Prevention of Autoimmune Diabetes and Induction of Beta-Cell Proliferation in NOD Mice by Hyperbaric Oxygen Therapy.

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<td>Manuscript ID:</td>
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<tr>
<td>Manuscript Type:</td>
<td>Original Article</td>
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<td>Date Submitted by the Author:</td>
<td>24-Feb-2012</td>
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| Key Words: | Autoimmunity, Hyperbaric Oxygen Therapy, NOD Mouse |
Prevention of Autoimmune Diabetes and Induction of Beta-Cell Proliferation in NOD mice by Hyperbaric Oxygen Therapy.

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Running title: Hyperbaric Oxygen Therapy Prevents T1D in NOD Mice

Abstract: 189 words | Manuscript: 3,330 words | Figures: 5 (1 BW + 4 color) | References: 49

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ABSTRACT

We evaluated the effects of Hyperbaric Oxygen Therapy (HOT) on autoimmune diabetes development in Non-Obese Diabetic (NOD) mice. Animals received no treatment or daily 60-min HOT 2.0ATA, 100% oxygen and were monitored for diabetes onset, insulitis, infiltrating cells, immune cell function, beta-cell apoptosis and proliferation. Cyclophosphamide-induced diabetes onset was reduced from 85.3% in controls to 48% following HOT-100% (p<0.005), and paralleled by lower insulitis. Spontaneous diabetes incidence reduced from 85% in controls to 65% in HOT-100% (p=0.01). Prediabetic mice receiving HOT-100% showed lower insulitis scores, reduced T-cell proliferation upon stimulation in vitro (p<0.03), increased CD62L expression in T-lymphocytes (p<0.04), reduced co-stimulation markers (CD40, DC80 and CD86) and MHC-II expression in dendritic cells (p<0.025), compared to controls. After autoimmunity was established, HOT was less effective. HOT-100% yielded reduced apoptosis (TUNEL+Insulin+ cells; p<0.01) and increased proliferation (bromodeoxyuridine incorporation; p<0.001) of insulin+ cells than controls. HOT reduces autoimmune diabetes incidence in NOD mice via increased resting T-lymphocytes and reduced activation of dendritic cells with preservation of beta-cell mass resulting from decreased apoptosis and increased proliferation. The safety profile and non-invasiveness makes HOT an appealing adjuvant therapy for diabetes prevention and intervention trials.
INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disorder caused by autoreactive T cells, which mediate the destruction of insulin-producing pancreatic beta-cells leading to life-long dependence on exogenous insulin. Methods to achieve and maintain normoglycemia are currently based on insulin therapy, diet and exercise. Unfortunately, while able to delay/prevent chronic complications of diabetes, intensive insulin therapy not always achieves tight daily glycemic control and associates with increased frequency of severe hypoglycemia.

An ideal treatment for T1D may combine strategies aiming at restoring self immune tolerance with others focused on preservation/restoration of functional beta-cell mass. Different approaches have been proposed (1), including prevention studies in high risk subjects, timely interventions at the time of diabetes onset, delayed interventions to restore self tolerance and beta-cell regeneration, and replacement of beta-cell mass via islet or pancreas transplantation (2). Desirable therapeutic regimens should be effective (alone or in combination), readily accessible, and void of severe risks for the patients (1).

Increasing data supports the multiple beneficial effects of hyperbaric oxygen therapy (HOT), which has been clinically used to improve oxygen supply to hypoperfused tissues (i.e., carbon monoxide exposure, embolism and ischemic events, diabetic ulcers, amongst other). Anti-inflammatory properties (3-7) and mobilization of bone marrow stem cells (BMSC) that are involved in tissue repair processes (8-11) have been attributed to HOT. The known safety profile and non-invasive nature of HOT with virtually absent side effects makes its use attractive for the treatment of autoimmune diseases (12; 13). In a murine Lupus model, HOT was associated with reduced mortality, decreased proteinuria, altered lymphocyte-subset redistribution, reduced anti-DNA antibody titers, and amelioration of immune-complex deposition (14).
The NOD mouse is widely utilized as a ‘preclinical’ model of T1D to assess therapeutic approaches able to prevent/halt autoimmune-mediated beta-cell loss, though the success in diabetes prevention have been difficult to translate to the clinical arena (15-17). Herein, we report that HOT can prevent/delay the onset of autoimmune diabetes in NOD mice and that this phenomenon is associated with reduced mononuclear cell infiltrating the islets and increased beta-cell proliferation.

MATERIALS AND METHODS

A complete description of the Materials and Methods is included in the Online Supplement (18-22).

RESULTS

Prevention of accelerated autoimmune diabetes onset in NOD mice by chronic HOT.

Cyclophosphamide (CyP) administration leads to accelerated diabetes onset in NOD mice (23-25). A single dose of CyP resulted in diabetes onset in 85.3% of control untreated mice (n=34; median=13.5 days, range:11-36)(Fig.1A-B). Application of 2.0ATA HOT-100% once or twice daily starting one-week prior to CyP treatment resulted in a sizable reduction of diabetes incidence, with only 48% (n=25; range:11-34 days; p<0.005 vs. control) and 40% (n=10; range:11-14 days; p<0.05 vs. control) of animals developing the disease, respectively (Fig.1A). Shorter HOT application (one week prior to, until one week after CyP) yielded only partial reduction of diabetes incidence to 70% (n=10; median=14 days, range:11-21; p=n.s.)(Fig.1A). These results suggested that chronic HOT was required in our setting, and that a single daily HOT was as effective as multiple administrations. Thus, subsequent studies used single daily HOT at 2.0ATA.

Other experiments evaluated different oxygen concentrations (Fig.1B). Diabetes onset occurred in 75% and 80% of mice receiving HOT-12% (n=8; median=13 days, range:10-14) and
HOT-21% (n=10; median=14 days, range:11-14), respectively (p=n.s. vs. control)(Fig.1B).

These data indicate that high oxygen concentration is required to observe protection in this model. Pressures >2.6ATA were not used due to observed discomfort and morbidity (not shown). Histopathology revealed statistically significant reduction of insulitis in HOT-100% mice compared to controls (Fig.1C). The mean insulitis score was 1.48±0.15 in control euglycemic (n=125 islets), 0.83±0.19 in HOT euglycemic (n=37; p<0.05 vs. control diabetic), 2.18±0.17 in control diabetic (n=76; p<0.05 vs. control euglycemic) and 1.32±0.16 in HOT diabetic (n=84; p<0.05 vs. control diabetic); One-way ANOVA: p<0.0001 (Fig.1C). Evaluation of markers of activation, resting and regulatory T-cells in blood, spleens and pLN from HOT-100% and untreated controls at days 0, 3, 6 and 9 after CyP administration yielded no remarkable differences (not shown).

**Prevention of spontaneous diabetes onset in NOD mice by chronic HOT.** Daily HOT (60-min, 2.0ATA) was administered chronically to female NOD mice starting at 4-weeks of age. Diabetes occurred in 85% of untreated controls (n=20; median=20.5 weeks, range:16-27). A dose-dependent protection was observed following HOT with diabetes development in 75% of mice receiving HOT-21% (n=10; median=23wks, range:17-32; p=n.s.) and in 65% of HOT-100% (n=20; median=29.5wks, range:19-33; p=0.01 vs. control; p=n.s. vs. HOT-21%)(Fig.2A).

Screening of serum Th1/Th2 cytokine levels at 10-wks of age showed only significantly higher circulating interleukin (IL)-10 in HOT-100% mice than controls (23.0±3.2 vs. 13.1±2.2pg/ml, respectively; p=0.02)(Fig.2B). Pancreata obtained from euglycemic HOT-100% animals at 25 weeks showed well-preserved islets with intense insulin immunoreactivity and minimal/no immune cell infiltrate (Fig.2C). Splenocytes from euglycemic HOT-100% mice displayed reduced *in vitro* proliferative response to mitogenic stimulation with anti-CD3 antibodies than controls (not shown). Also, the proportions of CD4+ T-cells expressing resting (CD4+CD62L+) (Fig.2D-E) and activation (CD4+CD69+) (Fig.2F-G) phenotypes in LN were
significantly higher and lower, respectively. Adoptive transfer of splenocytes from recently diabetic NOD mice into NOD.scid mice led to diabetes onset in 40% of mice (n=5), while none of the recipients of co-transfer (1:1 ratio) with splenocytes from euglycemic HOT-100% mice (n=5) developed diabetes (Fig.2H).

**Short-course HOT reduced insulitis and induced changes in immune cell function in prediabetic NOD mice.** To study the effect of HOT close to the time of diabetes onset, 13-wk old prediabetic female NOD mice were used. A 2-week course HOT-100% (60-minutes at 2.0ATA daily) associated with significantly improved insulitis scores (0.78±0.07; n=277; p<0.012) when compared to control mice (1.01±0.06; n=400 islets) (Fig.3A). The mononuclear cells infiltrating islets included CD3+ T cells, a small proportion of Tregs (Foxp3+) and B-cells (B220+) (Fig.3B). Flow cytometry analysis of splenocytes revealed unremarkable differences between HOT and controls when comparing T-cells (percent of CD4+, CD8+), Tregs (CD4+CD25+Foxp3+), overall proportions of CD11c+ cells, and antigen presenting cells (APC) (not shown). Expression of CD62L on CD8+ (Fig.3C), CD4+ (Fig.3D) and CD4+Foxp3+ (Fig.3E) T-cells was significantly higher in HOT than in untreated control mice. Significant reduced expression of co-stimulation markers CD86 (Fig.3F), CD80 (Fig.3G) and CD40 (Fig.3H), as well as MHC-II expression (Fig.3I) was observed in dendritic cells (DC) of mice receiving HOT than controls.

Assessment of the efficiency to present an islet peptide to antigen-specific CD4+ T-cells from TCR-restricted BDC2.5 mice by CD11c+ DC’s obtained from prediabetic NOD mice receiving or not 2-week course of HOT showed comparable results in vitro (not shown). Upon mitogenic stimulation via TCR engagement with anti-CD3 antibody, significantly reduced proliferation rates were observed in CD4+ cells obtained from HOT-treated mice than controls (p<0.03; Fig.3J-K).

