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Original Paper

Adoptive transfer of regulatory NKT lymphocytes ameliorates non-alcoholic steatohepatitis and glucose intolerance in ob/ob mice and is associated with intrahepatic CD8 trapping

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Abstract

The aim of this study was to determine the effect of adoptive transfer of regulatory natural killer T (NKT) lymphocytes on the metabolic disorder in leptin-deficient ob/ob mice, which feature depletion and defective function of NKT and CD4 lymphocytes. Leptin-deficient ob/ob mice were subjected to transplantation of 1×10^6 of either ob/ob or wild-type-derived NKT lymphocytes, or to transplantation of either ob/ob or wild-type-derived splenocytes. The effect on hepatic fat content was measured by magnetic resonance imaging (signal intensity index) and histology, using the steatohepatitis grading scale. The degree of glucose intolerance was measured by an oral glucose tolerance test (GTT). Adoptive transfer of wild-type or ob/ob-derived regulatory NKT cells led to a 12% decrease in hepatic fat content. A significant histological shift from macrosteatosis to microsteatosis was observed. Marked improvement in the GTT was noted in wild-type or ob/ob-derived NKT recipients. Metabolic effects were associated with a significant decrease in peripheral and intrahepatic CD4/CD8 lymphocyte ratios. Intrahepatic CD8 trapping was observed in all responders. Serum interleukin 10 levels decreased significantly. In conclusion, adoptive transfer of a relatively small number of regulatory NKT lymphocytes into ob/ob mice results in a significant reduction in hepatic fat content, a shift from macro to microsteatosis, and significant improvement in glucose intolerance. These effects were associated with decreased peripheral and intrahepatic CD4/CD8 ratios and decreased interleukin 10 levels. The results further support a role for regulatory NKT lymphocytes in the pathogenesis of non-alcoholic steatohepatitis in the leptin-deficient murine model.

Keywords: adoptive transfer; non-alcoholic steatohepatitis (NASH); NKT lymphocytes; leptin

Introduction

Natural killer T (NKT) lymphocytes are a unique subset of lymphocytes that co-express cell surface receptors characteristic of both T lymphocytes (eg CD3, α/β T cell receptor) and natural killer cells (eg NK1.1) [1]. NKT cells express T cell antigen receptor (TCR) composed of a single invariant TCR α-chain (Vα14-Jα281) and a highly skewed TCR β-chain (Vβ8, Vβ7, or Vβ2) [2]. The restriction and relative conservation of the TCR repertoire are thought to suggest that NKT lymphocytes are activated by a conserved ligand. These cells recognize glycolipid antigens or particular hydrophobic peptide presented by the major histocompatibility complex (MHC) class Ib molecule CD1d. The development of NKT cells is strictly restricted by CD1d. α-Galactosylceramide (α-GalCer, KRN-7000), a glycolipid that does not occur naturally in mammals, is a potent activator of both mouse and human NKT cells [3]. NKT cells are especially abundant in the liver, but their physiological function in this organ remains unclear [4]. It has been suggested that these cells serve as a link between the innate and adoptive immune systems, and play a role in the regulation of immune responses towards various exogenous and endogenous stimuli [1].

Leptin-deficient ob/ob mice, the animal model for non-alcoholic steatohepatitis, exhibit morbid obesity, hyperlipidaemia, glucose intolerance, and steatohepatitis [5]. These mice feature impaired cell

mediated immunity [6,7], reduced number of hepatic NKT lymphocytes [8], impaired function of hepatic Kupffer cells [9], reduced serum levels of interleukin (IL)10 and IL15, and increased levels of IL12 [10–12]. The reduced number of intrahepatic NKT lymphocytes may result from chronic oxidative stress that promotes increased apoptosis. Alternatively, altered secretion of IL15 by Kupffer cells, which is important for NKT cell differentiation, and decreased expression of leukocyte factor antigen 1, necessary for hepatic accumulation of CD4 + NKT lymphocytes, was also suggested to be responsible for this defect [13,14]. Leptin replenishment results in increased numbers of hepatic NKT lymphocytes, and partial reversal of the associated immune derangements [7]. These mice manifest extreme hepatic sensitivity to low doses of lipopolysaccharide [15] and a relative resistance to concanavalin A-induced hepatitis [16]. In humans, it has been recently demonstrated that monocytes of patients with non-alcoholic steatohepatitis (NASH), over-produced IL6, IL8, and tumour necrosis factor-α(TNF-α) [17].

