pugliese A, Malek TR. Selective IL-2 responsiveness of regulatory T cells through multiple intrinsic mechanisms supports the use of low-dose IL-2 therapy in type 1 diabetes. *Diabetes.* 2015; 64:2172–2183. [PubMed: 25576057]

Fig 1. GM-CSF-differentiated BMDCs derived from NOD mice express OX40L and induce Treg expansion *ex vivo*

A) BMDCs were isolated from the femur of NOD mice and cultured with GM-CSF for 7 days. GM-CSF differentiated-BMDCs were analyzed for CD11c and OX40L expression at day 0 (upper panel) and day 7 (lower panel) of GM-CSF culture. After 7 days of GM-CSF culture, about 35% of CD11c⁺ BMDCs express OX40L (lower right). **B)** CFSE labeled CD4⁺ T cells were co-cultured, in the absence of any exogenous antigen, with SpDCs (Control) or GM-CSF differentiated BMDCs from NOD mice. After 5 days of co-culture, cells were analyzed by FACS for Treg proliferation. **C)** CFSE labeled CD4⁺ T-cells were co-cultured with either splenic dendritic cells (SpDCs), or total, OX40L⁺ or OX40L⁻ enriched G-BMDCs for 5 days without exogenous antigen and analyzed by FACS for Treg proliferation. **D)** 1×10⁶ G-BMDCs or SpDCs were adoptively transferred via i.v. tail injection into 10-week-old NOD mice. 1 week post-transfer, spleens and PLNs were analyzed for Foxp3⁺Treg expansion (n=3).

Fig 2. Divergent effects of soluble OX40L treatment in 6- and 12-week-old NOD mice

A) Soluble OX40L treatment induces rapid onset of T1D in 12-week-old, but not in 6-week-old, NOD mice (12-week control $n = 6$, 12-week OX40L $n = 5$, 6-week control $n = 6$, 6-week OX40L $n = 6$). Plot showing incidence of hyperglycemia (Blood glucose > 200 mg/dl) in 12- or 6-week-old NOD mice that were left untreated or treated with OX40L (200 μ g/mouse/week $\times 3$ treatments). **B)** H&E stained pancreatic tissue sections from untreated and OX40L-treated 12- and 6-week-old NOD mice showing different degrees of lymphocytic infiltration and islet damage. **C)** Effect of OX40L treatment on Tregs in the spleen and the pancreatic lymph nodes from 12- or 6-week-old NOD mice, after three treatments of OX40L or compared to untreated controls. **D)** Bar graph comparing CD4⁺Foxp3⁺ populations in thymic tissues isolated from 12- or 6-week-old NOD mice, after three treatments of OX40L or compared to untreated controls ($n = 3$).

Fig 3. Divergent outcomes of OX40L treatment of 6-week vs. 12-week-old mice is specific to the NOD strain

A–B) Thymectomy of NOD mice was performed at 4-weeks of age. Mice were treated with OX40L (200ug/mouse×3 treatments, 1 treatment/week) starting at 6 weeks of age. **A)** Representative histogram showing analysis of CD4⁺Foxp3⁺ T-cells in spleen (top) and PLN (bottom) in treated and untreated thymectomized mice and **B)** Bar graphs showing statistical analysis of populations shown in A (n=3). **C)** Spleen (top), PLN (middle) and thymus (bottom) were isolated from 12 week old Balb/c mice, following three treatments with OX40L. Cells were analyzed for Foxp3 expression by FACS. **D)** Bar graphs showing statistical analysis of populations shown in C; (n=3); * indicates p< 0.05.

Fig 4. OX40L treatment increases autoreactive T cells in the PLN of 12-week-old NOD mice
A) Spleen (top) and PLN (bottom) were isolated from 12-or 6-week-old NOD mice, following three treatments with OX40L. Presence of autoreactive T cells was determined using InsB9-23 MHCII tetramers. **B)** Bar graphs are statistical representation of percentages of InsB9-23⁺ CD4⁺ T cells; (n=3). # indicates p<0.05 while comparing OX40L-treated and untreated within 6-week age groups and * indicates p<0.05 while comparing OX40L treated and untreated within 12-week age group as well as between OX40L-treated 6-week and OX40L-treated 12-week age groups.

