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Hyperbaric oxygen treatment attenuates the pro-inflammatory and immune responses in apolipoprotein E knockout mice

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Abstract Chronic hyperbaric oxygen (HBO) therapy significantly attenuates atherosclerosis in New Zealand white rabbits as well as the apoE knockout (KO) mice, independent of plasma lipid concentrations and lipoprotein profiles. Because atherosclerosis has many features of a chronic inflammatory disease, in which both cell-mediated and humoral immune responses participate, we examined the effect of HBO treatment on various aspects of the immune response.
We now demonstrate that in apoE KO mice, HBO treatment significantly reduces the circulating levels of antibodies to $_{MDA}$LDL, both in the IgG and IgM class, as well as the delayed-type hypersensitivity (DTH) response to oxLDL challenge. Furthermore, HBO treatment results in a profound attenuation in the production of pro-inflammatory cytokines in response to an inflammatory stimulus (LPS), which is accompanied by a marked increase in the constitutive production of the anti-inflammatory cytokine IL-10 by spleen cells, independent of antigen specificity, as indicated by polyclonal activation of T cells. Our results demonstrate that HBO treatment results in the dampening of T and B cell-mediated responses to oxLDL or inflammatory stimuli.

Introduction

There is mounting evidence that atherosclerosis is a chronic, immune-mediated inflammatory response to tissue damage in the arterial intima [1]. Tissue damage may result through increased formation of reactive oxygen species (ROS) in response to altered shear stress [2], ischemia/reperfusion or exposure to modified (oxidized) LDL [3]. ROS, through activation of the redox-sensitive transcription factors NF-κB and AP-1, induce the expression of adhesion molecules (ICAMs and VCAMs), chemotactic factors, pro-inflammatory cytokines and host-defense proteins in the vasculature [3]. Bioactive lipids in oxLDL induce the expression of CD-36 (oxLDL receptor) in macrophages [4]. The net result of these effects is an inflammatory response and a massive accumulation of macrophages and T-lymphocytes in the arterial wall [1]. Their continuous interaction with oxLDL leads to foam

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cell formation, continued production of pro-inflammatory cytokines and growth factors. These events promote cellular proliferation and sustain atherogenesis by a positive feedback loop mechanism.

We have previously shown that chronic hyperbaric oxygen (HBO) treatment of cholesterol-fed rabbits or apoE knockout (KO) mice significantly attenuates atherogenesis [5,6]. Moreover we demonstrated that the beneficial effect of HBO treatment is due to, at least in part, a substantial change in the redox environment of the arterial wall (and other tissues) to a more reductive environment. This is accomplished by the induction of a number of antioxidant enzymes as well as changes in reduced glutathione concentrations [6]. Thus one of the mechanisms through which HBO treatment may reduce atherosclerosis is by decreased formation of oxLDL, an important initial "pathogen" for this disease.

HBO treatment may also impact atherogenesis through its effects on the immune/inflammatory system. HBO treatment has been shown to reduce cytokine expression as well as mortality in animal models of septic shock and ischemia/reperfusion injuries [7–9]. The immunosuppressive effect of HBO treatment is also demonstrated by suppressed development of Th cells, decreased spleen and lymph node size, suppression of autoimmune symptoms, decreased responses to antigens, a decrease in circulating lymphocytes and leukocytes and increased allograft survival [10–13]. HBO treatment can promote Th2 cell differentiation and immune tolerance [14], which should have an anti-atherogenic effect [15–17]. In summary, HBO treatment may reduce the immune and inflammatory reactions at the vessel wall directly, by affecting the immune cell responses that contribute to these reactions, as well as indirectly, by reducing the formation of oxidized antigen (oxLDL).

The aim of the present study was therefore to establish that changes in the immune/inflammatory response represent a critical component of the beneficial effects of HBO treatment of experimental atherosclerosis. We demonstrate that in apoE KO mice, HBO treatment significantly reduces the circulating levels of antibodies to $_{MDA}$LDL, both in the IgG and IgM class, as well as the delayed hypersensitivity (DTH) response to oxLDL challenge. Furthermore, HBO treatment results in a profound attenuation in the production of pro-inflammatory cytokines in response to an inflammatory stimulus (LPS), accompanied by a marked increase in the production of the anti-inflammatory cytokine IL-10 by spleen cells.

