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A maternal high-fat diet may accelerate adipo-immunologic aging in offspring

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ABSTRACT

Aim: Maternal obesity and improper nutrition predispose offspring to chronic metabolic diseases. Although the frequency of these diseases increases with aging, the effect of a maternal high-fat diet on aged offspring remains elusive.

Main methods: C57BL/6J female mice were fed a high-fat (HF) diet or a control (CON) diet and then mated. All offspring remained with their birth dam until weaning at 3 weeks. After weaning, the offspring from the HF and CON diet-fed dams were given either the HF diet or CON diet, which resulted in four groups: CON/CON, CON/HF, HF/CON, and HF/HF. All mice were immunized with ovalbumin and then sacrificed at 70 weeks.

Key findings: The body weights in offspring from dam exposed to a HF diet were significantly higher than those in offspring from dam fed a CON diet in the early stage of life but then became lower in the later stage of life. The serum adiponectin levels were lower in offspring from dam exposed to a HF diet and were correlated with adiposity measured by visceral and subcutaneous fat mass. Non-alcoholic fatty liver disease was much more severe in the livers of offspring from the maternal HF groups. In particular, lobular inflammation and fibrosis were prominent in the HF/HF group. Regarding immunological parameters, senescence-associated T cells were increased, and natural killer T cells were decreased by the effect of both maternal and offspring HF diet.

Significance: We have demonstrated that a maternal high-fat diet may accelerate the adipo-immunologic aging process.

1. Introduction

Maternal health and nutritional status influence offspring health and vulnerability to diseases. Accumulating epidemiologic and experimental evidence strongly suggests that maternal obesity and improper nutrition predispose offspring to chronic disease [1,2].

Several studies in rodents have shown the effects of a maternal high-fat diet on the development of metabolic abnormalities in offspring. A phenotype that closely resembles human metabolic syndrome, increased adiposity, blood pressure, and abnormal serum lipid profiles, has been reported in offspring. Excess lipid accumulation in the liver and steatohepatitis have also been reported [3–5]. These long-term conditions cause serious diseases, such as coronary artery disease, cerebrovascular disease and liver cirrhosis. In particular, visceral adipose tissue (VAT) accumulation is an important factor that causes diabetes, hypertension, and dyslipidemia. Although the onset of these diseases increases with aging, the effect of the intrauterine environment on VAT accumulation and functional changes in aged offspring is not clear.

Immune function plays an important role in the pathological process of many diseases, including not only immune disorders but also obesity and related abnormalities. Aberrant immune function, such as the accumulation of proinflammatory immune cells, within VAT in obesity plays a major role in the development of systemic chronic, low-grade inflammation and limits the function of adipocytes, leading to imbalanced adipokine secretion. Aging is often associated with alterations in immune function, particularly with decreased adaptive immunity competence, increased proinflammatory traits, and an increased risk for autoimmunity. This condition is known as immunosenescence. The involution of the thymus with aging results in decreased T cell production and export. The decreased production and exportation of naïve phenotype ($CD44^{low}CD62L^{high}$) T cells and the enhanced homeostatic proliferation of memory phenotype ($CD44^{high}CD62L^{low}$) T cells are the most recognized changes in the immune system with age [6,7]. However, the effect of a maternal high fat-diet on metabolic and immunologic aging in offspring remains elusive.

On the other hand, it has been reported that a maternal high-fat diet enhanced offspring predisposition to steatohepatitis by changing

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mitochondrial dysfunction and altering lipogenesis gene expression [4,8,9]. Natural killer T (NKT) cells have various bioactivities, including the quick production of large quantities of cytokines. Healthy livers have NKT cells in the hepatic sinusoids, which are believed to act as essential links between innate and adaptive immunity [10]. Interactions between the pathology of non-alcoholic fatty liver disease (NAFLD) and NKT cells have been reported [11–13], however, the precise role of NKT cells in the progression from steatosis to NAFLD remains controversial. Moreover, the effects of NKT cells on offspring remain uncertain in steatohepatitis predisposition induced by a maternal high-fat diet.

In this study, we investigated the effect of a maternal high-fat diet on offspring over a long-term period and evaluated the effects on adipose tissue function, adaptive immunity competence, T cell population, and NAFLD by histological analysis in aged mice. Moreover, we determined that the immune system changes induced by a maternal high-fat diet could be associated with metabolic and inflammatory conditions in adipose tissue and NAFLD in offspring with aging.

2. Materials and methods

2.1. Mice

Eight-week-old female C57BL6/J mice (CLEA Japan, Inc., Tokyo, Japan) were given ad libitum access to a high-fat (HF) diets or control (CON) diets (Research Diets, Inc., New Brunswick, NJ, Table 1) and water and were kept in a light-, temperature-, and humidity-controlled environment throughout the experiment (12–12 h light-dark cycle, temperature 23 ± 1 °C, and 55 ± 5% relative humidity). This study was approved by the Institutional Animal Care and Use Committee of the Japan Women's University (approval number II15-6), and the animals were maintained in accordance with the Guidelines for the Proper Conduct of Animal Experiments by the Science Council of Japan.

2.2. Experimental design

At nine weeks old, female mice were mated in cages with male mice of the same strain. All offspring remained with their birth dams until weaning at 3 weeks. The diet given the eighth week was maintained until weaning. Only female offspring were used for further experiments. After weaning, the offspring from HF or CON diet-fed dams were randomly divided into to the HF or CON diet-fed group, which resulted in four groups: CON/CON (n = 8), CON/HF (n = 9), HF/CON (n = 9), and HF/HF (n = 11). The experimental design is shown in Fig. 1. Body weight and experimental diet intake were measured once a week until sacrifice. All mice received an intraperitoneal injection of 200 μg ovalbumin (OVA; Sigma, St Louis, MO) with aluminum potassium sulfate (Alum; 4 mg/0.5 mL in phosphate-buffered saline; Sigma) at both 67 and 69 weeks and were then sacrificed at 70 weeks of age (1 week after the last OVA injection) [14]. Seven-week-old female mice were used as a young control group (n = 8). The young group was given the CON diet, and an OVA injection was administered at both 9 and 11 weeks; then, the mice were sacrificed at 12 weeks of age (young group). All mice were sacrificed by cervical dislocation under isoflurane (for animal, Mylan EPD G.K., Tokyo, Japan) inhalation anesthesia. After blood collection, serum was obtained by centrifugation and stored at −80 °C.

