CD14 upregulation as a distinct feature of NAFLD after pancreatoduodenectomy

Running title: CD14 upregulation on Kupffer cells

Daisuke Satoh, Takahito Yagi, Takeshi Nagasaka, Susumu Shinoura, Yuzo Umeda, Ryuichi Yoshida, Masashi Utsumi, Takehiro Tanaka, Hiroshi Sadamori, Toshiyoshi Fujiwara

Daisuke Satoh, Takahito Yagi, Takeshi Nagasaka, Susumu Shinoura, Yuzo Umeda, Ryuichi Yoshida, Masashi Utsumi, Hiroshi Sadamori, Toshiyoshi Fujiwara, Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Takehiro Tanaka, Department of Pathology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

Author contributions: Satoh D performed the majority of experiments; Yagi T, Nagasaka T, Tanaka T, Shinoura S, Umeda U, Yoshida R, Utsumi M, Yoshida K, Hiroshi Sadamori H were involved in editing the manuscript; and Satoh D and Fuziwara T designed the study and wrote the manuscript.

Correspondence to: Daisuke Satoh, MD, Department of Gastroenterological Surgery Transplant, and Surgical Oncology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. ddds4863@gmail.com

Telephone: +81-86-235-7257 Fax: +81-86-221-8775

Received: Revised: Accepted: Published online

ABSTRACT

Aim: To investigate the pathogenesis of non-alcoholic fatty liver disease (NAFLD) after pancreatoduodenectomy (PD).

Methods: A cohort of 82 patients who underwent PD at Okayama University Hospital between 2003 and 2009 was enrolled and the clinicopathological features were compared between patients with and without NAFLD after PD. Computed tomography (CT) images were evaluated every 6 months after PD for follow-up. Hepatic steatosis was diagnosed on CT when hepatic attenuation values were ≤40 Hounsfield units. Liver biopsy was performed for 4 of 30 patients with NAFLD after PD who consented to undergo biopsies. To compare NAFLD after PD with NAFLD associated with metabolic syndrome, liver samples were obtained from 10 patients with NAFLD associated with metabolic syndrome [fatty liver, n=5; non-alcoholic steatohepatitis (NASH), n=5] by percutaneous ultrasonography-guided liver biopsy. Double-fluorescence immunohistochemistry was applied to examine CD14 expression as a marker of lipopolysaccharide (LPS)-sensitized macrophage cells (Kupffer cells) in liver biopsy specimens.

Results: The incidence of postoperative NAFLD was 36.6% (30/82). Univariate analysis identified cancer of the pancreatic head, sex, diameter of the main pancreatic duct, and dissection of the nerve plexus as factors associated with the development of NAFLD after PD. Those patients who developed NAFLD after PD demonstrated significantly decreased levels of serum albumin, total protein, cholesterol, and triglycerides compared to patients without NAFLD after PD, but no glucose intolerance or insulin resistance. Liver biopsy was performed in four patients with NAFLD after PD. All four patients showed moderate-to-severe steatosis and non-alcoholic steatohepatitis (NASH) was diagnosed in two. Numbers of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells) were counted in all biopsy specimens. The number of CD68+ cells in specimens of NAFLD after PD was significantly

increased from that in specimens of NAFLD associated with metabolic syndrome specimens, which indicated the presence of significantly more Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome. Similarly, more CD14+ cells, namely, LPS-sensitized Kupffer cells, were observed in NAFLD after PD than in NAFLD associated with metabolic syndrome. Regarding NASH, more CD68+ cells and CD14+ cells were observed in NASH after PD specimens than in NASH associated with metabolic syndrome. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in NASH after PD that in NASH associated with metabolic syndrome. These observations suggest that after PD, Kupffer cells and LPS-sensitized Kupffer cells were significantly upregulated not only in NASH, but also in simple fatty liver.

Conclusion: NAFLD after PD is characterized by both malnutrition and the up-regulation of CD14 on Kupffer cells. Gut-derived endotoxin appears central to the development of NAFLD after PD.

Key words: NAFLD; pancreatoduodenectomy; CD14; endotoxin; Kupffer cells

INTRODUCTION

The prevalence of non-alcoholic fatty liver disease (NAFLD) associated with metabolic syndrome is increasing worldwide[1,2]. NAFLD associated with metabolic syndrome is considered to be associated with chronic over-nutrition that results in the accumulation of visceral fat and obesity and is one of the most common forms of chronic liver disease[3]. NAFLD associated with metabolic syndrome is related to states such as severe obesity, impairment of glucose tolerance, and hyperlipidemia[4]. Indeed, several studies have shown strong relationships among NAFLD associated with metabolic syndrome, hepatic insulin resistance, and type 2 diabetes mellitus[5-7].