Materials and methods

Animals

Female apoE KO mice (back-crossed for 10 generations on a C57BL/6 background) were purchased from Jackson laboratories (Bar Harbor, Maine). Mice were maintained in a specific pathogen-free environment on a 12 h light, 12 h dark cycle. They were provided with standard rodent chow and water *ad libitum*. At 5 wks of age the apoE KO mice were assigned to four groups. Two groups of apoE KO mice received HBO treatment, one for a period of 5 wks ($n=15$) while the other for a period of 10 wks ($n=15$). The other two groups of apoE KO mice ($n=15$, each) remained untreated and were used as a control group for the comparison with the HBO-treated mice. The Institutional Animal Care and Use Committee approved all the procedures with animals.

HBO treatment

HBO treatment was administered in a specialized HBO chamber for animals. Mice, caged in groups of 5, were exposed to 100% oxygen for 90 min at 2.4 atm for 5 d/wk for 5 or 10 wks. The desired pressure in the chamber was reached slowly over 15 min, and after the 90 min treatment period pressure was released slowly over 15 min. Control untreated mice were handled exactly the same way, except they were not placed into the hyperbaric chamber.

Delayed-type hypersensitivity (DTH) response

DTH responses were examined following a challenge with oxLDL [18,19]. Human plasma LDL ($d=1.019–1.063$ g/ml) was oxidized by incubating with copper sulfate [6]. Forty-eight hours before the end of the HBO treatment period, mice were lightly anesthetized and ear lobe thickness was measured with a digital caliper. Acute ear skin edema was instituted by sub-dermal injection of 20 μl of oxLDL (20 μg) in the left ear. PBS (vehicle) alone was injected into the right ear. For DTH response, the percent change in ear thickness was calculated as follows: the increase in ear thickness (difference between that at 48 h after challenge and prior to challenge) was divided by the ear thickness prior to challenge; this was multiplied by 100 [19].

Preparation of spleen mononuclear cells

Mice were anesthetized by subcutaneous injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The abdomen and chest were opened, and the organs were perfused with ice-cold PBS, excised, blotted dry and weighed. Spleens were passed through a 250 μm mesh filter by gentle mashing to obtain a single cell suspension. Single cell suspensions were collected and washed by centrifugation at 200 *g* for 10 min. After washing, red blood cells were lysed by treatment with AKC (ammonium chloride potassium) lysis buffer. Spleen cells were re-suspended in 10 ml of sterile RPMI wash medium [RPMI 1640 without sodium bicarbonate (Hyclone), supplemented with 5% FBS (Hyclone), HEPES and antibiotic/antimycotic solution (Gibco)] and washed by centrifugation at 200 *g* for 10 min. The final spleen cell preparations were re-suspended in 10 ml of RPMI wash medium and the viable cell number was determined using a hemocytometer [20].

Analysis of splenic lymphocyte populations

Splenocytes (10^6/ml) were stained with anti-CD3-FITC, anti-CD4-PE, anti-CD8-PE and anti-B220-PE mAbs and analyzed on an EPICS XL-MCL flow cytometer (Beckman Coulter). Data collection was done using System 2 software (Beckman Coulter). Lymphocyte gates and detector voltages were set

using antibody-matched isotype controls (BD PharMingen, San Diego, CA). The proportion of each cell population was expressed as the percentage of the number of stained lymphocytes. We also determined the total number of specific lymphocyte populations by multiplying their percentage by the total number of lymphocytes isolated.

