Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2011.04511.x

Oral administration of immunoglobulin G-enhanced colostrum alleviates insulin resistance and liver injury and is associated with alterations in natural killer T cells

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Accepted for publication 21 October 2011
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Summary

Insulin resistance and metabolic syndrome are chronic inflammatory conditions that lead to hepatic injury and non-alcoholic steatohepatitis (NASH). Bovine colostrum has therapeutic effects in a variety of chronic infections. However its effectiveness in NASH was never studied. Natural killer T (NKT) cells have been shown to be associated with some of the pathological and metabolic abnormalities accompanying NASH in leptin-deficient (*ob/ob*) mice. In the present study, we used hyperimmune bovine colostrum to treat hepatic injury and insulin resistance and we also assessed the effects on NKT cells. We used *ob/ob* mice that were fed for 6 weeks with either 0·1 mg bovine colostrum prepared from non-immunized cows, 0·1 mg hyperimmune colostrum raised against a bacterial lipopolysaccharide (LPS) extract or 0·001, 0·1 or 1 mg of immunoglobulin (Ig)G purified from hyperimmune colostrum (IgG–LPS). NKT cells were phenotyped by flow cytometry, and hepatic injury and insulin resistance were assessed by measuring fasting glucose levels, glucose tolerance tests and liver enzymes. Fat accumulation was measured in the liver and plasma. Oral administration of hyperimmune colostrums decreased alanine aminotransferase (ALT) serum levels and serum triglycerides compared to controls. Glucose intolerance was also improved by the hyperimmune colostrum preparations. These results were accompanied by a decrease in serum tumour necrosis factor (TNF)-α levels following oral treatment with 0·1 or 1 mg of IgG–LPS. The beneficial effects of hyperimmune colostrums were associated with an increase in the number of splenic NKT cells. These data suggest that oral administration of hyperimmune colostrum preparations can alleviate chronic inflammation, liver injury and insulin resistance associated with NASH.

Keywords: colostrum, NASH, NKT cells, TNF-α, type 2 diabetes

Introduction

Disruption of the interface between inflammatory and metabolic pathways is central to the pathogenesis of chronic metabolic diseases, including type 2 diabetes and fatty liver disease [1–3]. Obesity, a feature of metabolic syndrome, is characterized by chronic activation of inflammatory pathways in peripheral tissues [1,2]. Obesity is accompanied by chronic, low-grade inflammation initiated by adipose tissue. Alterations in visceral fat are associated with insulin resistance, and its removal in rodent models of diabetes and obesity is sufficient to restore insulin sensitivity [4]. Chronic inflammation in visceral fat leads to metabolic abnormalities through the secretion of inflammatory cytokines, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, which induce insulin resistance and diabetes [5]. In contrast, adiponectin production in visceral fat increases insulin sensitivity and decreases glucose intolerance and diabetes, resulting in beneficial metabolic effects [6–8].

Chronic low-grade inflammation involves endotoxin derived from the gut flora. Interestingly, endogenous gut-derived bacterial endotoxin has been considered to be an important co-factor that mediates the pathogenesis of liver injury in non-alcoholic steatohepatitis (NASH) [9,10]. In one prospective study, increased circulating levels of lipopolysaccharide (LPS)-binding protein (LBP) were detected in obese patients with non-alcoholic fatty liver disease (NAFLD) [11]. Miele and colleagues discovered that

NAFLD in humans is associated with increased gut permeability, which was related to increased prevalence of small intestine bacterial overgrowth [12].

Obesity is also associated strongly with NASH. Fatty livers are unusually susceptible to injury induced by a secondary inflammatory stress, including that evoked by exposure to endogenous, intestine-derived LPS [13]. However, the mechanisms underlying the susceptibility of fatty liver are not well understood. Previous studies have shown that in *ob/ob* mice, natural killer T (NKT) cells play important roles in fatty liver vulnerability to LPS [14,15]. NKT cells are components of the innate immune system. They express both T cell surface marker (e.g. CD3) and NK cell surface marker (i.e. NK1.1). These cells originate in the thymus but accumulate predominately in the liver, where they regulate local T helper type 1 (Th1) and anti-inflammatory Th2 cytokine production by other mononuclear cells [16]. In leptin-deficient *ob/ob* mice the hepatic NKT cells are depleted [14], and the hypothesis that Küpffer cell dysfunction plays a central role in the immune dysfunction that reduces the hepatic NKT cell population in obese mice was evaluated [17].

Immunoglobulins (Igs) are the main immune components of the acquired immune system and are present in colostrum preparations. IgG is the major class of immunoglobulin present in ruminant milk, whereas IgA is the major immunoglobulin class present in human milk [18,19]. The immunological activity of IgGs in milk from cows immunized against human pathogens is similar to that of IgGs in human milk, which indicates the potential benefits of hyperimmune bovine milk in the human diet [20,21]. Recent studies have shown that daily supplementation of skimmed milk from cows immunized with human enteropathogenic microorganisms can reduce elevated blood cholesterol concentrations in patients with primary hypercholesterolaemia [22].

