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Original Paper

Amelioration of non-alcoholic steatohepatitis and glucose intolerance in ob/ob mice by oral immune regulation towards liver-extracted proteins is associated with elevated intrahepatic NKT lymphocytes and serum IL-10 levels

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Abstract

Non-alcoholic steatohepatitis (NASH) is a common cause of cryptogenic cirrhosis in the Western world. In an animal model of NASH, leptin-deficient ob/ob mice present with alterations in number and function of hepatic NKT and peripheral CD4 lymphocytes. Oral immune regulation is a method to alter the immune response towards orally administered antigens. To determine the effect of oral immune regulation towards liver-extracted proteins on the metabolic disorders in ob/ob mice, ob/ob mice and their lean littermates were orally administered liver extracts from wild-type or ob/ob mice or bovine serum albumin for 1 month. The effect of treatment on hepatic fat content was measured by magnetic resonance imaging (MRI) and using a histological steatohepatitis grading scale. Glucose tolerance was measured by an oral glucose tolerance test (GTT). T lymphocyte subpopulations were assessed by flow cytometry analysis. Induction of immune regulation by oral presentation of liver-extracted proteins resulted in a significant 18% reduction of the hepatic fat content in ob/ob mice fed with either wild-type or ob/ob liver extracts for 1 month. The MRI signal intensity index in treated mice decreased to 0.48 and 0.51, respectively, compared with 0.62 in BSA-fed controls ($p = 0.037$ and $p = 0.019$, respectively), while the histological steatohepatitis score decreased in both treated groups to 2.0, compared with 2.4 in BSA-fed controls ($p = 0.05$). A significant improvement in GTT was noted in treated ob/ob mice. These changes were accompanied by a marked increase in the intrahepatic NKT lymphocyte population in mice fed with proteins extracted from both wild-type and ob/ob mice (46.96% and 56.72%, respectively, compared with 26.21% in BSA-fed controls; $p < 0.05$) and a significant elevation in serum IL-10 levels. Oral immune regulation towards liver extracted proteins in leptin-deficient mice resulted in a marked reduction in hepatic fat content and improved glucose tolerance. This effect was associated with a significant increase in the intrahepatic NKT lymphocyte population and serum IL-10 levels, suggesting a Th1 to Th2 immune shift. Immune regulation towards disease-associated antigens holds promise as a new mode of therapy for NASH.

Keywords: oral immune regulation; NASH; NKT lymphocytes; ob/ob mice

Introduction

Non-alcoholic steatohepatitis (NASH) is a common cause of cryptogenic cirrhosis in the Western world. Immune factors play a role in the pathogenesis of this disorder. Leptin-deficient ob/ob mice suffer from morbid obesity, hyperlipidaemia, diabetes mellitus and severe non-alcoholic steatohepatitis [1,2]. Both immune and non-immune cells are associated with the pathogenesis of these abnormalities [3]. These mice feature impaired cell-mediated immunity [4], reduction in numbers of hepatic NKT lymphocytes [5], impaired function of hepatic Kupffer cells [6], reduced serum levels of IL-10 and IL-15 and increased levels of IL-12 [7–9]. These immune alterations are associated with relative sensitivity to low doses of LPS [10,11] and resistance to Concanavalin A-induced hepatitis [12]. Monocytes from patients with NASH over-produce IL-6, IL-8 and TNFα [9,13].

Oral immune regulation is an alteration of an immune response towards disease-associated antigens via oral administration of these or surrogate epitopes [13,14]. Immune regulation via oral administration of antigens has been shown, in both animals and humans, to prevent or alleviate immune-mediated disorders such as collagen-induced arthritis, experimental colitis, graft vs. host disease, uveitis, diabetes and experimental allergic encephalomyelitis [13–16].

The aim of the present study was to determine the effect of oral immune regulation towards liver-associated antigens on the metabolic and immunological derangements observed in leptin-deficient ob/ob mice. Oral immune regulation employing liver extracted proteins in leptin-deficient mice results in a marked reduction in hepatic fat content and improved glucose tolerance. This effect is associated with a significant increase in the intrahepatic NKT lymphocyte population and a Th2-type cytokine shift.