Table 1
Composition of experimental diet.

	Control diet (CON)		High fat diet (HF)	
	gram%	kcal%	gram%	kcal%
Protein	20	20	26	20
Carbohydrate	64	64	30	23
Fat	7	**16**	32	**56**
Total		100		100
kcal/g	4		5.2	
Ingredient	gram	kcal	gram	kcal
Casein	200	800	200	800
L-cystine	3	12	3	12
Corn starch	397.486	1590	0	0
Maltodextrin 10	132	528	124.5	498
sucrose	100	400	100	400
cellulose	50	0	50	0
Soybean oil	70	630	70	630
Lard	0	0	180	1620
t-Butylhydroquinone	0.014	0	0.014	0
AIN-93G mineral mix	35	0	35	0
AIN-93 vitamin mix	10	40	10	40
Choline bitartrate	2.5	0	2.5	0
Total	1000	4000	775.014	4000

2.3. Measurement of OVA-specific IgG and IgE

The amount of OVA-specific IgG antibody in the sera was determined by enzyme-linked immunosorbent assay (ELISA) [3]. Ninety-six-well plates were coated with OVA (120 μg/mL, 50 μL/well in 0.1 M $NaHCO_3$ buffer, pH 9.5) overnight at 4 °C. Each well was incubated with 300 μL blocking solution (3% BSA in PBS with 0.05% Tween 20) for 1 h at room temperature. One hundred microliters of sera (diluted 1:32000) was placed in each well, and the plates were incubated for 90 min at room temperature. Then, a biotin-labeled goat anti-mouse IgG antibody (1:8000 dilution; Zymed) and streptavidin-HRP (1:4000 dilution; Dako) were used for signal detection. The enzyme reaction uses *o*-phenylene diamine (Sigma, St Louis, MO) in a sodium-citrate buffer (pH 5.0) in the presence of 0.01% H_2O_2 in the dark. The reaction was stopped by the addition of 50 μl 2 N HCl, and the absorbance was measured at 492 nm. The data are presented as the optical density (OD) of non-coated wells subtracted from the OD of OVA-coated wells.

ELISA was used to determine the OVA-specific IgE antibody in the sera. Briefly, microplates were coated with OVA (120 μg/mL, 50 μL/well in 0.1 M $NaHCO_3$ buffer, pH 9.5) overnight at 4 °C. After sera incubation (100 μL, diluted 1:200), mixed biotin-labeled goat anti-mouse IgE antibody (1:1000) and streptavidin-HRP reagent (1:250, BD Pharmingen, San Diego, CA) were added and incubated at 37 °C for 2 h. The enzyme reaction and measurement were carried out as described for IgG.

2.4. Preparation and incubation of splenic lymphocytes

The spleens were removed from the mice aseptically and placed in RPMI 1640 medium (NISSUI, Tokyo, Japan) containing 5% heat-inactivated fetal calf serum (FCS). Cells were then diluted in medium to 1×10^7 cells/mL. Splenic lymphocytes (5×10^5 cells/100 μL per well) were cultured at 37 °C in 5% CO_2 in the presence of plate-bound anti-CD3 mAb (0.5 μg/mL, BD Pharmingen) with soluble anti-CD28 mAb (1 μg/mL, BD Pharmingen) to evaluate T cell receptor signaling or with 5 μg/mL concanavalin A (ConA, Difco Laboratories, Detroit, MI) or 10 μg/mL LPS (Difco Laboratories) to elicit a non-specific mitogenic response. The antigen-specific response was evaluated with 20 μg/mL OVA.

2.5. Splenic lymphocyte proliferation assessed by Alamar Blue assay

After 72 h of culture, the proliferative response of splenic lymphocytes was measured by Alamar Blue assay. Alamar Blue solution (Serotec, Oxford, UK) was added to the culture media, and the cells were incubated for 4 h at 37 °C in 5% CO_2. Fluorescence was measured at 544–590 nm. The fluorescence of each anti-CD3/CD28, mitogen- or OVA-stimulated well was compared with that of non-stimulated wells to determine lymphocyte proliferation [15].

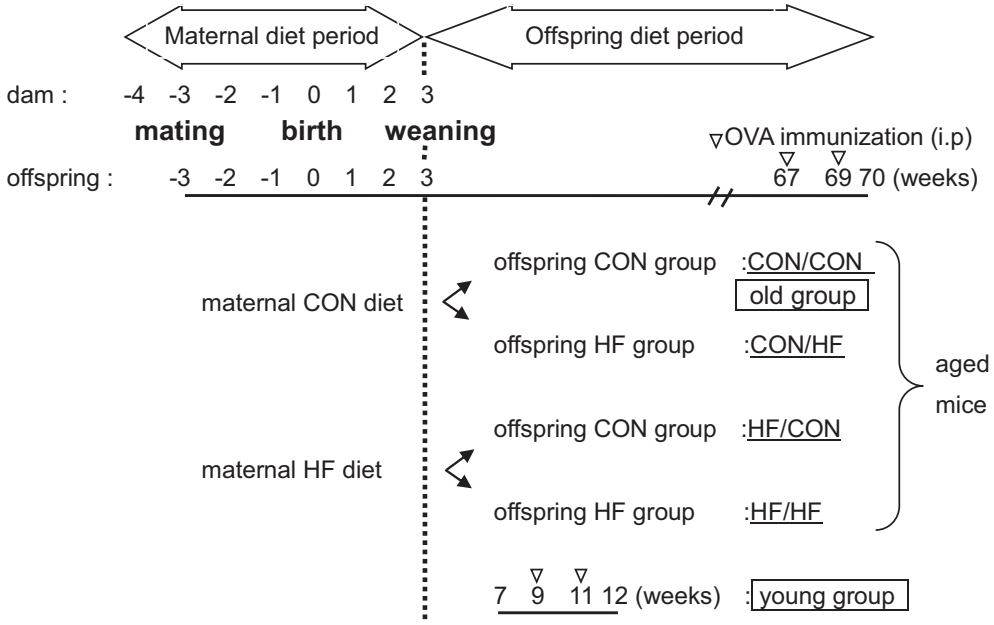

Fig. 1. Experimental design.
Mice were fed either a control (CON) or high-fat (HF) diet. The number of mice in each group was as follows; CON/CON (n = 8), CON/HF (n = 9), HF/CON (n = 9), and HF/HF (n = 11). OVA, ovalbumin.

