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Blockade of B-cell-activating factor signaling enhances hepatic steatosis induced by a high-fat diet and improves insulin sensitivity

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Chronic inflammation is an important contributor to the development and progression of metabolic syndrome. Recent evidence indicates that, in addition to innate immune cells, adaptive immune cells have an important role in this process. We previously showed that the serum level of B-cell-activating factor (BAFF) was increased in patients with nonalcoholic steatohepatitis. However, it is currently unknown whether BAFF and BAFF-R (BAFF-R) have a role in lipid metabolism in the liver. To address this issue, the role played by BAFF and BAFF-R signaling in the development of insulin resistance and hepatic steatosis was examined in BAFF-R$^{-/-}$ mice fed a high-fat diet (HFD). Furthermore, the effect of BAFF on lipid metabolism in hepatocytes was analyzed *in vitro*. BAFF-R$^{-/-}$ mice showed improvements in HFD-induced obesity and insulin resistance. In addition, the number of B cells, levels of serum IgG, and inflammation of visceral fat were reduced in these mice. However, the expression of steatogenic genes and fatty acid deposition in the liver was higher in these mice than in control mice. BAFF was also found to downregulate the expression of steatogenesis genes and enhance steatosis in hepatocytes through BAFF-R. Collectively, these data indicated that, in addition to its known functions in inflammation and glucose metabolism, BAFF has a protective role in hepatic steatosis by regulating lipid metabolism in the liver.
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Nonalcoholic fatty liver disease (NAFLD), a feature of metabolic syndrome of the liver, is one of the most common liver diseases worldwide.[1] Most patients with NAFLD have simple steatosis, which has a good prognosis. However, some patients develop nonalcoholic steatohepatitis (NASH), which is associated with the risk of cirrhosis, liver failure, and hepatocellular carcinoma.[2] The pathogenesis of NAFLD is currently thought to involve multiple steps, of which the first is the accumulation of liver fat.[3] In addition, hepatic steatosis has an impact on the acceleration of liver damage in patients with chronic hepatitis due to other factors, especially hepatitis C viral infections.[4] These findings indicate the importance of identifying the mechanisms of hepatic fat accumulation.

Chronic inflammation is one of the main factors contributing to impaired insulin sensitivity and metabolic syndrome. Macrophage infiltration of visceral adipose tissues (VATs) is an important event in the establishment of adipose tissue inflammation,[5] and T cells are also major participants in VAT inflammation.[6,7] Recently, several studies have indicated that B cells are important pathogenic effectors in metabolic syndrome, including in insulin resistance.[8,9]

B-cell-activating factor (BAFF; CD257) belongs to the tumor necrosis factor ligand family and has been found to promote the expansion and differentiation of the B-cell population, leading to increased serum immunoglobulin (Ig) levels.[10] BAFF specifically binds to BAFF-R (CD268).[11] Although BAFF is primarily expressed by myeloid cells, BAFF-R is expressed on B cells and subsets of T cells. BAFF$^{-/-}$ mice and BAFF-R$^{-/-}$ mice have similar phenotypes, exhibiting reduced numbers of mature B cells and impaired antigen-dependent antibody responses.[12]

Recently, we reported that the serum BAFF level is increased and that BAFF is preferentially expressed in the

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VATs in high-fat diet (HFD)-fed mice.[13] We also found that in these mice, BAFF-R was preferentially expressed in the VATs and liver,[13] in addition to B cells and plasma cells. Furthermore, we found that NASH patients have higher serum BAFF levels than simple steatosis patients.[14] Collectively, these data indicate that BAFF is involved in the induction of the 'second hit' that is thought to be required for NASH development mediated by BAFF-R. However, the direct roles played by BAFF and BAFF-R in hepatic fat accumulation have not been ascertained.

In this study, to determine the direct *in vivo* role played by BAFF and BAFF-R in the development of insulin resistance and hepatic steatosis, we studied the HFD-induced NAFLD model with BAFF-R$^{-/-}$ mice, which do not express BAFF-R.

MATERIALS AND METHODS

Animals

Male C57BL/6J wild-type (WT) mice and B6(Cg)-Tnfrsf13c^{tm1Mass}/J (BAFF-R$^{-/-}$) mice were purchased from CLEA Japan (Tokyo, Japan) and the Jackson Laboratory (Bar Harbor, ME, USA), respectively. They were maintained at the Department of Biological Resources, Integrated Center for Science, Ehime University, under controlled conditions. These 6-week-old mice were divided into two groups. The control group was fed a normal diet (ND; 13% fat, 26% protein, and 60% carbohydrates; 360 kcal/100 g). The HFD group was fed an HFD (60% fat, 20% protein, and 20% carbohydrates; 520 kcal/100 g; D12492; Research Diets, New Brunswick, NJ, USA), as described previously.[13] All animals received humane care, and the study protocols complied with the guidelines of Ehime University.

Serum was extracted from the whole blood of each mouse when being killed and stored at $-80\,^{\circ}$C. Liver and epididymal adipose tissues were harvested, submerged in RNA-later (Life Technologies Co., Carlsbad, CA, USA), and stored at $-80\,^{\circ}$C for storage.

Cell Culture

The Hepa1-6 cell line, which is derived from a BW7756 tumor in a C57L mouse, was purchased from DS Pharma Biomedical Japan (Osaka, Japan), and primary cultured hepatocytes isolated from the liver of an ICR mouse were purchased from Primary Cell (Hokkaido, Japan). The cells were cultured as described previously.[15,16] After 4 days of incubation, the Hepa1-6 cells were treated for 6 h with 100 ng/ml recombinant murine BAFF (R&D Systems, Minneapolis, MN, USA). In some experiments, the cells were treated for 6 h with 2 μg/ml murine BAFF-R Fc (Enzo Life Science, Plymouth Meeting, PA, USA), in which the extracellular domain of mouse BAFF-R is fused at the C terminus to a linker peptide and to the Fc portion of human IgG1.

To analyze lipid accumulation in hepatocytes, Hepa1-6 cells were exposed to 0.5 mM oleic acid (Wako Chemical Co., Osaka, Japan) in the absence or presence of murine BAFF (100 ng/ml) for 12 h. The amounts of triglycerides were determined using a Triglyceride E-Test (Wako Chemical Co.).