Materials and methods

Animals

Male ob/ob mice, 8 weeks old, and their lean littermates were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in laminar flow hoods in sterilized cages, given irradiated food and sterile acidified water, and kept on regular 12 h light–dark cycles. All animal experiments were carried out in accordance with the guidelines of the Hebrew University–Hadassah Institutional Committee for care and use of laboratory animals and with the Committee's approval.

Preparation and administration of oral antigens

Livers were removed from mice, cut into small pieces and homogenized mechanically. Following filtration through a 40 μm nylon cell strainer, intact cells were spun down and removed. Proteins were quantified by using a protein assay kit (Biorad, Munich, Germany).

Experimental groups

Ob/ob mice and their lean littermates were divided into six experimental and control groups ($n = 14$; Table 1). The dose and frequency of antigen administration were determined in a range that was previously suggested to be associated with induction of suppressor cells, and/or activation of regulatory cells [15]. Mice in groups A and D were given 20 μg/kg liver protein extracts derived from healthy C57bl mice. Mice in groups B and E were administered 20 μg/kg liver protein extracts derived from ob/ob mice, while groups C and F mice were given 20 μg/kg bovine serum albumin. Extracts were given by gavage every other day for a total of 1 month (15 feedings), followed by regular chow feeding to all mice for 1 additional month.

Table 1. Experimental and control groups ($n = 14$)

Group	Mouse strain	Oral antigen
A	ob/ob	C57Bl/6 liver extract
B	ob/ob	ob/ob liver extract
C	ob/ob	Bovine serum albumin
D	C57Bl/6	C57Bl/6 liver extract
E	C57Bl/6	ob/ob liver extract
F	C57Bl/6	Bovine serum albumin

Glucose tolerance test

Glucose tolerance test was performed on day 60 of the experiment in all mice. Mice were given glucose in an amount of 1g/kg weight, followed by serum glucose measurements every 15 min for 3 h, via blood collection from the tail vein under isoflurane anaesthesia. Glucose levels were measured with standard glucose test strips and a glucometer (Elite Co.).

MRI hepatic fat content measurement

Magnetic resonance imaging (MRI) was performed in all mice on day 60. Hepatic fat content was measured using the technique of double-echo chemical shift gradient-echo sequence. This method provides in-phase and opposed-phase images in a single acquisition for assessment/quantification of fat in mice livers. The T1-weighted opposed-phase MR imaging technique is sensitive for detection of relatively small proportions of fat in tissues [15–18]. All MR images were performed with a 1.5-T system (Sigma LX, GE, Milwaukee, USA). Double-echo MR imaging was performed with a repetition time (TR) of 125 ms, double-echo times (TEs) of 4 and 6.5 ms, and flip angle of 80°. Imaging parameters included section thickness of 3 mm, 13 cm field of view, 256×160 matrix, and one signal acquired by use of a knee coil. Transverse (axial) and coronal images were acquired at the level of the liver with a 3 mm section thickness and no intersection gap. Quantitative assessment of signal intensity (SI) of changes between in-phase and out of phase images was computed as described in previous studies. The SI index was calculated as follows: SI index $= (SI_{ip} - Si_{op})/SI_{ip}$, where SI_{ip} is SI on in-phase images and SI_{op} is SI on out-of-phase images. The SI index reflects the fraction of SI loss on out-of-phase images compared with the SI on in-phase images.

Hepatic aminotransferase and triglyceride measurement

Following decapitation, serum ALT and AST levels were measured using a standardized automated procedure. Serum triglyceride levels were measured using a spectrophotometer (Cobas DP-25), with a wavelength of 550 nm.

Liver steatohepatitis score

Liver segments from each mouse within all experimental and control groups were fixed in 10% buffered formaldehyde and embedded in paraffin wax for histological analysis. Five sections (5 μm) were stained with haematoxylin/eosin, and histological examination and the steatohepatitis grade scoring (NASH score) was performed [19].

Cytokine measurement

Serum cytokine levels, including IL-10, IL-12, IL-4, IL-6, TNFα and TGFβ were measured in all mice by a 'sandwich' ELISA method, using Genzyme Diagnostic kits (Genzyme Diagnostics, MA, USA) according to the manufacturer's instructions.