**Short-course HOT associated with reduced apoptosis and increased proliferation of β-cells in pre-diabetic NOD mice.** The proportion of TUNEL+Insulin+ cells (means±SEM)
assessed by fluorescence microscopy on pancreatic sections was 3.0±0.6% in untreated mice and 1.3±0.6% in HOT mice (p<0.01). In NOD.scid mice that lack the autoimmune process, comparable numbers of apoptotic β-cells were observed in both control (0.6±0.2%) or HOT groups (0.3±0.1%), which were significantly lower than immunocompetent NOD mice (Fig.4A-B), suggesting that the apoptosis is due to the autoimmune attack, and that reducing insulitis by HOT in turn reduced cell death.

The proportion of proliferating (BrdU+Insulin+) β-cells was 3.9±0.4% in untreated NOD mice and significantly increased to 9.4±0.7% after HOT (p<0.001). Similarly, the proportion of BrdU+Insulin+ cells in NOD.scid mice was increased from 3.5±0.4% to 6.2±0.6% after HOT (p<0.01)(Fig.4C-D). These data suggest that HOT may induce β-cell proliferation.

Lack of stem cell mobilization by HOT. Mobilization BMSC has been recognized to contribute to HOT-mediated protection in other models through induction of nitric oxide (NO) (8; 9; 26). We assessed BMSC mobilization in prediabetic NOD mice following 2-wk HOT-100%. Lineage-negative cells represented a very small proportion of mononuclear cells (<0.6%), with comparable c-kit+, Sca+, c-kit+Sca+, and Flk-1+ cells in the bone marrow and peripheral blood of HOT and control animals (not shown). No difference in CD34+, CD31+, CD34+CD31+ and CD34+Flk-1+ cells were detected in the marrow (not shown). In addition, in the CyP-accelerated autoimmunity model, treatment with the Nitric Oxide Synthase (NOS)-inhibitor L-NAME did not preclude attaining a reduction of diabetes incidence in animals receiving HOT (not shown), suggesting that the NO pathway may not be involved in our experimental setting.

Effects of HOT on diabetes progression and recurrence. Synergy has been reported after combining GLP-1 agonists with immunotherapy at the time of spontaneous diabetes onset in NOD mice (16; 17; 27). In our study, insulin therapy alone (n=12)(Fig.5A,D) or insulin+exenatide (n=3)(Fig.5B,D) resulted in spontaneous diabetes with a median of 18 (range:0-31) and 12 (range:11-33) days, respectively. Mice receiving HOT+exenatide (n=7)
developed hyperglycemia later than controls (median=32 days, range:17-45; \(p=0.02\) vs. insulin alone) except for one mouse displaying long-term islet function (Fig.5C-D).

Syngeneic NOD.scid islets transplanted into spontaneously diabetic NOD mice lost function invariably in untreated and HOT-100% groups with a median of 8 (range:5-12; \(n=10\)) and 7 (range:4-8; \(n=5\)) days, respectively (Fig.5E). Hyperglycemia recurred in 4-of-5 animals receiving HOT+EXN (median=7, range 6-7), with one mouse showing sustained function for >150 days. In this mouse, nephrectomy of the graft-bearing kidney promptly restored hyperglycemia. The explanted graft showed well-preserved islet morphology and insulin immunoreactivity in presence of peri-insular mononuclear cell infiltrate comprising B-cells (B220+) and mostly T-cells (CD3+) with numerous Tregs (Foxp3+) (Fig.5F). The pancreas (overtly diabetic for >3 months pretransplant) displayed only few small-size islet cell clusters with rare insulin immunoreactivity and minimal mononuclear cells (Fig.5G).

Administration of HOT-100% to NOD.scid mice starting 1-wk before adoptive transfer of splenocytes from spontaneously diabetic NOD mice resulted in hyperglycemia later (\(n=5\); median=62, range:49-85) than controls (\(n=5\); median=43, range:27-62 days) (Fig.5H). To assess the effect of HOT on the proliferation of autoreactive T-cells \textit{in vivo}, fluorescently labeled BDC2.5-Thy1.1+CD4+ cells were adoptively transferred into prediabetic female NOD mice receiving or not HOT-100% for two-weeks prior to inoculum (\(n=3\)/group); comparable degrees of proliferation (measured as fluorescent dye dilution three days later) were observed in pLN (Fig.5I) while no proliferation occurred in iLN (Fig.5J).

**DISCUSSION**

Immunomodulatory properties of HOT have been reported. In mice, HOT decreased CD8+CD4+ T-cells in thymus and of B220+ B-cells in spleen (13). In a model of graft-versus-host disease following BMSC transplantation in lethally-irradiated mice, HOT ameliorated recipients' survival that was associated with reduced CD4+ and CD8+ T-cell numbers, as well
as adhesion molecule expression (CD11a and CD18)(28). Reduced islet and human fetal pancreas immunogenicity has been reported after pre-treatment with high oxygen (29) resulting in indefinite survival upon allo- and xeno-transplantation (30; 31). Depletion of Langerhans cells in allogeneic murine corneas after HOT resulted in long-term acceptance after transplantation (32). Combinatorial use of cyclosporine and HOT prevented rejection of murine allogeneic skin grafts (33). It has been also proposed that hyperoxia may ameliorate the acute net proinflammatory response generated following ischemia-reperfusion injury via inhibition of polymorphonuclear lymphocyte rolling, adhesion, activation, and transmigration to tissues (34), and/or by ameliorating tissue hypoxia—a key trigger of inflammation (35).

In autoimmune-prone MRL-lpr/lpr mice HOT resulted in marked reduction of cellularity in otherwise enlarged spleens and lymph nodes (13). Reduced mortality was reported after HOT in a murine Lupus model that was characterized by decreased proteinuria, alterations in lymphocyte-subset redistribution, anti-DNA antibody titers, and amelioration of immune-complex deposition (14). Interestingly, HOT effectively treated a clinical case of severe, mutilating vasculitis refractory to conventional therapy (36). Together these data make a compelling case for the examination of HOT in the autoimmune diabetes setting.

The autoimmune process underlying T1D is the result of genetic predisposition, immunological defects and environmental factors concurring to the development of auto-reactive T-cells. An ideal treatment for T1D should preserve functional beta-cell mass from autoimmune attack possibly before (i.e., prevention in high-risk subjects) or at the time of diagnosis. Clinical trials have shown that preservation of c-peptide can be achieved in recently diagnosed T1D following immunotherapy, but the ultimate goal of a persistent restoration of function remains elusive thus far (1). Therefore, safe interventions able to modulate immune responses and preserve beta-cell mass long-term need to be explored.
In our study, HOT was associated with significantly reduced autoimmune diabetes incidence in NOD mice in both spontaneous and accelerated (CyP-induced) experimental models. This phenomenon appeared to be dependent on the duration of HOT and oxygen concentration administered. In the CyP model, diabetes occurred in only 48% of animals receiving HOT-100% and 85.3% of controls. Diabetes incidence in the experimental groups receiving depleted oxygen (e.g., HOT-21% and -12%) hyperbaric treatment was comparable to untreated controls, indicating that high oxygen is required to elicit the protective effect of HOT.

Significantly increased IL-10 levels were measured in the sera of NOD mice following 6-wk HOT-100%. In this group, spontaneous diabetes onset was significantly reduced/delayed and associated with lower insulitis scores when compared to controls. Long-term euglycemic HOT-100% mice showed increased frequency of resting CD4+CD62L+ and lower activated CD4+CD69+ T-cells in LNs than controls. Splenocytes from HOT-100% mice suppressed disease transfer when co-implanted with recent-onset diabetic cells, suggesting the presence of cellular subsets able to suppress autoreactive immune cells. In prediabetic NOD mice, a 2-wk course HOT-100% resulted in significantly higher numbers of well-preserved islets with reduced degrees of insulitis and peri-insulitis than controls. Moreover, HOT associated with significantly increased frequencies of resting T-lymphocytes expressing CD62L. Previous studies showed that DC-expanded islet specific (e.g., isolated from BDC2.5 NOD mice) CD4+CD25+CD62L+ Tregs efficiently prevented autoimmunity when adoptively transferred to prediabetic, 13-weeks old female NOD mice (37). Inoculum of CD4+CD25+CD62L+ T-cells from BDC2.5 NOD mice at the time of diabetes onset resulted in hyperglycemia reversal and sustained normoglycemia in 50% of female NOD mice (37). We observed that CD4+ T-cells displayed lower proliferation rates than control in response to polyclonal stimulation in vitro (via anti-CD3 antibody) following HOT. Also, HOT-100% resulted in a significant reduction in APC activation measured as lower expression of co-stimulation markers than controls. Collectively, these data point to decreased
immune activation in HOT mice, which may have contributed ultimately to the reduction of insulitis and protection from diabetes observed in our study. Notably, the degree of ‘generalized immunosuppression’ achieved in our experimental setting did not appear to represent a hazard for the treated mice that, despite chronic HOT, did not show increased morbidity and mortality.

HOT appeared less effective when the autoimmune process was already established. Interfering with established autoimmune diabetes has proven quite challenging also in recent clinical intervention trials at the time of T1D onset in which immunotherapy resulted in only a transient preservation of c-peptide (1). Recurrence of autoimmunity has been recognized difficult to control even in chronically immunosuppressed pancreas transplant recipients, in which selective beta-cell destruction was associated with the persistence and increased frequency of autoreactive T-lymphocytes and autoantibody titers refractory to relatively harsh rescue immunotherapies (38).

When administered soon after spontaneous diabetes onset, HOT combined with EXN delayed the progression of the disease that occurred in the majority of the mice. Adoptive transfer of diabetes in NOD.scid mice was delayed by HOT, but not prevented. When BDC2.5-Thy1.1+CD4+ cells were adoptively transferred in prediabetic NOD mice treated or not with HOT-100%, comparable rates of proliferation were observed in pLN. Although the latter model of TCR-restricted islet-antigen-specific T-cells may be too stringent to observe protection, being the response very acute (3 days), these data suggest inadequate suppression of already activated autoreactive T-cells and point to the need for combinatorial strategies to synergize with HOT after diabetes is established. Indeed, long-term function was observed only in few animals receiving HOT+EXN at onset and in the transplant setting. Treatments that increase incretin levels are already in clinical use. The use of EXN in combinatorial regimens was shown synergistic in diabetes intervention trials in NOD mice possibly through its cytoprotective effects
on beta-cells (16; 17; 27). In addition, anti-inflammatory properties of EXN have been recently described (39), which may also contribute preserving beta-cell mass. Nonetheless, the immunomodulating effects of HOT and EXN may be inadequate to counteract the autoimmune process, which could be overcome by combinatorial use of immunotherapy (i.e., T-cell depletion, co-stimulatory blockade, or modulation of inflammatory pathways, amongst others) to enhance the success rates, particularly after autoimmunity establishment.