Glucose, Pyruvate, and Insulin Tolerance Tests

Glucose and pyruvate tolerance were assessed using the intraperitoneal tolerance test. After overnight fasting, glucose (1.5 mg/g body weight) or pyruvate (2 mg/g body weight; Wako) was administered intraperitoneally. Blood samples were drawn from the tail vein at 0, 30, 60, 90, 120, and 150 min after administration.[17–19]

Insulin sensitivity was assessed using the insulin tolerance test. After 3 h of fasting, insulin (1 U/kg body weight; Eli Lilly, Indianapolis, IN, USA) was administered intraperitoneally, and blood samples were drawn from the tail vein at 0, 30, 60, 90, 120, and 150 min after administration.[19]

The glucose concentration and plasma insulin level were measured with a glucosensor (Glucose Pilot; Syntron Bioresearch, Carlsbad, CA, USA) and enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Kanagawa, Japan), respectively.

Histopathological and Immunohistochemical Examinations

Frozen liver tissues were cut into 5-μm-thick sections using a Cryostat OT (Hacker-Bright, Winnsboro, SC, UK) and fixed in neutral-buffered formalin. Paraffin-embedded sections of the liver were stained with hematoxylin and eosin. The liver lipids were stained with Sudan III. Histological examination was performed in a blinded manner by two experienced gastrointestinal pathologists with the histological scoring system for NAFLD.[20] Briefly, steatosis and inflammation scores ranged from 0 to 3, with 0 being within normal limits and 3 being most severe. Individual scores were assigned for each parameter.

Measurement of Hepatic Triglyceride and Cholesterol Contents

Hepatic triglyceride and cholesterol contents were measured at Skylight Biotech (Akita, Japan) using the Folch technique with Cholestest TG and Cholestest CHO kits (Sekisui Medical, Tokyo, Japan), respectively. The values were corrected for liver weight.

Biochemical Analysis of Serum and Measurement of B-Cell Counts

Serum IgG levels were measured using a mouse IgG enzyme-linked immunosorbent assay (Roche Diagnostics, Mannheim, Germany). Serum albumin, cholinesterase, cholesterol, triglyceride, and alanine aminotransferase (ALT) levels were measured using the Hitachi 7180 Auto Analyzer (Hitachi Ltd, Tokyo, Japan).

Leukocytes from among peripheral blood cells were counted using MYTHIC 18 (AT Will, Kanagawa, Japan). The cells were stained with fluorescein isothiocyanate anti-mouse

CD19 (clone 1D3) mAb or isotype controls (BD Bioscience). Flow cytometry was performed on a Becton Dickinson fluorescence-activated cell sorter (FACScalibur, Franklin Lakes, NJ, USA), and data acquisition was performed using CellQuest Software (Becton Dickinson). Data analysis was performed using FlowJo software (TreeStar Corporation, Ashland, OR, USA).

Measurement of Nuclear Factor-κB in Hepa1-6 Cell Nuclei

Hepa1-6 cells were treated with BAFF (100 ng/ml) for 6 h. Induction of p50, p52, RelA (p65), and RelB, a transcription factor of the nuclear factor (NF)-κB family, was analyzed using the TransAM Assay kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's protocol.

Real-Time Reverse Transcription Polymerase Chain Reaction

RNA was extracted from livers and epididymal adipose tissues using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) and RNeasy Plus Lipid kit (Qiagen), respectively. Reverse transcription reactions were performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), and real-time polymerase chain reaction analysis was performed using SYBR Green I (Roche Diagnostics, Basel, Switzerland) on LightCycler 480II (Roche Diagnostics). Primer sequences and annealing temperatures are detailed in Table 1. Gene expression data were normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Protein Sample Preparation

Hepa1-6 cells were washed twice with phosphate-buffered saline, and whole-cell protein samples were extracted using a radio-immunoprecipitation assay, as described previously.[13] Whole-cell lysates were sonicated, boiled at 95 °C for 5 min, and chilled on ice for 10 min. The protein concentration of the lysates was measured using the DC protein assay kit (Bio-Rad, Hercules, CA, USA).

Western Blot Analysis

Protein (30 μg) was placed in the wells of 4–12% Bis-Tris Gels (Invitrogen, Carlsbad, CA, USA) and resolved. The products were then blotted onto a polyvinylidene difluoride membrane using the NuPAGE transfer buffer and XCell SureLock (both from Invitrogen) according to the E-PAGE guide blotting protocol, available at www.invitrogen.com/epage. Next, they were incubated with the relevant antibody and detected using the Western Breeze Chromogenic Western Blot Immunodetection Kit (Invitrogen). The primary antibodies for fatty acid synthase (FAS) and GAPDH were purchased from Cell Signaling (Danvers, MA, USA), and those for BAFF-R were purchased from Abcam (Cambridge, UK).

Statistical Analyses

Data were analyzed using the JMP 8.0 software (SAS Institute, Cary, NC, USA). Values are presented as the mean ± s.e.m. Nonparametric Wilcoxon tests were used because the relatively small sample size undermined the distributional assumptions of parametric tests like the *t*-test. The means were statistically compared among the groups using one-way analysis of variance followed by Tukey's multiple comparison. Differences were considered significant at $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

RESULTS

Body Weight and Insulin Sensitivity of the HFD-Fed BAFF-R$^{-/-}$ Mice

BAFF-R$^{-/-}$ mice showed splenic deficiencies and significantly reduced splenic weights (0.068 ± 0.007 g; $n=10$) compared to WT mice (0.094 ± 0.003 g; $n=8$).

No difference was found in the body weights of WT and BAFF-R$^{-/-}$ mice who were fed the ND diet. However, the body weight of HFD-fed BAFF-R$^{-/-}$ mice was significantly lower than that of HFD-fed WT mice (Figure 1a). Bone lengths did not differ between WT and BAFF-R$^{-/-}$ mice that were fed the HFD (Figure 1b). Furthermore, the weight of the VAT did not differ between WT and BAFF-R$^{-/-}$ mice that were fed the HFD for 12 weeks, but the weights of inguinal fat and spleen were significantly lower in HFD-fed BAFF-R$^{-/-}$ mice (Figure 1c).

Next, we assessed insulin sensitivity in BAFF-R$^{-/-}$ mice fed the HFD. ND-fed BAFF-R$^{-/-}$ mice did not exhibit altered glucose metabolism (data not shown). Fasting blood glucose levels were significantly lower in HFD-fed BAFF-R$^{-/-}$ mice than in HFD-fed WT mice, although the fasting insulin levels were not different after HFD consumption for 12 weeks (Table 2). Compared with HFD-fed WT mice, HFD-fed BAFF-R$^{-/-}$ mice showed better glucose tolerance and insulin sensitivity on insulin challenge (Figure 2a). Responses to pyruvate did not differ between HFD-fed BAFF-R$^{-/-}$ mice and HFD-fed WT mice (Supplementary Figure 1a). Furthermore, mRNA expression of insulin receptor substrate (IRS)-1 and IRS-2 did not differ between these mouse groups (Supplementary Figure 1b), indicating that hepatic glucose homeostasis did not contribute substantially to systemic insulin sensitivity in our model.

