Treatment with alpha-galactosylceramide protects mice from early onset of nonalcoholic steatohepatitis: Role of intestinal barrier function

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Scope: The role of invariant natural killer T cells in the development of nonalcoholic steatohepatitis (NASH) has not yet been fully understood. Here, the effect of the invariant natural killer T-cell activator alpha-galactosylceramide (αGalCer) on the development of nonalcoholic fatty liver disease and intestinal barrier function was assessed in a mouse model of early Western-style diet (WSD) induced NASH.

Methods and results: Female C57BL/6J mice were either fed a liquid control diet or a liquid fructose-enriched WSD for 6 wk while being treated three times weekly with αGalCer (2 µg intraperitoneal) or vehicle. Indices of liver damage, glucose metabolism, and intestinal permeability were measured. Treatment with αGalCer markedly suppressed hepatic fat accumulation and inflammation while not affecting fasting glucose. The protective effects of αGalCer were associated with a protection against the increased translocation of bacterial endotoxins and the decreased protein levels of tight junction proteins occludin and zonula occludens 1 found in vehicle-treated mice while being fed a WSD.

Conclusion: Taken together, our data suggest that the protective effects of αGalCer against the development of a diet-induced NASH in mice are associated with a protection against the increased translocation of intestinal bacterial endotoxins associated with the development of NASH.

Keywords:
Endotoxin / Natural killer T cells / Small intestine / Tight junction proteins / Western-style diet

1 Introduction

Results of recent surveys suggest that nonalcoholic fatty liver disease (NAFLD) is by now one of the most common liver diseases in the United States, but also in Europe and Asia [1–3]. NAFLD comprises a broad spectrum of disease stages ranging from simple fatty liver to nonalcoholic steatohepatitis (NASH), fibrosis, and even hepatocellular carcinoma (for overview see [4]). Obesity and insulin resistance have been shown to be key triggers for the development of NAFLD (for overview see [4]). However, other facts such as genetic predisposition, physical inactivity, and the intake of a sugar and/or fat-rich diet especially when combined with an elevated cholesterol intake are also discussed to be critical factors in
the development of NAFLD (for overview see [5]). Furthermore, animal and human studies suggest that alterations of intestinal microbiota composition and barrier function may also be involved in the onset but also progression of NAFLD (for overview see [6]). Indeed, it has been shown in mouse models of NAFLD and NASH that chronic intake of a diet rich in fructose and/or fat is associated with changes of intestinal microbiota composition and a loss of tight junction proteins in the upper parts of the small intestine as well as increased portal endotoxin levels (for overview see [6]).

Invariant natural killer T (iNKT) cells, a subgroup of lipid-reactive T cells being part of the innate immune system [7], have repeatedly been suggested to be also critical in the development of liver diseases; however, knowledge on the role of these cells in the development and progression of NAFLD is still rather limited. Indeed, Adler et al. showed in a study with patients with moderate to severe hepatic steatosis (n = 6), patients with less pronounced stages of the disease (n = 11) and controls (n = 10), that the percentage of NKT cells in the liver was markedly higher in patients with moderate to severe hepatic steatosis than in patients with less pronounced stages of the disease or controls, whereas percentages of iNKT cells were unchanged between groups [8]. However, others have shown that in patients with severe steatosis (n = 3–5) the relative number of NKT cells per microscopic field was markedly decreased in comparison to controls (n = 3–5) [9]. Similar contradictory findings regarding the number of iNKT cells in livers have also been reported for rodent models of the disease (for overview [10]). Recent data suggest that iNKT cells may not only be critical in the development of liver disease, but may also modulate intestinal barrier function. Indeed, iNKT cells shown to be activated through recognition of self-lipid and/or proinflammatory mechanisms seem to play a central role in the bidirectional interaction of host and commensal microbiota and thereby in maintaining intestinal homeostasis and preventing impairments of intestinal barrier function (for overview see [11] and [12]). However, if an activation of iNKT cells in the gut through their ligand alpha-galactosylceramide (αGalCer) impacts intestinal barrier function and subsequently also the development of NAFLD remains to be determined.

In the present study, we determined in a mouse model of early NASH induced through feeding a so called Western-style diet (WSD) the effects of a continuous αGalCer treatment on the development of liver damage and markers of intestinal barrier function.