The aim of the present study was to evaluate the effect of adoptive transfer of NKT lymphocytes on the metabolic and immunological derangements observed in the leptin-deficient ob/ob mouse NASH model. Adoptive transfer of a relatively small number of regulatory NKT lymphocytes into ob/ob mice significantly reduced hepatic fat content and improved glucose intolerance. These effects were associated with a decreased peripheral and intrahepatic CD4/CD8 ratio and decreased IL10 levels. These results further support the notion that the NKT cells are a major component in the pathogenesis of NASH.

Methods

Animals

Male Ob/ob mice, 8-weeks old, and their lean littermates (+/−) were purchased from Jackson laboratories (Bar Harbor, ME, USA). All animals were housed in laminar flow hoods in sterilized cages, given irradiated food and sterile acidified water, and kept on regular 12 hour 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.

Splenic and NKT lymphocyte isolation

Donor mice were sacrificed at day 1 of the experiment and splenic lymphocytes were isolated and red blood cells removed as previously described [18]. Spleens were crushed through a stainless mesh (size 60, Sigma Chemical Co, St Louis, MO, USA). The cell suspension was placed in a 50 ml tube for 3 min and washed twice in cold phosphate buffered saline (PBS; 1250 rpm for 10 min), and debris was removed. Cells were re-suspended in PBS, passed through a nylon mesh presoaked in PBS, and unbound cells were collected. Cells were washed twice in 45 ml PBS (1250 rpm at room temperature). For splenocyte isolation, 20 ml of Histopaque® 1077 (Sigma Diagnostics, St Louis, MO, USA) was slowly placed underneath the cells suspended in 7 ml of PBS, in a 50 ml tube. The tube was centrifuged at 1640 rpm 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 rpm for 10 minutes). The viability by trypan blue staining was more than 95%. Cell separation was performed using magnetic cell sorting. NKT cells were isolated using both anti-CD3 and anti-NK1.1 beads, in accordance with the manufacturer's instructions (Miltenyl Biotec, Bergisch Gladbach, Germany).

Experimental groups

Donor mice

Donor ob/ob mice and C57bl mice were sacrificed on day 1 of the experiment, and splenocytes and NKT lymphocytes were isolated as described above.

Recipient mice

Recipient ob/ob mice were divided into five groups (n = 10, Table 1). Mice in control group A were not transplanted. Mice in groups B–E were transplanted on day 1 of the experiment without prior irradiation, with splenocytes of either wild-type or ob/ob origin (groups B and C, respectively), or with NKT lymphocytes of either wild-type or ob/ob origin (groups D and E, respectively).

Glucose tolerance test

The glucose tolerance test was performed on day 12 of the experiment on all mice in all experimental and control groups. Mice were orally given glucose in an amount of 1 g/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 the Elite glucose test strips and glucometer.

MRI hepatic fat content measurement

Magnetic resonance imaging (MRI) was performed on all mice on day 12. The hepatic fat content was

Table 1. Experimental groups (n = 10)

Group	Mice	Transplanted cells (1×10^6)
A	ob/ob mice	No transplantation
B	ob/ob mice	Wild-type splenocytes
C	ob/ob mice	ob/ob splenocytes
D	ob/ob mice	Wild-type NKT lymphocytes
E	ob/ob mice	ob/ob NKT lymphocytes

measured using the technique of double-echo chemical shift gradient-echo sequence that provides in-phase and opposed-phase images in a single acquisition for assessment/quantification of fat in mice livers. The T_1-weighted opposed-phase MRI technique is sensitive to the detection of relatively small proportions of fat in tissues [19–22]. All MR images were performed with a 1.5 T system (Sigma LX; GE, Milwaukee, USA). Double-echo MRI was performed with a repetition time of 125 ms, double echo times 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, with 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 measurements of signal intensity (SI) 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 the SI on in-phase images and SI_{op} is the 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.

Triglyceride measurement

At the end of the study, serum triglyceride levels were measured using a spectrophotometer (Cobas DP-25P).