Bovine colostrum (BC) is milk from lactating mammals that is secreted during the first 72 h after birth. BC differs from regular milk because it contains abundant bioactive components, including growth factors, Igs, lactoperoxidase, lysozyme, lactoferrin, nucleosides, vitamins, peptides and oligosaccharides, which have been shown to be of increasing relevance to human health [23,24]. Immediately postpartum, high concentrations of these immune components can be found in colostrum, with Igs making up approximately 5% of the total content. Colostrum is also rich in cytokines and other immune agents that provide bacteriostatic, bactericidal, anti-viral, anti-inflammatory and immunomodulatory protection against infection [25,26]. BC may also serve as an easy and safe method for generating antigen-specific antibodies, and it may also serve as a source of immune adjuvants, both of which have been shown to activate the innate system [27]. In fact, a recent study has shown that Igs in colostrum confer passive immunity to the ruminant neonate until its own immune system is developed [28]. While normal bovine colostrum has been shown to contain IgGs that are active against specific enteric pathogens, their specificity is dictated by previous systemic challenge, and the concentration is often too low to afford optimal protection [29].

The aim of this study was to evaluate the metabolic and immunological effects of hyperimmune colostrum preparations and IgG-enhanced colostrum fractions [from cows immunized against LPS from intestinal bacteria (*Escherichia coli*)] on metabolic syndrome abnormalities. Specifically, we determined the potential of orally administered colostrum preparation-derived antibodies to affect metabolic pathways and to explore associated effects on NKT cells. Our data indicate that oral administration of IgG-enhanced fractions specific for LPS alleviated the chronic inflammatory state of metabolic syndrome, thereby leading to improved blood glucose levels and reduced liver injury. These improvements were associated with increase of NKT cells.

Methods

Colostrum collection and processing

All colostrum preparations were prepared and provided by Immuron Ltd (Melbourne, VIC, Australia). To prepare each batch of colostrum powder, colostrum was collected appropriately from immunized or non-immunized cows and was frozen in individual bags. Next, for processing, it was thawed and pooled and the fat was removed. Each batch was subsequently pasteurized and then concentrated by ultra-filtration to reduce the volume before freeze-drying. The ultra-filtration step reduced the percentage of lactose in the final powder to less than 7%, compared to approximately 50% in skimmed milk powder. All colostrum preparations were manufactured and tested by an accredited testing laboratory (Dairy Technical Services, Melbourne, VIC, Australia) against specifications for the levels of IgG protein, moisture, lactose, fat, antibiotics and other microbiology parameters. All first-milking colostrum powder preparations were provided by Immuron Ltd and contained approximately 40% IgG (w/w). All freeze-dried bovine colostrum powders were emulsified in water and stored at 4°C until they were administered to mice.

Colostrum preparation

Three different freeze-dried bovine colostrum powders were tested. Colostrum derived from non-immunized cows was used as a control and designated as 'colostrum control'. LPS from enterotoxigenic *E. coli* (ETEC) colostrum was prepared from cows immunized with the most common varieties of ETEC and was designated as 'Imm124-E'. IgG purified from Imm124-E was prepared using a Prosep G column to purify colostrum powder. The colostrum powder was resuspended, and then colostrum whey was prepared by adjusting the pH

to 4·6, by mixing at 37°C for 2 h, by cooling and finally by centrifuging at 10 000 ***g*** for 30 min to remove casein. Colostral whey was then adjusted to pH 6·6 and diafiltrated against phosphate-buffered saline (PBS) using a 30 kD ultrafiltration membrane. The whey was filtered prior to purification using a 0·45 μm protein-G Sepharose column (GE Healthcare Australia Pty. Ltd., Rydalmere, NSW, Australia) using PBS as a running buffer and 50 mM citrate (pH 2·6) as an elution buffer. After elution, peak protein was neutralized to pH 7·0 by the addition of 1 M Tris (pH 8·0) and then diafiltrated against PBS and concentrated. For freeze-drying, 3·5 g of the purified IgG was mixed with a freeze-drying mix of 50·0 g Trehalose and 3·8 g borax and then freeze-dried. This preparation was designated by our laboratory as 'IgG–LPS', and contained approximately 6% purified IgG (w/w).

Coating antigens

Killed bacterial antigens from the single ETEC strain (O78) vaccine or from the multiple ETEC strain vaccine (O6, O8, O15, O25, O27, O63, O114, O115, O128, O148, O153 and O159 serotypes) were used as a source of antigen for coating enzyme-linked immunosorbent assay (ELISA) plates. Stock antigens were prepared according to the manufacturer's instructions (Allied Biotechnology, Kings Park, Australia). This procedure was also used to produce vaccines with which to immunize the cows for the Imm124-E colostrum. Antigens were stored at −20°C in aliquots of concentrations of 1·0 mg/ml. Antigens were used to coat ELISA plates at concentrations of 1×10^{-3} mg/ml for the single-strain antigen and 1×10^{-2} mg/ml for the multi-strain antigens. A 60 mM carbonate/bicarbonate buffer (pH 9·6) was used as the diluent.