2 Materials and methods

2.1 Animals and treatments

Female C57BL/6|J mice 6–8 wk old (Janvier SAS, Le-genest-St. Isle, France), shown in previous studies of our own group to be more susceptible to NAFLD induced by feeding a fructose rich diet [13], were housed in a specific pathogen free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the local Institutional Animal Care and Use Committee (V296/12EM). Calculation of number of cases was based upon previous findings [14–16] with “NAFLD activity score” and “hepatic triglycerides” being defined as primary readouts for the present study. After an adaption phase of 2 wk during which mice were fed ad libitum the liquid WSD or control diet, animals (control group and WSD-fed groups, n = 8 per group) were pair-fed the different liquid diets for additional 4 wk. Diets used and feeding protocols have been described in detail previously [14, 16]. Liquid diets were used to enable isocaloric feeding of groups. Mice were injected either vehicle (saline) or αGalCer (2 μg/mouse; concentration according to [17, 18]; Sigma–Aldrich Chemie GmbH, Germany) every 3 days intraperitoneal. Two weeks prior to sacrifice, mice were fasted for 6 h to determine fasting glucose levels. After 6 wk of feeding, mice were anesthetized with a mix of 100 mg ketamine and 16 mg xylazine/kg body weight, intraperitoneal, and blood was collected just prior to sacrifice from the portal vein. Portions of liver and upper parts of the small intestine were either snap frozen immediately, frozen-fixed in optimal cutting temperature (OCT) media (Medite, Germany) or fixed in neutral-buffered formalin for further use.

2.2 Clinical chemistry

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity in plasma were measured in a routine laboratory at the University Hospital of Jena using standard techniques on a multiparameter analyzer (Architect, Abbott GmbH & Co. KG, Wiesbaden, Germany).

2.3 Liver histology, neutrophil staining, and myeloperoxidase activity measurement

Hematoxylin and eosin staining was conducted to evaluate the grade of inflammation and steatosis via the NAFLD activity score as previously described [19]. Neutrophils in liver tissue were stained using a commercially available Naphthol AS-D Choloroacetate Esterase kit (Sigma Aldrich Chemie GmbH, Germany) and cell number was determined as previously detailed [19]. To determine myeloperoxidase (MPO) activity, the method detailed by Lima et al. was adapted to mice liver tissue [20].

2.4 Hepatic triglyceride determination

Hepatic triglycerides (TGs) were isolated by a lipid extraction of liver homogenate according to Folch (chloroform/methanol [2:1] extraction) and levels were measured.
using a commercial available kit (Randox, Germany) as further detailed by Kanuri et al. [21].

### 2.5 Immunohistochemical and immunofluorescence staining

For all stainings, paraffin-embedded tissue sections (4 µm; liver or duodenum) were used. Liver sections were immunohistochemically stained to detect CD3⁺ cells, whereas sections of duodenum were stained for zonula occludens 1 (ZO-1) and occludin with primary polyclonal antibodies (CD3⁺ cells, Santa Cruz Biotechnologies, USA; ZO-1 and occludin, Thermo Fisher Scientific, USA) as detailed previously [14,16]. In brief, following the incubation with the respective primary antibody, sections were incubated with a peroxidase linked secondary antibody and dianinobenzidine (Dianinobenzidine Envision Kit; Dako, Germany) or NovaRED (NovaRED Peroxidase [HRP] Substrate Kit; Vector Laboratories, USA).

To determine number of neutrophils and CD3⁺ cells, counts from eight fields of each tissue section in liver were used to determine means. To determine the extent of immunohistochemical staining of occludin and ZO-1 in sections defined as percent of the field area within the default color range, an image acquisition and analysis system incorporated in the microscope was applied. Data from eight fields of each tissue section in liver were used to calculate means. Photomicrographs of microscopic fields used to evaluate staining were captured at 630× (oil immersion) magnification.

