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Probiotics Antigens Stimulate Hepatic NKT cells

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Summary

Increasing evidence suggests gut flora play an important role in the pathogenesis of nonalcoholic fatty liver disease (NAFLD). Our previous studies show hepatic NKT cells play a significant role in the pathogenesis of NAFLD. In this study, we explore the mechanism by which modification of gut flora leads to the alteration of hepatic NKT cells and improvement of steatosis. Mice were fed HF to induce NAFLD. Some of them also received different dose of mixed strain probiotics (VSL#3); single strain probiotic (*B. infantis*) or antibiotics. Animal weight, glucose tolerance, liver steatosis and hepatic NKT cells were assessed. Lipid extracts from probiotics were tested their ability to activate NKT cells. Toll like receptor 4 knockout (TLR4 ko) mice were also evaluated for their responses to HF. High dose VSL#3 was much more effective than low dose VSL#3 and *B. infantis* for the improvement of hepatic NKT cell depletion and steatosis. The lipids extracted from VSL#3 stimulated NKT cells both *in vivo* and *in vitro*. In contrast, lipids from *B. infantis* decreased α-GalCer -mediated NKT cell activation *in vitro*, but were able to stimulate NKT cells. TLR4 ko mice have a similar effect towards HF-induced NKT cell depletion and obesity. These results suggest alterations in the gut flora have profound effects on hepatic NKT cells and steatosis, which are both strain specific and dose dependent, but not through TLR4 signaling. Furthermore, these data suggest probiotics may contain bacterial glycolipid antigens that directly modulate the effector functions of hepatic NKT cells.

Keywords

Probiotics; Nonalcoholic fatty liver disease; NKT cells; Steatosis; IL-2

Introduction

Obesity and its related nonalcoholic fatty liver disease (NAFLD) have emerged as a major health problem around the world. More than 60 million adults are obese[1] and 9.1 million individuals have NAFLD[2] in the US, the epicenter of this endemic. NAFLD encompasses a spectrum of entities including simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis[3]. Among the various environmental factors that might contribute to the rising incidence of obesity and NAFLD, dietary habits and gut flora merit particular consideration. Many studies have shown that changes in dietary fats are associated with an increased prevalence of NAFLD[4-6]. Intestinal bacteria are also known to play a critical role

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Conflict of interest
none.

in obesity and NAFLD[7]. Germ-free mice colonized with gut bacteria from obese mice had higher weight gain than mice colonized with gut bacteria from lean mice on the same diet[8]. Germ-free mice are also resistant to high fat diet-induced obesity related metabolic changes[9]. Although many physicochemical determinants may affect the composition of gut bacteria, dietary factors have the most significant effect [10, 11]. Despite strong evidence supporting the role of intestinal bacteria in the pathogenesis of obesity and metabolic dysfunction[12], there is little knowledge about the mechanisms by which altered intestinal bacteria contribute to obesity and NAFLD.

NKT cells are a group of unique lymphocytes that are predominantly in the liver, with significantly lower abundance in other lymphoid organs[13, 14]. Hepatic NKT cells are also implicated in the pathogenesis and disease progression of NAFLD, although the exact function of NKT cells in the pathogenesis of NAFLD remains controversial. Our studies and others have shown a reduction in NKT cells in mice with fatty liver disease [4, 15] and in the peripheral blood of patients with NAFLD[16]. Adoptive transfer of NKT cells improves steatosis and insulin resistance in a high fat (HF) diet-induced mouse model of NAFLD [17]. Despite mounting evidence demonstrating the importance of NKT cells in regulating hepatic immune responses in NAFLD, there is little knowledge of how NKT cells themselves are regulated. Recent studies have shown that bacterial antigens can serve as ligands for NKT cells[18-20]. In addition, probiotics, live bacteria that alter the host's microflora and exert health benefits [21], can also influence the composition of hepatic NKT cells [17]. This raises the possibility that endogenous gut microbiota and exogenous bacterial supplements (probiotics) may be able to regulate NKT cells, and in turn, modulate NAFLD.

In the current study, we evaluated the strain and dose effects of probiotics on hepatic NKT cells in a mouse model of NAFLD. More importantly, we explore the mechanism by which modification of gut flora by probiotics leads to the alteration of hepatic NKT cells and the improvement of steatosis. Results from this study may have profound therapeutic implications for the management of obesity related fatty liver disease and insulin resistance.