Splenic and hepatic lymphocyte isolation

Splenic lymphocytes were isolated and red blood cells removed as previously described [20]. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 ml cold PBS until it become pale. Livers were placed in a 10 ml dish in cold sterile PBS. Livers and spleens were crushed through a stainless steel mesh (size 60, Sigma Chemical Co., St. Louis, MO). The cells were resuspended in PBS and consequently placed through a nylon mesh pre-soaked in PBS, and unbound cells were collected. Cells were washed twice in 45 ml PBS (1250 r.p.m. at room temperature). For liver and spleen lymphocyte isolation, 20 ml Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) was slowly placed underneath the cells suspended in 7 ml PBS in a 50 ml tube. The tube was centrifuged at 1640 r.p.m. for 15 min at room temperature. Cells at the interface were collected, diluted in a 50 ml tube and washed twice with ice-cold PBS (1250 r.p.m. for 10 min). Approximately 1×10^6 cells/mouse liver were recovered. The viability by Trypan blue staining was greater than 95%.

Flow cytometry analysis for determination of CD4, CD8 and NKT lymphocyte subsets

Following lymphocyte isolation, triplicates of $2-5 \times 10^5$ cells/500 μl PBS were put into Falcon 2052 tubes and incubated with 4 ml 1% BSA for 10 min and centrifuged at 1400 r.p.m. for 5 min. The cells were resuspended in 10 μl FCS with 1 : 20 FITC : anti-mouse CD3 antibody, with 1 : 20 PE-anti mouse : CD4 antibody or 1 : 20 APC : anti-mouse CD8 antibody, or 1 : 20 FITC : anti-mouse NK1.1 antibody (NKR-P1C, Pharmingen, USA), and mixed every 10 min for 30 min. The cells were washed twice in 1% BSA and kept at 4 °C until reading. For the control group, 5 μl 1% BSA was added. Analytical cell sorting was performed on 1×10^4 cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson, Oxnard, CA). Gates were set on forward- and side-scatters to exclude dead cells and red blood cells. The data was analysed with Consort 30 two-colour contour plot program (Becton Dickinson), using the CELLQuest program.

Statistical analysis

Student's t-test was used for computation of data. Results are presented as p value, mean and STD.

Results

Effect of oral immune regulation with liver extracted proteins on liver fat content by MRI

Induction of oral immune regulation with liver extracted proteins significantly reduced the hepatic fat content in ob/ob mice fed with wild-type or ob/ob liver extracts than in BSA-fed mice. Within 30 days of treatment, an 18% decrease in hepatic fat content was noted in treated as compared with BSA-fed control ob/ob mice (Figure 1). The MRI SI index in treated ob/ob mice in groups A and B decreased to 0.48 ± 0.12 and 0.51 ± 0.08, respectively, compared with 0.62 ± 0.14 in BSA-fed controls in group C ($p = 0.037$ and $p = 0.019$, respectively). The MRI SI index was negligible in wild-type mice (0.09 ± 0.05, 0.09 ± 0.08 and 0.11 ± 0.08 in groups D, E and F, respectively) and was not altered by feeding with wild-type or ob/ob liver extract for groups D and E ($p = 0.25$ and $p = 0.41$, respectively). A representative image from a wild-type mouse of group F (Figure 2a) shows a normal-sized liver with negligible fat content (SI index = 0.08). The MRI image from a BSA-fed mouse of control group C shows an enlarged steatotic liver (Figure 2b, SI index = 0.63), while a reduction in SI index is demonstrated in a wild-type liver extract-fed ob/ob mouse of group A (Figure 2c, SI index = 0.50).