Histological examination

For each mouse, a single liver segment was fixed in 10% buffered formaldehyde and embedded in paraffin wax for histological analysis. Sections (5 μm) were stained with haematoxylin/eosin, and histological examination and steatohepatitis grade scoring was performed [23]. For histological determination: grade 0 = no steatosis, grade 1 = fat accumulation (micro or macro) in 5–33% of the hepatocytes, grade 2 = 33–66% of hepatocytes with fat droplets, grade 3 => 66% of hepatocytes with fat droplets. Scoring was done blindly be an expert pathologist.

Cytokine measurement

Serum cytokine levels, including IL10, IL12, IL4, IL6, TNF-α, and TGF-β were measured in all mice by a "sandwich" enzyme linked immunosorbent assay (ELISA) method, using Genzyme Diagnostic kits (Genzyme Diagnostics, MA, USA), according to the manufacturer's instructions.

Splenic and hepatic NKT/lymphocyte isolation for T cell population subset determination

All mice were sacrificed on day 12 of the experiment. Splenic lymphocytes and NKT cells were isolated and red blood cells removed as previously described [18]. Intrahepatic lymphocytes were isolated from all groups of mice at the end of the study. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 ml of cold PBS until it became pale. The connective tissue and gall bladder were removed, and livers were placed in a 10 ml dish in cold sterile PBS. Livers and spleens were crushed through a stainless mesh (size 60, Sigma Chemical Co). The cell suspension was placed in a 50 ml tube for 3 min, washed twice in cold PBS (1250 rpm for 10 min), and debris was removed. Cells were re-suspended in PBS, the cell suspension was passed through a nylon mesh presoaked in PBS, and unbound cells were collected. Cells were washed twice in 45 ml PBS (1250 rpm at room temperature). For liver and spleen lymphocyte isolation, 20 ml Histopaque® 1077 (Sigma Diagnostics) was slowly placed underneath the cells suspended in 7 ml PBS, in a 50 ml tube. The tube was centrifuged at 1640 rpm 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 rpm for 10 min). Approximately 1×10^6 cells/mouse liver were recovered. The viability by trypan blue staining was expected to be more than 95%.

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

Immediately after lymphocyte isolation, triplicates of $2–5 \times 10^5$ cells/500 μl PBS were put into Falcon 2052 tubes incubated with 4 ml of 1% bovine serum albumin (BSA) for 10 min, and centrifuged at 1400 rpm for 5 min. Cells were re-suspended in 10 μl fetal calf serum with 1 : 20 fluorescein isothiocyanate-anti-mouse CD3 antibody, 1 : 20 phycoerythrin-anti-mouse CD4 antibody, or 1 : 20 antigen-presenting cells-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. Cells were washed twice in 1% BSA, and kept at 4 °C until reading. For the control group, only 5 μl of 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, USA). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes was deducted from the levels obtained. Gates were set on forward and side scatters to exclude dead cells and red blood cells. The data were analysed with Consort 30 two-colour contour plot program (Becton Dickinson, Oxnard, CA, USA), or the CELLQuest program.

Statistical analysis

Student's t-test was used for computation of data. Results are presented as the p value, mean and SD. $p < 0.005$ was considered significant.

Results

Effect of adoptive transfer of NKT lymphocytes on liver fat content by MRI

Adoptive transfer of either wild-type or ob/ob NKT lymphocytes to ob/ob mice resulted in a significantly reduced hepatic fat content. Within 12 days of transplantation, an estimated 12% decrease was noted in the hepatic fat content in NKT cell-transplanted mice, as compared with control and ob/ob splenocyte-transplanted ob/ob mice (Figure 1). The MR SI index in ob/ob mice transplanted with either wild-type or ob/ob NKT lymphocytes, in groups D and E, decreased to 0.55 ± 0.05 and 0.59 ± 0.05, respectively, compared with 0.66 ± 0.05 in BSA controls from group A ($p < 0.001$ and $p = 0.06$, respectively). The MR SI index remained elevated in ob/ob splenocyte transplanted mice from group C (0.64 ± 0.06), and was mildly, but not significantly-, reduced in wild-type splenocyte-transplanted mice from group B (0.61 ± 0.05, $p = 0.25$). Representative MR images from non-transplanted ob/ob mice (Figure 2A) feature an enlarged liver with elevated hepatic fat content (SI index = 0.65). Images from wild-type NKT lymphocyte-transplanted ob/ob mice feature an enlarged liver, with a reduction noted in the SI index to 0.55 (Figure 2B).