2.6. Measurement of adipokine in sera and cytokine secretion by splenic lymphocytes

Serum adiponectin and leptin concentrations and the TNF-α secretion into the supernatants of cultured splenic lymphocytes stimulated with LPS were evaluated. ELISA was used to determine adiponectin, leptin and TNF-α levels using the DuoSet ELISA Development System (R &D Systems), according to the manufacturer's instructions. The culture supernatants were collected after a 48-h incubation, centrifuged and stored at −30 °C until analysis.

2.7. Flow cytometric analysis of splenic lymphocytes

Splenic lymphocyte suspensions (1×10^7 cells/mL) were treated with red blood cell lysis buffer for 7 min on ice. After the addition of 10 mL PBS containing 2% FCS and centrifugation (1400 rpm, 5 min), the pellets were resuspended in PBS containing 2% FCS. The cells were incubated with monoclonal antibodies for 30 min on ice to avoid placing the tubes in direct light. Flow cytometric analysis was performed on a Gallios instrument (Beckman Coulter Co., Ltd., Tokyo, Japan), and the results were analyzed using Kaluza 1.2 software (Beckman Coulter Co., Ltd.). The antibodies used were specific to CD3e (145-2C11; Sony); CD4 (GK1.5; Sony); CD8a (53–6.7; BD Bioscience); CD25 (PC61; iCyt); CD45RB (C363-16A; SONY); CD44 (IM7; BioLegend); CD62 L (MEL-14; eBioscience); PD-1 (RMP1-30; BioLegend); and NK1.1 (PK136; iCyt). We analyzed FOXP3, a transcriptional factor, using a FOXP3 Fix/Perm Buffer Set (BioLegend) and the Ab used was specific to FOXP3 (150D; BioLegend).

2.8. Histological analysis of the liver and NAFLD assessment

For the histological analysis, livers from aged and young mice were fixed in paraformaldehyde. Paraffin embedding and hematoxylin-eosin (HE) staining and Masson trichrome (MT) staining were performed by the Sapporo General Pathology Laboratory (Sapporo, Japan). The severity of (1) steatosis (macrovesicular lipid accumulation), and (2) lobular inflammation (lymphomonocytic infiltration) according to HE staining, and the severity of (3) fibrosis according to MT staining were evaluated and scored on a four- or five-point scale in a blinded manner (none = 0, mild = 1, moderate = 2, severe = 3 and very severe = 4). To minimize scoring bias, two researchers evaluated the scores independently after confirming the histological standard for each score.

NAFLD severity in the offspring livers was assessed using a modified steatosis, activity and fibrosis (SAF) score [16], which allows the scoring of individual features, including steatosis, disease activity and fibrosis. We evaluated steatosis severity with a 4-point scale of 0 to 3. In addition, disease activity was evaluated with lobular inflammation in this study. Steatosis and lobular inflammation (4-point scale of 0 to 3) were assessed by HE staining. Fibrosis was assessed by MT staining with a 5-point scale of 0 to 4.

2.9. Statistical analysis

The data are presented as the mean ± standard error. Statistical analysis was performed using IBM SPSS 22 (IBM Japan, Tokyo, Japan). For the statistical analysis of aging (the old group: CON/CON vs the young group), the Kolmogorov-Smirnov test was used to ascertain data normality. Normally distributed variables were compared by Student's t-test, and non-normally distributed variables were evaluated by the Mann-Whitney U test. The Spearman rank correlation was used to assess the associations between two variables. The results were considered statistically significant at $P < 0.05$. was used for all the statistical analyses. Two-way ANOVA was performed to determine any significant effects and interactions of the maternal diet and the offspring diet. When the interaction between the maternal diet and the offspring diet was statistically significant, a simple main effect test was used to make comparisons.

3. Results

3.1. The number of siblings

The number of siblings used in this study was 5–8 (7.5 ± 0.3 in control dam and 6.7 ± 0.4 in high-fat diet fed dam). Pups of the number of siblings 1–4 in the high-fat diet fed dam were excluded in this study (When all of them were included, the number of siblings of the high-fat diet fed dam was 5.6 ± 2.3).

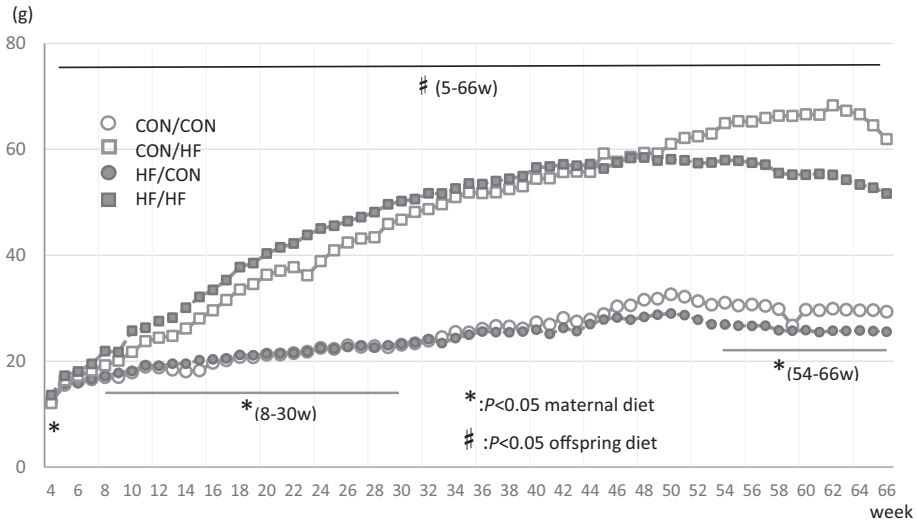

Fig. 2. Time course of body weight changes.
The body weights were significantly higher in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet when the mice were of 8 to 30 weeks of age. However, from 54 to 66 weeks, the body weights was significantly lower in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet. The body weights were significantly higher in offspring exposed to a HF diet after weaning than in offspring fed a CON diet at these points. The data are presented as the mean ± S.E. CON, control; HF, high-fat.