In-house ELISA

Imm124-E and IgG–LPS powders were weighed and resuspended vigorously in PBS-Tween at 40 mg/ml. Suspensions were vortexed repeatedly for 2–3 h at room temperature, and insoluble components were removed by centrifugation. Supernatants were then collected into fresh tubes for further dilution. Samples were diluted in 0·5% casein/PBS-Tween by serial fourfold steps to produce dilutions of 1/250, 1/1000, 1/4000 and 1/16 000 prior to assay. One hundred μl of coating antigen (described above) was dispensed into flat-bottomed 96-well plates (Nunc-Immuno, Nunc, Denmark) and then incubated overnight at 4°C. One ELISA plate was coated with single-strain antigen at a concentration of 1×10^{-3} mg/ml, and another plate was coated with multi-strain antigen at a concentration of 1×10^{-2} mg/ml. Following incubation, the plates were washed five times with PBS-Tween and then tapped dry on paper towels. Two hundred μl of blocking solution (5% casein/PBS) was added, and the plates were incubated for 2 h at 37°C to reduce non-specific binding. After washing, fourfold dilutions (1/250, 1/1000, 1/4000, 1/16 000, 1/64 000 and 1/256 000) of each sample and the controls were prepared in an uncoated 96-well plate using 0·5% casein/PBS-Tween as the diluent. One hundred μl of each dilution was removed from the dilution tray, transferred into the wells of the ELISA plate and incubated at 37°C for 2 h. All samples were tested at least twice to ensure accurate results. Positive and negative (no antibody) controls were included in each plate. Plates were washed five times with PBS-Tween and tapped dry. We used horseradish peroxidase (HRP)-conjugated rabbit anti-bovine IgG (Sigma-Aldrich, St Louis, MO, USA) as a secondary antibody, which was diluted to a final concentration of 1/4000 using 0·5% casein/PBS-Tween. Then, 100 μl of the secondary antibody was dispensed into the 96-well plates and incubated for 1 h at 37°C. Plates were washed five times, and 100 μl of substrate reagent was dispensed into the plates, which were then mixed and incubated at room temperature with gentle shaking. The substrate (KPL, 'Sure Blue') reaction was stopped by adding 100 μl of 1 M HCl. The optical density (OD) of each well was read at 450 nm on a plate reader (Labsystems 'Multiskan Ascent' ELISA) and analysed using Ascent Software, version 2·4.

Animals and experimental design

Male (aged 7–8 weeks), leptin-deficient, *ob/ob* mice on a C57BL/6 background were purchased from Harlan Laboratories (Indianapolis, IN, USA). Six groups of mice (six to eight mice per group) were orally administered either vehicle (DDW); 100 μg of colostrum control; 1, 100 or 1000 μg of IgG–LPS; or 100 μg of Imm124-E daily for 8 weeks and then killed. Mice were maintained in the Animal Core of the Hadassah-Hebrew University Medical School. All mice were administered standard laboratory chow and water *ad libitum* and maintained under a 12 h light/dark cycle. All animal experiments were performed according to the guidelines of the Hebrew University-Hadassah Institutional Committee for the Care and Use of Laboratory Animals and with the approval of the committee.

Measurements of serum lipids and liver enzymes

Serum triglycerides were measured at week 7. Total cholesterol levels and serum alanine aminotransferase (ALT) were measured after 4 weeks using a Reflovet Plus clinical chemistry analyser (Roche Diagnostics, GmbH, Mannheim, Germany).

Effects of colostrum preparations on insulin resistance

Ob/ob mice were monitored weekly for alterations in weight and blood fasting glucose levels. Glucose tolerance tests (GTTs) were performed at week 5 after overnight fasting. Glucose was administered orally (1·25 g per kg), and then serum glucose was measured from tail vein blood every

15 min for 3 h. Glucose levels were measured by a standard glucometer.

Fat content in the liver

Triglycerides (TGs) were extracted from snap-frozen livers using a modification of the Folch method. Hepatic TGs content was assayed spectrophotometrically using the GPO-Trinder kit (Sigma-Aldrich, Rehovot, Israel) and was normalized to the protein content in the homogenate.

Cytokine determination

Serum levels of TNF-α were determined by sandwich ELISA using a commercial kit according to the manufacturer's recommended instructions (Quantikine, R&D Systems, Minneapolis, MN, USA).

Isolation of splenocytes and intrahepatic lymphocytes

Spleens were kept in RPMI-1640 supplemented with fetal calf serum (FCS), and were then crushed through a 70-μm nylon cell strainer (BD Falcon, NJ, USA) and centrifuged (295 ***g*** for 7 min). Red blood cells were lysed in 1 ml of cold 155 mM ammonium chloride lysis buffer. Splenocytes were washed and resuspended in 1 ml of RPMI-1640 supplemented with FCS. Viability was assessed by trypan blue (exclusion was above 90%).