### 2.6 RNA isolation and real-time RT-PCR

RNA isolation and real-time RT-PCR were carried out as previously detailed by Kanuri et al. [21]. In brief, primers were added to a final concentration of 3 pmol in the PCR mix (SYBR Green® Supermix, Agilent Technologies, Germany). Primer sequences are shown in Table 1. The amplification reactions were carried out with an initial hold step (95°C for 10 min) and 40 cycles of a two-step (95°C for 30 s, 60°C for 60 s) or three-step PCR (95°C for 30 s, 60°C for 60 s, 72°C for 60 s) in a thermocycler (Agilent Technologies Stratagene Mx3005P, Germany). To determine the amount of target, the comparative CT method was applied, normalized to Eef2 or 18S, respectively, relative to a calibrator (2^-ΔΔCt). Melting curves and gel electrophoresis were used to verify the purity of the PCR products.

### 2.7 Endotoxin assay and plasminogen activator inhibitor-1 ELISA

Endotoxin levels in portal heparinized plasma were measured using a commercially available limulus amebocyte lysate assay with a concentration range of 0.015–1.2 EU/mL (Charles River, L’Arbresle, France) as described previously [22]. To
determine protein concentrations of plasminogen activator inhibitor-1 (PAI-1) in liver homogenate of mice, a commercially available mouse PAI-1 ELISA kit (Molecular Innovations, USA) was used following the instructions of the manufacturer.

2.8 Western blot

Protein lysates from duodenal tissue were prepared using Trizol (pegGOLD Trifast; Pqllab, Germany) as previously detailed [23]. Liver protein was extracted using an extraction buffer containing 1 M HEPES (pH 7.6), 1 M MgCl₂, 2 M KCl, 1 M DTT, and protease and phosphatase inhibitor cocktails (Sigma–Aldrich Chemie GmbH, Germany) as described in detail by Spruss et al. [24]. Protein lysates (40 μg protein/well) were separated in a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred to Hybond™-P polyvinylidene difluoride membranes (Bio-Rad Laboratories, USA). Duodenal protein lysates blots without signs of degradation as determined by Ponceau Red staining of blots (n = 4) were then probed with antibodies against occludin (Thermo Fisher Scientific), E-cadherin, or β-catenin (both BD Bioscience), whereas blots with liver tissue extracts were probed with alpha smooth muscle actin (αSMA; abcam. UK). For normalization, all blots were probed with an antibody against β-actin (New England Biolabs, Germany). Bands were visualized using a Super Signal Western Dura Chemiluminescent Substrate (Thermo Fisher Scientific). For detection and analysis a ChemiDoc MP System (Bio-Rad Laboratories) was used. Ponceau Red staining (Roth, Germany) was performed to ensure equal loading of blots.

2.9 Statistical analysis

Results are reported as means ± standard error of the mean (SEM). Grubb’s test was used to identify outliers before statistical analysis (GraphPad Prism Software, USA). Parametric t-test and Mann–Whitney test were used for the determination of statistical significance among treatment groups, while for testing for normality D’Agostino & Pearson omnibus normality test was applied (GraphPad Prism Software). P ≤ 0.05 was selected as the level of significance before the study.

3 Results

3.1 Effect of chronic intake of a WSD on CD1d and NK1.1 expression in the upper part of the small intestine

In line with previous findings of our own group, mice fed chronically a diet rich in fructose, fat, and cholesterol developed early signs of NASH within 6 wk of feeding [16]. Representative pictures of liver histology of mice fed a WSD for 6 wk are shown in Fig. 1. Also in line with our previous findings [14, 16], development of early NASH was associated with a loss of tight junction proteins in the upper parts of the small intestine and increased portal endotoxin levels (also see Figs. 2 and 3). These changes in the upper part of the small intestine were associated with significantly higher NK1.1 and markedly higher CD1d mRNA expression levels when compared to mice fed a control diet (see Table 2); however, differences for the latter did not reach the level of significance as expression varied considerable in some groups.