Significant reduction of beta-cell death was observed after HOT-100% treatment in prediabetic NOD mice. This is in keeping with the significantly lower insulitis scores observed in animals receiving HOT compared to controls, which, in turn, resulted in preservation of islet beta-cells from autoimmune destruction. Unexpectedly, a significant increase in beta-cell proliferation was observed following HOT-100%, even in immunodeficient NOD.scid mice that displayed 2-fold increase in beta-cell proliferation over baseline after HOT. Interestingly, the proportions of proliferating beta-cells were significantly lower in NOD.scid than in NOD mice, possibly as the result of a physiological increase in beta-cell proliferation occurring during the progression of insulitis in response to increased metabolic demand. Proliferation of beta-cells has been demonstrated in pancreatitis models in rodents (40), and it is possible that the inflammatory milieu in islets may activate survival/replication pathways in beta-cells as part of local tissue remodeling. Pancreatic islets are richly vascularized endocrine cell clusters representing ~1% of pancreas of which they receive ~20% of the blood supply. In mice, the appearance of endocrine cells in the embryonic pancreas coincides with the formation of new blood vessels, suggesting a key role of oxygen in the maturation of endocrine cells, which is supported by the observed enhanced maturation of pancreatic endocrine precursors in vitro into endocrine cells exposed to optimal oxygen concentrations (41), possibly via the modulation of the hypoxia-inducible factor-1α pathway (42). It is conceivable that high oxygen concentrations generated by HOT in the local microenvironment may enhance beta-cell mass and/or
regeneration in our model. Further studies are needed to understand the mechanisms underlying the increased replicative potential of beta-cells exposed to HOT. Beneficial effects of HOT on the metabolic control of patients with diabetes have been recently reported in nonrandomized studies lacking mechanistic data. Improved glycemic control and reduced insulin requirements have been reported in a pilot type 2 diabetes (T2D) trial in patients receiving HOT and intra-arterial injection of bone marrow mononuclear cells (11). Significant improvement of fasting glycemia, HbA1c levels, and HOMA-IR were reported following HOT for diabetic foot ulcers in patients with T2D (43). Improved metabolic function was also described in a large number of subjects with T1D undergoing HOT in addition to conventional therapy (44), and it has been suggested that HOT might ameliorate diabetes complications (45).

HOT may enhance tissue regeneration (46; 47) and repair processes (i.e., wound healing)(48; 49) as a result of the mobilization of marrow-derived stem cells via induction of increased nitric oxide levels (8; 9; 26). No significant differences were detected in our model to support this mechanism. This might reflect intrinsic differences in BMSC function in NOD mice, when compared to non-diabetes prone mouse strains. Also, our preliminary data suggest that the nitric oxide pathway is not involved in the beneficial effects observed following HOT, since treatment with the NOS-inhibitor L-NAME did not prevent the ability to reduce diabetes incidence in animals receiving HOT (not shown). Thus, other mechanisms may be operational in our model.

Collectively, our data suggest that HOT may reduce the incidence of autoimmune diabetes in NOD mice via reduction of insulitis possibly through the modulation of T-cell and DC functions, which in turn result in preservation of beta-cell mass via reduction of apoptosis and enhanced proliferation. Hyperbaric oxygen is a safe therapy that may be considered as part of combinatorial strategies to preserve/restore beta-cell mass and halting autoimmunity in future clinical trials. Since our results showed a stronger effect of HOT in prevention of T1D rather
than after disease onset, we envision its use for the treatment of subjects at high risk of developing T1D. In addition, the lack of side effects makes HOT an ideal candidate to be used in combinatorial therapies aimed at halting the progression of insulitis, for instance in conjunction with biologics that target immune cell function and/or agents that may enhance functional beta-cell mass. Furthermore, in depth understanding of the molecular pathways involved in the beneficial effects of HOT in T1D could help identifying potential targets for novel drugs in the future.

Author contribution: CR and AP conceived and designed the studies, analyzed data, and wrote manuscript. GF, CF, NB, RDM study design, research data, wrote manuscript. ALB and JSS study design, reviewed manuscript. EZA, JM, SV, and OU research data. Dr. Pileggi is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

ACKNOWLEDGEMENTS. This work was supported by the Diabetes Research Institute Foundation (www.DiabetesResearch.org) and in part by Converge Biotech, Inc., Miami, FL. The study relied heavily on the availability of shared infrastructures at the Diabetes Research Institute (DRI), including the Preclinical Cell Processing and Translational Models, the Imaging and the Flow Cytometry Cores (all partially supported also by the Juvenile Diabetes Research Foundation Intl.), and the University of Miami (UM) Miller School of Medicine’s Animal Care and Use Committee and Division of Veterinary Resources. The Authors are grateful to Drs. Alberto Pugliese (UM-DRI), Eckhard Podack (UM), Luca Inverardi (UM-DRI), Ricardo L. Pastori (UM-DRI), Rodolfo Alejandro (UM-DRI), Christopher A. Fraker (UM-DRI), Juan Dominguez-Bendala (UM-DRI) and George McNamara (UM-DRI), Pramod K. Srivastava (University of Connecticut), and Mark Anderson (University of California – San Francisco) for invaluable discussions.
Special thanks to Yelena Gadea (UM-DRI), Irayme Labrada (UM-DRI), Maite Lopez-Cabezas (UM-DRI) and Kevin Johnson (UM-DRI) for outstanding technical assistance.

**Funding:** This work was supported by a grant from the Diabetes Research Institute Foundation (to CR and AP) and in part by Converge Biotech, Inc. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of Interest Disclosure:** The Authors declare no conflict of interest related to the data presented in this manuscript. Converge Biotech, Inc. partially supported these studies. CR and AP are co-founders and members of the scientific advisory board of Converge Biotech, Inc. RDM, CR and AP are stock option holders in Converge Biotech Inc. There are no patents, products in development or marketed products to declare.

**REFERENCES**


FIGURE LEGENDS

Figure 1. Evaluation of hyperbaric oxygen therapy on accelerated diabetes onset in NOD mice. Prediabetic 10-week old female NOD mice were given a single intravenous dose of cyclophosphamide (CyP) on Day 0. A. Control mice (○; n=10) received no hyperbaric treatment (median=13.5 days, range:11-36). HOT consisted of 60-minutes sessions of 100% oxygen at 2.0ATA starting one week prior to CyP injection. Animals received HOT twice a day (bis in die, bid) for one (●; n=10; median=14 days; range:11-21) or four weeks (■; n=10; range:11-14 days), or once a day (quaque die, qd) for four weeks (□; n=25; range:11-34 days) after CyP administration. Nonfasting glycosuria and glycemia were monitored to determine the time of diabetes onset. *= Logrank test p<0.005; **p<0.5 vs. control. B. Single daily hyperbaric oxygen therapy (HOT; 60-minutes session, 2.0 ATA) at the indicated oxygen concentrations was started one week before CyP injection and continued daily. Time to diabetes onset after CyP injection in untreated controls (n=34), and animals exposed to hyperbaric therapy with incremental oxygen concentrations HOT-12% (resulting in tissue oxygen levels comparable of ambient air ~21% oxygen; n=8; median=13 days; range:10-14) , HOT-21% (ambient air; n=10; median=11-14 days; range:11-14) and HOT-100% (n=25). *= Logrank test p<0.05 vs. Control. C. Insulitis score on pancreatic sections of long-term euglycemic mice or after diabetes onset (Diabetic) in control and HOT 100% groups (6 sections per mouse, n=2-3 mice per group). Score 0 = no insulitis; 1 = polar and/or peri-insulitis; 2 = mild insulitis (<50% of the islet area infiltrated); 3 = severe insulitis (≥50% of the islet area infiltrated); and 4 = massive insulitis.
≥90% of the islet area infiltrated). The mean insulitis score was 1.48±0.15 in control euglycemic (n=125 islets), 0.83±0.19 in HOT euglycemic (n=37; p<0.05 vs. control diabetic), 2.18±0.17 in control diabetic (n=76; p<0.05 vs. control euglycemic) and 1.32±0.16 in HOT diabetic (n=84; p<0.05 vs. control diabetic); One-way ANOVA: p<0.0001.