3.2. Time course of body weight changes

The body weights were significantly higher in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet when the mice were 8 to 30 weeks of age. However, when the mice were 54 to 66 weeks, the body weight was significantly lower in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet. The body weights were significantly higher in offspring exposed to a HF diet after weaning than in the offspring fed a CON diet at these points (Fig. 2). In other words, the effect of the maternal HF diet was different at the early stage compared to the later stage of life. The week that the mice reached their maximum body weight in the experimental period was earlier in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet (Fig. 3). Although food intake was comparable among the four groups, the amount of energy ingested was higher in offspring exposed to a HF diet after weaning than in offspring fed a CON diet (data not shown).

3.3. Organ weights and body composition

The visceral fat pad weights (and percent visceral fat mass/body weight) were not different between the old group (CON/CON) and the young group. The subcutaneous and scapula brown fat weights (and percent fat mass/body weight) were higher in the old group (CON/CON) than in the young group. The gastrocnemius weights (and percent mass/body weight), the lean body mass index were significantly lower in the old group (CON/CON) than in the young group.

The percent visceral, subcutaneous, and brown fat pad weights (and percent mass/body weight) were significantly lower by the effect of maternal HF diet. On the other hand, the gastrocnemius weights were not changed, but the percent mass of gastrocnemius weight was increased by the effect of maternal HF diet.

The visceral, subcutaneous, and brown fat pad weights (and percent mass/body weight) were significantly increased in offspring exposed a HF diet. The gastrocnemius weights were significantly increased, however, the percent mass of gastrocnemius weight was significantly decreased by the offspring fed a HF diet.

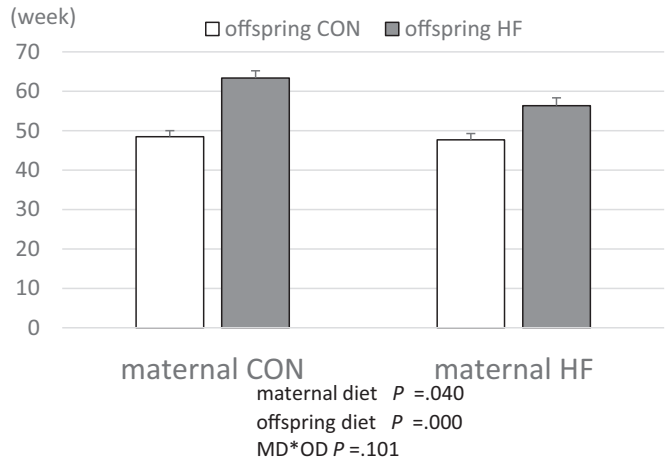

Fig. 3. Week when maximum body weight was reached.
The week that the mice reached their maximum body weight was earlier in offspring from dam exposed a HF diet than in offspring from dam fed a CON diet. CON, control; HF, high-fat.

3.4. Serum adipokines (Table 2)

Both adiponectin and leptin concentrations in the sera were higher in the old group (CON/CON) than in the young group. On the other hand, both were significantly lower in offspring from dam exposed to a HF diet. The leptin concentration was significantly increased by the effect of a HF diet in the offspring after weaning.

3.5. Correlation between serum adiponectin levels and body weight reductions (Fig. 4)

According to the regression analysis using data from all aged mice (CON/CON, HF/CON, CON/HF and HF/HF), serum adiponectin levels were negatively correlated with the body weight reductions which were calculated by subtracting the weight at sacrifice from the maximum weight of each mouse.

3.6. Immunological parameters (Table 3)

The proliferative responses of splenocytes stimulated with ConA were lower in the old group (CON/CON) than in the young group; these responses tended to be lower for anti-CD3/CD28 stimulation as well. The proliferative responses to ConA, anti-CD3/CD28, and LPS were significantly lower in the offspring fed the HF diet. In addition, there was no effect of the maternal HF diet for each stimulation condition. There was also no effect of OVA stimulation in this study. TNF-α secretion from splenocytes stimulated with LPS was higher in the old group (CON/CON) than in the young group. There were no significant differences by the effect of both maternal and offspring HF diet.

The OVA-specific IgG titer was significantly lower, and the IgE titer

Table 2
Body weight, organ weight, body composition, and serum adipokine.