Serum levels of albumin, cholinesterase, cholesterol, and triglycerides were not different between HFD-fed BAFF-R$^{-/-}$ mice and HFD-fed WT mice after HFD consumption for 12 weeks (Table 2).

B-Cell and Antibody Profiles in BAFF-R$^{-/-}$ Mice

We analyzed the profile of immune cells in the blood and VATs of BAFF-R$^{-/-}$ mice fed the HFD. The number of peripheral blood B cells was lower in HFD-fed BAFF-R$^{-/-}$ mice than in HFD-fed WT mice (Figure 2b). This difference was also found in BAFF-R$^{-/-}$ mice compared with WT mice

Table 1 Oligonucleotide sequences and annealing temperature for quantitative real-time RT-RCR

Primer	GenBank accession no.	Sequence 5′-forward-3′ 5′-reverse-3′	Location	Annealing temperature (°C)
GAPDH	NM_008084	TGCACCACCAACTGCTTA	nt498–515	63
		GGATGCAGGGATGATGTT	nt657–674	
F4/80	NM_010130	AGTACGATGTGGGGCTTTTG	nt35–54	60
		CCCCATCTGTACATCCCACT	nt77–96	
CD11b	NM_001082960	CAGTTCCCAGAGGCTCTCA	nt509–527	65
		CGGAGCCATCAATCAAGAAG	nt559–578	
CD11c	NM_021334	ATGGAGCCTCAAGACAGGAC	nt1725–1744	60
		GGATCTGGGATGCTGAAATC	nt1768–1787	
TNFα	NM_013693	TCTTCTCATTCCTGCTTGTGG	nt260–280	60
		GGTCTGGGCCATAGAACTGA	nt368–387	
BAFF-R	NM_028075	GAAACTGCGTGTCCTGTGAG	nt122–141	70
		CTGAGGCTGCAGAGCTGTC	nt189–207	
BCMA	NM_011608	TGGTCCTCTCTTTGGCACTT	nt320–339	70
		TGATCCTAGTCAGCTCGGTGT	nt443–463	
TACI	NM_021349	GAGCTCGGGAGACCACAG	nt265–282	67
		TGGTCGCTACTTAGCCTCAAT	nt348–368	
CD36	NM_016741	CGTCTACCCACCCAACGA	nt1148–1165	65
		AGAAACAGAGGCGCACCA	nt1220–1237	
FABP4	NM_024406	GAAAACGAGATGGTGACAAGC	nt385–405	60
		GCCCTTTCATAAACTCTTGTGG	nt439–460	
SREBP-1c	ENSMUST00000144942	CATGGATTGCACATTTGAAGA	nt111–131	65
		CGGGAAGTCACTGTCTTGGT	nt152–171	
FAS	NM_007988	GCTGCTGTTGGAAGTCAGC	nt767–785	70
		AGTGTTCGTTCCTCGGAGTG	nt823–842	
ACC	NM_133360	GCGTCGGGTAGATCCAGTT	nt6434–6452	60
		CTCAGTGGGGCTTAGCTCTG	nt6484–6503	
SCD-1	NM_009127	TTCCCTCCTGCAAGCTCTAC	nt566–585	60
		CAGAGCGCTGGTCATGTAGT	nt608–627	
ACS	NM_030210	GACCCGAGGCATTCCATA	nt1971–1988	60
		CAGCCATCACCTGCTTCAC	nt2020–2038	
PPARα	NM_011144	CACGCATGTGAAGGCTGTAA	nt794–813	67
		CAGCTCCGATCACACTTGTC	nt854–873	
MTP	NM_008642	TGTCAGAATGAAGGCTGCAA	nt1395–1414	60
		AGTCCTCCCAGGATCAGCTT	nt1440–1459	
ApoB	NM_009693	GGCACTGTGGGTCTGGAT	nt12 016–12 033	70
		TTCTTCTCTGGAGGGGACTG	nt12 082–12 101	
TGFβ-1	NM_011577	TGGAGCAACATGTGGAACTC	nt1358–1377	60
		CAGCAGCCGGTTACCAAG	nt1411–1428	
IL-6	NM_031168	ACAACCACGGCCTTCCCTACTT	nt92–113	63
		CACGATTTCCCAGAGAACATGTG	nt198–220	
IL-18	NM_008360	GACAACACGCTTTACTTTATACCTGA	nt73–84	70
		CAGTGAAGTCGGCCAAAGTT	nt99–112	

Table (Continued)

Primer	GenBank accession no.	Sequence 5′-forward-3′ 5′-reverse-3′	Location	Annealing temperature (°C)
MCP-1	NM_011333	CATCCACGTGTTGGCTCA	nt139–56	70
		GATCATCTTGCTGGTGAATGAGT	nt192–214	
IRS-1	NM_010570	CTATGCCAGCATCAGCTTCC	nt4597–4616	60
		TTGCTGAGGTCATTTAGGTCTTC	nt4667–4689	
IRS-2	NM_001081212	CGACTTCCTGTCCCATCACT	nt3918–3937	70
		GCTGGTAGCGCTTCACTCTT	nt3959–3978	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TNF: tumor necrosis factor; BAFF-R: B-cell-activating factor receptor; BCMA: B-cell maturation antigen; TACI: transmembrane activator, calcium-modulator, and cyclophilin ligand interactor; FABP: fatty acid-binding protein; SREBP: sterol regulatory element-binding protein; FAS: fatty acid synthase; ACC: acetyl-CoA carboxylase; SCD: stearoyl-CoA desaturase; ACS: acetyl-CoA synthetase; PPAR: proliferator-activated receptor; MTP: microsomal triglyceride transfer protein; ApoB: apolipoprotein B; TGF: transforming growth factor; IL: interleukin; MCP-1: monocyte chemotactic protein-1; IRS: insulin receptor substrate.