Flow cytometry for lymphocyte subsets

Flow cytometry was performed following splenocyte and hepatic lymphocyte isolation using 1×10^6 lymphocytes in 100 μl PBS with 0·1% bovine serum albumin (BSA). For surface staining, cells were incubated with fluorochrome-conjugated antibodies against the indicated cell surface markers (eBioscience, San Diego, CA, USA) at the recommended dilutions or with isotype control antibodies for 30 min at 4°C. The following cell surface anti-mouse antibodies were used: CD3-Pacific Blue and NK1.1-phycoerythrin (eBioscience). Cells were then washed twice and resuspended in 250 μl of PBS containing 1% BSA and stored at 4°C. Stained cells at a density of 1×10^6 in 250 μl of PBS containing 1% BSA were subsequently analysed using a fluorescence activated cell sorter (FACS) LSR II instrument (Becton Dickinson, San Jose, CA, USA) with FCS Express V3 software (DeNovo Software, Los Angeles, CA, USA). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes was subtracted.

Statistical analyses

In this study we used two comparison groups: water and colostrum control-treated mice. All treatments were compared to these two controls. Therefore we used the Tukey test, and a *P*-value of 0·05 or less was considered statistically significant.

Results

Determination of IgG titres in colostrum preparations

The immunization of cows with several strains of enterotoxigenic *E. coli* (ETEC) boosts the concentration of specific antibodies against these microbes in the blood and colostrum. For immunization, a patented killed vaccine was used to produce very high levels of specific antibodies against selected surface antigens from 13 of the most common strains of ETEC. The resulting antibodies were anti-LPS. An acceptable specific antibody titre requires equivalent or better ELISA titre results compared to the control colostrum preparations for both multiple- and single-strain ETEC antigens. The specific antibody titres in Imm124-E hyperimmune bovine colostrum powder and in IgG–LPS were analysed by a validated in-house ELISA against a pool of antigens from both the multiple ETEC strain vaccine (O6, O8, O15, O25, O27, O63, O114, O115, O128, O148, O153 and O159 serotypes) and the single-strain (O78) vaccine antigens. Figure 1 shows the results of this assay.

An average cut-off of approximately 0·2–0·4 OD was used to select the dilutions assigned as the end-points for the antibody titres for each sample.

Oral administration of IgG alleviated liver injury

There are many abnormalities characteristic of *ob/ob* mice; ALT augmentation is a known feature in these mice. Figure 2a shows the effects of oral administration of various doses of colostrum control, IgG–LPS and Imm124-E on ALT levels in *ob/ob* mice. A considerable reduction of ALT serum levels was measured in mice that received 1 mg of IgG–LPS. Figure 2b shows that treatment with a high dose (1 mg) of IgG or 100 μg of Imm124-E considerably reduced hepatic fat (TGs) levels in treated mice.

Oral administration of IgG–LPS or Imm124-E reduced serum TGs levels

Skimmed milk from cows immunized against a variety of human intestinal bacteria has been shown to exert a hypocholesterolaemic effect in humans [22]. Figure 3a shows that treatment with a high dose (1 mg) of IgG–LPS reduces serum TGs levels in mice significantly when compared to water-treated mice or to colostrum control mice. Also the treatment with Imm124-E colostrum reduces serum TG levels in mice when compared to water-treated mice or to colostrum control mice. Interestingly, the levels of serum TGs were elevated by low doses of IgG–LPS; however, this elevation did not reach statistical significance. Figure 3b

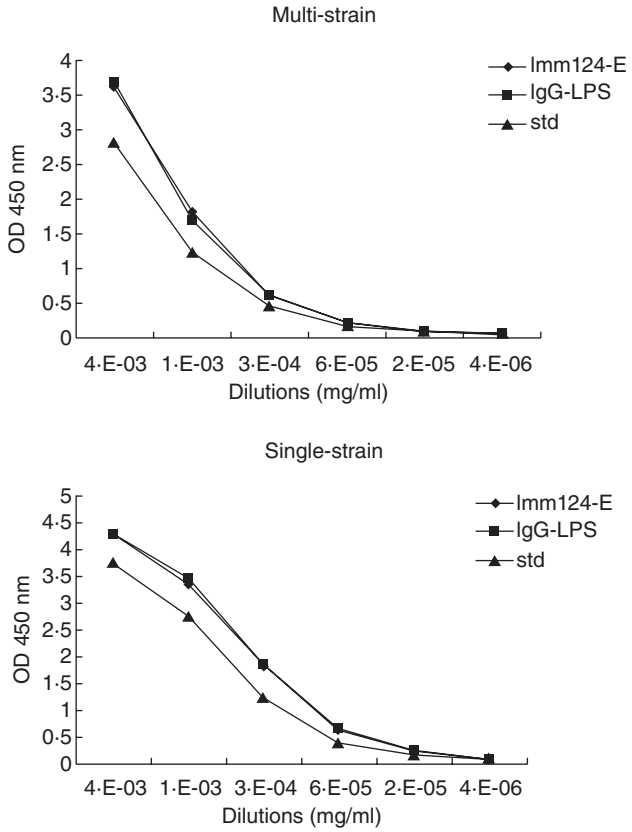

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) procedure for determining assay-specific antibody titre in colostrum preparations. An indirect ELISA was used to evaluate the specific antibody activity of immunoglobulin (IgG)–lipopolysaccharide (LPS) and Imm124-E colostrum preparations against the antigens used in both the multi-strain and single-strain vaccines. ELISA plates were coated with antigens, as described in the Methods section. Fourfold dilutions (1/250, 1/1000, 1/4000, 1/16 000, 1/64 000 and 1/256 000) of each sample and control were prepared. The optical density (OD) was read at 450 nm on a plate reader.

indicates that none of the treatments affected total cholesterol levels.