**Figure 2. Impact of hyperbaric oxygen therapy on spontaneous diabetes onset in female NOD mice.**  
**A.** Daily hyperbaric oxygen therapy (HOT; 60-minutes session, 2.0 ATA) at the indicated oxygen concentrations was started at 4 weeks of age and continued for the duration of the follow-up. Control animals received no hyperbaric treatment. The graph indicates the time to diabetes onset in untreated controls (n=20; median=20.5 weeks, range:16-27), and animals exposed to HOT 21% (ambient air; n=10; median=23wks, range:17-32; p=n.s.) and 100% (n=20; median=29.5wks, range:19-33). *= Logrank test: p< 0.01 vs. control.  
**B.** Serum levels of interleukin (IL)-10 were higher in mice undergoing HOT-100% (23±3.2 pg/ml; n=9) than in untreated controls (13.1±2.2 pg/ml; n=9) at 10-weeks of age (6-weeks since initiation of HOT). Data for each reading and mean (bar) are shown. Unpaired, two-tailed t-test: p=0.02.  
**C.** Representative microscopic images of pancreatic sections from euglycemic mice in the HOT-100% group at 25 wks of age. Hematoxilin and eosin (H&E) staining and immunofluorescent confocal microscopy for insulin (INS, red), glucagon (GLC, green) and nuclear staining (DAPI, blue)  
**D-E.** Expression of CD62L in CD4+ cells in inguinal (iLN) and pancreatic (pLN) lymph nodes of control (Ctrl) and HOT-100% (HOT) mice; * p<0.01.  
**F-G.** Expression of activation marker CD69 in CD4+ cells in inguinal (iLN) and pancreatic (pLN) lymph nodes of control (Ctrl) and HOT-100% (HOT) mice (n=2-3 per group; data expressed as mean±SEM; * p<0.01).  
**H.** Proportion of NOD.scid mice developing diabetes after adoptive transfer of 20x10^6 NOD splenocytes obtained from recently diabetic NOD mice only (40% n=5) or in combination (1:1 ratio) with splenocytes from euglycemic HOT-100% (0%;n=5) at the end of the follow-up.
Figure 3. **Impact of short course hyperbaric oxygen therapy in pre-diabetic female NOD mice.** Single daily hyperbaric oxygen therapy (HOT; 60-minutes sessions, 2.0 ATA) was given to pre-diabetic, 13-week old female NOD mice for two weeks. Control animals received no hyperbaric treatment. **A.** Insulitis score on pancreatic sections in control and HOT 100% groups. The mean insulitis score was 1.01±0.06 in control (n=400 islets) and 0.78±0.07 in HOT (n=277; p<0.012), respectively (at least 3 sections per mouse, n=6 mice per group). Data is presented as mean proportion of islets in a given score per section. **B.** Pancreatic sections from animals in the two groups; hematoxylin and eosin (H&E) stain, and immunohistochemistry with antibodies directed to, CD3+ T-cells, Treg cells expressing Foxp3 and the B-cell marker B220, respectively. **C-I.** Flowcytometry of splenocytes from HOT-100%-treated or control mice. Increased CD62L expression in HOT-100% treated mice vs. controls in T lymphocytes CD8+ (72.7±4.0 vs. 59.1±9.0; p<0.03)(C), CD4+(58.7±2.3 vs. 50.4±6.1; p<0.04)(D), and CD4+Foxp3+ Tregs (44.5±3.5 vs. 36.5±4.2; p<0.03)(E). Representative histograms for each cell subset (upper panels) and overall distribution (n=4-5 mice per condition). Significant reduction in expression of co-stimulation markers CD80 (49.1±1.5% vs. 59.0±1.3%; p=0.001)(F), CD86 (11.1±1.0% vs. 15.6±1.0%; p=0.018)(G) and CD40 (72.7±1.6% vs. 83.7±3.5%; p=0.020)(H) in CD11b+CD11c+ splenic DC's from HOT-100% mice vs. controls(n=5 mice per condition), as well as expression of MHC class II in CD11b-CD11c+ splenic DC's (62.7±1.2% vs. 69.5±2.1%; n=5 mice per condition; * p<0.025)(I). **J-K.** In vitro proliferation of CD4+cells from HOT-100% treated or control mice exposed to anti CD3 stilmulation. Dilution of celltrace dye (J), and [³H]-Thymidine incorporation (counts per minute, cpm). *p<0.03 (K).

Figure 4. **Impact of a short course hyperbaric oxygen therapy on β cell apoptosis and proliferation in pre-diabetic female NOD mice.** Single daily hyperbaric oxygen therapy (HOT 100%; 60-minutes sessions, 2.0 ATA) was given to pre-diabetic, 13-weeks old female NOD or NOD.scid (age- and sex-matched) mice for two consecutive weeks. Control animals received...
no hyperbaric treatment. **A.** Proportion of apoptotic β-cells. Data are presented as percent TUNEL+Insulin+ cells per islet and are representative of at least 7-12 islets per animal and 6 animals per group. Data are presented as mean±SEM. One-way ANOVA *p<0.0001; Newman-Keuls Multiple Comparison Test *p<0.05; **p<0.01. **B.** Representative immunofluorescence micrographs of pancreatic sections from control and HOT groups immunolabeled with anti-insulin antibody (red fluorescence), TUNEL (apoptosis, green fluorescence) and DAPI (nuclei, blue). White arrowheads indicate TUNEL-positive nuclei. **C.** Proportion of proliferating β-cells based on BrdU incorporation over a period of one week. Data are presented as percent Insulin+BrdU+ cells per islet and are representative of at least 7-12 islets per animal and 6 animals per group. Data are expressed as mean±SEM. One-way ANOVA *p<0.0001; Newman-Keuls Multiple Comparison Test *p<0.01; **p<0.001. **D.** Representative immunofluorescence micrographs of pancreatic sections from prediabetic NOD mice undergoing HOT 100% or no treatment immunolabeled with anti-insulin (white color), anti-BrdU (proliferating cell’s nuclei, green), and anti-B220 (B lymphocytes, red) antibodies and DAPI (nuclei, blue).

**Figure 5. Impact of hyperbaric oxygen therapy on diabetes progression and recurrence.**

**A-D.** Non-fasting blood glucose profile of recently diabetic NOD mice that were treated with either Insulin therapy only (INS) by the use of subcutaneous insulin pellets (n=12)(A), Insulin and Exenatide (EXN), administered at 1.5ug/day for 14 days via a mini-osmotic pump (n=3)(B), or HOT-100%, Insulin and Exenatide (n=7)(C). **D.** Time of recurrence of overt hyperglycemia after initiating therapy. Median survival times were 18 (range:0-31), 12 (range:11-33) and 32 days (range:17-45; Logrank test *p=0.02 vs. insulin alone) for groups receiving INS, INS+EXN, or INS+EXN+HOT, respectively. **E.** Syngeneic islet graft survival in diabetic NOD mice recipients of NOD.scid islets under the kidney capsule. Recurrence of diabetes occurred in untreated (--) and HOT-100% (○) groups with a median of 8 (range:5-12; n=10) and 7 (range:4-8; n=5) days, respectively. In the HOT-100%+Exenatide 4/5 mice developed diabetes promptly
(median=7 days, range 6-7), and one maintained graft function for >150 days. **F.** Histological assessment of islet graft in mouse maintaining syngeneic graft function for >150 days; Hematoxylin and eosin (H&E), Immunostaining for insulin (red fluorescence), T-cells (CD3), Tregs (Foxp3) and B-cells (B-220). **G.** Pancreas H&E for same mouse. **H.** Diabetes incidence in NOD.Scid mice treated (●) or not (○) with HOT-100% after transfer of splenocytes from recently diabetic NOD mice. Median survival time were 62 days (range:49-85; n=5) and 43 (range:27-62; n=5) for HOT-treated and controls respectively. \( p=NS \). **I-J.** Proliferation of autoreactive cells *in vivo*. CD4+ cells from BDC2.5-Thy1.1+ were labeled with cell-trace dye and transferred into prediabetic NOD mice. After three days, cell trace dye dilution, evaluated by flow cytometry, was comparable in pancreatic lymph nodes of HOT-100% and control mice (n=5 per group; \( p=NS \))(I), while no proliferation was observed in inguinal lymph nodes (J).
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ONLINE SUPPLEMENT

MATERIALS AND METHODS

Animals. Studies were performed under protocols approved by the University of Miami Animal Care and Use Committee. NOD/MrkTac mice (Taconic), NOD.CB17-Prkdc<sup>scid</sup>/J mice (NOD.scid; Jackson Laboratories), and Thy1.1-BDC2.5 TCR transgenic NOD mice (kindly provided by Dr. Zhibin Chen) were housed at the Division of Veterinary Resources of the University of Miami medical campus in Virus Antibody-Free rooms into micro-isolated cages, exposed to 12-hour light/dark cycle with <i>ad libitum</i> access to autoclaved food and water.

Hyperbaric Treatment. Mice were transferred into Plexiglas cages that were placed into small animal hyperbaric chambers (RSI-B11; Reimers Systems). Control animals were handled in parallel in a similar manner without hyperbaric treatment. Each hyperbaric session started by applying increasing pressures over a 5-minute period followed by 60-minute continuous exposure to either 100% oxygen (HOT-100%), pressurized ambient air (21% oxygen, HOT-21%) or oxygen-depleted air mixture (12% oxygen, HOT-12%, to achieve ~21% oxygen tension in tissues, that is normoxia) at 2.0 atmospheres absolute (ATA). The pressure in the chamber was slowly reduced over 5-minutes before opening it.

Diabetes monitoring. Animals were monitored 3 times a week for glycosuria (Diastix; Bayer); if positive, glycemic levels were confirmed and tested thereafter on whole blood (tail vein pricking) using portable glucometers (OneTouchUltra2; Lifescan)(1). Diabetes was defined as nonfasting glycemic values ≥300mg/dl on two consecutive readings.

Experimental models.

Accelerated autoimmune diabetes onset was studied in 10-week old female NOD mice received (day 0) single cyclophosphamide (CyP; 200mg/kg; Sigma-Aldrich) injection intraperitoneally. Experimental groups received daily HOT-100%, HOT-21%, HOT-12% or no hyperbaric treatment, starting one week before CyP injection. Notably, CyP is activated through
oxygen-dependent P450 system and therefore high oxygen tensions may enhance its activity. To avoid this potential bias, HOT was not administered on the day of CyP injection in our studies. Animals were monitored for diabetes onset for at least 4 weeks.

**Spontaneous autoimmune diabetes** onset was evaluated in female NOD mice exposed to either daily HOT-100%, HOT-21% or no treatment starting on week 4 of age up to 35 weeks. Also, prediabetic 13-week old female NOD and NOD.scid mice underwent or not a 2-week course HOT-100% to study its impact on insulitis and islet cell proliferation.

**Intervention at the time of spontaneous diabetes onset** in female NOD mice was started after detection of glycosuria and confirmed elevation of nonfasting glycemia ≥250mg/dl on two consecutive days. One-to-two insulin pellets (LinShin) were implanted subcutaneously within few days from diabetes onset. Animals received 2-week glucagon-like peptide (GLP)-1 analog treatment (1.5µg/day, Exenatide, EXN; Amylin) subcutaneously (osmotic pump; Alzet) with or without HOT-100%. Recurrence of hyperglycemia during insulin therapy and after exhaustion of insulin pellets was assessed.

**Recurrence of autoimmunity.** Pancreatic islets isolated from NOD.scid mice were implanted under the kidney capsule of spontaneously diabetic NOD mice treated or not with HOT-100% starting two weeks before transplant (day 0) with or without EXN (1.5µg/day via intraperitoneal osmotic pump for 2-weeks starting on day 0). Nonfasting glycemic values monitored to determine the recurrence of hyperglycemia after transplantation.