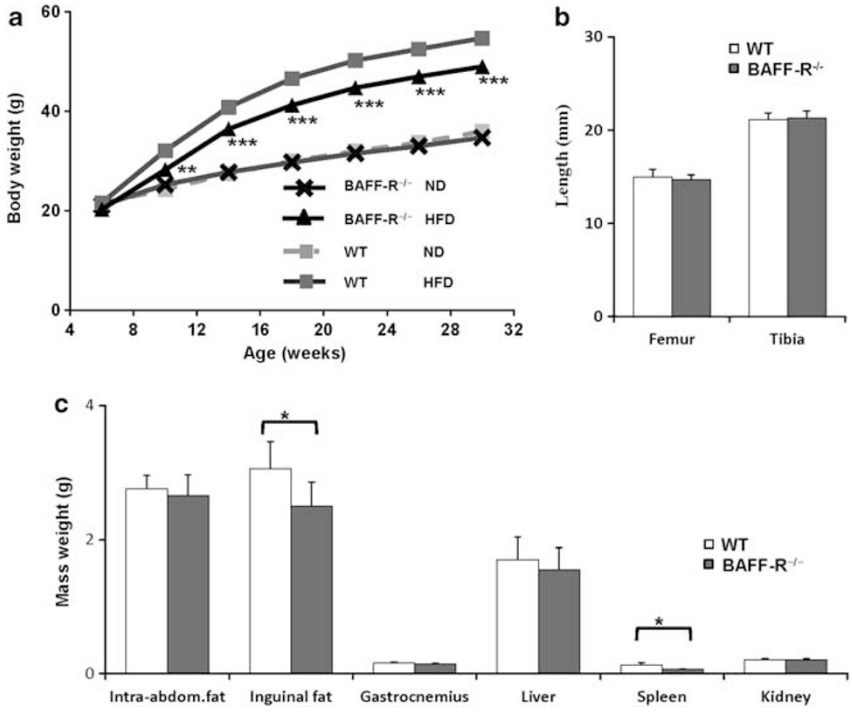

Figure 1 Metabolic and immunological profiles of high-fat diet (HFD)-fed B-cell-activating factor receptor (BAFF-R)$^{-/-}$ mice. (**a**) Weight gain in C57BL/6J (wild-type (WT)) and BAFF-R$^{-/-}$ mice on a normal diet (ND) or HFD ($n=30$–35 per group). (**b**) Bone length of mice fed the HFD for 12 weeks ($n=7$ per group). (**c**) Intra-abdominal fat, inguinal fat, gastrocnemius muscle, liver, spleen, and kidney masses from mice fed the HFD for 12 weeks ($n=7$ per group). Error bars in the graphs indicate means ± s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

fed the ND. Furthermore, serum IgG levels were significantly lower in HFD-fed BAFF-R$^{-/-}$ mice than in HFD-fed WT mice (Figure 2b). Moreover, the expression of macrophage markers, such as F4/80, CD11b, CD11c, and TNF-α, in the epididymal adipose tissues was lower in HFD-fed BAFF-R$^{-/-}$ mice than in HFD-fed WT mice after 12 weeks of HFD consumption (Figure 2c).

Hepatic Steatosis in BAFF-R$^{-/-}$ Mice Fed the HFD

Surprisingly, we found that serum ALT levels in HFD-fed BAFF-R$^{-/-}$ mice were significantly higher than those in HFD-fed WT mice (Figure 3a). Furthermore, the liver fat content in BAFF-R$^{-/-}$ mice was also higher than that in WT mice, although the extent of inflammatory cell infiltration did not differ between groups (Figure 3b and c). Similarly,

Table 2 Glucose metabolism profiles of HFD-fed BAFF-R$^{-/-}$ mice

	WT	BAFF-R$^{-/-}$
Blood glucose (mg/dl)	212.3 ± 12.2	159.1 ± 7.5*
Serum insulin (pg/mg)	2123.3 ± 117.5	2036.4 ± 70.5
Serum albumin (g/dl)	3.1 ± 0.07	3.1 ± 0.05
Serum cholinesterase (IU/l)	27.8 ± 1.0	27.1 ± 1.3
Serum cholesterol (mg/dl)	176.8 ± 2.8	183.4 ± 7.2
Serum triglyceride (mg/dl)	64.3 ± 4.5	50.3 ± 4.9

Various parameters were measured in HFD-fed mice after 12 weeks of feeding ($n = 8$ per group). Results are expressed as mean ± s.e.m. *$P < 0.05$, compared to WT.
*$P < 0.05$, compared with WT.

analysis of liver lipid contents showed that HFD-fed BAFF-R$^{-/-}$ mice had significantly higher liver triglyceride contents than HFD-fed WT mice after 12 weeks of HFD consumption. However, liver cholesterol levels did not differ between groups (Figure 3d).

Gene Expression in the Liver of BAFF-R$^{-/-}$ Mice

BAFF-R, transmembrane activator, calcium modulator, cyclophilin ligand interactor (TACI; CD267), and B-cell maturation factor (BCMA; CD269) have been identified as receptors for BAFF on B cells and plasma cells.[21] We analyzed the expression of these receptors in the livers of WT and BAFF-R$^{-/-}$ mice. As shown in Figure 4a, the expression of BAFF-R was higher than that of TACI and BCMA in WT mice. Furthermore, no difference was found in the expression levels of TACI and BCMA between WT and BAFF-R$^{-/-}$ mice.

To address the mechanisms underlying liver steatosis in BAFF-R$^{-/-}$ mice, we analyzed the expression of genes related to lipid metabolism in the livers of BAFF-R$^{-/-}$ and WT mice. The expression of the gene encoding fatty acid-binding protein (FABP) 4, which is related to uptake and transportation and increases with fatty deposition, was increased in the livers of the BAFF-R$^{-/-}$ mice (Figure 4b). In addition, an increase in the mRNA levels of proteins involved in steatogenesis, such as FAS and acetyl-CoA carboxylase (ACC), was observed in the livers of HFD-fed BAFF-R$^{-/-}$ mice. The mRNA level of acetyl-CoA synthetase (ACS), which activates fatty acids, was also increased in the livers of HFD-fed BAFF-R$^{-/-}$ mice (Figure 4c). While the expression of sterol regulatory element-binding protein (SREBP)-1c and stearoyl-CoA desaturase (SCD)-1 in the livers of ND-fed BAFF-R$^{-/-}$ mice was significantly higher than that of ND-fed WT mice, no difference was found in the expression of these molecules in the liver of WT and BAFF-R$^{-/-}$ mice fed the HFD. In addition, the mRNA levels of SREBP-1c, FAS, ACC, SCD-1, and ACS in the liver was not different between ND- and HFD-fed WT mice; however, SCD-1 expression in the liver was significantly lower in HFD-fed mice than ND-fed BAFF-R$^{-/-}$ mice (Figure 4c).