Oral administration of colostrum improved insulin resistance

Colostrum was shown to reduce blood sugar levels, and this was attributed (at least in part) to stimulation by insulin-like growth factor (IGF)-1 [30]. Accordingly, Fig. 4a shows that already after a 1-week colostrum supplementation (colostrum control) in *ob/ob* mice lowers fasting blood glucose levels. This effect is even greater after 3 and 6 weeks of treatment. However, these changes did not reach statistical significance. High doses of IgG–LPS and Imm124-E exerted a significant effect ($P < 0{\cdot}05$) only after 3 weeks. Interestingly, after 1 week of treatment, fasting glucose levels in the 100-μg IgG–LPS-treated mice were increased significantly; however, these levels dropped after another 2 weeks of treatment, but rose after another 2 weeks. Figure 4b shows the results of the glucose tolerance test (GTT) performed during the fifth week. Low-dose IgG–LPS-treated mice exhibited significantly decreased glucose levels after 15 min, when compared to water and to colostrum control-treated mice.

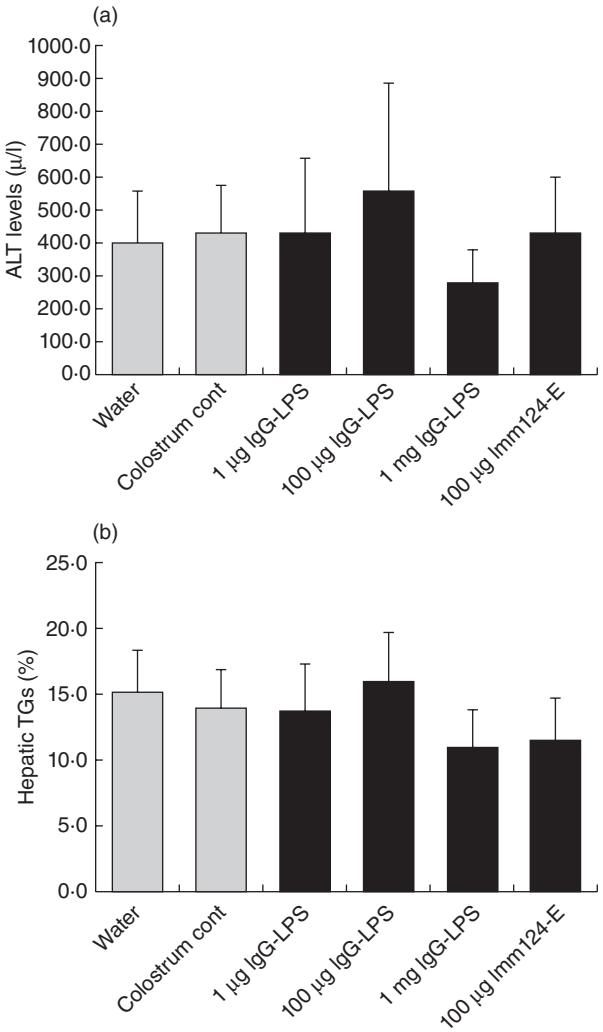

Fig. 2. (a) High-dose immunoglobulin (IgG)–lipopolysaccharide (LPS) abrogated hepatic injury in *ob/ob* mice. Serum ALT levels were measured in mice after 4 weeks of oral treatment with various colostrum preparations or water (vehicle). Data are shown as the mean ± standard deviation (s.d.) of nine to 13 mice in each group. Comparisons were made to water and to colostrum control. Similar results were obtained for two independent experiments. (b) High-dose IgG–LPS and Imm124-E decreased hepatic TG content in *ob/ob* mice. Eight weeks after oral treatment with various colostrum preparations or vehicle, mice were killed and their livers were harvested immediately. Triglycerides (TGs) were extracted from snap-frozen livers and then assayed spectrophotometrically. The number of milligrams of TGs in each sample was calculated on the basis of liver mass, and the amounts are expressed as percentages (mg TGs/g liver). Data are shown as the mean ± s.d. of six to 10 mice for each group. Comparisons were made to water and to colostrum control.