**Adoptive transfer** of splenocytes (20x10^6) from recent-onset diabetic NOD mice into NOD.scid mice undergoing or not HOT was also performed. In other experiments, >90% fractions of fluorescently-labelled (Celltrace, Invitrogen) CD4+Thy1.1+ T-cells obtained by positive selection (MACS®, Miltenyi) from BDC2.5 TCR-transgenic NOD mice (TCR-specific for an islet antigen in the context of MHC class II, l-Ag7)(2) were adoptively transferred (1.2x10^6 cells/mouse) into
naïve NOD female mice undergoing or not HOT-100% and their frequency assessed by flow cytometry in pancreatic lymph nodes explanted 3 days later.

**Cytokine Detection.** Serum from experimental animals was assayed for Th1/Th2 cytokines using the Bio-Plex Pro™ Mouse Cytokine 8-plex Assay on a BioPlex instrument (BioRad), as per manufacturer instructions.

**Flow Cytometry Analysis.** Phenotype and functional state of immune cell subsets from spleens, pancreatic lymph nodes (pLN) and peripheral blood were assessed, as described (3). Peripheral blood mononuclear cells (PBMC) were purified from whole blood on Ficoll-Paque™ Plus gradient (GE Healthcare Bio-Sciences AB). Spleen and pLN were collected and single cell suspensions filtered through nylon mesh. Bone marrow cell suspensions were obtained by flushing femurs and tibias (4). Red blood cells were lysed (ACK Lysis Buffer). Cells ($10^6$) were then incubated 20-minutes at 4°C with rat anti-CD16/32 (clone 24G2) and successively with specific primary antibodies conjugated with different fluorochromes (BD-Pharmingen) in FACS buffer (30-minutes, 4°C). Samples were acquired using a LSR-II instrument and analyzed with FACSDiva Software (BD-Pharmingen) at the DRI Flow Cytometry Core.

**Immune cell functional assays in vitro.** Enrichment of splenic CD4+ T-lymphocytes or CD11c+ dendritic cells (DC) was achieved by positive selection using specific surface antibodies and magnetic beads (MACS, Miltenyi) followed by cell sorting on a BD FACSaria instrument to attain final purity of ≥95%.

**Histopathology.** Pancreata were collected and stored in 10% buffered formalin solution or frozen at -80°C (Tissue-Tek® OCT Compound). Four-µm thick, formalin-fixed, paraffin-embedded sections (5/organ) were stained with hematoxylin and eosin (H&E) and assessed blindly by light microscopy. Scoring criteria: 0=no insulitis; 1=peri-insulitis (infiltration restricted to the periphery of islets); 2=mild insulitis (<50% of the islet area infiltrated); 3=severe insulitis
Mice received 5'-bromo-2'-deoxy-uridine (BrdU; 1mg/mL; Sigma-Aldrich) in the drinking water for 2-weeks and incorporation in proliferating cells was assessed by fluorescence microscopy on paraffin sections using specific antibody (1:50; Accurate)(5). To discriminate between endocrine and immune cells, co-staining was performed with guinea pig anti-insulin (1:100; DAKO) and rat anti–CD45/B220 (1:100; eBioscience). Secondary antibodies: goat anti-guinea pig AlexaFluor-488 (1:200) and goat anti-rat AlexaFluor-568 (1:200; Invitrogen).

Immunohistochemistry was performed on paraffin-embedded tissue sections. Slides were pre-treated with 10mM citrate at pH 6.0 (Biocare Medical) or 1mM EDTA (pH 8.0) in a steam pressure cooker for antigen retrieval. Primary antibodies: rabbit anti–CD3 (1:1500 in EDTA; CellMarque), rat anti–CD45/B220 (1:100), rat anti–FoxP3 (1:25; eBioscience). Secondary rabbit anti-rat (1:750; DAKO) was used for Foxp3 detection. Hematoxylin and Dako Envision plus system-HRP (DAB) were used. Images were taken on Leica DMLB microscope (Leica Application Suite v.3.6.0). Apoptosis was assessed using DeadEnd Fluorometric TUNEL system (Promega). Digital images were acquired using a Zeiss Apotome microscope. Analysis was performed with Metamorph software.

**Statistical analysis.** Survival curves were compared by Logrank test using Prism 5.0 (Graphpad) and data expressed as median with range. Two-tailed, unpaired Student’s t-test two group comparison, and one-way analysis of variance (ANOVA) for multiple comparisons were used. All *in vitro* determinations are means±standard error of the means (SEM) from at least three independent conditions. Results were considered statistically significant if \( p<0.05.\)

**REFERENCES**


Prevention of Autoimmune Diabetes and Induction of Beta-Cell Proliferation in NOD mice by Hyperbaric Oxygen Therapy.

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Running title: Hyperbaric Oxygen Therapy Prevents T1D in NOD Mice

Abstract: 189 words | Manuscript: 3,330 words | Figures: 5 (1 BW + 4 color) | References: 49

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ABSTRACT

We evaluated the effects of Hyperbaric Oxygen Therapy (HOT) on autoimmune diabetes development in Non-Obese Diabetic (NOD) mice. Animals received no treatment or daily 60-min HOT 2.0ATA, 100% oxygen and were monitored for diabetes onset, insulitis, infiltrating cells, immune cell function, beta-cell apoptosis and proliferation. Cyclophosphamide-induced diabetes onset was reduced from 85.3% in controls to 48% following HOT-100% ($p<0.005$), and paralleled by lower insulitis. Spontaneous diabetes incidence reduced from 85% in controls to 65% in HOT-100% ($p=0.01$). Prediabetic mice receiving HOT-100% showed lower insulitis scores, reduced T-cell proliferation upon stimulation in vitro ($p<0.03$), increased CD62L expression in T-lymphocytes ($p<0.04$), reduced co-stimulation markers (CD40, DC80 and CD86) and MHC-II expression in dendritic cells ($p<0.025$), compared to controls. After autoimmunity was established, HOT was less effective. HOT-100% yielded reduced apoptosis (TUNEL+Insulin+ cells; $p<0.01$) and increased proliferation (bromodeoxyuridine incorporation; $p<0.001$) of insulin+ cells than controls. HOT reduces autoimmune diabetes incidence in NOD mice via increased resting T-lymphocytes and reduced activation of dendritic cells with preservation of beta-cell mass resulting from decreased apoptosis and increased proliferation. The safety profile and non-invasiveness makes HOT an appealing adjuvant therapy for diabetes prevention and intervention trials.
INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disorder caused by autoreactive T cells, which mediate the destruction of insulin-producing pancreatic beta-cells leading to life-long dependence on exogenous insulin. Methods to achieve and maintain normoglycemia are currently based on insulin therapy, diet and exercise. Unfortunately, while able to delay/prevent chronic complications of diabetes, intensive insulin therapy not always achieves tight daily glycemic control and associates with increased frequency of severe hypoglycemia.

An ideal treatment for T1D may combine strategies aiming at restoring self immune tolerance with others focused on preservation/restoration of functional beta-cell mass. Different approaches have been proposed (1), including prevention studies in high risk subjects, timely interventions at the time of diabetes onset, delayed interventions to restore self tolerance and beta-cell regeneration, and replacement of beta-cell mass via islet or pancreas transplantation (2). Desirable therapeutic regimens should be effective (alone or in combination), readily accessible, and void of severe risks for the patients (1).

Increasing data supports the multiple beneficial effects of hyperbaric oxygen therapy (HOT), which has been clinically used to improve oxygen supply to hypoperfused tissues (i.e., carbon monoxide exposure, embolism and ischemic events, diabetic ulcers, amongst other). Anti-inflammatory properties (3-7) and mobilization of bone marrow stem cells (BMSC) that are involved in tissue repair processes (8-11) have been attributed to HOT. The known safety profile and non-invasive nature of HOT with virtually absent side effects makes its use attractive for the treatment of autoimmune diseases (12; 13). In a murine Lupus model, HOT was associated with reduced mortality, decreased proteinuria, altered lymphocyte-subset redistribution, reduced anti-DNA antibody titers, and amelioration of immune-complex deposition (14).
The NOD mouse is widely utilized as a ‘preclinical’ model of T1D to assess therapeutic approaches able to prevent/halt autoimmune-mediated beta-cell loss, though the success in diabetes prevention have been difficult to translate to the clinical arena (15-17). Herein, we report that HOT can prevent/delay the onset of autoimmune diabetes in NOD mice and that this phenomenon is associated with reduced mononuclear cell infiltrating the islets and increased beta-cell proliferation.

MATERIALS AND METHODS

A complete description of the Materials and Methods is included in the Online Supplement (18-22).

RESULTS

Prevention of accelerated autoimmune diabetes onset in NOD mice by chronic HOT.

Cyclophosphamide (CyP) administration leads to accelerated diabetes onset in NOD mice (23-25). A single dose of CyP resulted in diabetes onset in 85.3% of control untreated mice (n=34; median=13.5 days, range:11-36)\(^{(\text{Fig.1A-B})}\). Application of 2.0ATA HOT-100% once or twice daily starting one-week prior to CyP treatment resulted in a sizable reduction of diabetes incidence, with only 48% (n=25; range:11-34 days; \(p<0.005\) vs. control) and 40% (n=10; range:11-14 days; \(p<0.05\) vs. control) of animals developing the disease, respectively \((\text{Fig.1A})\). Shorter HOT application (one week prior to, until one week after CyP) yielded only partial reduction of diabetes incidence to 70% (n=10; median=14 days, range:11-21; \(p=\text{n.s.}\))\((\text{Fig.1A})\). These results suggested that chronic HOT was required in our setting, and that a single daily HOT was as effective as multiple administrations. Thus, subsequent studies used single daily HOT at 2.0ATA.

Other experiments evaluated different oxygen concentrations \((\text{Fig.1B})\). Diabetes onset occurred in 75% and 80% of mice receiving HOT-12% (n=8; median=13 days, range:10-14) and
HOT-21% (n=10; median=14 days, range:11-14), respectively (p=n.s. vs. control)(Fig.1B). These data indicate that high oxygen concentration is required to observe protection in this model. Pressures >2.6ATA were not used due to observed discomfort and morbidity (not shown). Histopathology revealed statistically significant reduction of insulitis in HOT-100% mice compared to controls (Fig.1C). The mean insulitis score was 1.48±0.15 in control euglycemic (n=125 islets), 0.83±0.19 in HOT euglycemic (n=37; p<0.05 vs. control diabetic), 2.18±0.17 in control diabetic (n=76; p<0.05 vs. control euglycemic) and 1.32±0.16 in HOT diabetic (n=84; p<0.05 vs. control diabetic); One-way ANOVA: p<0.0001 (Fig.1C). Evaluation of markers of activation, resting and regulatory T-cells in blood, spleens and pLN from HOT-100% and untreated controls at days 0, 3, 6 and 9 after CyP administration yielded no remarkable differences (not shown).