3.2 Effect of αGalCer treatment on body weight, liver damage, and markers of insulin resistance

Despite similar caloric intake and body weight, but also liver to body weight ratio (n.s. for all three parameters between WSD and WSD + αGalCer), number of fat infiltrated hepatocytes showing predominantly microvesicular fat accumulation, but also hepatic TG concentration were significantly lower in livers of WSD-fed mice treated with αGalCer when compared to WSD-fed mice treated with vehicle (p < 0.05, see Fig. 1). Indeed, hepatic TG concentration was almost at the level of controls. In line with these findings, plasma activity levels of ALT and AST were also significantly lower in WSD-fed mice treated with αGalCer when compared to vehicle-treated WSD-fed mice (ALT: ~−50 %, AST ~−44 %, p < 0.05 for both, see Table 3). Number of inflammatory foci and neutrophils in liver were also significantly lower in WSD-fed mice treated with αGalCer than in those of vehicle-treated WSD-fed mice (p < 0.05 for both, see Fig. 1 and Table 3). In line with the reduced number of neutrophils in livers of WSD-fed mice treated with αGalCer, the MPO activity was also significantly lower in livers of these mice compared to vehicle-treated mice fed a WSD (p < 0.05, see Fig. 1). Number of CD3⁺ cells in liver was by trend lower in livers of WSD-fed mice treated with αGalCer when compared to vehicle-treated mice fed the WSD (p = 0.052). Both, expression of PAI-1 mRNA and protein were also lower in livers of WSD-fed mice treated with αGalCer when compared to WSD-fed mice treated with vehicle (RNA ~−37 %, protein: ~−58 %, p < 0.05 for both, see Fig. 4). However, markers of fibrosis and severe liver damage such as mRNA expression of Col1α1, TGF-β, and IFN-γ as well as protein levels of α-SMA were similar between the two WSD-fed groups regardless of additional treatment (see Table 4 and Supporting Information Fig. 1 for representative picture of α-SMA Western blot). In line with these findings, Indian hedgehog and patched being markers of the hedgehog signaling pathway shown before to be involved in activation of hepatic stellate cells and development of fibrosis (for overview see [25] and [26]) were also similar between the two WSD-fed groups (see Table 4). Furthermore, while fasting blood glucose levels were similar between groups, expression of insulin receptor substrate (IRS) 1 and IRS-2 mRNA in livers of αGalCer-treated mice
fed a WSD were markedly higher than those of vehicle-treated mice fed the WSD (IRS-1: \( p = 0.069 \), IRS-2: \( p < 0.05 \), see Fig. 4).

### 3.3 Effect of \( \alpha \text{GalCer} \) treatment on tight junction proteins in small intestine, bacterial endotoxin levels in portal vein, and the toll-like receptor 4 signaling cascade in the liver

Repeated administration of \( \alpha \text{GalCer} \) was not associated with changes of CD1d and NK1.1 mRNA expression in small intestinal tissue of WSD-fed mice (see Table 2). In contrast, protein levels measured by immunohistochemical staining of the tight junction proteins occludin and ZO-1 were significantly lower in small intestinal tissue of WSD-fed mice treated with vehicle when compared to those mice fed a WSD while being treated with \( \alpha \text{GalCer} \) (\( p < 0.05 \) for both, see Figs. 2 and 3). Indeed, in the latter, protein levels of both tight junction proteins were almost at the level of animals fed a liquid control diet (see Figs. 2 and 3). Similar differences were also found when staining occludin, ZO-1 and E-cadherin with immunofluorescence. Representative pictures of these staining are shown in Fig. 2. Furthermore, protein levels of occludin and E-cadherin as determined by Western blot were also higher in duodenal samples of WSD-fed mice treated with \( \alpha \text{GalCer} \) when compared to vehicle-treated mice fed a WSD; however, as we were only able to analyze protein levels in 4 samples per group the level of significance was not reached when comparing WSD-fed groups (see Fig. 3). In contrast, protein levels of \( \beta \)-catenin in duodenal tissue did not differ between groups (see Fig. 3). Expressions of occludin and ZO-1 were similar between groups; however, expression varied considerable within groups (see Table 5). Levels of bacterial endotoxin in portal plasma were also significantly lower in \( \alpha \text{GalCer} \) treated mice fed a WSD when compared to vehicle treated mice fed a WSD (\( p < 0.05 \), see Fig. 2). Furthermore, mRNA expression levels of toll-like receptor 4 (TLR-4), but also of myeloid differentiation primary response gene 88 (MyD88) were higher in livers of vehicle-treated mice fed a WSD when compared to WSD-fed mice treated with \( \alpha \text{GalCer} \); however, as data varied considerable...
Figure 2. Protein staining of tight junction proteins occludin and ZO-1 and the adherens junction protein E-cadherin of mice fed control diet, WSD, or WSD with repeated αGalCer injections (intraperitoneal) for 6 wk. (A) Representative photomicrographs (400×) of immunohistochemical occludin and ZO-1 protein staining. (B) Representative photomicrographs (630×) of immunofluorescent staining of occludin, ZO-1, and E-cadherin. αGalCer, alpha-galactosylceramide; WSD, Western-style diet; ZO-1, zonula occludens 1.