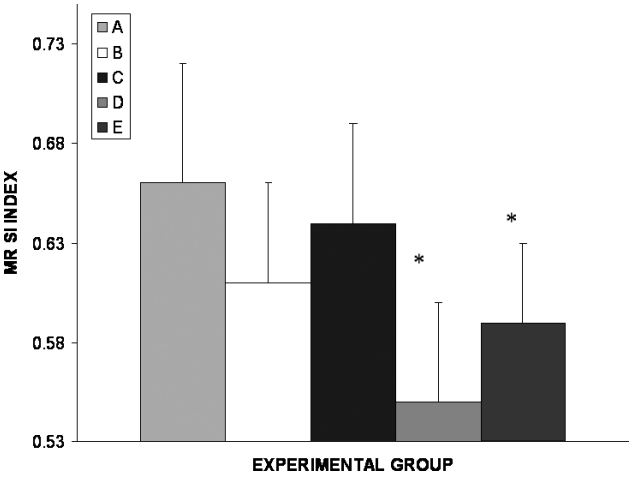

Figure 1. MR signal intensity (SI) index, demonstrating increased hepatic fat content in control ob/ob mice (group A) and ob/ob mice transplanted with ob/ob splenocytes (group C). Ob/ob mice transplanted with either wild-type (group D) or ob/ob (group E) NKT lymphocytes showed a significant reduction in hepatic fat content. The SI index in mice transplanted with wild-type splenocytes (group B) manifested a moderate, but non-significant, reduction

Effect of adoptive transfer of NKT lymphocytes on liver histology

Histological examination of livers of mice from the different groups featured a shift from a mixed micro and macrovesicular steatosis in group A control mice (Figure 3A) to a mainly microvesicular steatosis pattern in wild-type and ob/ob NKT cell-transplanted mice (groups D and E, respectively, Figure 3B). The histological steatohepatitis grading scale was not significantly different between the groups, with a steatohepatitis grading scale of 3.03 ± 0.1, 2.00 ± 0.1, 2.50 ± 0.52, 2.16 ± 0.4, 2.44 ± 0.52 for groups A–E, respectively.

Effect of adoptive transfer of NKT lymphocytes on mice body weight

No statistically significant changes in body weight were noted between the groups, with average weights of 49.78 ± 3.47 g, 51.22 ± 3.41 g, 45.46 ± 14.54 g, 51.06 ± 2.04 g, 49.27 ± 2.43 g for groups A–E, respectively, (p = NS).

Effect of adoptive transfer of NKT lymphocytes on serum triglyceride levels

No statistically significant changes in serum triglyceride levels were noted between the groups, with values of 1.70 ± 0.52 mmol/l, 2.86 ± 1.20 mmol/l, 1.32 ± 0.32 mmol/l, 1.90 ± 0.42 mmol/l, and $2.22 \pm$