Materials and Methods

Animal experiments

Adult male wild type C57BL6 mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were fed commercial high fat diets (HF diet; 60% of the total of calories from fat) or normal diet (ND diet; 11% of the total of calories from fat, BioServ, Frenchtown, NJ) for 12 weeks. All mice were maintained in a temperature-controlled and light-controlled facility and allowed to consume water and food ad libitum. Some HF diet fed mice also received either high dose (1.5×10^9 colonies /mouse /day) or low dose (1.5×10^8 colonies /mouse /day) VSL#3 probiotics (a mixture of viable, lyophilized bifidobacteria, lactobacilli and Streptococcus thermophilus, Danisco – USA - Madison Plant, Madison, WI), or single strain probiotic *Bifidobacterium infantis* (Align, Procter&Gamble, Cincinnati, Ohio), or antibiotics (KCV, a mixture of kanamycin, colistin and vancomycin, Sigma, St. Louis, MO) for the last 4 weeks. Some ND fed mice also received lipid extracts from probiotics VSL#3 or Align *B. infantis* (see below for detail) intraperitoneally (i.p.). Toll-like receptor 4 knock out (TLR4 ko) mice with C57BL6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and also evaluated for their response to HF diet. All animal experiments fulfilled the National Institutes of Health and Johns Hopkins University criteria for the humane treatment of laboratory animals.

Liver histology and triglyceride content

Thin slices of liver tissue were stained with hematoxylin and eosin (H&E). Ten 200× light microscope fields were assessed on each section and scored for the severity of steatosis [17]. Total lipids were extracted from frozen liver tissue according to published method [22]. Hepatic triglyceride content was measured using a kit according to manufacturer's instructions (Sigma – Aldrich #TR0100).

Glucose tolerance test (GTT) and HOMA-IR

Mice were given glucose (1.5g/kg, i.p.) following overnight fasting. Glucose was measured in blood collected from the tail vein using the OneTouch® ultra® monitoring system with a range of 20 to 600 mg/dL (LifeScan) at various time points (from 0 min to 120 mins after the glucose injection). The insulin concentration in mouse serum was determined by an ultra sensitive mouse insulin ELISA kit according to the manufacturer's instructions (Crystal Chem INC). Insulin resistance was evaluated by the homeostasis model assessment method (HOMA-IR) [23]. HOMA - IR are calculated using the formula: HOMA-IR (mmole/L × μU/ml) = fasting glucose (mmol/L) × fasting insulin (μU/ml)/22.5 [24].

Isolation and cell surface labeling of hepatic mononuclear cells (HMNCs)

HMNCs were isolated as previous described [4] and then labeled with CD3 and CD1d tetramer (NIH tetramer facility) loaded with a ligand (PBS-57, an analogue of alpha-GalCer). After surface labeling, HMNCs were evaluated by flow cytometry (Becton Dickinson, Palo Alto, CA). The data were analyzed using Cell Quest software (Becton Dicksinson).

CFSE proliferation assay

HMNCs were isolated and incubated for 10 min at 37°C with 2.5 μM carboxyfluorescein succinimidyl easter (CFSE) using the CellTrace™ CFSE cell proliferation kit following the manufacturer's instructions (Invitrogen, Eugene, OR). CFSE-labeled HMNCs were washed with PBS / 0.1% FBS and cultured with aAPCs loaded with VSL#3 extract or unloaded empty beads (used as a control). After 3 days co-culture, cells were harvested, washed and stained with NKT tetramer. NKT cell proliferation was evaluated by flow cytometry. The data were analyzed using Flowjo software (Tree Star, INC, Ashland, OR).

Probiotic lipid extraction and antigen presentation

9×10^{11} VSL#3 or 2.1×10^{10} Align *B. infantis* were dissolved into 18 ml water. 2:1 methanol /chloroform were used to extract bacterial lipids. Extracts were centrifuged at 3,000 rpm for 15 min at room temperature. The supernatant containing the lipid were dried and resuspended in PBS. The total lipid contents were measured using a total lipid kit according to the manufacturer's protocol (Biotron Diagnostics, Hemet, California). CD1d-Ig based artificial antigen presenting cells (aAPCs) were generated according to the method described by Webb [25]. Briefly, CD1d-Ig (DimerXI; BD Biosciences) was mixed with epoxy beads (Dynabeads, M-450, Epoxy, Invitrogen, Grand Island, NY). These CD1d-Ig beads were loaded with either 5 μg/ml of α-GalCer (Enzo Life Sciences, Plymouth Meeting, PA) or different concentrations of probiotic lipid extracts. After extensive washing, aAPCs loaded with α-GalCer or probiotic lipid extract (unloaded empty beads were used as a control) were co-cultured with the NKT cell hybridoma, DN32.D3, for 16-24 hr at 37 °C in 96 well U-bottom plates (2×10^5 aAPCs mixed with 5×10^4 NKT cells in each well). IL-2 released by NKT hybridoma cells was measured by standard ELISA using an IL-2 ELISA kit according to the manufacturer's instructions (Biolegend, San Diego, CA), was used to indicate NKT cell activation.