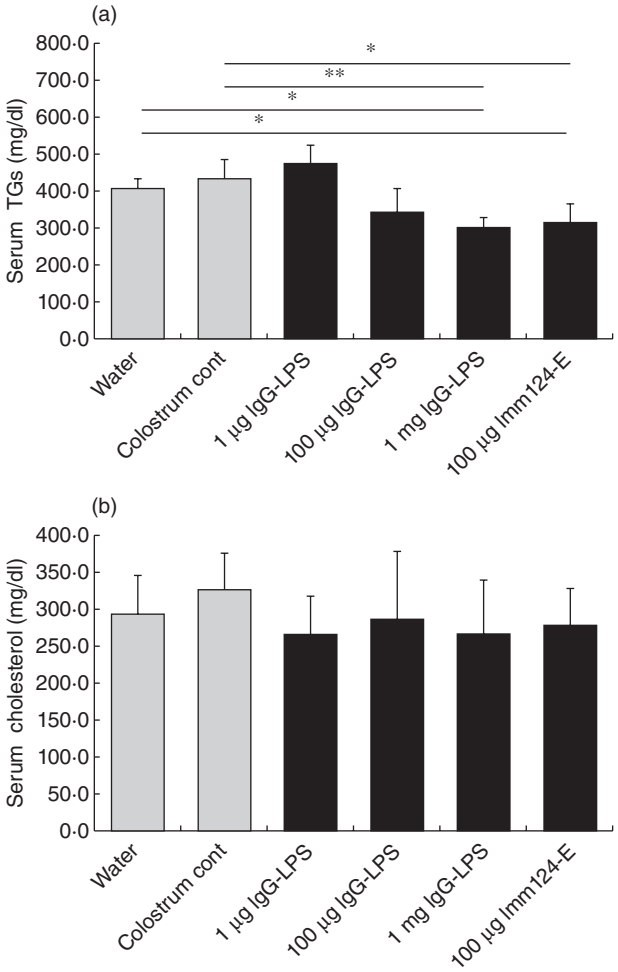

Fig. 3. (a) High-dose immunoglobulin (IgG)–lipopolysaccharide (LPS) and Imm124-E decreased serum triglycerides (TGs) in *ob/ob* mice. Serum TGs levels were measured in mice after 4 weeks of oral treatment with various colostrum preparations or vehicle. Data are shown as the mean ± standard deviation (s.d.) of three to six mice for each group. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with water and with colostrum control. (b) Colostrum treatment did not affect serum cholesterol levels in *ob/ob* mice. Total serum cholesterol levels in *ob/ob* mice were determined after 4 weeks of oral treatment with various colostrum preparations or vehicle. Data are shown as the mean ± s.d. of six to 12 mice for each group. Similar results were obtained for two independent experiments. Comparisons were made to water and to colostrum control.

No effect on body weight was noted in any of the treated groups. Thus, the noted improvement in insulin resistance was independent of weight (data not shown).

Oral administration of hyperimmunized colostrum decreased serum TNF-α levels

Extensive evidence supports a central role of TNF-α and other proinflammatory cytokines in the development of obesity-associated insulin resistance and fatty liver. Circulating, as well as liver and adipose tissue levels, of TNF-α are increased in animal models of obesity and NASH [31,32]. Figure 5 demonstrates the effect of the various colostrum preparations on serum TNF-α levels. In mice treated with IgG–LPS (100 μg or 1 mg), a marked decrease in serum TNF-α levels was observed, suggesting an anti-inflammatory effect caused IgG–LPS-enhanced colostrum.

Oral administration of hyperimmune colostrum increased splenic $CD3^{+}NK1.1^{+}$ cells

Figure 6 shows the effects of IgG–LPS and Imm124-E colostrum preparations on surface staining of $CD3^{+}NK1.1^{+}$ cells in the spleen. We also examined the distribution of splenic $CD4^{+}CD25^{+}Foxp3^{+}$ lymphocytes and found that these cells were elevated considerably in the spleen after treatment with 1 μg of IgG–LPS or 100 μg of Imm124-E; however, no significant results were recorded between treated groups (data not shown).

Discussion

Many studies support the concept that end-organ damage in NASH is an inflammatory condition [2]. The consequences of this systemic inflammation include type 2 diabetes mellitus, atherosclerosis and NAFLD [2].

Colostrum of bovine origin has been known in popular medicine for many years. Bovine colostrums (BC) has properties similar to human colostrum in stimulating the immune system, and more than one of the many present constituents seems to be involved. However, the main documented actions of BC include various anti-bacterial and anti-viral effects, but the effectiveness of BC was not investigated in many NASH and related insulin resistance studies.

In the present study, we determined whether oral administration of hyperimmune colostrum preparations could improve insulin resistance, fatty liver disease and high levels of plasma lipids in *ob/ob* mice. Here, we described an associated innate immune pathway that was activated in NASH. Our data show that daily oral administration of IgG-enhanced colostrum (IgG–LPS) and Imm124-E alleviated liver damage and insulin resistance in *ob/ob* mice. The beneficial effects of oral administration of IgG–LPS and Imm124-E were associated with promotion of NKT cells in the spleens of treated mice.

Biological response modifiers are also present in the hyperimmune colostrum, but their concentration in the present study was not evaluated. Such information, however, is probably essential for determining the exact effect on the modulation of the immune response.