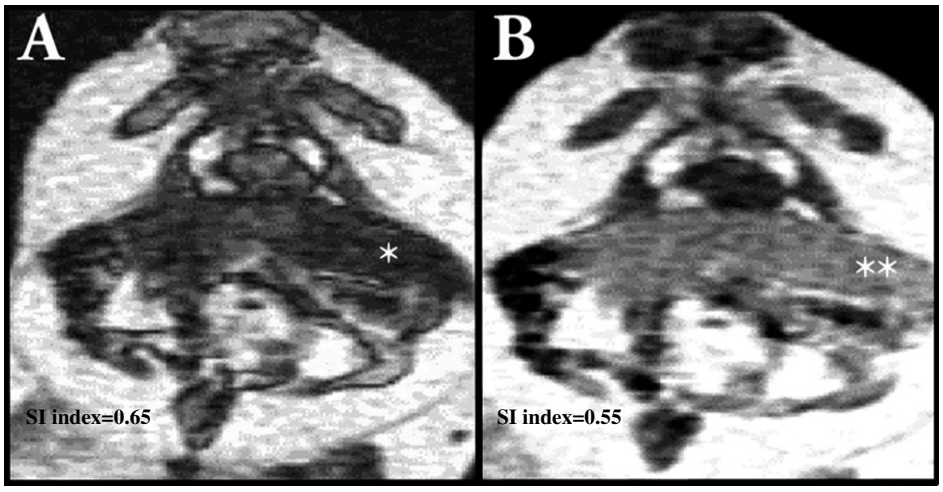

Figure 2. Demonstrative MR images depicting an enlarged ob/ob liver from control group A with elevated SI index of 0.65 (A) as compared with an enlarged ob/ob liver from treatment group D with reduced SI index of 0.55 (B)

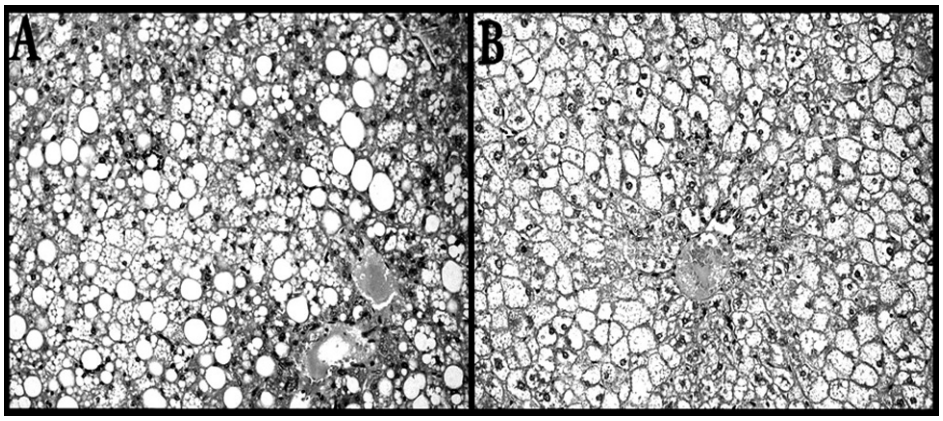

Figure 3. Representative histological hepatic images depicting a hepatic section from an ob/ob mouse from control group A (A) with a severe mixed microvesicular and macrovesicular steatosis pattern, as compared with a liver section from an ob/ob mouse from treatment group D (B) featuring a shift to a purely microvesicular steatosis pattern