On the other hand, NAFLD sometimes develops after pancreatoduodenectomy (PD)[8]. Tanaka *et al.* recently reported that NAFLD after PD was characterized by non-obesity and a lack of both hyperlipidemia and insulin resistance[9]. The pathogenesis of NAFLD after PD may thus differ from the pathogenesis of NAFLD associated with metabolic syndrome.

Recently, Tilg *et al.* proposed a new model, suggesting that many hits may act in parallel to finally result in liver inflammation, with gut- and adipose tissue-derived factors in particular playing a central role[10]. It was reported that the clinical features of the patients with NAFLD after PD are similar to those found in the murine NASH model induced by a methionine-choline-deficient (MCD) diet[9]. In mice fed the MCD diet, portal endotoxemia due to an impaired gut barrier caused by the MCD diet was observed[11]. We therefore hypothesized that gut–derived lipopolysaccharide (LPS) was associated with NAFLD after PD. LPS might trigger the release of inflammatory cytokines from Kupffer cells, in turn mediating severe hepatic steatosis and liver injury after PD. The process by which LPS activates Kupffer cells seems to be mediated by LPS-binding protein, CD14, and toll-like receptor 4[12]. We therefore focused on the CD14 expression on Kupffer cells from liver specimens in cases of NAFLD after PD.

We examined the prevalence, clinical and histological features, and expression of CD14 as a marker of LPS-sensitized Kupffer cells in liver tissues obtained from patients with either NAFLD associated with metabolic syndrome or NAFLD after PD.

MATERIALS AND METHODS

Between February 2003 and August 2009, a total of 100 patients underwent PD at Okayama University Hospital, Okayama, Japan. Of these, 18 patients were excluded from the study because they were unavailable for regular follow-up computed tomography (CT) of the abdomen. The remaining 82 patients (49 men and 33 women) were enrolled in this study. There were no patients with preoperative NAFLD based on CT and laboratory findings. The mean age at the time of surgery was 63 years (range, 31-85 years). Histological diagnosis was pancreatic carcinoma in 32 patients, intraductal papillary-mucinous neoplasm in 27, bile duct carcinoma in 3, and others in 20. In terms of surgical procedures, conventional PD was employed for 73 patients and pylorus-preserving PD for 9 patients. For patients with adenocarcinoma of the pancreatic head, we routinely performed dissection of the nerve plexus around the superior mesenteric artery (SMA), leaving the left side of the SMA near the origin intact. A modification of Child's method was employed for digestive reconstruction.

Body mass index (BMI) was determined for all patients and blood examinations were performed before and every 3 months after PD. Follow-up was continued for more than 12 months in all cases. Routine blood examinations included fasting lipid, blood glucose, and insulin levels, and levels of hemoglobin (HbA1c), asparate aminotransferase (AST), alanine aminotransferase (ALT), total protein, albumin, and cholinesterase.

CT images were obtained using a 16-multidetector CT scanner (GE Yokogawa, Tokyo, Japan) without intravenous contrast medium. The raw data set was reconstructed at 5-mm thickness. CT images were evaluated every 6 months after PD for follow-up. Hepatic steatosis was diagnosed on CT when hepatic attenuation values were ≤ 40 Hounsfield units. According to this criterion, 30 of 82 patients who underwent PD were found to have newly appearing hepatic steatosis.

Liver biopsy was performed for 4 of 30 patients with NAFLD after PD who consented to undergo biopsies [fatty liver, n=2; non-alcoholic steatohepatitis (NASH), n=2]; in these two men and two women, the mean age was 61 (52-73) years old, histological diagnosis was pancreatic carcinoma in one patient, intraductal papillary-mucinous neoplasm in two, and serous cyst adenoma in one, and all of them underwent PD. To compare NAFLD after PD with NAFLD associated with metabolic syndrome, liver samples were obtained from 10 patients with NAFLD associated with metabolic syndrome (fatty liver, n=5; NASH, n=5) by percutaneous ultrasonography-guided liver biopsy; these four men and six women had a mean age of 52 (40-64) years. In 10 patients, NAFLD associated with metabolic syndrome was diagnosed by the following criteria: (1) the absence of regular intake of alcohol and past history of abdominal surgery; (2) negative results for hepatitis B virus surface antigen and anti-hepatitis C virus antibodies; and (3) the absence of other types of chronic liver disease.