Prevention of spontaneous diabetes onset in NOD mice by chronic HOT. Daily HOT (60-min, 2.0ATA) was administered chronically to female NOD mice starting at 4-weeks of age. Diabetes occurred in 85% of untreated controls (n=20; median=20.5 weeks, range:16-27). A dose-dependent protection was observed following HOT with diabetes development in 75% of mice receiving HOT-21% (n=10; median=23wks, range:17-32; p=n.s.) and in 65% of HOT-100% (n=20; median=29.5wks, range:19-33; p=0.01 vs. control; p=n.s. vs. HOT-21%)(Fig.2A).

Screening of serum Th1/Th2 cytokine levels at 10-wks of age showed only significantly higher circulating interleukin (IL)-10 in HOT-100% mice than controls (23.0±3.2 vs. 13.1±2.2 pg/ml, respectively; p=0.02)(Fig.2B). Pancreata obtained from euglycemic HOT-100% animals at 25 weeks showed well-preserved islets with intense insulin immunoreactivity and minimal/no immune cell infiltrate (Fig.2C). Splenocytes from euglycemic HOT-100% mice displayed reduced in vitro proliferative response to mitogenic stimulation with anti-CD3 antibodies than controls (not shown). Also, the proportions of CD4+ T-cells expressing resting (CD4+CD62L+)(Fig.2D-E) and activation (CD4+CD69+)(Fig.2F-G) phenotypes in LN were
significantly higher and lower, respectively. Adoptive transfer of splenocytes from recently diabetic NOD mice into NOD.scid mice led to diabetes onset in 40% of mice (n=5), while none of the recipients of co-transfer (1:1 ratio) with splenocytes from euglycemic HOT-100% mice (n=5) developed diabetes (Fig.2H).

**Short-course HOT reduced insulitis and induced changes in immune cell function in pre-diabetic NOD mice.** To study the effect of HOT close to the time of diabetes onset, 13-wk old prediabetic female NOD mice were used. A 2-week course HOT-100% (60-minutes at 2.0ATA daily) associated with significantly improved insulitis scores (0.78±0.07; n=277; p<0.012) when compared to control mice (1.01±0.06; n=400 islets) (Fig.3A). The mononuclear cells infiltrating islets included CD3+ T cells, a small proportion of Tregs (Foxp3+) and B-cells (B220+) (Fig.3B). Flow cytometry analysis of splenocytes revealed unremarkable differences between HOT and controls when comparing T-cells (percent of CD4+, CD8+), Tregs (CD4+CD25+Foxp3+), overall proportions of CD11c+ cells, and antigen presenting cells (APC)(not shown). Expression of CD62L on CD8+ (Fig.3C), CD4+ (Fig.3D) and CD4+Foxp3+ (Fig.3E) T-cells was significantly higher in HOT than in untreated control mice. Significant reduced expression of co-stimulation markers CD86 (Fig.3F), CD80 (Fig.3G) and CD40 (Fig.3H), as well as MHC-II expression (Fig.3I) was observed in dendritic cells (DC) of mice receiving HOT than controls.

Assessment of the efficiency to present an islet peptide to antigen-specific CD4+ T-cells from TCR-restricted BDC2.5 mice by CD11c+ DC’s obtained from prediabetic NOD mice receiving or not 2-week course of HOT showed comparable results *in vitro* (not shown). Upon mitogenic stimulation via TCR engagement with anti-CD3 antibody, significantly reduced proliferation rates were observed in CD4+ cells obtained from HOT-treated mice than controls (p<0.03; Fig.3J-K).

**Short-course HOT associated with reduced apoptosis and increased proliferation of β-cells in pre-diabetic NOD mice.** The proportion of TUNEL+Insulin+ cells (means±SEM)
assessed by fluorescence microscopy on pancreatic sections was 3.0±0.6% in untreated mice and 1.3±0.6% in HOT mice (p<0.01). In NOD.scid mice that lack the autoimmune process, comparable numbers of apoptotic β-cells were observed in both control (0.6±0.2%) or HOT groups (0.3±0.1%), which were significantly lower than immunocompetent NOD mice ([Fig.4A-B]), suggesting that the apoptosis is due to the autoimmune attack, and that reducing insulitis by HOT in turn reduced cell death.

The proportion of proliferating (BrdU+Insulin+) β-cells was 3.9±0.4% in untreated NOD mice and significantly increased to 9.4±0.7% after HOT (p<0.001). Similarly, the proportion of BrdU+Insulin+ cells in NOD.scid mice was increased from 3.5±0.4% to 6.2±0.6% after HOT (p<0.01) ([Fig.4C-D]). These data suggest that HOT may induce β-cell proliferation.

**Lack of stem cell mobilization by HOT.** Mobilization BMSC has been recognized to contribute to HOT-mediated protection in other models through induction of nitric oxide (NO) (8; 9; 26). We assessed BMSC mobilization in prediabetic NOD mice following 2-wk HOT-100%. Lineage-negative cells represented a very small proportion of mononuclear cells (<0.6%), with comparable c-kit+, Sca+, c-kit+Sca+, and Flk-1+ cells in the bone marrow and peripheral blood of HOT and control animals (not shown). No difference in CD34+, CD31+, CD34+CD31+ and CD34+Flk-1+ cells were detected in the marrow (not shown). In addition, in the CyP-accelerated autoimmunity model, treatment with the Nitric Oxide Synthase (NOS)-inhibitor L-NAME did not preclude attaining a reduction of diabetes incidence in animals receiving HOT (not shown), suggesting that the NO pathway may not be involved in our experimental setting.

**Effects of HOT on diabetes progression and recurrence.** Synergy has been reported after combining GLP-1 agonists with immunotherapy at the time of spontaneous diabetes onset in NOD mice (16; 17; 27). In our study, insulin therapy alone (n=12) ([Fig.5A,D]) or insulin+exenatide (n=3) ([Fig.5B,D]) resulted in spontaneous diabetes with a median of 18 (range:0-31) and 12 (range:11-33) days, respectively. Mice receiving HOT+exenatide (n=7)
developed hyperglycemia later than controls (median=32 days, range:17-45; \( p=0.02 \) vs. insulin alone) except for one mouse displaying long-term islet function (Fig.5C-D).

Syngeneic NOD.scid islets transplanted into spontaneously diabetic NOD mice lost function invariably in untreated and HOT-100% groups with a median of 8 (range:5-12; n=10) and 7 (range:4-8; n=5) days, respectively (Fig.5E). Hyperglycemia recurred in 4-of-5 animals receiving HOT+EXN (median=7, range 6-7), with one mouse showing sustained function for >150 days. In this mouse, nephrectomy of the graft-bearing kidney promptly restored hyperglycemia. The explanted graft showed well-preserved islet morphology and insulin immunoreactivity in presence of peri-insular mononuclear cell infiltrate comprising B-cells (B220+) and mostly T-cells (CD3+) with numerous Tregs (Foxp3+) (Fig.5F). The pancreas (overtly diabetic for >3 months pretransplant) displayed only few small-size islet cell clusters with rare insulin immunoreactivity and minimal mononuclear cells (Fig.5G).

Administration of HOT-100% to NOD.scid mice starting 1-wk before adoptive transfer of splenocytes from spontaneously diabetic NOD mice resulted in hyperglycemia later (n=5; median=62, range:49-85) than controls (n=5; median=43, range:27-62 days) (Fig.5H). To assess the effect of HOT on the proliferation of autoreactive T-cells in vivo, fluorescently labeled BDC2.5-Thy1.1+CD4+ cells were adoptively transferred into prediabetic female NOD mice receiving or not HOT-100% for two-weeks prior to inoculum (n=3/group); comparable degrees of proliferation (measured as fluorescent dye dilution three days later) were observed in pLN (Fig.5I) while no proliferation occurred in iLN (Fig.5J).

**DISCUSSION**

Immunomodulatory properties of HOT have been reported. In mice, HOT decreased CD8+CD4+ T-cells in thymus and of B220+ B-cells in spleen (13). In a model of graft-versus-host disease following BMSC transplantation in lethally-irradiated mice, HOT ameliorated recipients’ survival that was associated with reduced CD4+ and CD8+ T-cell numbers, as well
as adhesion molecule expression (CD11a and CD18)(28). Reduced islet and human fetal pancreas immunogenicity has been reported after pre-treatment with high oxygen (29) resulting in indefinite survival upon allo- and xeno-transplantation (30; 31). Depletion of Langerhans cells in allogeneic murine corneas after HOT resulted in long-term acceptance after transplantation (32). Combinatorial use of cyclosporine and HOT prevented rejection of murine allogeneic skin grafts (33). It has been also proposed that hyperoxia may ameliorate the acute net proinflammatory response generated following ischemia-reperfusion injury via inhibition of polymorphonuclear lymphocyte rolling, adhesion, activation, and transmigration to tissues (34), and/or by ameliorating tissue hypoxia—a key trigger of inflammation (35).

In autoimmune-prone MRL-<sup>lpr/lpr</sup> mice HOT resulted in marked reduction of cellularity in otherwise enlarged spleens and lymph nodes (13). Reduced mortality was reported after HOT in a murine Lupus model that was characterized by decreased proteinuria, alterations in lymphocyte-subset redistribution, anti-DNA antibody titers, and amelioration of immune-complex deposition (14). Interestingly, HOT effectively treated a clinical case of severe, mutilating vasculitis refractory to conventional therapy (36). Together these data make a compelling case for the examination of HOT in the autoimmune diabetes setting.