Polyclonal stimulation of $CD3^+$ lymphocytes

T cells were stimulated *in vitro*, as previously described [21]. A 50 µl aliquot of purified anti-CD3 mAb (10 µg/ml, 145-2C11, BD PharMingen), diluted in PBS, was added to a sterile 96-well flat bottom microtiter plates and incubated overnight at 4 °C. Anti-CD28 mAb (37.51, BD PharMingen) was also added to the cell cultures at a concentration of 1.25 µg/ml. Plates were gently washed with sterile RPMI 1640 culture medium (Hyclone). Whole and purified lymphocyte fractions of lung and splenic mononuclear cells were placed in wells suspended in 100 µl of culture media at a final concentration of 2×10^6 cells/ml and incubated for four days at 37 °C and 5% CO_2 in air. Supernatants were collected and stored at −80 °C until assayed for IL-10 levels.

In vivo LPS-elicited cytokine production by spleen cells

Cytokine secretion by spleen cells was determined with or without prior *in vivo* stimulation with LPS. Before sacrifice, both HBO-treated and untreated groups of mice were subdivided into two groups ($n=5$): one was treated with LPS (100 µg i.p., Sigma) 4 h before sacrifice, while the other was injected with equivalent amount of PBS. Upon sacrifice, spleen cell suspensions were placed in 96-well plates and suspended in 200 µl of culture medium [RPMI 1640 (Hyclone) 5% FBS (Hyclone), HEPES and antibiotic/antimycotic solution (Gibco)] at a final concentration of 2×10^6 cells/ml and incubated at 37 °C and 5% CO_2. After 18 h of incubation the supernatants were harvested and stored at −80 °C until IL-1β, IL-6, IL-10, IL-12, IFNγ and TNFα levels were determined using ELISA (PeproTech, Rocky Hill, NJ).

Detection of anti-malondialdehyde-modified LDL antibodies

Plasma was collected from the retro-orbital sinus into heparinized capillary tubes. The levels of IgM and IgG autoantibodies binding to native and malondialdehyde modified LDL ($_{MDA}$LDL) were assayed as previously described [22]. ELISA plates were coated with diluted LDL preparations (5 µg/ml) overnight at 4 °C, followed by blocking with 5% BSA/PBS for 2 h at room temperature. 100 µl of diluted plasma (using 1:1600 and 1:100 dilution for IgM and IgG class respectively) were added to the wells and incubated at 4 °C overnight. The amount of bound Ig was revealed by incubating with a goat anti-mouse IgM or IgG conjugated with biotin, followed by incubation with avidin-conjugated horseradish peroxidase. Color was developed by the addition of substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Moss, Inc., Pasadena, CA) and the OD_{630} was determined. Specific antibody to $_{MDA}$LDL was defined as the difference between O.D. readings obtained with $_{MDA}$LDL and with native LDL.

Statistical analyses

Data are presented as mean ± SEM. Two-tailed Student's *t* test was used to identify significant differences between two groups (HBO-treated and untreated), whereas comparisons between multiple groups were analyzed by one-way ANOVA followed by Tukey's multiple mean comparison test. A probability $p \le 0.05$ was accepted as statistically significant. All analyses were performed with StatView 4.5 (Abacus Concepts, Berkley, CA) or Prism 4.0 (Graphpad Software, Inc., San Diego, CA).

Results

HBO treatment of apoE KO mice has no apparent adverse effects

There were no adverse signs observed in HBO-treated or untreated apoE KO mice. In addition to observing grooming behavior and appearance we also measured body weights as well as weights of individual organs after sacrifice. At the end of 5 or 10 wks of HBO treatment all groups of mice had similar weights. There were no differences in individual organ weights (spleen, liver or lung) and HBO treatment had no effect on the total number of cells in the spleen ($11.6\pm1.0\times10^7$ cells/ml in untreated vs. $11.8\pm1.4\times10^7$ cells/ml in HBO-treated mice). The percentages of lymphocyte subpopulations ($CD3^+CD4^+$ Th, $CD3^+CD8^+$ T, and $B220^+$ B cells) in spleens were also not affected by HBO treatment.