LPS and endogenous gut-derived bacterial endotoxins have been suggested to play a role in NASH. Increased permeability in liver disease appears to be caused by disruption of intracellular tight junctions in the intestine. This has been shown to correlate with a high prevalence of small intestine

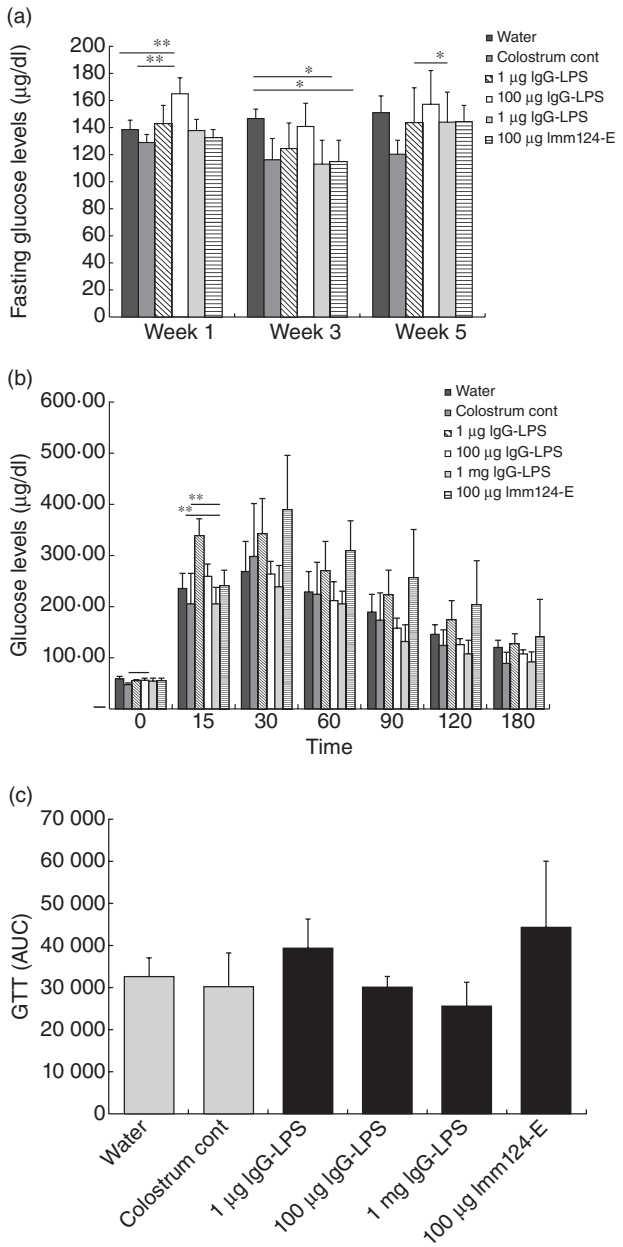

Fig. 4. (a) Colostrum preparations decreased fasting plasma glucose levels in *ob/ob* mice. Fasting glucose levels were monitored after 1, 3 and 5 weeks of treatment with various colostrum preparations. Glucose levels were measured in tail vein blood by a standard glucometer. Data are shown as the mean ± standard deviation (s.d.) of six mice for each group. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with water and with colostrum control. (b) High-dose immunoglobulin IgG–LPS ameliorated glucose intolerance in *ob/ob* mice. Oral glucose tolerance test (OGTT) was performed during week 5 after an overnight fast. Glucose was administered orally (1·25 g/kg body weight), and serum glucose levels in tail vein blood were measured every 15 min for 3 h using a standard glucometer. Glucose values are presented in absolute values. Data symbols represent average ± s.d. For each time-point, six mice were analysed. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with water and with colostrum control. (c) High-dose IgG–LPS ameliorated glucose intolerance in *ob/ob* mice. Oral GTTs were performed during week 5 after an overnight fast. Glucose was administered orally (1·25 g/kg body weight), and serum glucose levels in tail vein blood were measured every 15 min for 3 h using a standard glucometer. Results are presented as area under the curve (AUC). Data symbols represent average ± s.d. For each time-point, five to six mice were analysed.

bacterial overgrowth [12]. Another study discovered that a lifestyle that included high-intensity and high-volume exercise induced favourable changes in chronic inflammatory markers associated with endotoxin treatment [33]. A recent study explored the efficacy of a probiotic compound for attenuating hepatic and intestinal injuries in a mouse model of sepsis. In this report, orally administered probiotics prevented liver and intestinal damage through a peroxisome proliferator-activated receptor γ (PPAR)-dependent mechanism [34]. Taken together, these results suggest that a similar mechanism may underlie some of the observations described in the current study, which may be associated with endotoxin pathogenesis.

Most interesting was the significant effect of hyper-immune colstrum praparations on serum TGs. This lipid-lowering effect was also observed in the liver. These effects were accompanied by a decrease in serum TNF-α levels. Previous study has shown in humans that skimmed milk from immunized cows can cause a hypocholesterolaemic effect [22]. However, the mechanism by which a specifically increased IgG–LPS content in colostrum lowers serum lipids is not clear. Nevertheless, it is known that bovine antibodies resists proteolysis in the stomach and retains specific antibody activity after passage through the gastrointestinal tract [29,35].