Statistical analysis

All values are expressed as mean ± SD. Multiple comparisions were evaluated by ANOVA (SPSS 11.5 for windows, SPSS Inc, Chicago, Illinois). The paired group means were compared by t-test using Microsoft Excel (Microsoft, Redmond, WA). *P* values of less than 0.05 were considered statistically significant.

Results

High dose VSL#3 improves obesity and hepatic steatosis in HF diet fed mic

We have previously shown that probiotics improve NAFLD caused by HF diet [17]. To better understand the mechanisms by which the probiotics result in convalescence, we investigated the effects of different doses and evaluated whether the effects on NAFLD were strain specific. HF diet fed wild type mice were treated with high or low doses of VSL#3 (mix strains of probiotics) and a high dose single strain of *B. infantis*. We also treated HF diet fed mice with a mixture of antibiotics. High doses of VSL#3 significantly reduced HF-induced obesity (Figure 1A). Although low dose of VSL#3 also improved weight gain in HF diet fed mice but the result didn't reach a statistical significance. In the mean time, high dose of *B. infantis* and a mixture of antibiotics had little impact on animal weight (Figure 1A). High doses of VSL#3 also significantly improved glucose tolerance test (Figure 1B) and reduced serum insulin level (Figure 1C). Low dose of VSL#3 had slightly improved glucose tolerance test (Figure 1B), but no effect in serum insulin level (Figure 1C). *B. infantis* reduced serum insulin level (Figure 1C), but significantly worsened glucose tolerance test (Figure 1B) while antibiotic mixture had little impact in glucose tolerance test (Figure 1B) and significant higher serum insulin level (Figure 1C). Since HOMA-IR is the best indicator for insulin resistance[26, 27], it was calculated by using above parameters. High doses of VSL#3 significantly improved HF-induced insulin resistance as reflected by HOMA-IR while low dose of VSL#3 and *B.infantis* had little effect, and the antibiotic mixture even slightly worsened HOMA-IR, respectively (Figure 1D). For HF-induced hepatic steatosis, high doses of VSL#3 significantly reduced hepatic triglyceride content (Figure 1E) and improved histology (Figure 1F). Low dose of VSL#3 slightly improved steatosis on histology (Figure 1F), but didn't reach statistical significance on hepatic triglyceride content (Figure 1E), while *B. infantis* and the antibiotic mixture had no effect in hepatic triglyceride content and histological steatosis.

Treatment with high dose VSL#3 increases the percentage of hepatic NKT cells

Previously, probiotics were shown to improve HF diet-induced hepatic NKT cell depletion [4, 17]. In the current study, we demonstrate that the effect of probiotics on hepatic NKT cells was also dose and strain dependent. Only high dose VSL#3 improves hepatic NKT cell depletion in HF diet fed mice, while low dose VSL#3, single strain *B. infantis* and antibiotics had little impact (Figure 2).

Probiotic antigens stimulate NKT cells

To determine the potential mechanism of probiotics stimulation of NKT cells, lipid extractions were made from VSL#3 and *B. Infantis*. The extracts were loaded to aAPCs and then co-cultured with NKT hybridomas. IL-2 released from the NKT hybridomas reflected the stimulation of NKT cells by probiotic antigens. The VSL#3 extracts stimulated NKT cells in a dose dependent manner (Figure 3). *B. Infantis* extracts had little effect on NKT cells (Figure 3A). Both extracts showed competitive binding against α-GalCer to stimulate NKT cells. To further determine whether probiotic antigens can stimulate NKT cells *in vivo*, low dose of lipid extractions from VSL#3 or *B. Infantis* were administrated by i.p. to mice. Both VSL#3 and *B. Infantis* lipid extract administration *in vivo* stimulated hepatic NKT cells

(Figure 3B), while α-Galcer *in vivo* caused hepatic NKT cell anergy as previously published [28]. This result suggests that the lipids from probiotics and α-Galcer may have different mechanisms to induce NKT cells stimulation in vivo. To further investigate the mechanism of probiotic induced NKT cell stimulation, a NKT cell proliferation assay was performed with CFSE labeling. Primary NKT cells showed increased proliferation after stimulated with VSL#3 extraction (Figure 3C). The result confirms that the lipids from VSL#3 stimulate NKT cells and increase their proliferation.