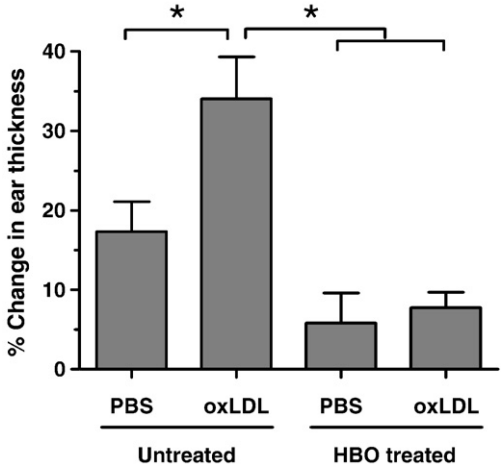

Figure 1 The inhibitory effect of chronic HBO treatment on DTH responses to oxLDL challenge. The DTH response of untreated and 10 wks HBO-treated apoE KO mice to oxLDL and PBS was measured. The results are presented from 2 experiments, as the mean ± SEM ($n=7$ for each group of untreated mice, and $n=8$ for each group of HBO-treated mice). * denotes significant differences ($p \le 0.05$) between indicated groups.

HBO treatment suppresses DTH response against oxLDL in apoE KO mice

We examined the effect of HBO on DTH responses against oxLDL in apoE KO mice. Mice were challenged by injection of oxLDL in their left ears, and as a control, the right ears of the mice were challenged with PBS. Forty-eight hours later the change in ear thickness was determined. As shown in Figure 1, ear thickness increased significantly (doubled) in untreated mice after challenge with oxLDL ($p \leq 0.05$), while in the HBO-treated mice no difference was observed. There was a trend for a slightly greater response in the ears of untreated mice challenged with PBS than in HBO-treated mice, but this was not statistically significant. However, oxLDL responses in untreated mice were significantly greater than in HBO-treated mice, indicating that HBO treatment significantly dampened the DTH response.

HBO treatment reduces the levels of autoantibodies to $_{MDA}$LDL

The levels of autoantibodies to $_{MDA}$LDL have been shown to reflect the levels of oxidatively modified LDL in circulation and correlate with the extent of atherosclerosis in mouse models [22]. As shown in Figure 2, 10 wks HBO treatment significantly reduced the circulating levels of antibodies to $_{MDA}$LDL ($p<0.005$), both in IgG (44%) and IgM (40%) classes. Antibody responses against $_{MDA}$LDL were similarly lower in mice treated for 5 wks with HBO, when compared to untreated mice (data not shown). Thus, HBO treatment resulted in lower antibody responses against $_{MDA}$LDL in apoE KO mice.

HBO treatment increases IL-10 production by stimulated splenic T cells

To determine if HBO treatment results in increased production of the anti-inflammatory cytokine IL-10 after T cell stimulation, spleen cells from HBO-treated and untreated mice were polyclonally stimulated with an anti-CD3 antibody. As shown in Figure 3, splenic cells secreted significantly greater amounts of IL-10 in response to polyclonal stimulation. Splenic cells from HBO-treated mice however secreted higher levels of IL-10 than those from untreated mice both in the absence or presence of the antibody. Thus, the stimulation of T cells from HBO-treated mice, regardless of antigen specificity, resulted in higher levels of IL-10 expression, suggesting that HBO treatment has a broad effect on the adaptive immune responses.

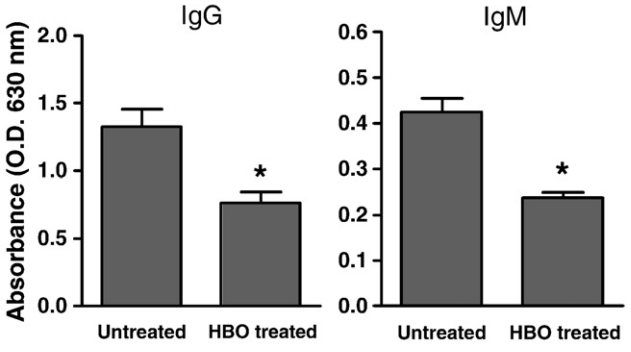

Figure 2 HBO treatment reduces the levels of autoantibodies to $_{MDA}$LDL. The levels of IgM and IgG against oxLDL were measured in untreated and 10 wk HBO-treated apoE KO. O.D. indicates optical density at 630 nm, corrected for antibody binding to native LDL. The results are presented as the mean ± SEM ($n=5$ in each group). * denotes significant differences ($p \leq 0.05$) in antibody levels between HBO-treated and untreated groups of mice.