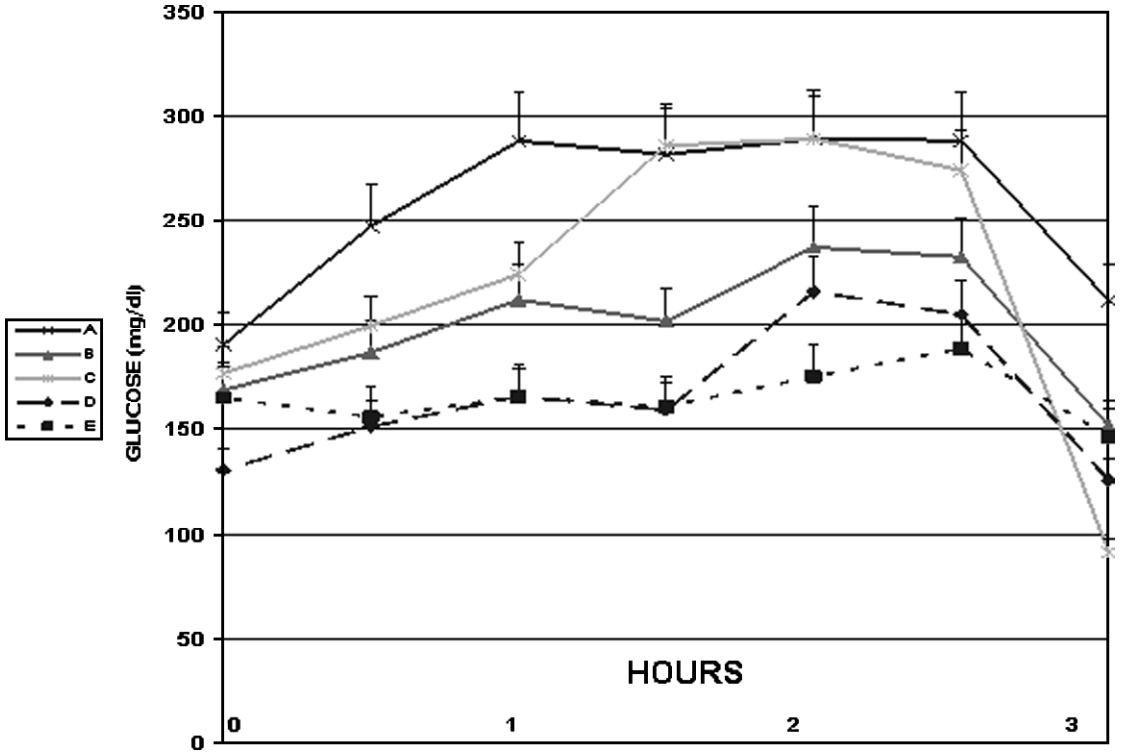

Figure 4. The glucose tolerance test (GTT) was abnormal in control ob/ob mice with marked insulin resistance (group A). In contrast, ob/ob mice transplanted with either wild-type-derived or ob/ob-derived NKT lymphocytes from groups D and E, respectively, demonstrated significantly reduced glucose levels throughout the three-hour test, ($p < 0.001$ for all GTT measurements)

0.32 mmol/l for groups A–E, respectively, ($p =$ NS).

Effect of adoptive transfer of NKT lymphocytes on glucose tolerance test

The glucose tolerance test (GTT) was abnormal in control ob/ob mice with marked insulin resistance (glucose 281 mg/dl [15.6 mmol/l] at 1.5 h, in group A, Figure 4). In contrast, ob/ob mice transplanted with either wild-type-derived or ob/ob-derived NKT lymphocytes from groups D and E, respectively, demonstrated significantly reduced glucose levels throughout the three-hour test (glucose levels of 8.7 mmol/l and 8.4 mmol/l at 1.5 h, for groups D and E, respectively, $p < 0.001$ for all GTT measurements). Serum glucose levels in mice in these groups were similar to those of wild-type mice. Ob/ob mice transplanted with ob/ob splenocytes in group C had glucose levels that were similar to those of control ob/ob mice (285 mg/dl [15.8 mmol/l] at 1.5 h). Glucose levels throughout the GTT of ob/ob mice that were transplanted with wild-type-derived splenocytes from group B, were significantly reduced ($p = 0.029$), with glucose levels ranging between levels of control ob/ob mice and levels of

NKT lymphocyte-transplanted ob/ob mice (205 mg/dl [11.4 mmol/l] at 1.5 h).

Effect of adoptive transfer of NKT lymphocytes on intrahepatic lymphocyte subsets

Adoptive transfer of NKT lymphocytes was associated with marked intrahepatic CD8 trapping. An increase of the proportion of intrahepatic CD8+ cells was observed in all responders, with the CD8 lymphocyte subset increasing to 49.12%, 45.3%, and 49.13%, in groups B, D, and E, respectively, as compared with 3.61% and 8.03%, in groups A and C, respectively ($p < 0.005$, Figure 5). A significant decrease in peripheral to intrahepatic CD4/CD8 lymphocyte ratio was noted (0.13, 0.76 for groups D and E, compared with 1.47 and 1.57, in groups A and C, respectively, $p = 0.05$). Group B featured a CD4/CD8 ratio of 0.72, significantly lower than that of group A, $p < 0.05$).

Effect of adoptive transfer of NKT lymphocytes on serum cytokine level

Adoptive transfer of NKT lymphocytes significantly decreased serum IL10 levels (9.4 and 0 pg/ml) in mice from groups D and E, as compared with 75.5 pg/ml in control ob/ob mice, respectively, $p = 0.01$). Group B featured a statistically insignificant trend towards decreases in IL10 levels (25.8 pg/ml, $p = 0.18$). No significant changes were noted between the groups in serum IL4, IL6, interferon γ (IFNγ), and TNF-α levels.