Fig 5. OX40L treatment leads to increase in different subsets of Tregs in 6- and 12-week-old NOD mice

A–B) OX40 Expression in the thymus and periphery of 12- and 6-week-old NOD mice: Thymus, spleen and PLNs from 12- and 6-week-old NOD mice were stained with OX40 and CD4 specific antibodies to determine surface expression of OX40 on CD4⁺ T cells and analyzed by FACs. **A)** Representative histograms, **B)** Bar graphs showing OX40 expression on CD4⁺ T-cells in control and OX40L treated mice at different age groups (left panel) and Foxp3 expression within CD4⁺OX40⁺ T cells between control and OX40L treated mice at different age groups (right panel) (n=3). **C)** Thymus (top), spleen (middle), PLNs (bottom), from OX40L or untreated control mice were analyzed for stable Tregs (Foxp3⁺CD103⁺CD38⁻) and labile Tregs (Foxp3⁺CD103⁻CD38⁺). **D)** Shows group statistics; (#) indicates statistical significance between 6-week-old OX40L vs. 6-week-old controls; (n=3) (*) indicates statistical significance (p<0.05) between 12-week-old OX40L vs. 12-week-old controls.

Fig 6. 12-week NOD mice exhibit reduced ability to sustain Foxp3 expression in activated Tregs

A) Increased conversion of Foxp3⁺Tregs from 12 week old NOD mice *ex vivo*. Sorted CD25⁺ CD4⁺Tregs were stained for Foxp3 to determine the purity of the sorted population isolated from spleens or PLNs of 12- or 6-week-old NOD mice (Top panel). Tregs were then CFSE labeled and co-cultured with indicated total splenocytes, in the presence or absence of cell stimulation cocktail (PMA/ionomycin). After 24 hrs, cells were analyzed for Foxp3 expression in the CFSE⁺ population. **B)** Sorted CD4⁺CD25⁺Tregs were stained with Foxp3 to determine the purity of the population (Top). These sorted Tregs were CFSE labeled and co-cultured with splenocytes either from their own age group (middle) or from both age groups as indicated, in the presence or absence of PMA as indicated. After 24 hrs, cells were analyzed for Foxp3 expression in the CFSE⁺ population.

Fig. 7. Reduced IL-2 production in 12-week splenocytes is correlated with increased IL-17 production

CD4⁺CD25⁺ Tregs were isolated from 6- or 12-week-old NOD mice, CellTrace labeled and co-cultured back with total splenocytes, in the presence cell stimulation cocktail (PMA/ionomycin). **A**) After 24 hrs, cells were analyzed for Foxp3 expression in the CellTrace⁺ population by FACS. **B**) Bar graphs showing average IL-2 concentration in the supernatants of stimulated 6- and 12-week-old splenocyte cultures (n=3). **C**) IL-2 prevents the conversion of Tregs in *ex vivo* cultures: Sorted CD4⁺CD25⁺ Tregs from 12- or 6-week-old mice were CFSE labeled and co-cultured with respective total splenocytes, in the absence or presence of PMA/Ionomycin and/or IL-2. Bar graphs showing Foxp3 expression in the CFSE⁺ populations after 24 hours. **D**) Representative dot plots (left panel) and bar graphs (right panel) showing IFN- γ , IL-4 and IL-17 expression by CD4⁺ T-cells 24 hours after stimulation as analyzed by FACS (n=3). **E**) Quantitative RT-PCR analysis showing fold change in the mRNA levels of different pro-inflammatory cytokines in stimulated splenocytes from 12-week-old NOD mice compared to 6-week-old NOD mice (n=3). ** indicates p-value<0.01, * indicates p-value<0.05.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
IFN- γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-12 α	CCCTTGCCCTCCTAAACCAC	AAGGAACCCTTAGAGTGCTTACT
IL-12 β	TGGTTTGCCATCGTTTGCTG	ACAGGTGAGGTTCACTGTTTCT
TNF- α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-4	GGTCTCAACCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-5	CTCTGTTGACAAGCAATGAGACG	TCTTCAGTATGTCTAGCCCCTG
IL-6	TAGTCCTTCCTACCCCAATTCC	TGGTCCTTAGCCACTCCTTC
IL-17	TTTAACTCCCTTGGCGCAAAA	CTTTCCTCCGCAATTGACAC
IL-21	GGACCCTTGTCTGTCTGGTAG	TGTGGAGCTGATAGAAGTTCAGG