Disruption of TLR4 signaling does not protect HF diet induced NKT cell depletion and metabolic dysfunction

We further examined whether toll-like receptor 4 (TLR4) played any role in high fat diet-induced hepatic NKT cell depletion. Wild type and TLR4 knockout mice were fed normal or HF diet. Hepatic NKT cells and metabolic profiles were measured. Disrupting TLR4 had no impact on HF diet-induced hepatic NKT cell depletion (Figure 4A) nor on HF diet-induced obesity (Figure 4B), insulin resistance or hepatic steatosis (Figure 4E, F). There is a slight increased glucose intolerance in TLR4 KO mice fed normal diet (Figure 4C). However, it didn't reach a statistical significance. HOMA-IR also showed no different between wt and TLR4 ko groups on insulin resistance (Figure 4D).

Discussion

Intestinal bacteria are known to play a critical role in obesity and NAFLD[7]. Colonization of germ-free mice with conventional gut bacteria causes a significant increase in body fat, despite a decrease in food intake[29]. In the current study, we show that modification of intestinal bacteria with probiotics has significant impact on liver NKT cells in a dose and strain dependent manner. We also show that the effects of probiotics on hepatic NKT cells are likely, at least, partially due to probiotic antigens that can direct stimulate NKT cells, although the overall effect of probiotics may still be the balance of endogenous bacteria. In addition, VSL#3 has much bigger impact on hepatic NKT cells *in vivo* than single strain *B. infantis*, even *B. infantis* is part of VSL#3 mixture, suggest that the interaction between different strains among probiotics or between host and probiotic may also play an important role.

The exact role of NKT cells in the pathogenesis of NAFLD is still controversial. Although studies from our groups and others show a reduction of NKT cells in HF diet induced mouse fatty liver [4, 15] and in the peripheral blood of patients with NAFLD [16], another study reported increased hepatic NKT cells in methionine choline-deficient (MCD) diet-induced NASH[30]. There is also a report demonstrating that the number of hepatic NKT cells are increased in patients with steatosis [31]. These controversies are most likely due to diverse NKT cell populations and to the lack of reliable and standard methods to detect NKT cells. More importantly, animal NAFLD models generated by different diets are likely to have different intestinal bacteria, since dietary factors have the most significant influence on the composition of gut bacteria [10, 11]. A recent study showed that the phenotypes and functions of NKT cells are directly depended on gut bacterial antigen presented to them [32]. HF and MCD diets fed mice are likely to have great difference in their gut bacterial content. Our current study supports the notion that alteration of intestinal bacteria can modulate hepatic NKT cells. Further studies are currently underway in our lab to understand the effect of different diets in gut bacterial composition and regulation of hepatic NKT cells. We also are studying the mechanism of which *B. Infantis* lipid extract had different effect on hepatic NKT cells *in vitro vs in vivo*. This is likely due to antigen modification and presentation when bacterial lipid extract was injected *in vivo*.

Similarly, dietary factors may also explain the controversial role of TLR4 in the pathogenesis of NAFLD. Previously, some studies have shown that TLR4 is involved in the development of MCD diet-induced [33], as well as fructose-induced [34] NAFLD. However, in our current study, we observe no difference in metabolic profiles, include steatosis, between wild type and TLR4 ko mice fed a HF diet. Again, different diets may alter intestinal bacteria thus having different effects on TLR4 signaling. Other studies also showed that the NKT cell responsiveness to gut bacteria didn't require Toll-like receptor signals [32].

In summary, our study investigates the potential mechanisms by which dietary factors alter intestinal bacteria and lead to the formation NAFLD. Modification of intestinal bacteria by probiotics directly influences hepatic NKT cells that contribute to the pathogenesis of NAFLD. Our observation that probiotics can enhance NKT cell function has potentially broader implications for the treatment of various liver diseases including NAFLD and autoimmune diseases.