The expression of genes related to oxidation, very low-density lipoprotein production, liver fibrosis, and inflammation did not differ between HFD-fed BAFF-R$^{-/-}$ mice and HFD-fed WT mice (Figures 4d and e). While the reduced interleukin (IL)-6 expression in the livers of HFD-fed mice compared with those from ND-fed mice was observed both in WT and BAFF-R$^{-/-}$ mice, the expression of IL-6 did not differ between HFD-fed BAFF-R$^{-/-}$ mice and HFD-fed WT mice (Figure 4d).

In addition, we confirmed that FAS protein levels increased in the livers of BAFF-R$^{-/-}$ mice (Figure 4f).

BAFF Decreased the Expression of Lipogenesis-Related Genes in Hepa1-6 Cells

We previously reported that BAFF-R is expressed in 3T3-L1 adipocytes.[13] Here, we found that Hepa1-6 cells and primary cultured murine hepatocytes also expressed BAFF-R (Figure 5a). We then examined whether BAFF-R exerted its signaling function in hepatocytes. It has been reported that BAFF preferentially activates the non-canonical NF-κB pathway through ligation with BAFF-R on B cells and adipocytes.[22] We found that NF-κB2 and RelB were induced in Hepa1-6 cells treated with BAFF (Figure 5b). Finally, we investigated the effect of exogenous BAFF on hepatocytes. BAFF treatment downregulated the expression of genes encoding proteins related to steatogenesis, such as FAS, ACC, and SCD-1, in Hepa1-6 cells (Figure 5c). Furthermore, these changes were inhibited by treatment with BAFF-R Fc (Figure 5d). Cell viability was not affected by BAFF treatment (data not shown).

Finally, we analyzed the role of BAFF in lipid accumulation in an *in vitro* model of hepatic steatosis. Hepa1-6 cells were exposed to oleic acid (0.5 mM) in the absence or presence of BAFF (100 ng/ml) for 12 h. Lipid accumulation reduced by treatment with BAFF (Figure 5e).

DISCUSSION

We previously showed that BAFF impairs insulin sensitivity and is associated with NAFLD severity,[13,14] indicating that this molecule is an important factor in lipid and glucose metabolism. In this study, as expected, HFD-fed BAFF-R$^{-/-}$ mice showed lower body weight gain. This finding may be attributed to the inguinal fat deposition in these mice as the spleen contributes very little to body weight (Figure 1c). HFD-fed BAFF-R$^{-/-}$ mice showed lower fasting glucose levels than HFD-fed WT mice (Table 2). Furthermore, HFD-fed BAFF-R$^{-/-}$ mice showed better glucose tolerance and insulin sensitivity after insulin challenge that HFD-fed WT mice (Figure 2a). ND-fed BAFF-R$^{-/-}$ mice did not exhibit altered glucose metabolism (data not shown), suggesting that these metabolic effects required an HFD to be manifested.

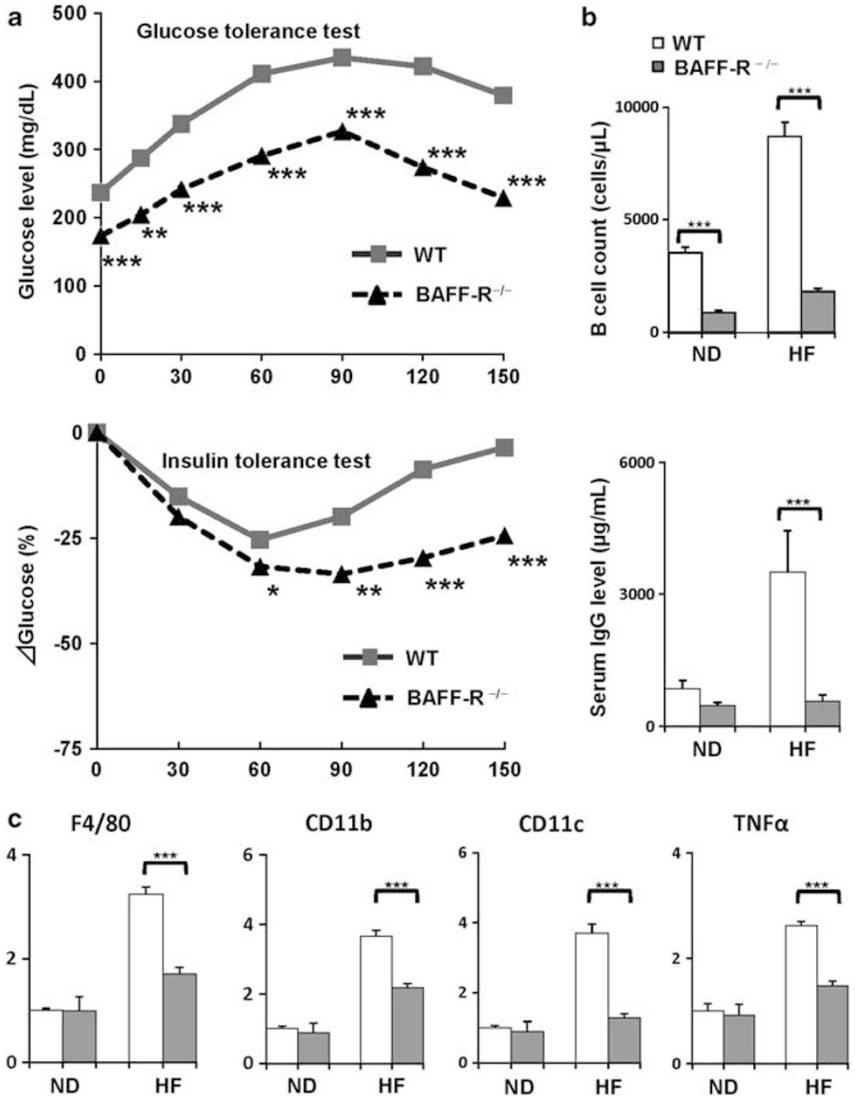

Figure 2 Glucose metabolism and immunological profiles of high-fat diet (HFD)-fed B-cell-activating factor receptor (BAFF-R)$^{-/-}$ mice. (**a**) Results of a glucose tolerance test and insulin tolerance test for HFD-fed mice after 12 weeks of feeding ($n=5$ per group). (**b**) Numbers of B cells in the peripheral blood and serum immunoglobulin G (IgG) levels after 12 weeks of normal diet (ND) or HFD consumption ($n=8$ per group). (**c**) Gene expression in epididymal adipose tissues of mice after 12 weeks of ND or HFD consumption. Gene expression data were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were expressed as a ratio to those obtained for WT mice after 12 weeks of ND feeding ($n=8$ per group). Error bars in the graphs indicate means ± s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$. TNF, tumor necrosis factor.