The autoimmune process underlying T1D is the result of genetic predisposition, immunological defects and environmental factors concurring to the development of autoimmune T-cells. An ideal treatment for T1D should preserve functional beta-cell mass from autoimmune attack possibly before (i.e., prevention in high-risk subjects) or at the time of diagnosis. Clinical trials have shown that preservation of c-peptide can be achieved in recently diagnosed T1D following immunotherapy, but the ultimate goal of a persistent restoration of function remains elusive thus far (1). Therefore, safe interventions able to modulate immune responses and preserve beta-cell mass long-term need to be explored.
In our study, HOT was associated with significantly reduced autoimmune diabetes incidence in NOD mice in both spontaneous and accelerated (CyP-induced) experimental models. This phenomenon appeared to be dependent on the duration of HOT and oxygen concentration administered. In the CyP model, diabetes occurred in only 48% of animals receiving HOT-100% and 85.3% of controls. Diabetes incidence in the experimental groups receiving depleted oxygen (e.g., HOT-21% and -12%) hyperbaric treatment was comparable to untreated controls, indicating that high oxygen is required to elicit the protective effect of HOT.

Significantly increased IL-10 levels were measured in the sera of NOD mice following 6-wk HOT-100%. In this group, spontaneous diabetes onset was significantly reduced/delayed and associated with lower insulitis scores when compared to controls. Long-term euglycemic HOT-100% mice showed increased frequency of resting CD4+CD62L+ and lower activated CD4+CD69+ T-cells in LNs than controls. Splenocytes from HOT-100% mice suppressed disease transfer when co-implanted with recent-onset diabetic cells, suggesting the presence of cellular subsets able to suppress autoreactive immune cells. In prediabetic NOD mice, a 2-wk course HOT-100% resulted in significantly higher numbers of well-preserved islets with reduced degrees of insulitis and peri-insulitis than controls. Moreover, HOT associated with significantly increased frequencies of resting T-lymphocytes expressing CD62L. Previous studies showed that DC-expanded islet specific (e.g., isolated from BDC2.5 NOD mice) CD4+CD25+CD62L+ Tregs efficiently prevented autoimmunity when adoptively transferred to prediabetic, 13-weeks old female NOD mice (37). Inoculum of CD4+CD25+CD62L+ T-cells from BDC2.5 NOD mice at the time of diabetes onset resulted in hyperglycemia reversal and sustained normoglycemia in 50% of female NOD mice (37). We observed that CD4+ T-cells displayed lower proliferation rates than control in response to polyclonal stimulation in vitro (via anti-CD3 antibody) following HOT. Also, HOT-100% resulted in a significant reduction in APC activation measured as lower expression of co-stimulation markers than controls. Collectively, these data point to decreased
immune activation in HOT mice, which may have contributed ultimately to the reduction of insulitis and protection from diabetes observed in our study. Notably, the degree of ‘generalized immunosuppression’ achieved in our experimental setting did not appear to represent a hazard for the treated mice that, despite chronic HOT, did not show increased morbidity and mortality.

HOT appeared less effective when the autoimmune process was already established. Interfering with established autoimmune diabetes has proven quite challenging also in recent clinical intervention trials at the time of T1D onset in which immunotherapy resulted in only a transient preservation of c-peptide (1). Recurrence of autoimmunity has been recognized difficult to control even in chronically immunosuppressed pancreas transplant recipients, in which selective beta-cell destruction was associated with the persistence and increased frequency of autoreactive T-lymphocytes and autoantibody titers refractory to relatively harsh rescue immunotherapies (38).

When administered soon after spontaneous diabetes onset, HOT combined with EXN delayed the progression of the disease that occurred in the majority of the mice. Adoptive transfer of diabetes in NOD.scid mice was delayed by HOT, but not prevented. When BDC2.5-Thy1.1+CD4+ cells were adoptively transferred in prediabetic NOD mice treated or not with HOT-100%, comparable rates of proliferation were observed in pLN. Although the latter model of TCR-restricted islet-antigen-specific T-cells may be too stringent to observe protection, being the response very acute (3 days), these data suggest inadequate suppression of already activated autoreactive T-cells and point to the need for combinatorial strategies to synergize with HOT after diabetes is established. Indeed, long-term function was observed only in few animals receiving HOT+EXN at onset and in the transplant setting. Treatments that increase incretin levels are already in clinical use. The use of EXN in combinatorial regimens was shown synergistic in diabetes intervention trials in NOD mice possibly through its cytoprotective effects.
on beta-cells (16; 17; 27). In addition, anti-inflammatory properties of EXN have been recently described (39), which may also contribute preserving beta-cell mass. Nonetheless, the immunomodulating effects of HOT and EXN may be inadequate to counteract the autoimmune process, which could be overcome by combinatorial use of immunotherapy (i.e., T-cell depletion, co-stimulatory blockade, or modulation of inflammatory pathways, amongst others) to enhance the success rates, particularly after autoimmunity establishment.

Significant reduction of beta-cell death was observed after HOT-100% treatment in prediabetic NOD mice. This is in keeping with the significantly lower insulitis scores observed in animals receiving HOT compared to controls, which, in turn, resulted in preservation of islet beta-cells from autoimmune destruction. Unexpectedly, a significant increase in beta-cell proliferation was observed following HOT-100%, even in immunodeficient NOD.scid mice that displayed 2-fold increase in beta-cell proliferation over baseline after HOT. Interestingly, the proportions of proliferating beta-cells were significantly lower in NOD.scid than in NOD mice, possibly as the result of a physiological increase in beta-cell proliferation occurring during the progression of insulitis in response to increased metabolic demand. Proliferation of beta-cells has been demonstrated in pancreatitis models in rodents (40), and it is possible that the inflammatory milieu in islets may activate survival/replication pathways in beta-cells as part of local tissue remodeling. Pancreatic islets are richly vascularized endocrine cell clusters representing ~1% of pancreas of which they receive ~20% of the blood supply. In mice, the appearance of endocrine cells in the embryonic pancreas coincides with the formation of new blood vessels, suggesting a key role of oxygen in the maturation of endocrine cells, which is supported by the observed enhanced maturation of pancreatic endocrine precursors in vitro into endocrine cells exposed to optimal oxygen concentrations (41), possibly via the modulation of the hypoxia-inducible factor-1α pathway (42). It is conceivable that high oxygen concentrations generated by HOT in the local microenvironment may enhance beta-cell mass and/or
regeneration in our model. Further studies are needed to understand the mechanisms underlying the increased replicative potential of beta-cells exposed to HOT. Beneficial effects of HOT on the metabolic control of patients with diabetes have been recently reported in nonrandomized studies lacking mechanistic data. Improved glycemic control and reduced insulin requirements have been reported in a pilot type 2 diabetes (T2D) trial in patients receiving HOT and intra-arterial injection of bone marrow mononuclear cells (11). Significant improvement of fasting glycemia, HbA1c levels, and HOMA-IR were reported following HOT for diabetic foot ulcers in patients with T2D (43). Improved metabolic function was also described in a large number of subjects with T1D undergoing HOT in addition to conventional therapy (44), and it has been suggested that HOT might ameliorate diabetes complications (45).

HOT may enhance tissue regeneration (46; 47) and repair processes (i.e., wound healing)(48; 49) as a result of the mobilization of marrow-derived stem cells via induction of increased nitric oxide levels (8; 9; 26). No significant differences were detected in our model to support this mechanism. This might reflect intrinsic differences in BMSC function in NOD mice, when compared to non-diabetes prone mouse strains. Also, our preliminary data suggest that the nitric oxide pathway is not involved in the beneficial effects observed following HOT, since treatment with the NOS-inhibitor L-NAME did not prevent the ability to reduce diabetes incidence in animals receiving HOT (not shown). Thus, other mechanisms may be operational in our model.

Collectively, our data suggest that HOT may reduce the incidence of autoimmune diabetes in NOD mice via reduction of insulitis possibly through the modulation of T-cell and DC functions, which in turn result in preservation of beta-cell mass via reduction of apoptosis and enhanced proliferation. Hyperbaric oxygen is a safe therapy that may be considered as part of combinatorial strategies to preserve/restore beta-cell mass and halting autoimmunity in future clinical trials. Since our results showed a stronger effect of HOT in prevention of T1D rather
than after disease onset, we envision its use for the treatment of subjects at high risk of developing T1D. In addition, the lack of side effects makes HOT an ideal candidate to be used in combinatorial therapies aimed at halting the progression of insulitis, for instance in conjunction with biologics that target immune cell function and/or agents that may enhance functional beta-cell mass. Furthermore, in depth understanding of the molecular pathways involved in the beneficial effects of HOT in T1D could help identifying potential targets for novel drugs in the future.

**Author contribution:** CR and AP conceived and designed the studies, analyzed data, and wrote manuscript. GF, CF, NB, RDM study design, research data, wrote manuscript. ALB and JSS study design, reviewed manuscript. EZA, JM, SV, and OU research data. Dr. Pileggi is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

**ACKNOWLEDGEMENTS.** This work was supported by the Diabetes Research Institute Foundation (www.DiabetesResearch.org) and in part by Converge Biotech, Inc., Miami, FL. The study relied heavily on the availability of shared infrastructures at the Diabetes Research Institute (DRI), including the Preclinical Cell Processing and Translational Models, the Imaging and the Flow Cytometry Cores (all partially supported also by the Juvenile Diabetes Research Foundation Intl.), and the University of Miami (UM) Miller School of Medicine’s Animal Care and Use Committee and Division of Veterinary Resources. The Authors are grateful to Drs. Alberto Pugliese (UM-DRI), Eckhard Podack (UM), Luca Inverardi (UM-DRI), Ricardo L. Pastori (UM-DRI), Rodolfo Alejandro (UM-DRI), Christopher A. Fraker (UM-DRI), Juan Dominguez-Bendala (UM-DRI) and George McNamara (UM-DRI), Pramod K. Srivastava (University of Connecticut), and Mark Anderson (University of California – San Francisco) for invaluable discussions.
Special thanks to Yelena Gadea (UM-DRI), Irayme Labrada (UM-DRI), Maite Lopez-Cabezas (UM-DRI) and Kevin Johnson (UM-DRI) for outstanding technical assistance.

**Funding:** This work was supported by a grant from the Diabetes Research Institute Foundation (to CR and AP) and in part by Converge Biotech, Inc. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of Interest Disclosure:** The Authors declare no conflict of interest related to the data presented in this manuscript. Converge Biotech, Inc. partially supported these studies. CR and AP are co-founders and members of the scientific advisory board of Converge Biotech, Inc. RDM, CR and AP are stock option holders in Converge Biotech Inc. There are no patents, products in development or marketed products to declare.