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List of abbreviations

NAFLD	nonalcoholic fatty liver disease
HF	high fat diet
VSL#3	mixed strain probiotics
B. infantis	*bifidobacterium infantis*
TLR4 ko	toll like receptor 4 knockout
NASH	nonalcoholic steatohepatitis
ND	normal diet
High dose	1.5×10^9 colonies /mouse /day
Low dose	1.5×10^8 colonies /mouse /day
KCV	a mixture of kanamycin, colistin and vancomycin
GTT	Glucose tolerance test
HOMA-IR	homeostasis model assessment method
aAPCs	antigen presenting cells
TLR4	toll-like receptor four
MCD	methionine choline-deficient

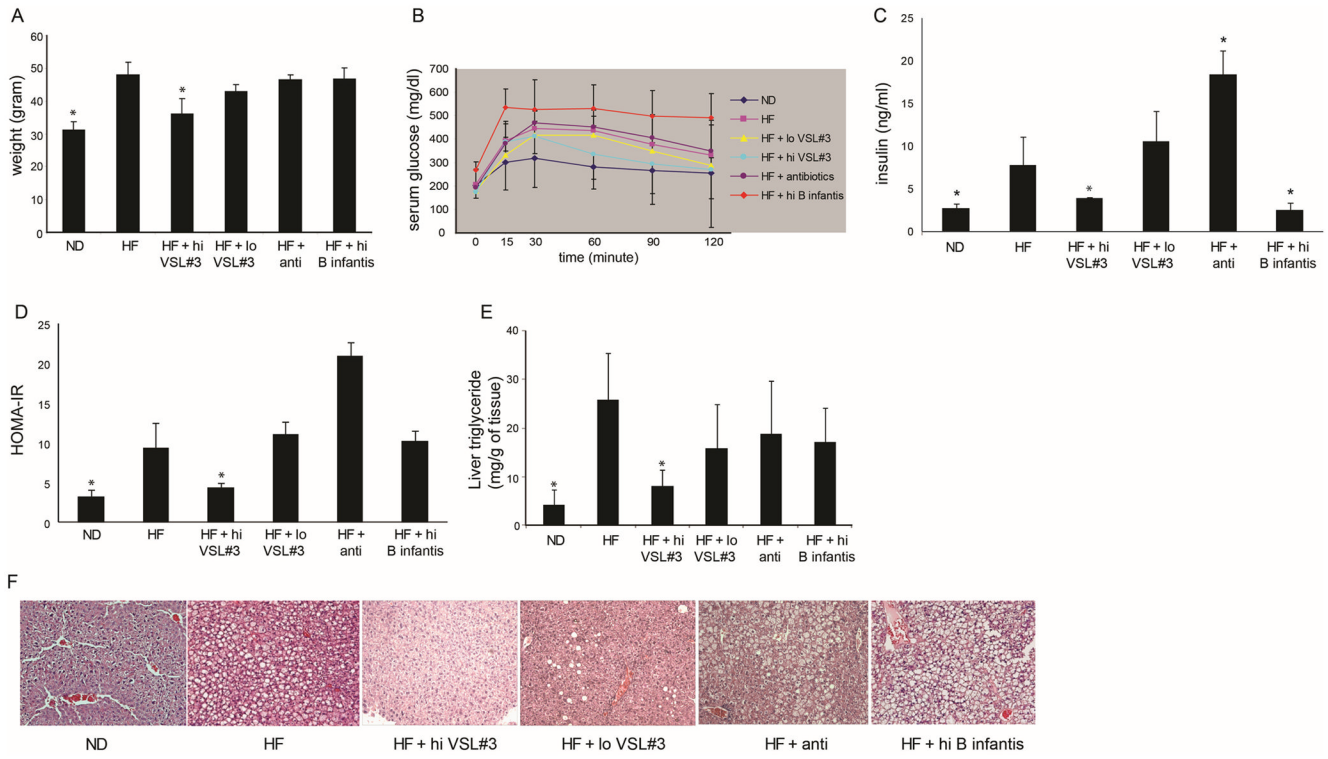

Figure 1. The dose and strain dependent effects of probiotics on HF diet-induced obesity, insulin resistance and hepatic steatosis

WT C57BL/6 mice were fed either ND or HF diet and some of HF fed mice were also treated with high dose VSL#3 (HF+hi VSL#3), or low dose VSL#3 (HF+lo VSL#3), or antibiotics (HF+anti) or high dose of *B. infantis* (HF+hi B infantis), or continue ND or HF diet. (A) Animal weight; (B) Glucose tolerance tests; (C) serum insulin level; (D) Insulin resistance measured by HOMA-IR; (E) Hepatic triglyceride content; (F) Representative H&E of liver histology. * $p<0.05$ vs HF diet group.