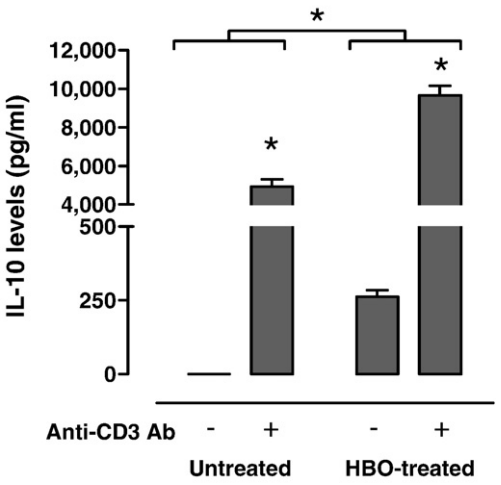

Figure 3 HBO treatment increases IL-10 production by stimulated splenic T cells. Splenocytes were collected from untreated mice or mice treated with HBO for 10 weeks. Polyclonal activation of T cells was accomplished with anti-CD3 and anti-CD28 antibodies for 4 days, and supernatants were collected and analyzed for IL-10 levels by ELISA. Values represent mean ± SEM ($n=5$ in each group). * denotes significant differences ($p \leq 0.05$) in cytokine expression between unstimulated and stimulated cells of indicated groups of mice.

Inflammatory cytokine responses by splenocytes are reduced by HBO treatment

To determine if the effects of HBO treatment were limited to adaptive immune responses against oxLDL, the response to *in vivo* LPS administration was assessed. Mice (HBO-treated or untreated) were treated with LPS (i.p.) 4 h prior to sacrifice. Spleen cells were then removed and their *in vitro* capacity to secrete inflammatory cytokines was measured. As expected, and shown in Figure 4, LPS treatment stimulated cytokine secretion by splenocytes regardless of the HBO treatment (all except IL-10). HBO treatment however significantly reduced the *in vitro* production of pro-inflammatory cytokines (IL-1ß, IL-12, TNFα and IFNγ $p \leq 0.05$) by spleen cells from LPS-treated mice. A similar trend was observed with splenocytes from unstimulated mice. In direct contrast to the pro-inflammatory cytokines, the production of IL-10, an anti-inflammatory cytokine, was significantly higher from splenocytes from HBO-treated mice. Interestingly, there was no significant increase in IL-10 production by cells from either group of animals in response to