in the vehicle-treated group, differences did not reach level of significance (TLR-4: \( p = 0.386 \), MyD88: \( p = 0.091 \); see Table 5).

4 Discussion

During the last decades the prevalence of NAFLD has increased world-wide [1–3]. Indeed, it is discussed that by 2020 NAFLD may be the leading cause of liver transplantation in the United States [27]. Results of recent years suggest that changes in innate immunity may be a key factor in triggering but also amplifying the development of NAFLD [28]. Several studies suggest that number and activity of iNKT cells may be altered during the development of NAFLD and may be involved in the development of this disease (for overview see [10]). Results of more recent studies suggest that NKT cells may modulate hepatic injury through mechanisms involving gut microbiota and intestinal barrier function [29]. In the present study, using a mouse model of pair-feeding a liquid WSD to induce early NASH, we determined the effect of a repeated administration of αGalCer, a glycolipid shown before to be a potent and specific activator of mouse and human iNKT cells, on the development of early NASH. Of course, liquid diets depending on their composition, for example, fiber content and source bear a risk of inducing alterations of intestinal barrier function as they may affect bacterial translocation and even intestinal microbiota [30–32]. However, in the present study markers of intestinal barrier function in controls were similar to those of chow fed control animals (unpublished data), whereas alterations found in WSD-fed mice were in accordance with those reported earlier for feeding a solid WSD [33]. Repeated administration of αGalCer to WSD-fed mice markedly attenuated the onset of early signs of NASH. Indeed, hepatic steatosis and TG accumulation were markedly lower in livers of αGalCer-treated mice fed a WSD. Furthermore, markers of hepatic inflammation such as number of inflammatory foci, neutrophils, MPO activity, and CD3+ cells, but also expression of PAI-1 in livers and plasma ALT and AST activity were almost at the level of control diet-fed mice in WSD-fed mice treated with αGalCer. Syn et al. showed before in Jax18−/− and Cd1d−/− mice fed a MCD diet to induce fibrosis, that manipulating NKT cells and dependent signaling pathways may at least in part attenuate the development of hepatic fibrosis [34]. However, in the present study, markers of fibrosis and genes involved in the hedgehog signaling pathway shown to be involved in the development of fibrosis and severe liver damage (for overview see [26]) were not altered between WSD-fed groups, suggesting that αGalCer administration had no effect on the
Figure 3. Small intestinal protein levels of tight junction and adherens junction proteins and endotoxin levels in portal plasma of mice fed control diet, WSD, or WSD with repeated αGalCer injections (intraperitoneal) for 6 wk. Densitometric analysis of (A) occludin staining and (B) ZO-1 protein staining. Representative blots and densitometric analysis of (C) occludin, (D) E-cadherin, and (E) β-catenin Western blots normalized to β-actin and (F) endotoxin concentrations in portal plasma. Data are expressed as means ± SEM. Mean value was significantly different from that of the WSD group: *p < 0.05. n = 4 for Western blot analyses. αGalCer, alpha-galactosylceramide; C, control; WSD, Western-style diet; ZO-1, zonula occludens 1.

Table 2. CD1d and NK1.1 mRNA expression in small intestinal tissue in mice fed control diet, WSD, or a WSD with repeated αGalCer injections (intraperitoneal) for 6 wk

<table>
<thead>
<tr>
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<th>Control (C)</th>
<th>WSD</th>
<th>WSD + αGalCer</th>
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<tbody>
<tr>
<td>CD1d mRNA expression (% of control)a)</td>
<td>100.0 ± 10.9</td>
<td>149.4 ± 27.0</td>
<td>104.6 ± 30.1</td>
</tr>
<tr>
<td>NK1.1 mRNA expression (% of control)a)</td>
<td>100.0 ± 22.1</td>
<td>272.8 ± 50.5b)</td>
<td>201.7 ± 67.6</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM.

a) For both parameters control and WSD were also statistically compared.
b) p < 0.05 compared with control mice.
αGalCer, alpha-galactosylceramide; C, control diet; WSD, Western-style diet.