	Young (12 weeks)	old(70 weeks)				Statistic			
		Maternal CON		Maternal HF					
		Offspring CON CON/CON	Offspring HF CON/HF	Offspring CON HF/CON	Offspring HF HF/HF	Young vs old (CON/CON)	Maternal diet	Offspring diet	MD*OD
Body weight(g)	19.4 ± 0.32	27.2 ± 0.66[a]	58.2 ± 2.65[b]	23.8 ± 0.44[a]	40.0 ± 3.97[c]	**0.001**	**< 0.001**	**< 0.001**	**0.010**
Visceral fat pad (mg)	45 ± 6	290 ± 44[a]	2163 ± 111[b]	122 ± 15[a]	854 ± 168[c]	0.105	**< 0.001**	**< 0.001**	**0.001**
Visceral fat pad/BW	2.3 ± 0.28	7.9 ± 2.12[a]	35.1 ± 1.55[b]	5.1 ± 0.64[a]	19.2 ± 2.46[c]	0.105	**< 0.001**	**< 0.001**	**0.008**
Subcutaneous fat pad (mg)	72 ± 5	269 ± 26[a]	1280 ± 131[b]	133 ± 11[a]	563 ± 186[c]	**< 0.001**	**0.002**	**< 0.001**	**0.032**
Subcutaneous fat pad/BW	3.7 ± 0.27	9.9 ± 0.84	21.5 ± 1.87	5.6 ± 0.43	12.0 ± 2.25	**< 0.001**	**< 0.001**	**< 0.001**	0.130
Brown adipose tissue (mg)	54 ± 6	121 ± 7	433 ± 52	76 ± 6	258 ± 72	**< 0.001**	**0.037**	**< 0.001**	0.204
Brown adipose tissue/BW	2.8 ± 0.27	4.4 ± 0.21	7.4 ± 0.75	3.2 ± 0.21	5.7 ± 0.86	**< 0.001**	**0.032**	**< 0.001**	0.783
Gastrocnemius (mg)	117 ± 3	133 ± 4	149 ± 10	130 ± 3	152 ± 7	**< 0.001**	0.996	**0.006**	0.607
Gastrocnemius/BW	6.0 ± 0.16	4.9 ± 0.13[a]	2.6 ± 0.13[b]	5.4 ± 0.09[a]	4.0 ± 0.30[c]	**< 0.001**	**< 0.001**	**< 0.001**	**0.027**
Liver(mg)	968 ± 57	1211 ± 42	1951 ± 74	1214 ± 85	1844 ± 131	**0.004**	**0.597**	**< 0.001**	**0.581**
Liver/BW	50.0 ± 2.5	44.6 ± 1.0	34.3 ± 2.6	51.0 ± 3.1	47.6 ± 2.5	**0.077**	**0.001**	**0.012**	**0.192**
Serum adiponectin (μg/mL)	6.4 ± 1.13	14.7 ± 1.74	16.5 ± 2.27	12.2 ± 1.12	11.8 ± 1.40	**0.001**	**0.039**	0.673	0.493
Serum leptin (ng/mL)	0.58 ± 0.05	5.00 ± 0.72[a]	30.40 ± 3.43[b]	1.51 ± 0.30[a]	10.57 ± 4.38[c]	**< 0.001**	**0.001**	**< 0.001**	**0.014**

CON, control diet; HF, high fat diet; MD, maternal diet; OD, offspring diet.
Values with different superscript are significantly different. Bold numbers indicate significant difference with $P < 0.05$.

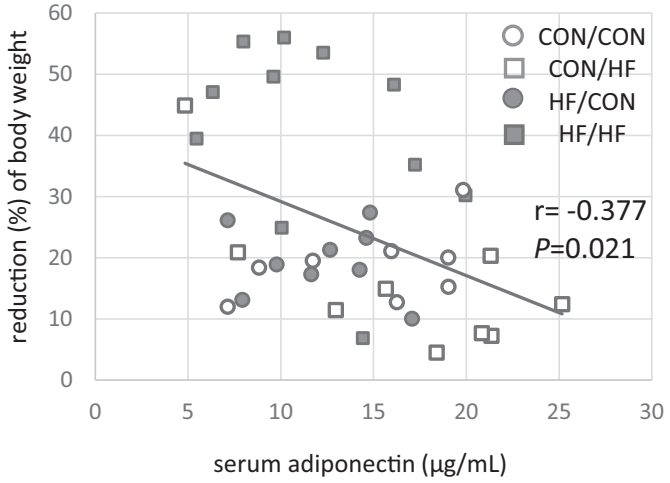

Fig. 4. Correlation between serum adiponectin levels and body weight reductions.
The body weight reductions, which were calculated by subtracting the weight at sacrifice from the maximum weight of each mouse. According to the regression analysis using data from all aged mice, serum adiponectin levels were negatively correlated with the body weight reductions. CON, control; HF, high-fat.

was higher in the old group (CON/CON) than in the young group. The IgG and IgE titers were significantly lower in offspring fed a HF diet after weaning than in offspring fed a CON diet. In addition, no significant difference was observed for the maternal HF diet.

The flow cytometry (FCM) analysis of T cell subsets indicated that the naïve/memory ratio and NK cells were lower in the old group (CON/CON) than in the young group. On the other hand, regulatory T cells, PD-1$^+$CD44^{high}CD62 L^{low}CD4$^+$T cells and NKT cells were significantly higher in the old group than in the young group. In addition, the maternal and offspring HF diet significantly increased the PD-1$^+$CD44^{high}CD62 L^{low} (memory phenotype, MP) CD4$^+$T cells. NKT cells were significantly lower by the effect of maternal and offspring HF diet.

3.7. Histological analysis of the liver (Fig. 5)

Liver histological findings in the offspring were assessed. Morphological analysis was performed by HE staining. Fibrosis was assessed by MT staining. A normal hepatic structure without lipid accumulation was observed in the livers from the young group (Fig. 5a). In the livers from the old group (CON/CON), mild fibrosis changes without steatosis and lobular inflammation were observed upon aging (Fig. 5b). The CON/HF group livers showed severe steatosis (Fig. 5c). In the HF/CON group livers, moderate lobular inflammation (Fig. 5d) and fibrosis were observed despite little lipid accumulation. In the HF/HF group, severe lobular inflammation and fibrosis, as well as mild steatosis, were observed (Fig. 5e–f).

3.8. Assessment of NAFLD severity (Table 4)

NAFLD severity in offspring livers was assessed using the modified SAF score [16,17]. Offspring from dam fed a CON diet (CON/CON, HF/CON) showed little steatosis. In offspring fed a CON diet after weaning, the maternal HF diet induced lobular inflammation, and worsened fibrosis (HF/CON). In offspring exposed to a HF diet, steatosis was milder in the HF/HF group than in the CON/HF group. On the other hand, lobular inflammation and fibrosis were much more severe in the HF/HF group than in the CON/HF group.