Studies on the inflammatory process have mainly focused on innate cells, particularly macrophages; however, recent evidence suggests that adaptive cells also have an important role in this process. Duffaut *et al*[8] reported that B-cell accumulation in VATs occurred as early as 3 weeks after the initiation of an HFD, before any modulation of fat mass and accumulation of T cells and macrophages. In addition, Winer *et al*[9] recently showed that HFD-fed B-cell-deficient mice (Ig μ heavy-chain knockout mice) have fewer proinflammatory M1 macrophages, as was found in this study (Figure 2c), and that these mice show improved glucose and insulin tolerance when fed an HFD. They also showed that the B cells of HFD-fed mice showed elevated secretion of IgG, an antibody that induces VAT inflammation and abnormal glucose metabolism.[9] In this study, we showed that the serum IgG level was significantly lower in HFD-fed BAFF-R$^{-/-}$ mice than in HFD-fed WT mice (Figure 2b). Taken together, these data indicate that a reduction in the number of B cells and the IgG levels affects the improvement in insulin sensitivity in HFD-fed BAFF-R$^{-/-}$ mice. Kyaw *et al*[23] also reported that atherosclerosis was markedly reduced in HFD-fed BAFF-R$^{-/-}$ apoprotein (Apo) E$^{-/-}$ mice compared with HFD-fed BAFF-R$^{+/+}$ ApoE$^{-/-}$ mice. In addition, we previously showed that the interaction of BAFF and BAFF-R in VAT leads to impaired insulin sensitivity via inhibition of insulin signaling pathways and alterations in adipokine

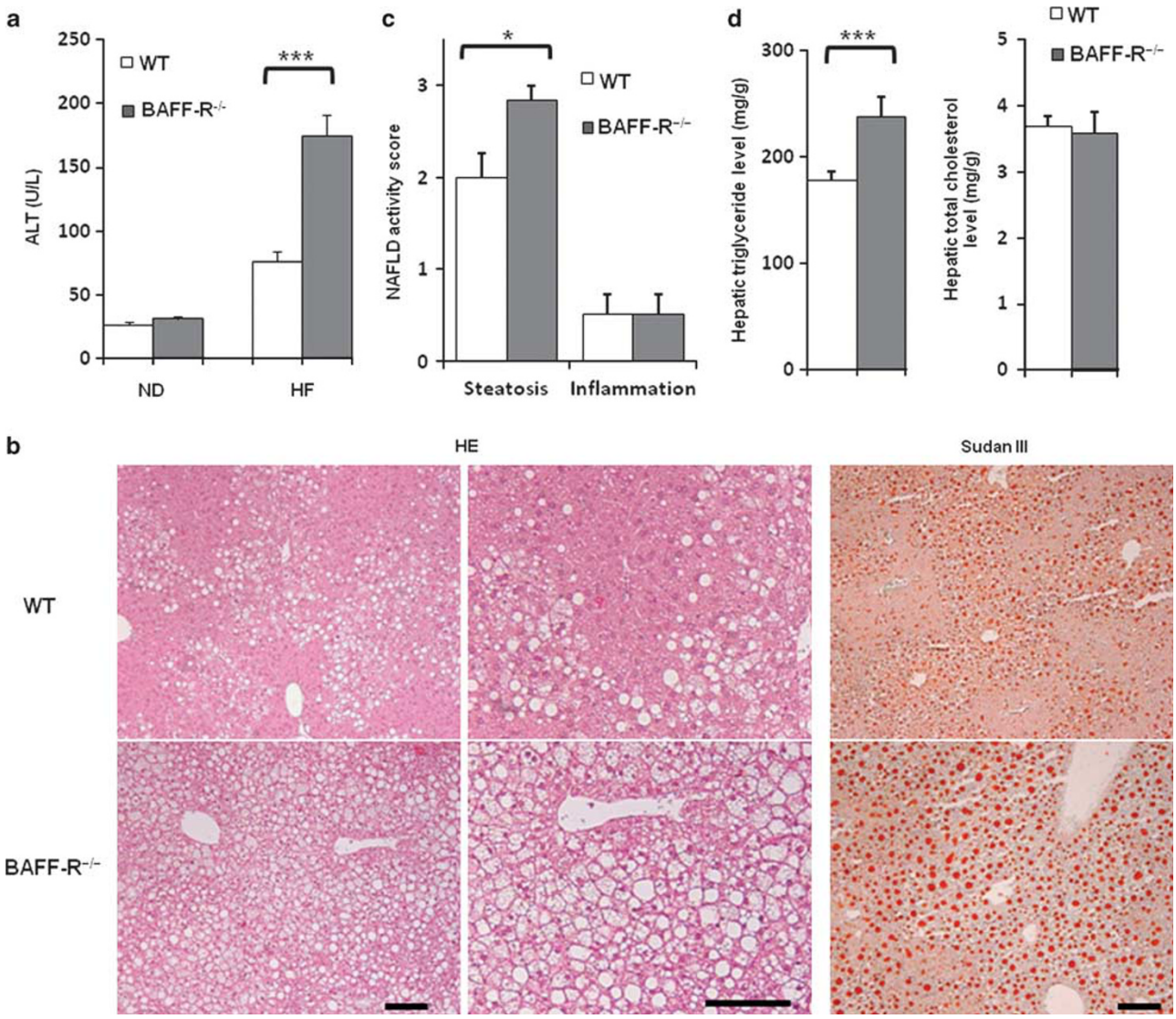

Figure 3 Liver steatosis in high-fat diet (HFD)-fed B-cell-activating factor receptor (BAFF-R)$^{-/-}$ mice. (**a**) Serum alanine aminotransferase (ALT) levels of normal diet (ND)- or HFD-fed mice after 12 weeks of feeding ($n=8$ per group). (**b**) Hematoxylin and eosin (H&E) staining (left and middle) and Sudan III (right) staining of sections of livers from mice after 12 weeks of HFD consumption; scale bar, 200 μm. (**c**) Nonalcoholic fatty liver disease (NAFLD) histological activity scores of HFD-fed mice after 12 weeks of feeding ($n=8$ per group). (**d**) Hepatic triglyceride and cholesterol levels of mice after 12 weeks of HFD consumption ($n=5$ per group). Error bars in the graphs indicate means ± s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$.

production.[13] These metabolic effects may also contribute to insulin sensitivity in HFD-fed BAFF-R$^{-/-}$ mice.