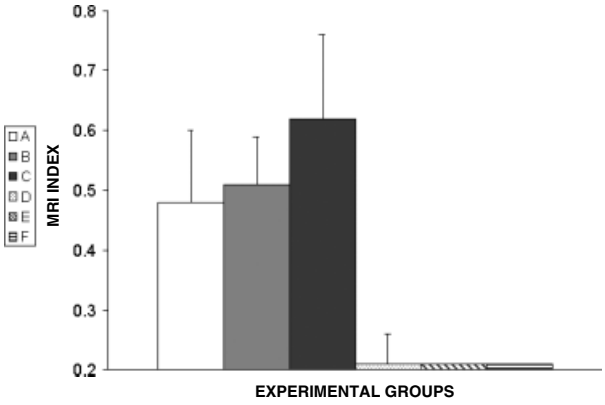

Figure 1. MRI signal intensity index, reflecting hepatic fat content, was significantly higher in BSA-fed ob/ob mice (group C), in comparison to wild-type mice (groups D–F). Ob/ob mice fed with either wild-type (group A) or ob/ob (group B) liver extract featured a significant reduction in hepatic fat content

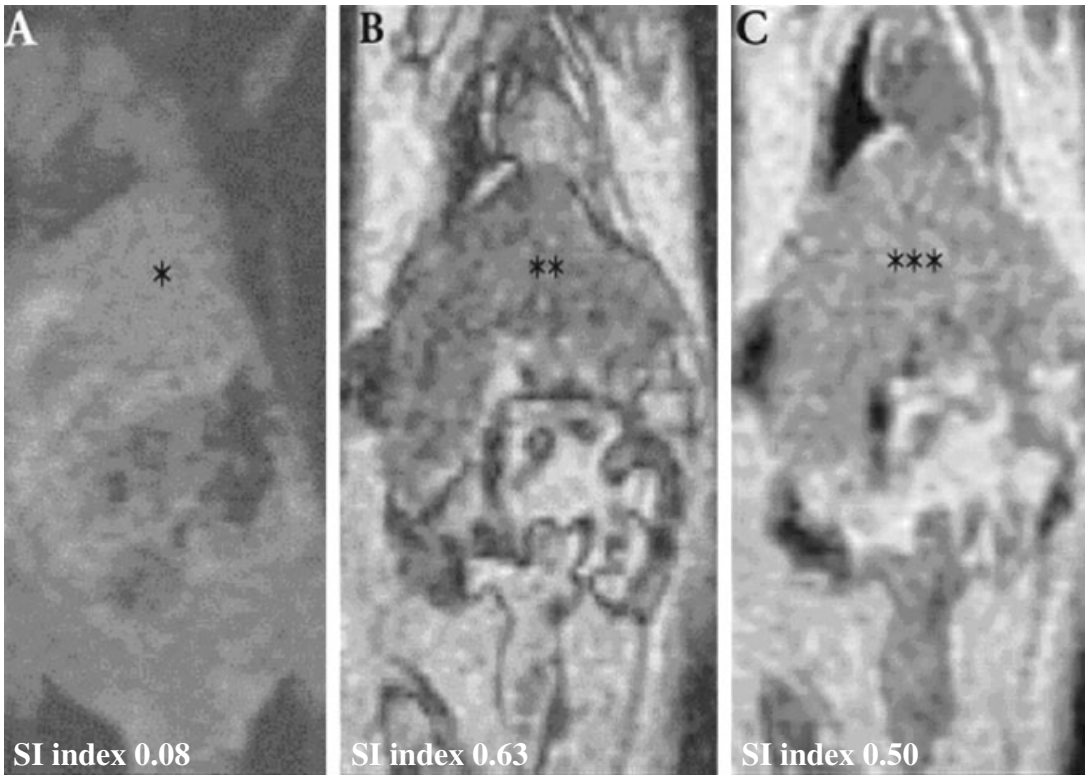

Figure 2. MRI images depicting normal sized wild-type liver with low SI index of 0.08 (2A, asterisk marking the liver); enlarged ob/ob liver of BSA-fed control group C with elevated SI index of 0.63 (2B, double asterisk marking the liver); enlarged ob/ob liver of treatment group A demonstrating reduced SI index of 0.50 (2C, triple asterisk marking the liver)

Effect of oral immune regulation with liver extracted proteins on liver NASH score

Induction of oral immune regulation towards liver extracted proteins reduced the histological NASH score in both wild-type liver-fed ob/ob mice (Group A), and ob/ob liver-fed ob/ob mice (Group B), in comparison with BSA-fed ob/ob mice (Group C) (2.0 ± 0.1, 2.0 ± 0.55 and 2.4 ± 0.77, respectively, $p = 0.05$). All three wild-type mice groups demonstrated a low histological score, regardless of antigen type used (0 in groups D, E and F; Figures 3, 4a). Histological examination demonstrated a shift from a confluent pattern of micro- and macrovesicular steatosis (Figure 4b) to a patchy pattern comprising areas of steatosis interposed within wide areas of essentially normal liver tissue (Figure 4c).