4. Discussion

In the present study, the body weights of offspring were higher in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet for the first half of their life. On the other hand, during the latter period of the experiments, offspring from dam exposed to a HF diet had lower body weights than offspring from dam fed a CON diet.

It was reported that a maternal high-fat diet induces obesity and metabolic dysfunction in offspring. The mechanisms of such phenomena have been explained by an increased risk of overeating induced by increased hypothalamic peptide-producing neurons proliferation [18], and impaired thermogenic function of brown adipose tissue in offspring [19]. These studies were performed on a young generation of offspring, and the effects on aged offspring have not been studied. In this study, we observed the influence of a maternal HF diet on offspring for a long-term period (66 weeks). The results indicated that the effects of the maternal HF diet on offspring body weight were different between the first half and the latter period.

In the body composition analysis in this study, the percent visceral, subcutaneous fat pad, and brown adipose tissue weights were significantly lower in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet. The gastrocnemius weights,

Table 3
Immunological parameter.

	young (12weeks)	old(70weeks)				Statistic			
		maternal CON		maternal HF					
		offspring CON	offspring HF	offspring CON	offspring HF	young vs old	maternal diet	offspring diet	MD*OD
		CON/CON	CON/HF	HF/CON	HF/HF	(CON/CON)			
Splenocytes proliferation									
ConA	0.990 ± 0.120	0.582 ± 0.106	0.360 ± 0.065	0.743 ± 0.093	0.278 ± 0.055	**0.023**	0.618	< **0.001**	0.134
LPS	1.055 ± 0.154	0.719 ± 0.143	0.555 ± 0.097	0.835 ± 0.119	0.450 ± 0.074	0.132	0.960	**0.015**	0.310
anti-CD3/CD28	1.026 ± 0.127	0.680 ± 0.121	0.360 ± 0.061	0.694 ± 0.091	0.195 ± 0.046	0.069	0.352	< **0.001**	0.270
OVA	0.367 ± 0.140	0.199 ± 0.079	0.331 ± 0.052	0.223 ± 0.052	0.226 ± 0.083	0.291	0.567	0.338	0.360
Cytokine secretion by splenobytes (by LPS stimulation)									
TNF-α (pg/mL)	56.2 ± 12.1	109.0 ± 12.8	113.7 ± 15.7	127.7 ± 11.3	130.8 ± 22.2	**0.010**	0.458	0.645	0.860
OVA- specific antibody titer									
IgG	1.038 ± 0.058	0.781 ± 0.062	0.402 ± 0.109	0.882 ± 0.114	0.350 ± 0.064	**0.009**	0.788	< **0.001**	0.399
IgE	0.009 ± 0.002	0.076 ± 0.024	0.028 ± 0.010	0.049 ± 0.014	0.026 ± 0.009	**0.015**	0.325	**0.018**	0.369
Flowcytometory analysis of splenic lymphocytes									
T cell (%)	22.6 ± 1.05	12.8 ± 0.79	12.7 ± 1.03	12.3 ± 0.69	14.0 ± 1.58	< **0.001**	0.710	0.485	0.860
CD4/CD8	1.11 ± 0.02	1.34 ± 0.18	1.05 ± 0.14	1.11 ± 0.10	1.26 ± 0.16	0.377	0.929	0.663	0.191
Naïve/Memory	1.35 ± 0.11	0.17 ± 0.05	0.08 ± 0.02	0.21 ± 0.03	0.07 ± 0.02	< **0.001**	0.730	< **0.001**	0.389
regulatory T cell	10.07 ± 0.59	13.87 ± 1.44	15.66 ± 1.95	14.40 ± 0.90	18.15 ± 2.76	**0.029**	0.481	0.201	0.647
PD-1^{+} MP CD4^{+}T cell	32.3 ± 3.40	66.7 ± 3.23	74.2 ± 3.91	77.0 ± 1.96	82.6 ± 2.87	< **0.001**	**0.041**	**0.005**	0.749
NK cell (%)	1.74 ± 0.11	1.32 ± 0.14	2.14 ± 0.25	1.24 ± 0.09	2.11 ± 0.20	**0.032**	0.764	< **0.001**	0.888
NKT cell (%)	0.84 ± 0.04	1.47 ± 0.15	0.90 ± 0.14	0.91 ± 0.08	0.88 ± 0.14	**0.003**	**0.042**	**0.033**	0.050

CON, control diet; HF, high fat diet; MD, maternal diet; OD, offspring diet; SI, stimulation index; OD, optical density; MP, memory phenotype. Bold numbers indicate significant difference with $P < 0.05$.

however, were not affected by the maternal HF diet. Accordingly, the loss of body weight in the maternal HF groups during the latter period may be due mainly to a decrease in fat tissue volume.

In aging, it has been reported that adipose tissue becomes dysfunctional when the differentiation pathway of preadipocytes to mature adipocytes becomes impaired, this results in dysfunctional adipocytes that are less able to store fat [20], and a decrease in adipose depot size is observed, which is due to reductions in cell size [21]. At the same time, there is also an age-related increase in serum free fatty acids (FFAs) due to the reduced capacity of adipose depots to store FFAs [20,22]. Decreases in adipose depot size and increased FFAs levels can be explained by the altered adipocyte maturation process. It has been reported that in advanced-age C3B6F1 mice, body weight and percent fat mass were lower than those in late-middle-aged mice [23]. The results in this study, which show decreases in body weight and fat tissue volume in offspring from dam exposed to a HF diet in the latter period, suggest that a maternal high-fat diet accelerates the aging process of adipose tissue function in offspring. In addition, offspring born to dams exposed to a HF diet had increased percent liver weight per body weight irrespective of the type of offspring diet after weaning. As a result, it has been reported that ectopic fat accumulation occurs in organs other than adipose tissue, especially in the liver. We speculated that the decrease in fat accumulation capacity of adipose tissue promoted hepatic ectopic fat accumulation.

In this study, serum adiponectin levels and proinflammatory cytokine TNF-α secretion by splenocytes stimulated with LPS were higher in aged mice than in the young mice. Moreover, the serum adiponectin levels were correlated with adiposity measured by visceral and subcutaneous fat mass and percent body weight.