Specimens were fixed in 40 g/L neutral-buffered formaldehyde, cut at 4-μm thickness, and stained using hematoxylin and eosin or the Azan-Mallory method. Histological findings were assessed in a blinded fashion by an independent pathologist. Histological diagnosis of NASH was made based on the presence of macrovesicular steatosis, hepatocyte ballooning, and lobular inflammation.

For double immunofluorescence, tissue array slides were deparaffinized and soaked in 0.01 M citrate buffer (pH 6.0) at 90°C for 30 min for antigen retrieval. Samples were treated with 10 mg/ml bovine serum albumin (BSA) to inhibit non-specific antibody binding, then incubated with primary murine monoclonal antibody to CD68 (Kp-1, dilution 1:500; Dako, Glostrup, Denmark) for 1 h at 37°C. After washing three times with phosphate-buffered saline (PBS) (pH 7.2), samples were incubated with Cy5-labeled secondary rabbit polyclonal antibody to murine immunoglobulin G for 30 min at 37°C. For the second

immunoreaction, a similar procedure was used: samples were treated with 10 mg/ml BSA, then incubated with another primary antibody to CD14 (dilution 1:100; Zymed Laboratories, San Francisco, CA), then incubated with fluoresceinisothiocyanate isomer (FITC)-labeled secondary antibody. After washing with PBS, Cy5-labeled and FITC-labeled samples were examined using a fluorescence microscope (SZX12; Olympus, Tokyo, Japan).

Frequencies of double-positive cells were determined by histology experts counting these cells in entire specimens. Double-positive cells in the hepatic lobule were counted in five high-power fields (original magnification 60×). Cell counts are expressed as the mean of a sum ± standard deviation (SD) in each specimen.

Statistical Analyses

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). Continuous variables are expressed as mean ± SD and the statistical significance of differences was determined using Student's *t* test. Comparisons between groups were made using the χ^2 test for categorical variables. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The median follow-up for the 82 patients who underwent PD was 840 days (range, 183-2,553 days). The frequency of NAFLD after PD was 36.6% (30/80). Clinical pre- and intraoperative data for patients with NAFLD (n=30, 36.6%) and patients without NAFLD (n=52, 63.4%) are summarized in **Table 1**. Univariate analysis identified cancer of the pancreatic head, sex, diameter of the main pancreatic duct (MPD), and dissection of the nerve plexus as factors associated with the development of NAFLD after PD.

Clinical postoperative data for patients with and without NAFLD are summarized in **Table 2**. The BMI, serum levels of total protein and albumin, and levels of cholinesterase, total cholesterol, and insulin were significantly lower in patients with NAFLD after PD than in patients without NAFLD. Homeostasis model assessment for insulin resistance (HOMA-IR) values, as an indicator of insulin resistance, and serum levels of triglycerides and HbA1c did not differ between these two groups.

At 6 months postoperatively, 17 of the 30 patients (57%) with NAFLD after PD showed increased blood levels of AST. Of these 17 patients, we performed liver biopsies to pathologically examine specimens from four patients [mean serum levels of AST was 67 (54-80) U/L] who consented to undergo biopsies. Histopathological examination diagnosed two cases as stage 2 NASH according to the Brunt criteria[13], and two cases as moderate-to-severe macrovesicular steatosis.

In the 10 patients with NAFLD associated with metabolic syndrome, the mean serum level of AST was 56 (35-112) U/L. Five of 10 patients showed moderate-to-severe macrovesicular steatosis and the remaining five patients showed stage 2 NASH in histological examination.

Numbers of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells) were counted in all biopsy specimens (**Fig. 1**). The mean number of CD68+ cells per individual was 50.6 ± 4.0 in