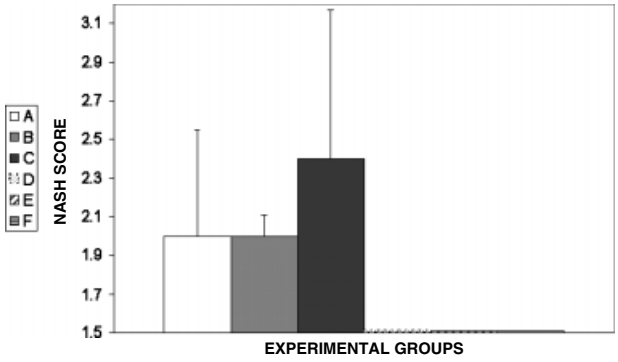

Figure 3. Pathological hepatic NASH score demonstrating extremely low levels of hepatic steatosis in wild-type mouse groups D–F, as opposed to a high level of steatosis in BSA-fed ob/ob mice (group C). Ob/ob mice fed with either wild-type (group A) or ob/ob liver extract (group B) feature a significant reduction in steatosis

Effect of oral immune regulation with liver extracted proteins on glucose tolerance test

Induction of oral immune regulation towards liver extracted proteins significantly improved glucose tolerance (GTT). Ob/ob mice, fed with either wild-type (group A) or ob/ob liver-extracted proteins (group B), demonstrated significantly reduced glucose levels throughout the GTT, compared with BSA-fed mice from control group C (serum glucose levels decreased after 2 h to 96 and 99 mg/dl in groups A and B mice, respectively, compared with 179 mg/dl in group C mice; $p < 0.05$ for all measurements throughout GTT). Glucose levels during GTT in liver extract-fed ob/ob mice (groups A and B) were not significantly different from those of their lean littermates (group F). Glucose tolerance test results were normal in the three wild-type mouse groups D, E and F, regardless of the fed antigen (Figure 5), while glucose levels throughout the test were significantly elevated in group C ob/ob mice fed with BSA ($p < 0.001$ for all time points).

Effect of oral immune regulation with liver extracted proteins on triglyceride levels

Triglyceride levels among untreated wild-type mice, ob/ob liver extract-fed wild-type mice and C57Bl