Previous reports have documented a negative correlation between adiposity and circulating adiponectin levels [23]. Adiponectin secretion is decreased by obesity and restored by decreases in adipocytes size following weight loss. It has also been confirmed that adiponectin expression is induced in local inflammatory sites [24–26]. Moreover, adiponectin expression stimulated by TNF-α reappeared in the A549 airway epithelial cell line [25]. Cells other than adipocytes also secrete adiponectin in response to inflammation.

Centenarians have higher adiponectin levels that are associated with longevity. However, in older individuals aged 65 or more, adiponectin is associated with higher mortality. Adiponectin dysregulation in older individuals may be due to a loss of circulating adiponectin function or a response to increased inflammatory processes [27]. It has been suggested that adiponectin is associated with higher mortality, if the adiponectin level is high in response to inflammatory states in old age.

In this study, the serum adiponectin concentrations were lower in offspring from dam exposed to a HF diet than in offspring from dam fed a CON diet irrespective of the type of diet consumed by the offspring after weaning. The serum adiponectin concentrations were as low as both the visceral and subcutaneous fat tissue volumes in offspring from dam exposed to a HF diet (Table 2). According to the regression analysis using data from all aged mice, adiponectin levels were negatively correlated with body weight reductions from the maximum weight of each mouse to their weight at sacrifice (Fig. 4). A maternal high-fat diet has been reported to increase proinflammatory phenotype macrophage infiltration, upregulate proinflammatory cytokine expression in adipose tissue [28], and stimulate proinflammatory cytokine production by splenocytes [3]. In this way, a compensatory increase in serum adiponectin levels may not have occurred despite the fact that offspring from dam exposed to a HF diet were in an inflammatory state. At least, adiponectin secretion recovery was not observed with the decrease in fat tissue weight, and the relationship between fat tissue volume and adiponectin secretion was different between the aged offspring and the young offspring.

It has been reported that the immune function varies with aging. Functional diminution in antigen-specific immune response, reduced vaccination efficacy, and a proinflammatory traits occurred, and these effects are known as immunosenescence. The involution of the thymus with aging results in decreased T cell production and export. The decreased production and exportation of naïve phenotype (CD44^{low}CD62 L^{high}) T cells and the enhanced homeostatic proliferation of memory phenotype (CD44^{high}CD62 L^{low}) T cells are the most recognized changes

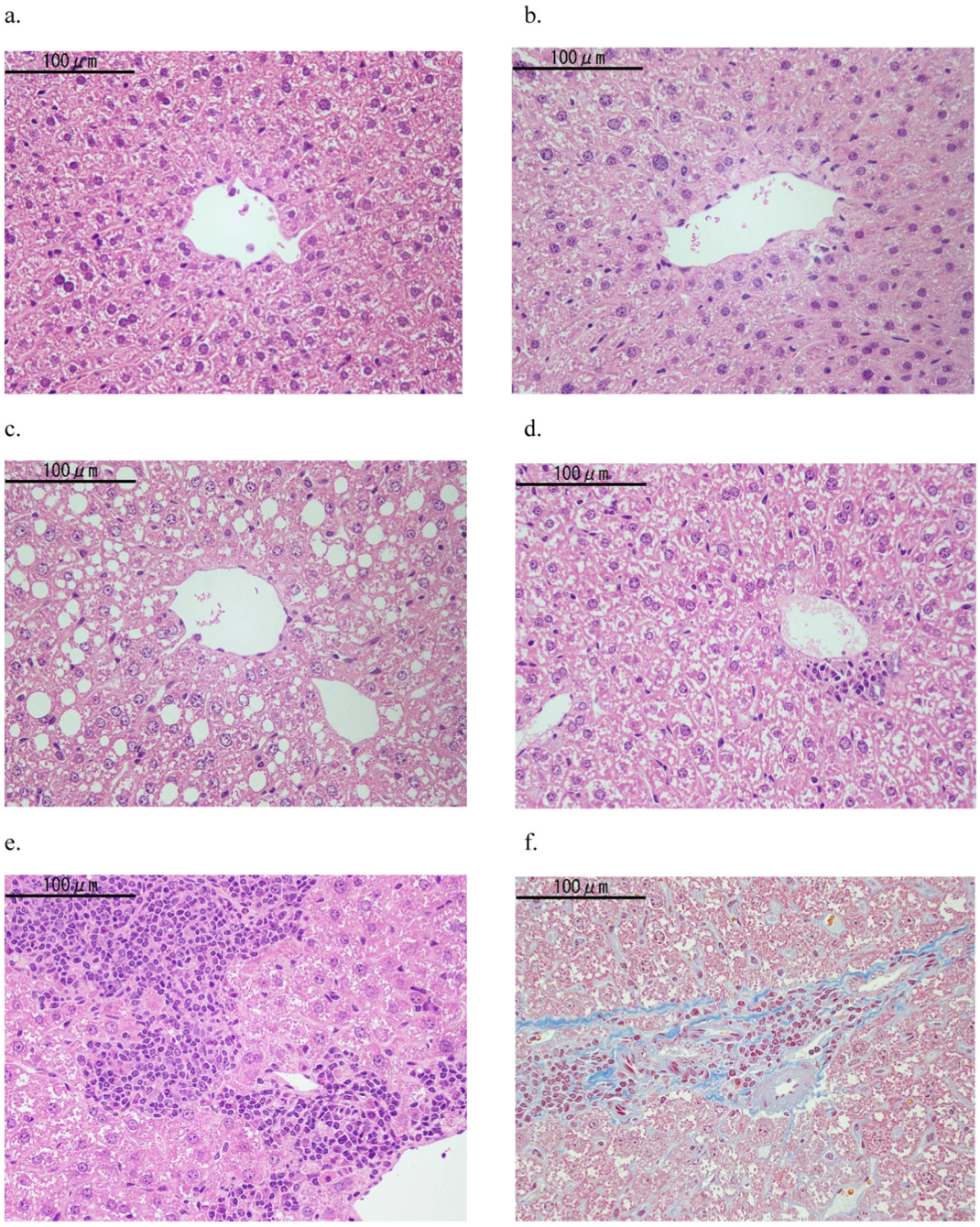

Fig. 5. Histological analysis (a-f) of offspring livers.
a. Young group, hematoxylin-eosin (HE) staining
b. CON/CON group, HE staining,
c. CON/HF group showing severe steatosis, HE staining,
d. HF/CON group showing little steatosis but lobular inflammation, HE staining,
e. HF/HF group showing severe lobular inflammation, HE staining,
f. HF/HF group showing severe fibrosis, Masson trichrome staining.