Our previous study[13] analyzed the role of BAFF in obesity and VAT inflammation using the HFD-fed mice model. Although we did not evaluate liver pathologies in the previous study, we speculated that hepatic steatosis would be ameliorated in HFD-fed BAFF-R$^{-/-}$ mice.[13] Surprisingly, hepatic fat deposition was found to be enhanced in BAFF-R$^{-/-}$ mice in the HFD-induced NAFLD model examined in this study (Figure 3c), although the extent of liver inflammation did not differ between test and control animals, indicating that the increased ALT level in HFD-fed BAFF-R$^{-/-}$ mice was predominantly caused by liver steatosis and not by liver inflammation. Triglycerides accumulate in the liver because of an imbalance among the uptake, transportation, biosynthesis, activation, export, and oxidation of fatty acids. DNA microarray analysis to compare gene expression levels between the livers of BAFF-R$^{-/-}$ mice and WT mice revealed that the expression of genes associated with fat transportation, biosynthesis, and activation was higher in the livers of BAFF-R$^{-/-}$ mice than WT mice (data not shown). We confirmed some of these changes using reverse transcription polymerase chain reaction (Figure 4) in this study.

The expression of IL-6 and SCD-1 in the liver differed between HFD- and ND-fed BAFF-R$^{-/-}$ mice. Lower IL-6 expression was also found in the livers of HFD-fed WT mice;

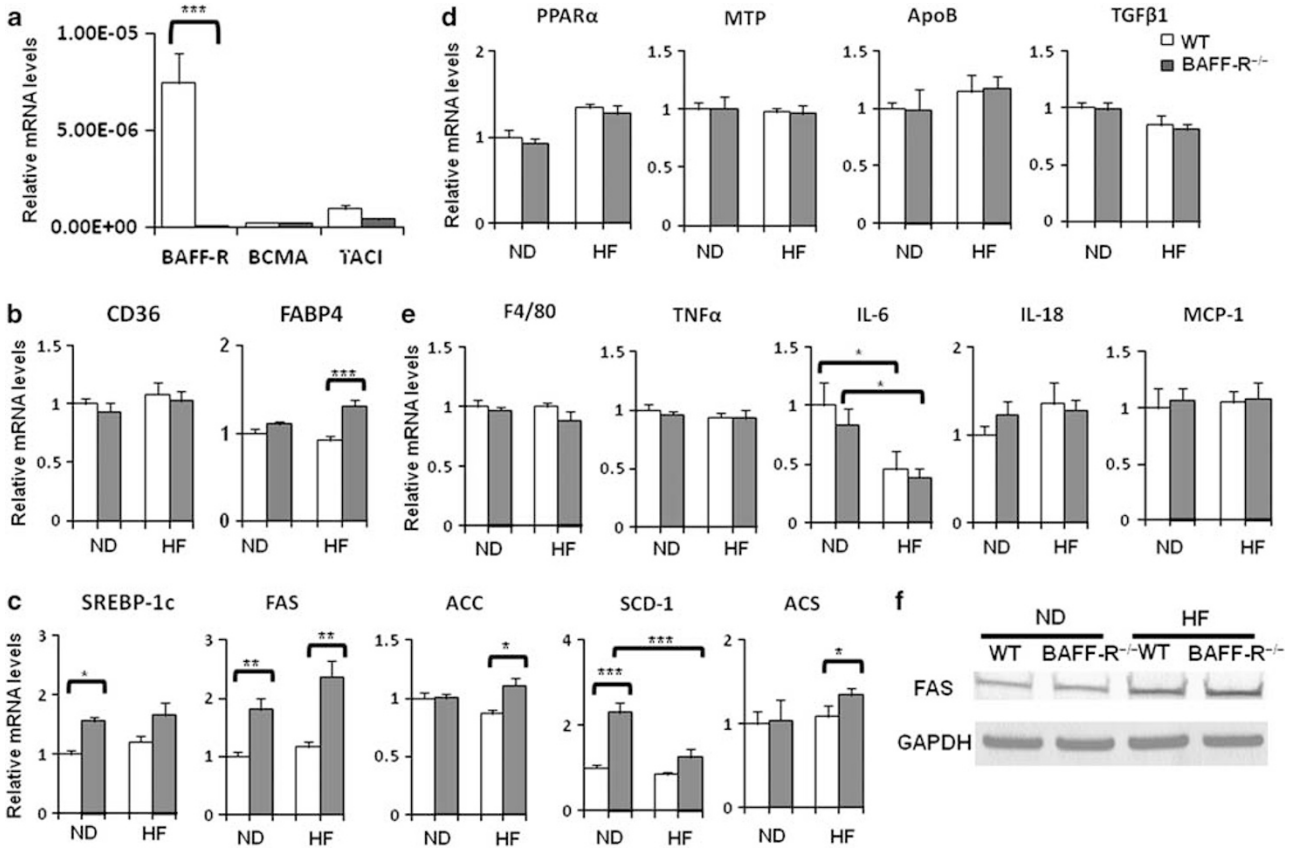

Figure 4 Gene expression in mouse livers after 12 weeks of normal diet (ND) or high-fat diet (HFD) consumption. (**a**) Expression of B-cell-activating factor receptor (*BAFF-R*), transmembrane activator, calcium modulator, cyclophilin ligand interactor (*TACI*), and B-cell maturation factor (*BCMA*) genes. The data are normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. (**b**–**e**) Expression of genes associated with uptake and transportation (**b**), biosynthesis and activation (**c**), oxidation, very low-density lipoprotein production, fibrosis (**d**), and inflammation (**e**). (**f**) Fatty acid synthase (FAS) protein expression in mouse livers after 12 weeks of ND or HFD consumption was analyzed by western blotting. Gene expression data were normalized to those of GAPDH and were expressed as a ratio to those obtained for the wild-type (WT) mice after 12 weeks of ND feeding ($n = 7$ each group). Error bars in the graphs indicate means ± s.e.m. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. ACC, acetyl-CoA carboxylase; ACS, acetyl-CoA synthetase; Apo, apoprotein; FABP, fatty acid-binding protein; IL, interleukin; MCP-1, monocyte chemotactic protein-1; MTP, microsomal triglyceride transfer protein; PPAR, peroxisome proliferator-activated receptor; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein; TGF, transforming growth factor; TNF, tumor necrosis factor.

this may be the effect of the HFD. On the other hand, SREBP-1c and SCD-1 expression in the liver did not differ between HFD- and ND-fed WT mice. The exact reasons for the difference in liver SREBP-1c and SCD-1 expression are not understood. However, discrepancies have been found in liver SREBP-1c and SCD-1 expression in experimental models of fatty liver.[24–26] Further studies are necessary to clarify this matter.