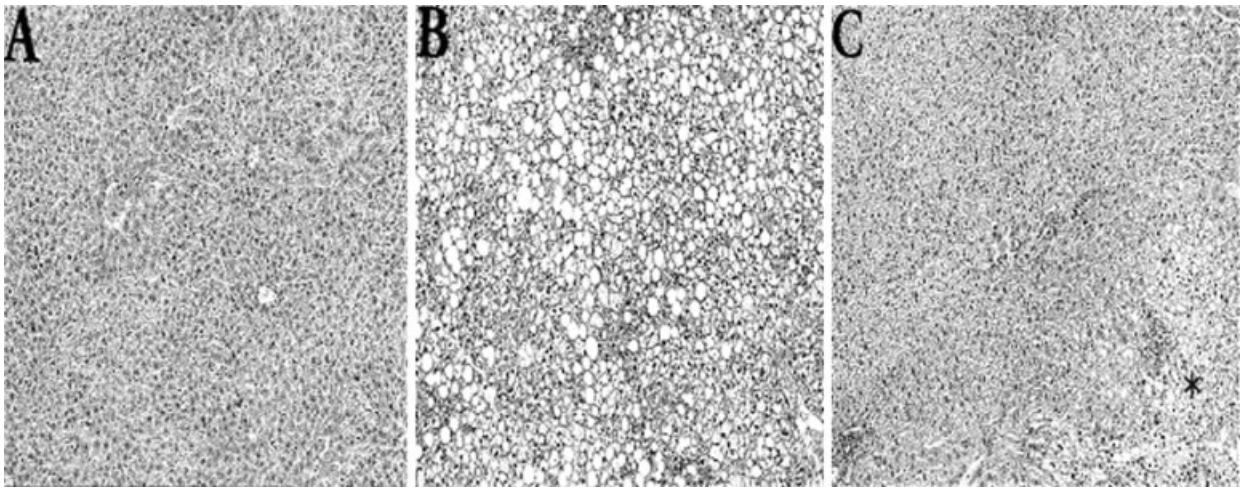

Figure 4. Pathological hepatic images (HandE, ×10), depicting a normal wild-type hepatic section (4A), hepatic section of an ob/ob mouse of BSA-fed control group C (4B) with severe micro- and macrovesicular steatosis, and a liver section of an ob/ob mouse of treatment group A (4C) featuring a marked reduction in steatohepatitis, with a remaining patchy area of steatosis (asterisk)

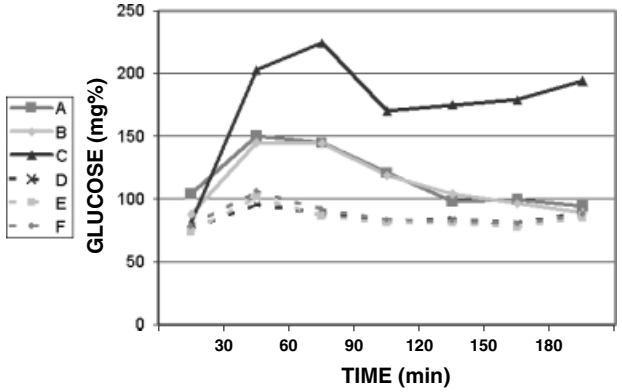

Figure 5. Glucose tolerance test (GTT), which was normal in wild-type mouse groups D–F and showed a diabetic pattern in ob/ob mice (group C). Ob/ob mice fed with either wild-type (group A) or ob/ob (group B) liver extract featured a marked improvement in glucose levels throughout the GTT

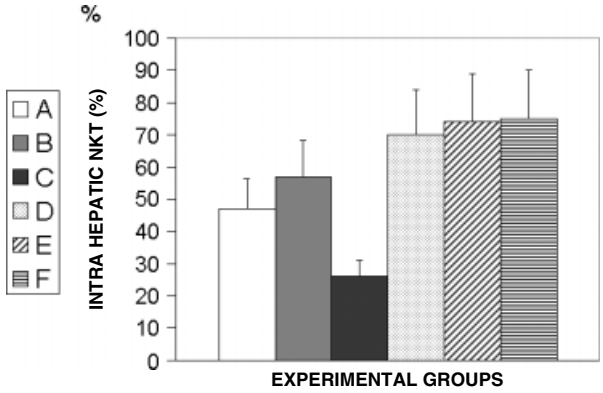

Figure 6. Intrahepatic NKT lymphocyte percentages depicting a high normal hepatic NKT cell population in wild-type mice (groups D–F), in comparison with low hepatic NKT lymphocyte levels in BSA-fed ob/ob mice (group C). Ob/ob mice fed with either wild-type (group A) or ob/ob (group B) liver extract featured a marked increase in hepatic NKT lymphocyte populations in comparison with BSA-fed mice in group C

liver extract-fed wild-type mice (100 ± 49 mg/dl, 53 ± 13 mg/dl and 87 ± 69 mg/dl, respectively) were lower than those of BSA-fed ob/ob mice (160.2 ± 145.2 mg/dl). Ob/ob mice fed with either wild-type liver extract (group A) or ob/ob liver extract (group B) demonstrated lower triglyceride levels (128 ± 38.9 mg/dl and 125 ± 39 mg/dl; $p = 0.44$ and $p = 0.4$, respectively).