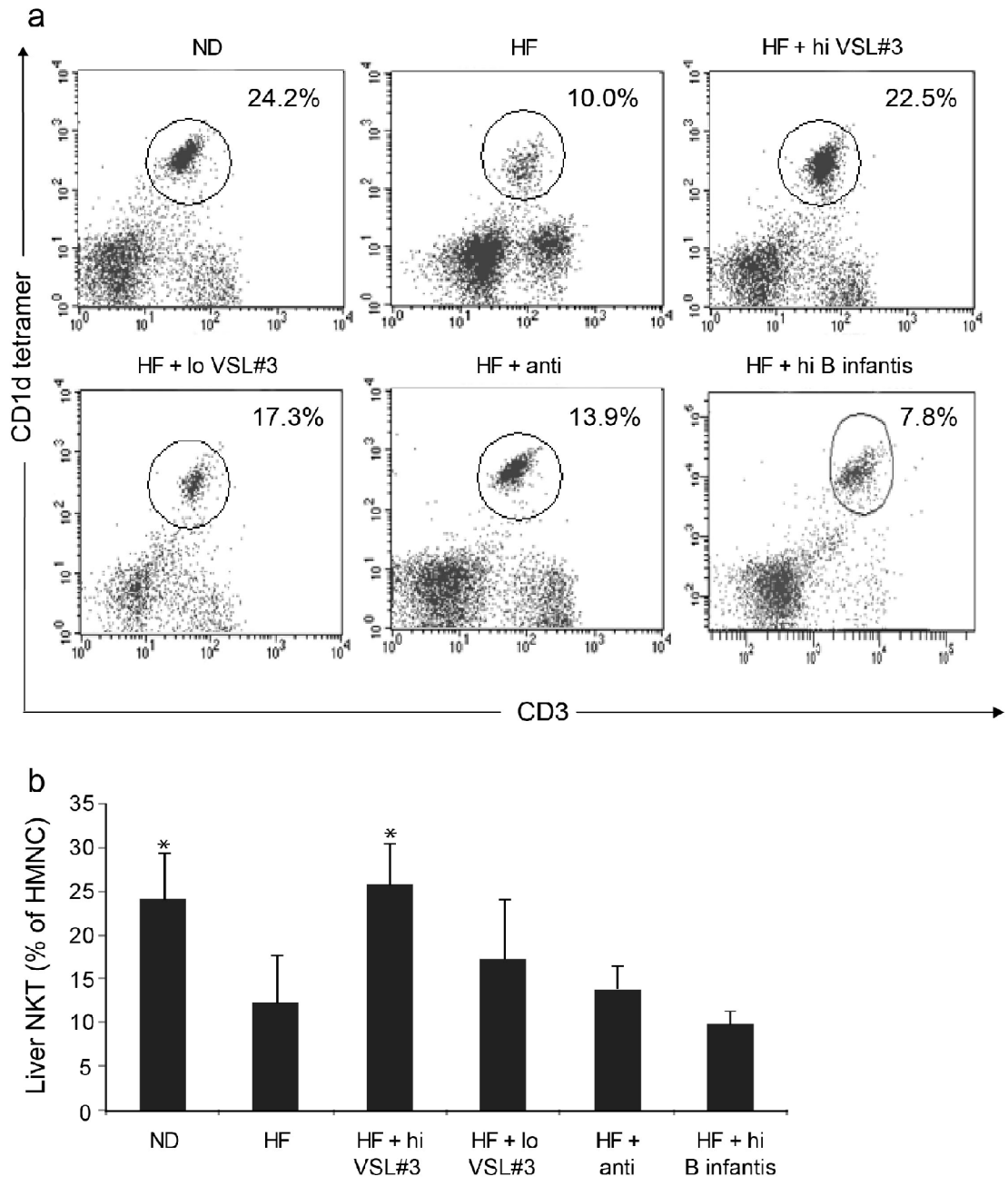

Figure 2. The dose and strain dependent effect of probiotics on hepatic NKT cells

Animals were treated as described in Fig. 1. Hepatic mononuclear (HMNC) cells were isolated and labeled with various surface markers. (A) A representative dot plot of NKT cell staining (gated on total HMNC); (B) Means ± SD of three independent experiments (n=5 per group). * $p<0.05$ vs HF diet group.