4.1 The protective effects of an αGalCer-treatment on the development of early NASH are associated with a protection against the increased translocation of bacterial endotoxin and loss of tight junction proteins in the upper parts of the small intestine

Studies in humans and animals strongly suggest that alterations of intestinal microbiota composition and intestinal barrier function subsequently leading to an enhanced translocation of bacterial endotoxins into the portal vein are critical in the development of NAFLD [6]. Results of recent studies suggest that innate immunity and herein especially iNKT cells may be critical in maintaining intestinal homeostasis, thereby also protecting the liver from the development of bacteria-associated diseases (for review see [11]). However, it also has been shown that commensal bacteria are critical in development of fibrosis in this disease model. Furthermore, while not affecting overall fasting glucose metabolism, the hepatoprotective effect of the αGalCer treatment on WSD-induced liver damage was also associated with a marked induction of genes involved in hepatic insulin signaling. These data suggest that the beneficial effects of αGalCer on the liver might have at least in part improved hepatic glucose metabolism. These findings are in line with our own, but also previous findings of others showing that the protection of livers from the development of NAFLD was associated with an induction of insulin receptor expression and dependent signaling cascades [35,36]. Furthermore, several studies suggest that the adoptive transfer of NKT cells or treatment with glycolipid antigens may not only reduce hepatic steatosis, but also improve markers of glucose intolerance in ob/ob mice suffering from NAFLD [37, 38].

Taken together, our data suggest that repeated administration of the iNKT activator αGalCer markedly protects mice from the development of early phases of NASH induced by feeding a WSD. However, as the feeding period used in the present study was rather short, in total only 6 wk and animals only developed early signs of NASH, it cannot be ruled out that effects found in the present study might differ if duration of feeding was extended and more severe phases of the disease are reached. This needs to be addressed in further studies.
Table 3. Daily caloric uptake, body and liver weight, liver to body weight ratio, and parameters of liver damage in mice fed control diet, WSD, or a WSD with repeated αGalCer injections (intraperitoneal) for 6 wk.

<table>
<thead>
<tr>
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<th>C</th>
<th>WSD</th>
<th>WSD + αGalCer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily caloric uptake (kcal/g body weight)</td>
<td>0.46 ± 0.01</td>
<td>0.47 ± 0.01</td>
<td>0.49 ± 0.01</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>21.7 ± 0.8</td>
<td>21.6 ± 0.3</td>
<td>20.7 ± 0.3</td>
</tr>
<tr>
<td>Absolute weight gain (g)</td>
<td>0.9 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>0.9 ± 0.0</td>
<td>1.2 ± 0.0</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>Liver to body weight ratio (%)</td>
<td>4.2 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>72.5 ± 9.8</td>
<td>92.6 ± 7.6</td>
<td>86.3 ± 4.4</td>
</tr>
<tr>
<td>Inflammation (using NAS)</td>
<td>0.14 ± 0.12</td>
<td>0.60 ± 0.19</td>
<td>0.13 ± 0.07a)</td>
</tr>
</tbody>
</table>
| Steatosis (using NAS)                 | 0.07 ± 0.07| 1.25 ± 0.16| 0.56 ± 0.14a|)
| ALT (U/L)b)                           | 27.6 ± 2.1 | 57.0 ± 6.6 | 28.3 ± 6.7a)|
| AST (U/L)c)                           | 52.6 ± 7.4 | 101.5 ± 9.5| 57.0 ± 11.0a)|
| CD3+ cells (per microscope field)c)   | 14.4 ± 2.8 | 22.5 ± 3.6 | 12.1 ± 2.3   |

Data are expressed as means ± SEM.
a) $p < 0.05$ compared with WSD-fed mice.
b) $n = 3–6$ as some plasma samples were hemolytic.
c) $n = 6$ as quality of tissue sections was not suitable for determination of CD3+ cells.