Effect of oral immune regulation using liver extracted proteins on intrahepatic NKT lymphocyte population

Induction of oral immune regulation towards liver extracted proteins led to a significant increase in the proportion of intrahepatic NKT cells. Proportions of intrahepatic NKT lymphocytes in the three wild-type mouse groups D, E and F were in the high–normal range (69.96%, 74.3% and 76.92%, respectively; Figure 6). As previously described, group C ob/ob BSA-fed mice featured significantly reduced levels of intrahepatic NKT lymphocytes in comparison to their lean littermates of group F (26.21%, $p < 0.01$). In contrast, both wild-type liver extract-fed (group A) and ob/ob-liver extract-fed (group B) mice demonstrated a marked increase in the intrahepatic NKT lymphocyte population (46.96% and 56.72%, respectively; $p < 0.05$) as compared with BSA-fed mice in group C. The intrahepatic NKT : CD8 lymphocyte ratio increased significantly in treated ob/ob mouse groups (1.76 and 2.12, compared with 0.96 in BSA-fed ob/ob mice; $p < 0.05$). No statistically significant changes were noted in either peripheral NKT lymphocytes or peripheral and intrahepatic CD4 and CD8 T lymphocyte subpopulations.

Effect of oral immune regulation using liver extracted proteins on serum cytokine levels

Induction of oral immune regulation towards liver extracted proteins led to a significant increase in IL-10 serum levels. Serum IL-10 levels in the three wild-type

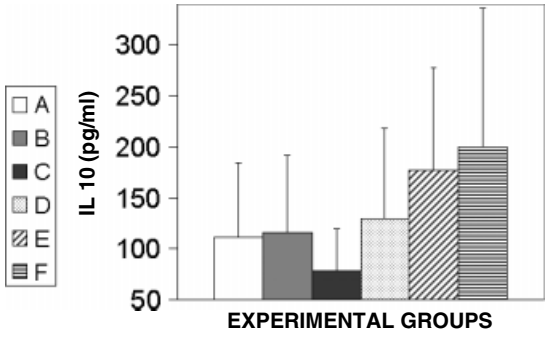

Figure 7. Serum IL-10 levels, which were normal in wild-type mice (groups D–F) in comparison with low levels in BSA-fed ob/ob mice (group C). Ob/ob mice fed with either wild-type (group A) or ob/ob (group B) liver extract featured a significant increase in serum IL-10 levels

mouse groups (groups D–F) ranged within normal levels, although a large variability was noted (129.3 ± 88, 177.6 ± 100, 200 ± 136, respectively; Figure 7). In comparison to BSA-fed ob/ob mice of group C (79.4 pg/ml ± 40.9), IL-10 levels were increased in both wild-type liver extract-fed and ob/ob-liver extract-fed mice (111.8 ± 73 and 116.2 ± 76, $p = 0.12$ and $p = 0.07$, for groups A and B, respectively). No statistically significant differences were noted in body weight, aminotransferase levels, serum IL-4, IL-6, IFNγ and TNFα levels and CD4 and CD8 T lymphocyte subpopulations.

Discussion

Oral immune regulation via oral administration of either wild-type liver extract or ob/ob-liver extracted proteins in a leptin-deficient murine model significantly reduced intrahepatic fat content, as measured by MRI and by liver steatohepatitis histological score and improved glucose tolerance. The metabolic improvement was associated with an increase in hepatic NKT lymphocyte number and serum IL-10 levels. These results suggest that immune modulation may play a role in the pathogenesis of the metabolic disturbances of NASH.

NKT lymphocytes are a regulatory subset of lymphocytes that co-express cell surface receptors characteristic of both NK and T lymphocytes [21,22]. This subset of lymphocytes secretes large amounts of IFNγ or IL-4, thus influencing the Th1–Th2 immune balance [23]. Although a natural activating ligand for NKT cells has not been identified, α-galactosylceramide (α-GalCer, KRN-7000) is a potent activator of both mouse and human NKT cells [24]. Upon its administration, robust cytokine production occurs [25]. NKT cell deficiency was associated with diabetes susceptibility in NOD mice, while replenishment of NKT cells in these mice resulted in inhibition of diabetes [26,27]. In experimental allergic encephalomyelitis, activation of NKT attenuated the disease [28], and in an animal model of systemic lupus erythematosus, a selective reduction in NKT population preceded the development of autoimmune phenomena [29]. In experimental colitis, NKT lymphocytes were suggested to play a role in both pathogenesis and tolerance induction [30]. In humans, NKT cells were suggested to play a role in the pathogenesis of several immune-mediated disorders [31–34].