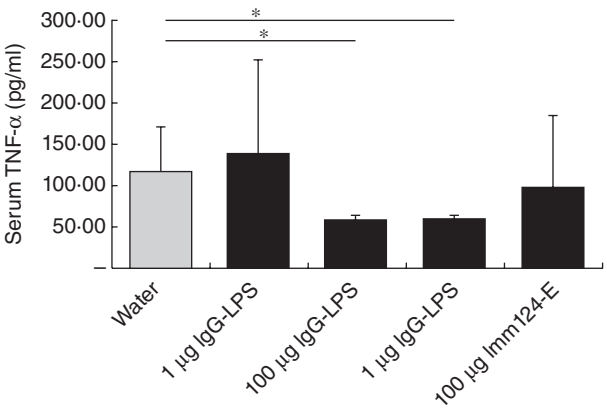

Fig. 5. Medium and high-dose immunoglobulin (IgG)–lipopolysaccharide (LPS) decreased serum tumour necrosis factor (TNF)-α in *ob/ob* mice. Serum TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA). Data are shown as the mean ± standard deviation (s.d.) of five to six mice for each group. Comparisons were made to water-treated mice. Significance in Student's *t*-test was considered as $P < 0{\cdot}05$.

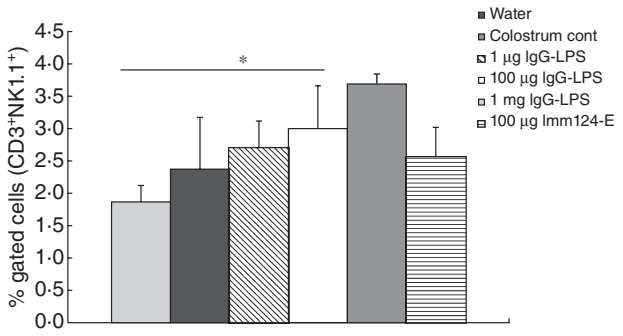

Fig. 6. (a) High- and low-dose immunoglobulin (IgG)–lipopolysaccharide (LPS) and Imm124-E promoted NKT cells in spleens of *ob/ob* mice. Eight weeks after treatment with various colostrum preparations or vehicle, mice were killed and their spleens removed. Splenocytes were prepared as described in the Methods section. One million cells were analysed for the expression of CD3 and NK1.1. The numbers of purified $CD3^+NK1.1^+$ cells were calculated. Data are shown as the mean ± standard deviation (s.d.) of six mice for each group. $^*P < 0{\cdot}05$, compared with water and with colostrum control.

Because NKT cells have been suggested to be important in the development of fatty liver in the *ob/ob* mouse model [14] we examined changes in the distribution of these cells in treated mice and found that all colostrum treatment promoted the expression of these cells in the spleen of treated animals. An interesting mechanism may link between the absence of hepatic NKT cells in *ob/ob* mice and the induction of these cells in the spleen of IgG–LPS-treated mice. Alterations in Küpffer cell activity have been proposed in the livers of *ob/ob* mice [17]. This also may play a role in the effect of IgG–LPS. Such a mechanism may be associated with probable decrease bacterial translocation. However, more investigations should be made in order to shed more light on the specific machinery which enables hyperimmune colostrums to exert its beneficial effect on the innate arm in NASH.

A chronic inflammation state is typical of obesity and type 2 diabetes and occurs at metabolically relevant sites, such as liver, muscle and adipose tissue [2]. Colostrum was shown to reduce blood sugar levels, and this was attributed (at least in part) to stimulation by IGF-1 [30]. Lactoferrin and some other constituents in BC may also take part in the effect which was exerted by BC [18]. Indeed, treatment with colostrum control was valuable for reducing blood glucose levels, even for longer periods of time than hyperimmune colostrums, thereby demonstrating the benefits of the various components, not IgGs, included in BC. However, time-related changes in glucose levels were observed in treated *ob/ob* mice and significant changes were not recorded after 5 weeks of treatment. Further studies with non-obese diabetic (NOD) mice or different models for insulin resistance are warranted.

Our data show that high doses of IgG–LPS were superior to low doses. These effects may be attributed to the specific activity of the IgG fraction against the human gut microorganisms used to produce the hyperimmunity in cows. It is generally accepted that the mechanisms underlying the beneficial effects of hyperimmune colostrum (cows immunized with human enteropathogenic *E. coli*) involve mediating microbial adherence to intestinal epithelial cells [22]. However, the present data support an active role for these antibodies.

Taken together, the results in the present study demonstrate alleviation by hyperimmune colostrums on several abnormalities characteristic to NASH. These improvements were accompanied with alteration of some innate cells that is known to play a role in NASH.

Disclosure

Y. Ilan is the medical director of Immuron. Victor Wong, Brian Muller, Grant Rawlin are employees of Immuron. This study was supported in part by Immuron Ltd.

Acknowledgement

This work was supported in part by a grant from the Roman-Epstein Liver Research Foundation (Y.I.).

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