Discussion

Adoptive transfer of NKT lymphocytes into leptin-deficient mice ameliorated steatosis and glucose intolerance within 12 days of cell transplantation. This effect was associated with an intrahepatic CD8 lymphocyte trapping, and a concomitant decrease in serum IL10 levels. These results further support a role for NKT lymphocytes in the pathogenesis of NASH in the leptin-deficient murine model.

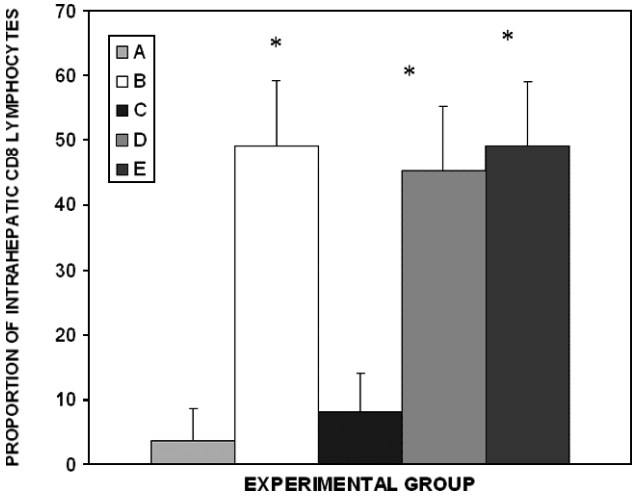

Figure 5. Intrahepatic CD8+ cell proportion (%). Intrahepatic CD8+ lymphocyte trapping noted in ob/ob mice transplanted with either wild-type (group D) or ob/ob (group E) NKT lymphocytes, as compared with control ob/ob mice (group A) and ob/ob mice transplanted with ob/ob splenocytes (group C). Ob/ob mice transplanted with wild-type splenocytes (group B) featured similar significant hepatic CD8+ trapping. $^*p < 0.005$

NKT cells are a regulatory subset of lymphocytes with unique immune modulatory functions towards various exogenous and endogenous stimuli [24]. NKT cell deficiency is associated with diabetes susceptibility in NOD mice, while replenishment of NKT cells in these mice results in inhibition of diabetes [25]. In experimental allergic encephalomyelitis, activation of NKT cells attenuated the disease, and in an animal model of systemic lupus erythematosus, it has been suggested that a selective reduction of the NKT population preceded the development of autoimmune phenomena [25]. In experimental colitis, NKT lymphocytes are important in both pathogenesis of inflammation and tolerance induction [26]. NKT cells play a role in antitumour activity, including direct tumour cell lysis, cytokine induced cytotoxicity [27], and non-MHC- restricted rejection of tumor cells [28–30]. A reduced number of NKT lymphocytes are found in livers of tumour-harbouring mice. In humans, it was suggested that NKT cells play a role in the pathogenesis of insulin-dependent diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis [31–35].

The results of the present study demonstrate that adoptive transfer of NKT lymphocytes harvested from either wild-type or ob/ob origin, results in improvement of hepatic steatosis within a relatively short period of time. This improvement was accompanied by a shift from a mixed microvesicular–macrovesicular steatosis pattern, to a microvesicular steatosis pattern. Although the nature of this shift is unknown, it may represent an early step in histological reduction of hepatic steatosis. Amelioration of steatosis was associated with a significant improvement in glucose intolerance. Slightly better results were obtained with transplantation of wild-type NKT lymphocytes in comparison with ob/ob lymphocytes, suggesting a quantitative, in addition to a qualitative, defect in NKT lymphocytes in this model. The relatively small number (1×10^6 per mouse) of transplanted NKT cells required for the observed effect, further supports the role of NKT cells as a regulatory subset of lymphocytes in ob/ob mice. The partial response of ob/ob mice to wild-type splenocytes, but not to ob/ob splenocytes, may represent a dose effect, as higher numbers of transplanted NKT cells were associated with a more pronounced metabolic effect. As splenic T lymphocytes possess approximately 5% NKT cells, mice transplanted with 1×10^6 wild-type splenocytes were exposed to 5×10^4 NKT cells. Ob/ob mice transplanted with wild-type splenocytes demonstrated partial improvement in both hepatic steatosis and glucose intolerance. Their MRI SI index, glucose levels, and IL10 levels, ranged between values observed in controls (group A) and mice transplanted with NKT lymphocytes (groups D

and E). Mice transplanted with 1×10^6 ob/ob splenocytes (group C), on the other hand, were exposed to much lower numbers of NKT cells, as ob/ob mice are known to be severely deficient in NKT lymphocytes. These mice showed no metabolic and immunological improvements.