In addition, we examined the direct effects of BAFF on hepatocytes and found that BAFF treatment suppressed the expression of steatogenesis genes and decreased lipid accumulation in Hepa1-6 cells exposed to oleic acid (Figure 5e). Inhibition of the steatogenesis gene was reversed by treatment with BAFF-Fc (Figure 5d). These data indicate that BAFF-R signaling in hepatocytes leads to the downregulation of steatogenesis gene expression.

Liver fatty acids are derived from *de novo* lipogenesis and plasma-free fatty acids. Although the contribution of *de novo* lipogenesis in the liver is very small, it has been reported that it is elevated in NAFLD,[27,28] and that the expression of ACC and FAS in the liver is increased in NAFLD patients,[29] as shown in the HFD-fed BAFF-$R^{-/-}$ mice in this study (Figure 4c). These data collectively indicate that the rate of fatty acid synthesis in the liver increases in HFD-fed BAFF-$R^{-/-}$ mice, despite the accumulation of fatty acids, and that the interaction between BAFF and BAFF-R interaction on hepatocytes may inhibit *de novo* lipogenesis in the liver.

Recent studies have shown that steatogenesis proteins, including FAS, promote the development and malignant potential of cancer.[30] Furthermore, inhibition of ACS amplifies antitumor effects.[31] The data in this study indicated that BAFF-R signaling on hepatocytes may have a role in the development and progression of liver cancer; nonetheless, further studies are necessary to substantiate this conclusion.

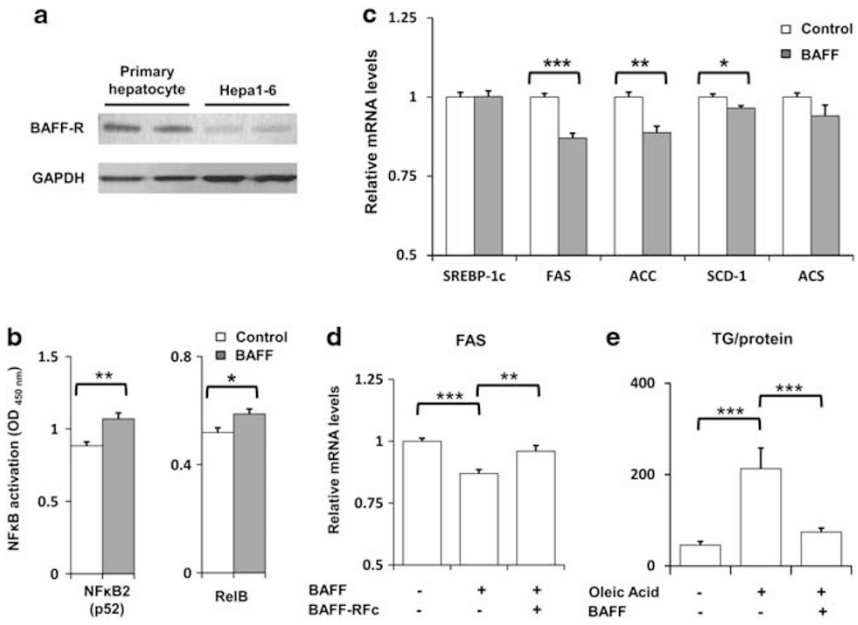

Figure 5 Effects of B-cell-activating factor (BAFF) on hepatic lipogenesis in Hepa1-6 cells. (**a**) BAFF receptor (BAFF-R) protein expression in Hepa1-6 cells and primary murine hepatocytes were analyzed by western blotting. (**b**) Induction of nuclear factor-κB (NF-κB) in Hepa1-6 cells treated with BAFF ($n=8$ per group). (**c**) Changes in gene expression in Hepa1-6 cells treated with BAFF ($n=10$ per group). (**d**) Fatty acid synthase (FAS) mRNA expression in Hepa1-6 cells treated with BAFF and BAFF-R Fc. Gene expression data were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (**e**) Lipid accumulation in Hepa1-6 cells exposed to oleic acid in the presence or absence of BAFF ($n=8$ per group). Error bars in the graphs indicate means ± s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$. ACC, acetyl-CoA carboxylase; ACS, acetyl-CoA synthetase; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein; TG, triglycerides.

We previously showed that the serum BAFF level was increased in NASH patients.[14] The present data seem to be inconsistent with those of our previous study. However, Bigorgne *et al*[32] showed that steatosis increases lymphocyte recruitment to the liver. Furthermore, BAFF has been reported to be a chemotactic agent of B cells[33] and may contribute to B-cell recruitment in livers in simple steatosis, followed by liver inflammation. As our HFD feeding model showed liver steatosis but little inflammation and fibrosis, our data indicated that BAFF signaling is one of the important factors related to the progression from NAFLD to NASH ('second hit'), but not liver steatosis ('first hit').

In this study, the serum BAFF concentrations in BAFF-R$^{-/-}$ mice increased to approximately three times those in WT mice (data not shown). We analyzed the expression levels of three previously identified BAFF-Rs and found that BAFF-R may be the main functional receptor that binds BAFF in the liver. However, other receptors, including as-of-yet unidentified ones, cannot be completely disregarded. In addition, our previous study[13] and the current data indicated that the BAFF–BAFF-R interaction in the liver may differ from that in the VAT. Further studies are necessary to examine their possible roles in these different tissues.

In summary, blockade of BAFF signaling improved insulin resistance in HFD-fed mice, but enhanced liver steatosis by activating fat biosynthesis in the liver. Several therapeutic agents that target BAFF are currently under clinical trial for autoimmune diseases,[34] but a question remains regarding their long-term and adverse effects. Metabolic markers should be carefully monitored in patients with autoimmune diseases taking such agents, particularly those who have obesity as a comorbidity.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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