in the immune system with age [6,7]. However, the total number of peripheral T cells is not affected by aging because of the homeostatic proliferation of memory phenotype (MP, $CD44^{high}CD62\ L^{low}$) T cells. As a result, the T cell population shows a progressive shift from naïve to memory cells with age [7]. It is thought that the shift of the T cell population contributes to immunosenescence. We examined antigen-specific immune responses and T cell populations affected by aging. OVA-specific IgG and IgE antibody titers were changed by aging, however, maternal HF diet influences were not observed. In proliferative reactions, OVA reactivity was not changed by the maternal HF diet or aging. In other words, the maternal HF diet did not affect the antibody-specific reactions in aged offspring in this study. On the other hand, the FCM analysis of the T cell subset of splenic lymphocytes indicated that the naïve/memory ratio was significantly decreased and $PD\text{-}1^+$MP $CD4^+$T cells was significantly increased by aging. Moreover, $PD\text{-}1^+$MP $CD4^+$T cells were significantly higher in offspring from dam exposed to a HF diet than offspring from dam fed a CON diet. $PD\text{-}1^+$ MP $CD4^+$T cells have been reported to be senescence-associated T cell (SA-

Table 4
Assenment of liver morphology.

	Steatosis (0-3)	Lobular Inflammation (0-3)	Fibrosis (0-4)
CON/CON (old)	0	0	1
CON/HF	3	1	2
HF/CON	0	1	2
HF/HF	2	3	3
young	0	0	0

Modified SAF score was used [16,17]. For each group (n = 4), the mean score for each characteristic is given.

T cell) and are increased by aging and in high-fat diet-induced obesity in mice [29]. Although the nature of the SA-T cell antigen has not been clarified, it is suggested that environments such as adipose tissue inflammation increase SA-T cells because these cells are increased in diet-induced obesity adipose tissue. In addition, it has been reported that palmitate in a high fat diet increases inflammatory effector memory $CD4^+$ T cells. This change occurred before the obesity state was induced [30]. The current study suggests that the environmental changes induced by metabolic stress or adipose tissue aging increase SA-T cells.

According to the histological analysis and assessment of NAFLD severity, the HF/HF group was predisposed to developing steatohepatitis rather than simple steatosis (Fig. 5e–f). In contrast, the CON/HF group, which was exposed to a HF diet during only the postweaning period, exhibited severe steatosis without severe inflammation and fibrosis (Fig. 5c). Therefore, exposure to a HF diet during both the perinatal and postweaning periods may induce worse conditions than HF diet exposure during the postweaning period only. Although the CON/CON and the HF/CON groups exhibited little steatosis, only the HF/CON group exhibited lobular inflammation and moderate fibrosis (Fig. 5d). It has been reported that a maternal HF diet primes obesity and hepatic lipid accumulation in adult offspring. The mechanisms of this phenomenon were demonstrated to involve mitochondrial dysfunction, and altered lipogenesis gene expression [4,8,9].

In this study, steatosis evaluated by microscopy was milder in the HF/HF group than in the CON/HF group. It was suggested that hepatocyte function was decreased by lobular inflammation and fibrosis under the influence of aging and as a result, steatosis was milder in the HF/HF group than in the CON/HF group. Taken together, we have demonstrated that the maternal HF diet, regardless of the offspring diet, exacerbates lobular inflammation and fibrosis in NAFLD in aged offspring. In the liver, the activation of the innate immune system has a critical role in maintaining homeostasis and liver regeneration, as well as disease pathogenesis [10]. NKT cells show various bioactivities, including the quick production of large quantities of cytokines. The healthy liver has NKT cells present in the hepatic sinusoids, which are believed to act as essential links between innate and adaptive immunity [10]. In humans, changes in the frequency of peripheral and parenchymal NKT cells have been correlated with NAFLD progression. Steatosis is associated with decreased numbers of liver NKT cells, and an immunohistochemical evaluation of human nonalcoholic steatohepatitis (NASH) biopsy sections showed decreased numbers of NKT cells as the steatosis grade increased [11]. There are other reports that patients with NASH have livers enriched with NKT cells [12,13]. Their precise role in the progression from steatosis to NAFLD, however, remains controversial. In this study, the NKT cell number was significantly decreased by the effect of maternal and offspring HF diet consumption. Remarkably, in the HF/CON group, lobular inflammation and fibrosis were observed without steatosis. Thus, we speculate that a maternal HF diet initiates changes in the normal innate immune system to maintain homeostasis (such as reduction of NKT cell ratio) in the liver and causes inflammation and fibrosis of the liver in aged offspring irrespective of the type of offspring diet after weaning. In addition, increased a proinflammatory traits by immunological aging (increased SA-T cell) and decreased adiponectin, as a hepato-protective cytokine, may have influenced the exacerbate of the liver.

5. Conclusions

We have demonstrated that a maternal high-fat diet may accelerate the aging process in adipose tissue, increase senescence-associated T cells in splenic lymphocytes, and induce histological NAFLD changes in the livers of aged offspring. These alterations may be related to each other; however, our study is a one-point analysis of aged offspring, and we cannot confirm the causality of each alteration. It will be necessary to investigate the time course changes in offspring, and to analyze the effects of a maternal high-fat diet on aging of adipose tissue function and immunological changes in offspring.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

AI, KS: design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. EH, KT data collection. UM: data analysis and data interpretation.

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