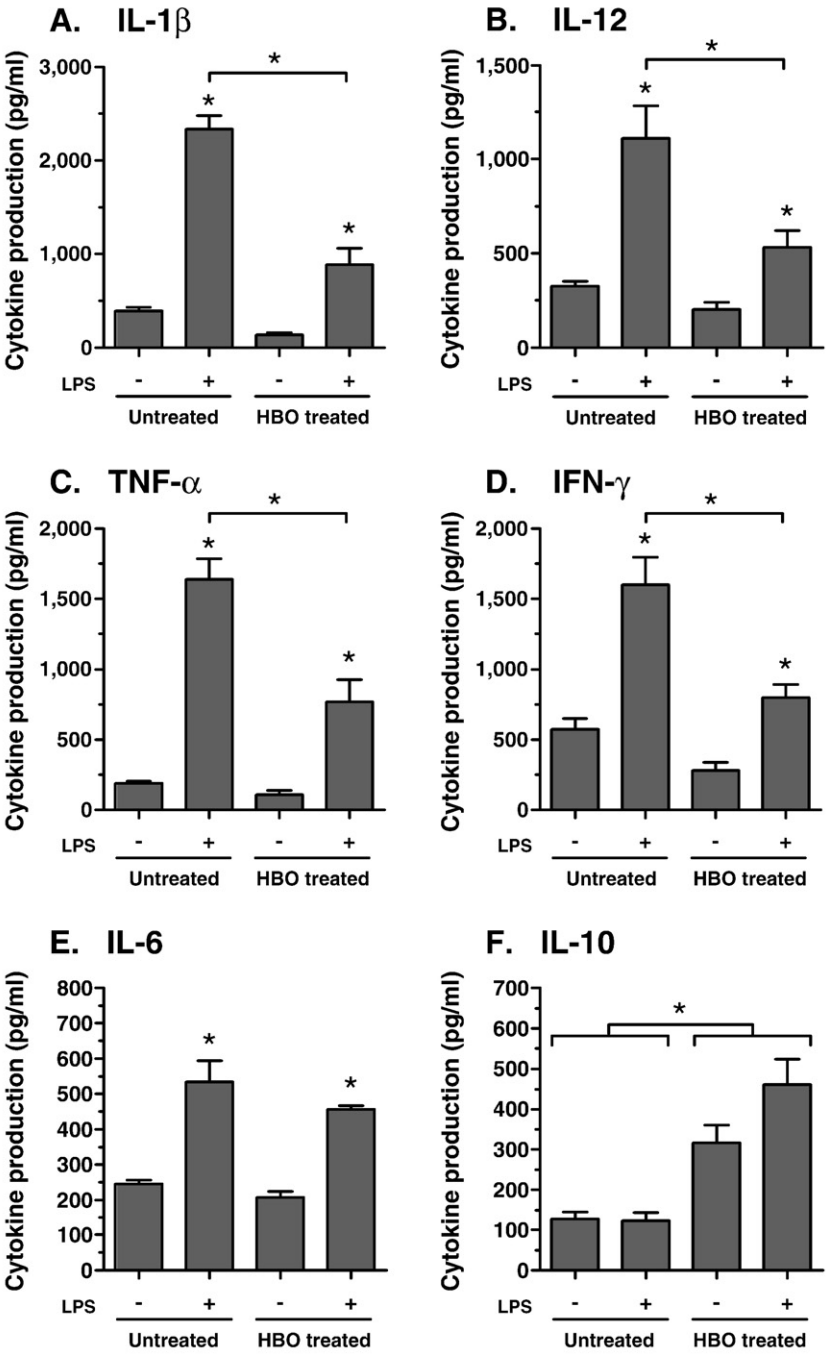

Figure 4 Inflammatory cytokine production by spleen cells is affected by HBO treatment. The animals were either injected with LPS (100 mg, i.p.) or PBS and 4 h later spleen cells were isolated and cultured. Splenocytes were cultured for 18 h and supernatants were collected and analyzed for IL-1ß (A), IL-12 (B), TNFα (C), IFNγ (D), IL-6 (E), and IL-10 (F) by ELISA. Values represent mean ± SEM ($n = 5$ in each group). * denotes significant differences ($p \leq 0.05$) in cytokine levels between indicated groups of mice.

LPS exposure. Similar results were obtained in mice treated for 5 wks with HBO (data not shown).

Discussion

ApoE KO mice represent an accepted and extensively used model of atherosclerosis. Our own previous data provide convincing evidence that HBO treatment, as used in the present experiments, results in a substantial reduction of the disease, as measured by aortic lesion formation or cholesterol content in the absence of substantial changes in their plasma lipoprotein profile [5,6]. We also demonstrated that reduced atherogenesis in HBO-treated mice is accompanied, somewhat paradoxically, by a significant shift in the tissue redox state to a more reductive environment. This appears to be

accomplished by an induction of a number of antioxidant enzymes and an increase in the reduced glutathione concentrations [6]. Since the involvement of the immune system in atherogenesis is well-accepted [1,15,17,23], we examined the effects of HBO treatment on this factor in the development of atherosclerosis.