αGalCer, alpha-galactosylceramide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control diet; NAS, NAFLD activity score; WSD, Western-style diet.

the regulation of innate immunity and NKT cells [29, 39]. In the present study, we found that the development of early signs of NASH were associated with an induction of markers of NKT cells as well as the lipid antigen presenting CD1d in the upper parts of the small intestine, suggesting that NKT cells in the small intestine might affect the development of NAFLD. Data on the effects of different diets on markers but even more so the activity of iNKT cells in the small intestine are rather limited. However, it has been suggested that intake of zinc but also polyphenols may alter markers of the innate immune response.

![Figure 4](image_url)

**Figure 4.** PAI-1 and markers of insulin resistance in livers of mice fed control diet, WSD, or WSD with repeated αGalCer injections (intraperitoneal) for 6 wk. (A) Normalized PAI-1 mRNA expression and (B) PAI-1 protein concentrations in the liver. (C) Normalized IRS-1 and (D) IRS-2 mRNA expression in liver. Data are expressed as means ± SEM. Mean value was significantly different from that of the WSD group: * $p < 0.05$. αGalCer, alpha-galactosylceramide; C, control; IRS, insulin receptor substrate; PAI-1, plasminogen activator inhibitor-1; WSD, Western-style diet.
Table 4. Expression of markers of fibrosis and hedgehog ligands in liver tissue of mice fed control diet, WSD, or WSD with repeated αGalCer injections (intraperitoneal) for 6 wk.

<table>
<thead>
<tr>
<th>mRNA expression (% of control)</th>
<th>C</th>
<th>WSD</th>
<th>WSD + αGalCer</th>
</tr>
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<tbody>
<tr>
<td>TGF-β</td>
<td>100.0 ± 11.6</td>
<td>162.6 ± 14.4</td>
<td>169.9 ± 18.1</td>
</tr>
<tr>
<td>Col1a1</td>
<td>100.0 ± 17.3</td>
<td>155.7 ± 16.8</td>
<td>213.9 ± 41.9</td>
</tr>
<tr>
<td>α-SMA relative protein concentration</td>
<td>0.54 ± 0.03</td>
<td>0.61 ± 0.09</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100.0 ± 12.3</td>
<td>225.7 ± 32.7</td>
<td>314.8 ± 70.0</td>
</tr>
<tr>
<td>Ihh mRNA expression (% of control)</td>
<td>100.0 ± 10.4</td>
<td>140.7 ± 17.1</td>
<td>142.1 ± 11.3</td>
</tr>
<tr>
<td>Ptc mRNA expression (% of control)</td>
<td>100.0 ± 11.6</td>
<td>128.3 ± 11.7</td>
<td>167.7 ± 17.9</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. α-SMA Western blots were normalized to β-actin. αGalCer, alpha-galactosylceramide; α-SMA, alpha-smooth muscle actin; C, control diet; Col1a1, collagen, type I, alpha 1; Ihh, Indian hedgehog; IFN-γ, interferon gamma; Ptc, patched; TGF-β, transforming growth factor beta; WSD, Western-style diet.