The metabolic improvement seen in the present trial after adoptive transfer of NKT lymphocytes was accompanied by redistribution of lymphocytes and by an increase in intrahepatic CD8 trapping. It has been suggested that the liver plays a major role in immune regulation. The heavy hepatic antigenic load, consisting of harmless food-derived and intestinal-derived antigens, and also potentially pathogenic bacterial antigens, necessitates an ability of the intrahepatic subset of lymphocytes to change its immune profile between an anti-inflammatory tolerogenic and a pro-inflammatory profile. The liver is a meeting place for regulatory lymphocytes, antigen presenting dendritic cells, endothelial cells, and peripheral lymphocytes that interact with each other and numerous intestinal antigens. Although the liver is a common site for metastasis [36], the risk of rejection of an allogeneic liver transplantation is less than for rejection of other transplanted organs [37]. The liver has an ability to preferentially trap activated CD8+ lymphocytes, which usually outnumber CD4+ lymphocytes [38]. It has been suggested that NKT cells are important in this process [18,39]. During inflammatory conditions, marked changes in hepatic lymphocyte subsets are noted, with a decrease in NKT cells and an increase in the CD4/CD8 ratio [40,41]. An opposite shift occurs during liver regeneration, following partial hepatectomy [42].

The observed metabolic changes in the present study were associated with a significant decrease in serum IL10 levels. This reduction may represent part of the immune modulatory effect, or a feedback response to hepatic CD8 redistribution. Ob/ob mice feature several distinct cytokine aberrations. In concanavalin A hepatitis and experimental arthritis models, ob/ob mice had reduced serum levels of IL10 and IL15, and increased levels of IL12 [10–12]. In contrast, in the autoimmune encephalomyelitis model, these mice had a Th2-type anti-inflammatory cytokine response, manifested by a reduced IL2 and IFNγ secretion. Leptin replenishment reverses these cytokine paradigms [43].

The exact mechanism by which NKT cells induced the metabolic effect observed in the present study is not yet clear. Possible mechanisms include activation of functionally defective hepatic Kupffer cells, reduction of chronic inflammation noted in ob/ob mice, or attenuation of the self-reinforcing cycle of inhibitor kappa beta kinase beta (IKKβ)-NFκB-TNF-α pathway. The concomitant improvement in both steatosis and glucose intolerance after transplantation of NKT lymphocytes, supports a close interaction between steatosis and insulin resistance in NASH [44]. Local alteration of TNFα signalling could play a part in amelioration of both steatosis and insulin resistance. TNF receptor knockout ob/ob mice feature improved insulin sensitivity, and administration of anti-diabetic drugs Metformin and Pioglitazone, which block TNF-α signalling, results in improved steatosis [45,46]. As hepatic NKT lymphocytes may affect secretion of TNF-α in hepatectomy models [47], replenishment of ob/ob mice with normally functioning subpopulations of NKT cells could have contributed to the noted metabolic improvement in these mice, via this pathway.

It is worth noting that in the present study, total levels of CD4, CD8, and NKT lymphocytes were measured, a method that potentially masks more subtle changes in these cell subsets. Adoptive transfer of the NKT lymphocyte population may reflect recruitment and/or concomitant inhibition of specific NKT subpopulations, which may be held responsible for the noted effect. In addition, as differentiation between subtypes of CD4 lymphocytes was not performed, a role for other types of regulatory cells (eg CD4 + CD25+) in this process cannot be ruled out. In addition, as mice were sacrificed relatively shortly after NKT lymphocyte transplantation, longer periods of follow-up may have resulted in a more significant immunological and metabolic response.

Adoptive transfer of NKT lymphocytes from either wild-type or ob/ob donor mice resulted in a metabolic improvement of NASH. These findings support a major role for the immune system, and, in particular, for NKT regulatory lymphocytes in the pathogenesis of NASH. These results may give rise to future use of immune manipulations of this subset of regulatory lymphocytes as a therapeutic tool in NASH.

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