HBO treatment of apoE KO mice resulted in a reduction in antigen-specific antibodies of the IgG and IgM class to $_{MDA}$LDL. The lower antibody response in HBO-treated mice may be due to a) decreased formation of antigen (oxLDL), or b) suppression of the B-cell mediated antibody production. Our data cannot distinguish between these two possibilities. Although our earlier results [6] demonstrate a decrease in the oxidative environment of the tissues of HBO-treated mice (and thus presumably decreased formation of oxLDL), it should be noted that decreased titers are already observed after 5 weeks of treatment. This would appear to be a relatively short time to reduce the antigen concentration, which in turn would lead to reduced antibody expression. It would appear more likely that HBO treatment inhibits directly B-cell mediated antibody secretion. In this regard it is interesting to point that in lupus-prone mice HBO treatment suppressed the autoantibody production and deposition of immune complexes in the kidney [10,24].

The effect of HBO treatment on the cell-mediated (DTH) responses against oxLDL was even more dramatic than the autoantibody responses. Although untreated mice exhibited a DTH response after exposure to oxLDL, HBO-treated mice failed to do so. DTH responses are examples of cell-mediated inflammatory responses that are mediated by antigen-specific Th1 cells. The lack of a DTH response in HBO-treated mice is likely due to the suppression of the Th1 cell responses. Consistent with our results, others have also reported that the exposure of experimental animals to HBO suppressed several cell-mediated immune responses, such as allograft rejection and DTH responses to tuberculin protein [9–13,25,26]. Furthermore, a number of studies indicate that T lymphocyte mediated responses against oxLDL play an important role in the initiation of the inflammatory reactions that participate in atherogenesis (for review see [1]). Thus, although HBO treatment of apoE KO mice resulted in a lower humoral and cell-mediated immune responses against modified LDL, the lack of a DTH response, in contrast to antibody response against modified LDL, indicates that HBO treatment preferentially dampens Th1-type cell-mediated pro-inflammatory immune responses against oxLDL.

Since HBO treatment impacts the cytokine responses to polyclonal activators (anti-CD3 Ab), the effects of HBO are independent of antigen specificity. Thus stimulation of T cells from HBO-treated mice, regardless of antigen specificity, results in higher levels of IL-10 expression. These observations suggest that HBO has a broad effect on adaptive immune responses. In support, HBO treatment leads to significant changes in cytokine production by LPS-stimulated splenocytes. Decreased production of IL-1ß, IFNγ, TNFα and IL-12 is consistent with a reduction in inflammatory responses. Since IFNγ and IL-12 are involved in the differentiation of Th cells to Th1 cell, these data further suggest that these cytokine responses contribute to the lack of a DTH response to oxLDL in the HBO-treated mice. Importantly, there was increased production of IL-10 by splenocytes from HBO-treated mice independent of LPS stimulation. Thus, HBO treatment has a broad effect on adaptive and innate immunity and promotes IL-10 production, independent of antigen specificity. Since one of the major functions of IL-10 is to dampen inflammatory and other immune reactions, the stimulation of IL-10 expression by HBO treatment is likely responsible for the suppression of cell-mediated and other immune responses associated with DTH and other inflammatory reactions, including atherosclerosis.

In summary, HBO treatment results in a substantial reduction in the severity of atherosclerosis in apoE KO mice. The results of this study demonstrate that HBO treatment promotes this reduction, in part, by shifting the balance between pro- and anti-inflammatory responses. Our results are supported by observations of increased atherogenesis in IFNγ- or IL-12-treated apoE KO mice and in IL-10 KO mice; conversely, reduced disease is observed following IL-10 over-expression or adoptive transfer of IL-10 expressing lymphocytes [27–30]. HBO treatment results in a preferential reduction in DTH, IL-12 and IFN-γ responses, while at the same time an increase in IL-10 expression, independent of antigen specificity. IL-10 can impact several pathways in the development of atherosclerosis, including the inhibition of ROS production, pro-inflammatory cytokine and chemokine production, expression of cell adhesion molecules and the presentation of antigens to T cells [31–33]. The rise in IL-10 levels may therefore be the key to the many of the effects of HBO treatment that slow the progression of atherosclerosis and other inflammatory conditions.

Acknowledgments

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