immune system in the small intestine [40,41]. We further found that in the upper parts of the small intestine repeated administration of αGalCer to WSD-fed mice attenuated the loss of the tight junction proteins occludin and ZO-1 at the level of protein concentration as determined by immunohistochemical staining. Immunofluorescent staining of occludin, ZO-1 and adherence junction protein E-cadherin also suggests similar differences between groups. As we could only analyze four intestinal tissue samples, protein levels of occludin and E-cadherin determined by Western blot did not differ significantly between the two WSD-fed groups. However, we have shown before that immunohistochemical staining of occludin in is line with protein concentrations of this tight junction protein when determined by Western blot [22]. Protein levels of β-catenin did not differ between groups. It has been shown before by others, too, that a dietary intervention associated with alterations of ZO-1 and E-cadherin protein levels did not affect β-catenin protein concentrations in the gut mucosa [42]. Furthermore, mRNA of occludin expression was similar between groups. These findings are in line with those of others that showed protein concentration of tight junction proteins might not be solely regulated at the level of mRNA expression [43], but rather posttranscriptional modifications such as phosphorylation might be involved in the regulation of tight junction proteins in intestinal tissue and subsequently intestinal barrier function [44]. In line with previous studies targeting intestinal tight junction proteins in the upper parts of the small intestine [22,43], the protection against the loss of tight junction proteins in this part of the intestinal tract was associated with a marked protection against the increased bacterial endotoxin levels in portal plasma found in mice only fed the WSD. Furthermore, mRNA expression of MyD88 in the liver was also by trend lower in αGalCer-treated WSD-fed mice, whereas the reduction of TLR-4 mRNA expression did not reach the level of significance. It has been suggested that CD1d, probably through activation of NKT cells, is a critical factor in bacterial colonization [45]. Results of other studies suggest that intestinal microbiota composition can affect iNKT cell phenotype and function in mice [46]. Furthermore, it has also been shown that repeated administration of αGalCer may cause NKT anergy in mice [47]. However, it remains to be determined if the expression of CD1d and NK1.1 in the small intestine is affected by NKT anergy resulting from the repeated administration of αGalCer to WSD-fed mice. Furthermore, it also remains to be determined if the treatment with αGalCer alters intestinal microbiota composition in mice fed a WSD, thereby maybe adding to the beneficial effects of αGalCer on the liver found in the present study. Taken together, our data suggest that the protective effects of a treatment with the iNKT cell activator αGalCer on the development of a WSD-induced NAFLD in the present study may at least in part have resulted from a protection against the loss of tight junction proteins in the upper parts of the small intestine and subsequently the increased translocation of bacterial endotoxins into the portal vein. The results of the present study

Table 5. Markers of intestinal permeability in small intestinal and liver tissue in mice fed a control diet, a WSD, or a WSD with repeated αGalCer injections (intraperitoneal) for 8 wk.

<table>
<thead>
<tr>
<th>mRNA expression (% of control)</th>
<th>C</th>
<th>WSD</th>
<th>WSD + αGalCer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small intestine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occludin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.0 ± 36.0</td>
<td>200.0 ± 74.4</td>
<td>101.6 ± 35.0</td>
</tr>
<tr>
<td>ZO-1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.0 ± 27.7</td>
<td>269.3 ± 85.1</td>
<td>137.9 ± 44.5</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MyD88</td>
<td>100.0 ± 7.8</td>
<td>121.5 ± 4.3</td>
<td>110.9 ± 4.0</td>
</tr>
<tr>
<td>TLR-4</td>
<td>100.0 ± 3.7</td>
<td>226.2 ± 32.0</td>
<td>169.0 ± 9.4</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. Occludin and ZO-1 expression was normalized to 18S, MyD88, and TLR-4 to Eef2.

<sup>a</sup> n = 5–8. αGalCer, alpha-galactosylceramide; C, control diet; MyD88, myeloid differentiation primary response gene 88; TLR, toll-like receptor; ZO-1, zonula occludens 1; WSD, Western-style diet.
by no means preclude that αGalCer in other settings and during later phases of the disease may also directly affect the liver/cells within the liver or other tissues/organs, for example, visceral adipose tissue, thereby contributing a protection against the development or progression of NAFLD. Rather, our data suggest that αGalCer and iNKT cells through so far not fully understood molecular mechanisms are involved in protecting mice from an increased translocation of bacterial endotoxin associated with the chronic intake of a diet rich in fructose, fat, and cholesterol.

5 Conclusion

In summary, results of our study suggest that changes of intestinal barrier function and increased translocation of bacterial endotoxin found in settings of a WSD-induced early NASH may at least in part result from alterations in the innate immune system in the upper parts of the small intestine and herein especially of iNKT cells. Our data further suggest that treatment with the iNKT cell activator αGalCer may in part attenuate the development of early signs of NASH through mechanism involving a protection against the increased translocation of bacterial endotoxin. However, further studies are needed (1) to determine molecular mechanisms involved in the changes of the intestinal innate immune systems and especially iNKT cells in the development of NAFLD and (2) to determine if similar mechanism are also involved in the development of NAFLD in humans.

AJE conducted research, analyzed data, and wrote paper, CS, CJJ, AB, KH, and JP conducted research and analyzed data; IB designed research, wrote paper, and had primary responsibility for final content. All authors read and approved the final manuscript.

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6 References


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