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FIGURE LEGENDS

Figure 1. Evaluation of hyperbaric oxygen therapy on accelerated diabetes onset in NOD mice. Prediabetic 10-week old female NOD mice were given a single intravenous dose of cyclophosphamide (CyP) on Day 0. **A.** Control mice (○; n=10) received no hyperbaric treatment (median=13.5 days, range:11-36). HOT consisted of 60-minutes sessions of 100% oxygen at 2.0ATA starting one week prior to CyP injection. Animals received HOT twice a day (bis in die, bid) for one (●; n=10; median=14 days, range:11-21) or four weeks (■; n=10; range:11-14 days), or once a day (quaque die, qd) for four weeks (□; n=25; range:11-34 days) after CyP administration. Nonfasting glycosuria and glycemia were monitored to determine the time of diabetes onset. *= Logrank test p<0.005; **p<0.5 vs. control. **B.** Single daily hyperbaric oxygen therapy (HOT; 60-minutes session, 2.0 ATA) at the indicated oxygen concentrations was started one week before CyP injection and continued daily. Time to diabetes onset after CyP injection in untreated controls (n=34), and animals exposed to hyperbaric therapy with incremental oxygen concentrations HOT-12% (resulting in tissue oxygen levels comparable of ambient air ~21% oxygen; n=8; median=13 days, range:10-14), HOT-21% (ambient air; n=10; median=11-14 days, range:11-14) and HOT-100% (n=25). *= Logrank test p<0.05 vs. Control. **C.** Insulitis score on pancreatic sections of long-term euglycemic mice or after diabetes onset (Diabetic) in control and HOT 100% groups (6 sections per mouse, n=2-3 mice per group). Score 0 = no insulitis; 1 = polar and/or peri-insulitis; 2 = mild insulitis (<50% of the islet area infiltrated); 3 = severe insulitis (≥50% of the islet area infiltrated); and 4 = massive insulitis
(≥90% of the islet area infiltrated). The mean insulitis score was 1.48±0.15 in control euglycemic (n=125 islets), 0.83±0.19 in HOT euglycemic (n=37; p<0.05 vs. control diabetic), 2.18±0.17 in control diabetic (n=76; p<0.05 vs. control euglycemic) and 1.32±0.16 in HOT diabetic (n=84; p<0.05 vs. control diabetic); One-way ANOVA: p<0.0001.

Figure 2. Impact of hyperbaric oxygen therapy on spontaneous diabetes onset in female NOD mice. A. Daily hyperbaric oxygen therapy (HOT; 60-minutes session, 2.0 ATA) at the indicated oxygen concentrations was started at 4 weeks of age and continued for the duration of the follow-up. Control animals received no hyperbaric treatment. The graph indicates the time to diabetes onset in untreated controls (n=20; median=20.5 weeks, range:16-27), and animals exposed to HOT 21% (ambient air; n=10; median=23wks, range:17-32; p=n.s.) and 100% (n=20; median=29.5wks, range:19-33). * = Logrank test: p<0.01 vs. control. B. Serum levels of interleukin (IL)-10 were higher in mice undergoing HOT-100% (23±3.2 pg/ml; n=9) than in untreated controls (13.1±2.2 pg/ml; n=9) at 10-weeks of age (6-weeks since initiation of HOT). Data for each reading and mean (bar) are shown. Unpaired, two-tailed t-test: p=0.02. C. Representative microscopic images of pancreatic sections from euglycemic mice in the HOT-100% group at 25 wks of age. Hematoxilin and eosin (H&E) staining and immunofluorescent confocal microscopy for insulin (INS, red), glucagon (GLC, green) and nuclear staining (DAPI, blue) D-E. Expression of CD62L in CD4+ cells in inguinal (iLN) and pancreatic (pLN) lymph nodes of control (Ctrl) and HOT-100% (HOT) mice; * p<0.01. F-G. Expression of activation marker CD69 in CD4+ cells in inguinal (iLN) and pancreatic (pLN) lymph nodes of control (Ctrl) and HOT-100% (HOT) mice (n=2-3 per group; data expressed as mean±SEM; * p<0.01). H. Proportion of NOD.scid mice developing diabetes after adoptive transfer of 20x10^6 NOD splenocytes obtained from recently diabetic NOD mice only (40% n=5) or in combination (1:1 ratio) with splenocytes from euglycemic HOT-100% (0%;n=5) at the end of the follow-up.
Figure 3. Impact of short course hyperbaric oxygen therapy in pre-diabetic female NOD mice. Single daily hyperbaric oxygen therapy (HOT; 60-minutes sessions, 2.0 ATA) was given to pre-diabetic, 13-week old female NOD mice for two weeks. Control animals received no hyperbaric treatment. A. Insulitis score on pancreatic sections in control and HOT 100% groups. The mean insulitis score was 1.01±0.06 in control (n=400 islets) and 0.78±0.07 in HOT (n=277; p<0.012), respectively (at least 3 sections per mouse, n=6 mice per group). Data is presented as mean proportion of islets in a given score per section. B. Pancreatic sections from animals in the two groups; hematoxylin and eosin (H&E) stain, and immunohistochemistry with antibodies directed to, CD3+ T-cells, Treg cells expressing Foxp3 and the B-cell marker B220, respectively. C-I. Flowcytometry of splenocytes from HOT-100%-treated or control mice. Increased CD62L expression in HOT-100% treated mice vs. controls in T lymphocytes CD8+ (72.7±4.0 vs. 59.1±9.0; p<0.03)(C), CD4+ (58.7±2.3 vs. 50.4±6.1; p<0.04)(D), and CD4+Foxp3+ Tregs (44.5±3.5 vs. 36.5±4.2; p<0.03)(E). Representative histograms for each cell subset (upper panels) and overall distribution (n=4-5 mice per condition). Significant reduction in expression of co-stimulation markers CD80 (49.1±1.5% vs. 59.0±1.3%; p=0.001)(F), CD86 (11.1±1.0% vs. 15.6±1.0%; p=0.018)(G) and CD40 (72.7±1.6% vs. 83.7±3.5%; p=0.020)(H) in CD11b+CD11c+ splenic DC’s from HOT-100% mice vs. controls (n=5 mice per condition), as well as expression of MHC class II in CD11b-CD11c+ splenic DC’s (62.7±1.2% vs. 69.5±2.1%; n=5 mice per condition; * p<0.025(I). J-K. In vitro proliferation of CD4+cells from HOT-100% treated or control mice exposed to anti CD3 stimulation. Dilution of celltrace dye (J), and [3H]-Thymidine incorporation (counts per minute, cpm). *p<0.03 (K).

Figure 4. Impact of a short course hyperbaric oxygen therapy on β cell apoptosis and proliferation in pre-diabetic female NOD mice. Single daily hyperbaric oxygen therapy (HOT 100%; 60-minutes sessions, 2.0 ATA) was given to pre-diabetic, 13-weeks old female NOD or NOD.scid (age- and sex-matched) mice for two consecutive weeks. Control animals received
no hyperbaric treatment. **A.** Proportion of apoptotic β-cells. Data are presented as percent TUNEL+Insulin+ cells per islet and are representative of at least 7-12 islets per animal and 6 animals per group. **Data are presented as mean±SEM.** One-way ANOVA $p<0.0001$; Newman-Keuls Multiple Comparison Test $^*p<0.05$; $^{**}p<0.01$. **B.** Representative immunofluorescence micrographs of pancreatic sections from control and HOT groups immunolabeled with anti-insulin antibody (red fluorescence), TUNEL (apoptosis, green fluorescence) and DAPI (nuclei, blue). White arrowheads indicate TUNEL-positive nuclei. **C.** Proportion of proliferating β-cells based on BrdU incorporation over a period of one week. Data are presented as percent Insulin+BrdU+ cells per islet and are representative of at least 7-12 islets per animal and 6 animals per group. **Data are expressed as mean±SEM.** One-way ANOVA $p<0.0001$; Newman-Keuls Multiple Comparison Test $^*p<0.01$; $^{**}p<0.001$. **D.** Representative immunofluorescence micrographs of pancreatic sections from prediabetic NOD mice undergoing HOT 100% or no treatment immunolabeled with anti-insulin (white color), anti-BrdU (proliferating cell’s nuclei, green), and anti-B220 (B lymphocytes, red) antibodies and DAPI (nuclei, blue).

**Figure 5. Impact of hyperbaric oxygen therapy on diabetes progression and recurrence.**

**A-D.** Non-fasting blood glucose profile of recently diabetic NOD mice that were treated with either Insulin therapy only (INS) by the use of subcutaneous insulin pellets (n=12)(A), Insulin and Exenatide (EXN), administered at 1.5ug/day for 14 days via a mini-osmotic pump (n=3)(B), or HOT-100%, Insulin and Exenatide (n=7)(C). **D.** Time of recurrence of overt hyperglycemia after initiating therapy. Median survival times were 18 (range:0-31), 12 (range:11-33) and 32 days (range:17-45; Logrank test $p=0.02$ vs. insulin alone) for groups receiving INS, INS+EXN, or INS+EXN+HOT, respectively. **E.** Syngeneic islet graft survival in diabetic NOD mice recipients of NOD.scid islets under the kidney capsule. Recurrence of diabetes occurred in untreated (→) and HOT-100% (○) groups with a median of 8 (range:5-12; n=10) and 7 (range:4-8; n=5) days, respectively. In the HOT-100%+Exenatide 4/5 mice developed diabetes promptly
(median=7 days, range 6-7), and one maintained graft function for >150 days. F. Histological assessment of islet graft in mouse maintaining syngeneic graft function for >150 days; Hematoxylin and eosin (H&E), Immunostaining for insulin (red fluorescence), T-cells (CD3), Tregs (Foxp3) and B-cells (B-220). G. Pancreas H&E for same mouse. H. Diabetes incidence in NOD.Scid mice treated (●) or not (○) with HOT-100% after transfer of splenocytes from recently diabetic NOD mice. Median survival time were 62 days (range:49-85; n=5) and 43 (range:27-62; n=5) for HOT-treated and controls respectively. p=NS. I-J. Proliferation of autoreactive cells in vivo. CD4+ cells from BDC2.5-Thy1.1+ were labeled with cell-trace dye and transferred into prediabetic NOD mice. After three days, cell trace dye dilution, evaluated by flow cytometry, was comparable in pancreatic lymph nodes of HOT-100% and control mice (n=5 per group; p=NS)(I), while no proliferation was observed in inguinal lymph nodes (J).