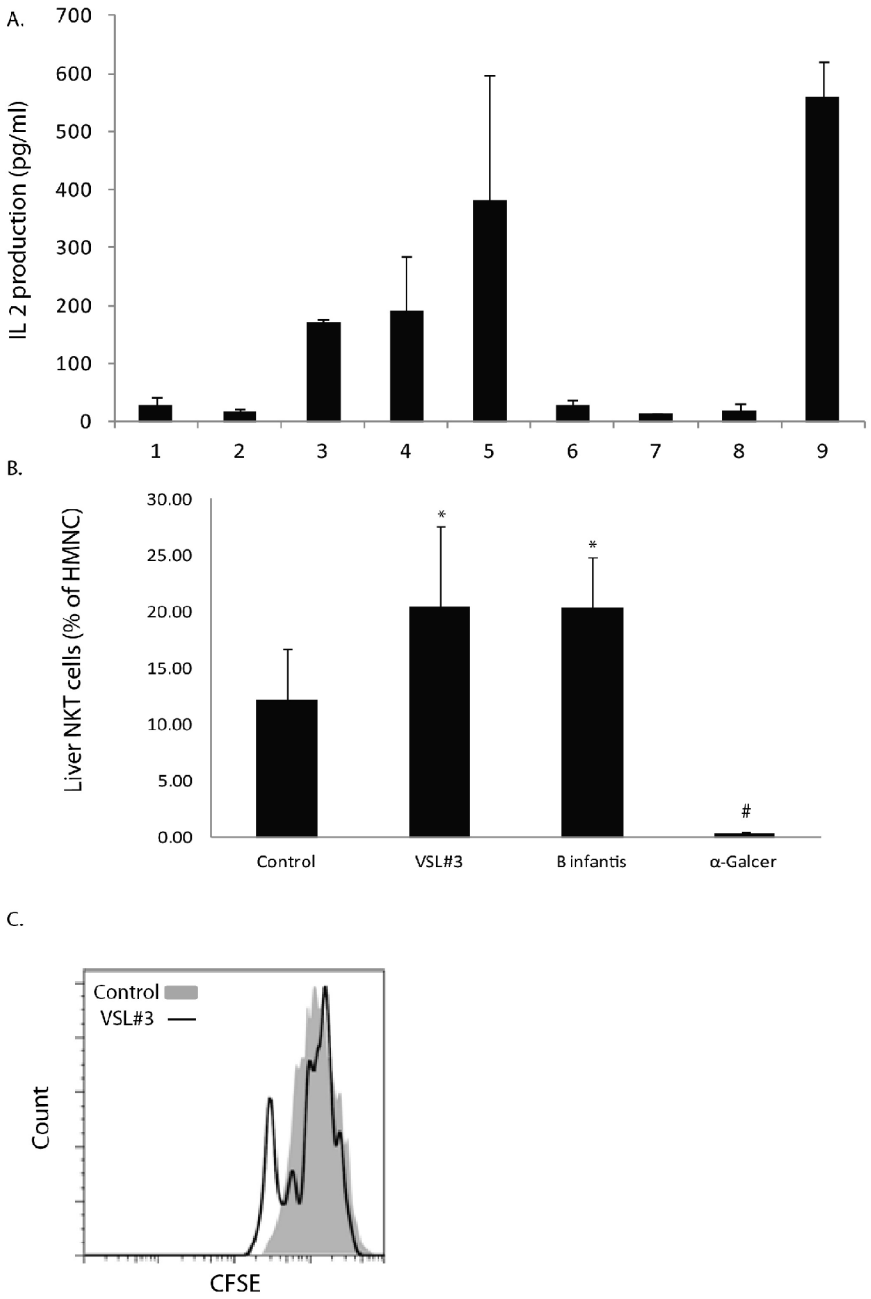

Figure 3. Probiotic antigens stimulate NKT cells *in vitro* and *in vivo*

(A) Bacterial glycolipids extracted were co-cultured with NKT hybridoma. IL-2 released by NKT hybridoma indicates their activation. 1 = media only; 2 = aAPCs only; 3 = aAPCs loaded with low dose VSL#3 extract; 4 = aAPCs loaded with high dose VSL#3 extract; 5 = aAPCs loaded with high dose VSL#3 extract plus α-GalCer; 6 = aAPCs loaded with low dose *B. infantis* extract; 7 = aAPCs loaded with high dose *B. infantis* extract; 8 = aAPCs loaded with high dose *B. infantis* extract plus α-GalCer; and 9 = aAPCs loaded α-GalCer. (B) Lipid extracts from VSL#3 or *B.Infantis*, or α-Galcer (2 μg/mouse) were injected to C57BL6 wt mice fed normal diet. After 24 hours, the animals were sacrificed and their hepatic NKT cells were evaluated as described in Fig 2. Means ± SD of the percentages of hepatic NKT cells (gated on CD3+ and CD1d Tetramer+) among HMNCs are shown (n=5

per group). (C) A representative histogram of NKT cell proliferation assay. HMNCs were labeled with CFSE and stimulated with aAPCs loaded with VSL#3 lipid extract or unloaded empty beads. *$p<0.025$, #$p<0.002$ vs control.