Leptin-deficient ob/ob mice feature a dysfunctional immune system, manifested by depletion of hepatic NKT lymphocytes, and impaired function of hepatic Kupffer cells [6–8]. Reduction in numbers of hepatic NKT lymphocytes may result from chronic oxidative stress, or altered IL-15 secretion by Kupffer cells, which is important in NKT differentiation, or decreased expression of leukocyte factor antigen 1, required for hepatic accumulation of $CD4^+$ NKT lymphocytes [35,36]. In addition, a role for non-immune cells in the pathogenesis of the disease was suggested [1,37]. Leptin replenishment results in increased numbers of hepatic NKT lymphocytes, and partial reversal of these immune derangements [4]. Amelioration of NASH and glucose intolerance by oral immune regulation towards liver antigens in the present study was associated with a significant increase in the intrahepatic NKT lymphocyte population. These data suggest that NKT cells may be important in the regulation of the metabolic derangement in this model [30,38].

Oral immune regulation was shown to be associated with modulation of one of several regulatory cells, including NKT lymphocytes, dendritic cells, and $CD4^+$ $CD25^+$ lymphocytes [29]. The beneficial effect of oral immune regulation may be secondary to overcoming a quantitative or qualitative defect in NKT regulatory lymphocytes, or correction of an antigen presentation defect, or modulation of other immune components, such as dendritic cells or hepatic macrophages. In the present study, amelioration of NASH and glucose intolerance via oral immune regulation towards either wild-type or ob/ob liver antigens was associated with an increase of serum IL-10 levels. This increase may reflect an immune shift from a Th1-predominant response to a Th2 response.

A bystander effect involving reactivity to multiple closely-related antigens plays a role in oral immune regulation [39]. As regulatory cells secrete non-antigen specific cytokines after being triggered by a fed antigen, they may suppress inflammation in the microenvironment where the fed epitope is localized. In the present study, similar results were obtained following oral administration of either ob/ob or wild-type liver-extracted proteins. These data support the existence of the target antigen in both healthy and fatty livers. Alternatively, an antigen in healthy livers may be closely related to the disease-specific antigen that serves as a surrogate antigen to an as-yet unidentified epitope.

In the present trial, the dose of the crude hepatic extract was determined in a range that was previously suggested to be associated with induction of suppressor cells and/or activation of regulatory cells [15].

Antigen feeding for 1 month was followed by feeding with regular chow for an additional month. This was aimed to avoid a direct metabolic effect inflicted by ingested antigens. The significant immune and metabolic changes observed using this protocol may reflect development of immune memory mechanisms-that exert a long-term effect beyond the antigen exposure period.

The correlation between immune modulation and metabolic effects of oral immune regulation is in accordance with recently published studies. Increased levels of TNFα were shown to result in both lipolysis, free fatty acid accumulation, and reduced expression of mRNA for glucose transporter [40]. Increased β-peroxidation, on the other hand, resulted in activation of the NF-κB pathway, leading to secretion of pro-inflammatory cytokines. Improvement in both steatosis and glucose tolerance supports an association between steatosis and insulin resistance in NASH [41]. It has been previously demonstrated that TNF receptor knockout ob/ob mice feature improved insulin sensitivity, and that administration of the anti-diabetic drugs Metformin and Pioglitazone, which block TNFα signalling, improves steatosis [42,43].

A 'two-hit' theory has been proposed for the pathogenesis of NASH in humans [44]. The first hit consists of a tendency for development of hepatic steatosis, due to genetic predisposition, obesity, hyperglycaemia and hyperlipidaemia. The second hit results in the development of steatohepatitis and eventual hepatic fibrosis, from oxidative stress, and induction of pro-inflammatory cytokines such as TNFα. The findings of the present study suggest that immune modulation may result in improvement of steatohepatitis and may also ameliorate the currently regarded primary pathogenic events, steatosis and insulin resistance.

In summary, oral immune regulation with liver extracted proteins is a relatively simple and side effect-free method that led to a significant metabolic improvement in the ob/ob murine model. Further studies are needed to determine the disease specific antigens and to elucidate the role of intracellular pathways in this process.

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