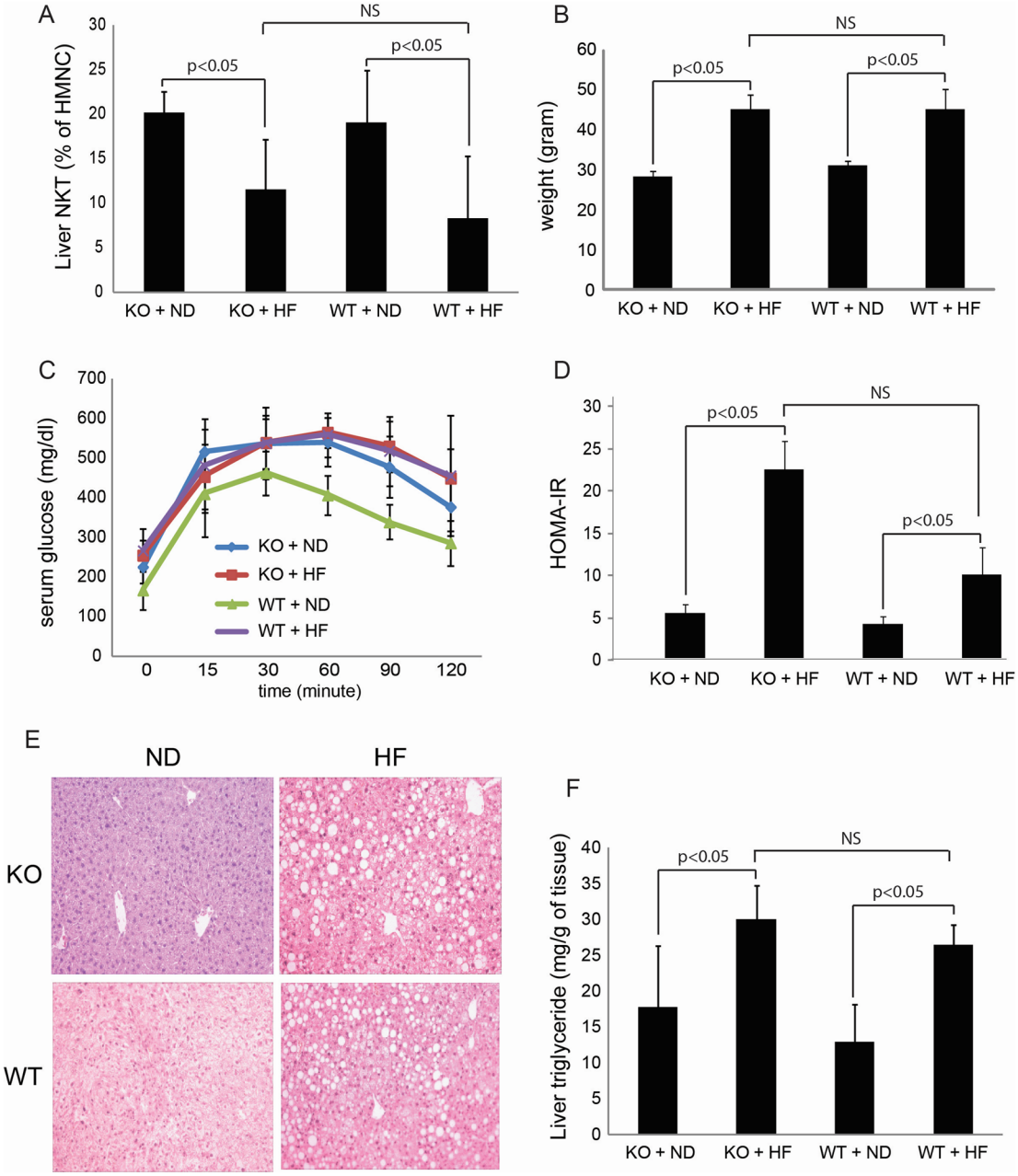

Figure 4. Disrupting TLR4 signaling did not protect mice from HF diet-induced NAFLD

Wild type and TLR4 knock out C57BL6 mice were fed ND or HF diet for 12 weeks. (A) Hepatic NKT cell content; (B) Animal weight; (C) Glucose tolerance tests; (D) Insulin resistance as measured by HOMA-IR; (E) Representative H&E staining of liver histology; (F) Hepatic triglyceride content. Results were means ± SD (n=5 per group)