specimens from NAFLD associated with metabolic syndrome and 104.3 ± 13.3 in specimens from NAFLD after PD, indicating the presence of significantly more Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome ($P < 0.001$) (**Fig. 2**). CD14+ cells were observed in specimens from NAFLD associated with metabolic syndrome and in specimens from NAFLD after PD, with mean positive cell counts of 0.6 ± 0.3 and 13.5 ± 4.2, respectively, showing more LPS-sensitized Kupffer cells in NAFLD after PD than in NAFLD associated with metabolic syndrome ($P < 0.001$) (**Fig. 2**). We also attempted to classify NAFLD into simple fatty liver or NASH. As for simple fatty liver, mean numbers of CD68+ cells and CD14+ cells per individual were 51.0 ± 6.8 and 0.4 ± 0.2, respectively, in specimens from fatty liver associated with metabolic syndrome and 116.0 ± 10.7 and 20.5 ± 2.5, respectively, in specimens from fatty liver after PD. This indicated the presence of more Kupffer cells and more LPS-sensitized Kupffer cells in specimens from simple fatty liver after PD than in specimens from simple fatty liver associated with metabolic syndrome ($P < 0.001$, CD68+ cells; $P < 0.001$, CD14+ cells). Regarding NASH, the mean numbers of CD68+ cells and CD14+ cells per individual were 50.2 ± 7.4 and 0.8 ± 0.6, respectively, in specimens from NASH associated with metabolic syndrome and 92.5 ± 19.5 and 6.5 ± 0.5, respectively, in specimens from NASH after PD. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in specimens from NASH after PD than in specimens from NASH associated with metabolic syndrome (CD68+ cells, $P < 0.05$; CD14+ cells, $P < 0.05$) (**Fig. 3**). These observations suggest that after PD, Kupffer cells and LPS-sensitized Kupffer cells were significantly upregulated not only in NASH, but also in simple fatty liver.

DISCUSSION

Conventional NAFLD is thought to be caused by excessive nutrition, lipid metabolic disorder, and insulin resistance, as a part of metabolic syndrome[14]. However, the present study demonstrated that NAFLD after PD was related to non-obese status, malnutrition, and lack of hyperlipidemia and insulin resistance. These findings suggest that the mechanisms underlying NAFLD after PD differ from those causing NAFLD associated with metabolic syndrome. Recent studies have also suggested that pancreatic exocrine insufficiency may cause NAFLD after PD[8,9]. Although only univariate analyses were examined in our study, pancreatic cancer was associated with the development of NAFLD, which is considered to lead to impaired pancreatic exocrine functions due to obstruction of the MPD, in turn resulting in obstructive distal pancreatic atrophy and fibrosis.

The clinical features of patients with NAFLD after PD resemble those in mice with methionine- and choline-deficient diet (MCD)-induced NASH, in terms of the absence of obesity, insulin resistance, and hypocholesterolemia[15]. Increased fatty acid uptake and decreased hepatic export of triglycerides in the form of very-low-density lipoprotein (VLDL) represent two important mechanisms contributing to MCD-induced NASH[15,16]. Malabsorption of essential amino acids such as choline due to pancreatic exocrine insufficiency may result in the development of NAFLD after PD.

NAFLD is often observed in patients showing hypoinsulinemia. Insulin inhibits lipolysis in adipose tissue and decreases the flux of free fatty acids (FFA) into plasma. In the absence of adequate insulin secretion, plasma FFA levels could conceivably be elevated due to increased adipose tissue lipolysis and the liver would then be unable to adequately couple triglycerides to apoprotein B or secrete VLDL-triglyceride, resulting in hepatic steatosis[17]. In this study, serum insulin concentrations were significantly lower in NAFLD

after PD compared with non-NAFLD after PD. Insufficiency of insulin may be another factor contributing to the development of NAFLD after PD.

We performed liver biopsy in four patients with NAFLD after PD, diagnosing two cases of NASH. Kupffer cell counts have been shown to play important roles in the pathogenesis of NASH[18]. Within the liver, Kupffer cells are major sources of TNF-α produced in response to LPS[12,19,20]. The process by which LPS activates Kupffer cells seems to be mediated by LPS-binding protein, CD14, and toll-like receptor 4[21]. We therefore focused on CD14 expression in liver specimens from NAFLD after PD, because the promoter polymorphism of CD14 has been reported as a risk factor for both alcoholic and non-alcoholic steatohepatitis[22]. CD14 expression on Kupffer cells is low in the healthy human liver[23,24], but increases in the presence of inflammatory liver disease[25]. Expression of CD14 on Kupffer cells can be upregulated with LPS[26,27]. A previous study reported that TNF-α production was decreased in genetically engineered CD14-deficient mice by downregulating sensitivity to LPS[28]. In contrast, the CD14 transgenic mice that overexpress CD14 on monocytes showed increased sensitivity to LPS[29]. These changes in CD14 expression could represent a mechanism regulating liver sensitivity to LPS toxicity.

The present study demonstrated that Kupffer cells were significantly more common and LPS-induced CD14+ Kupffer cells were upregulated in NAFLD after PD specimens compared to NAFLD associated with metabolic syndrome specimens. Furthermore, even in simple fatty liver after PD specimens, more Kupffer cells and more LPS-sensitized Kupffer cells were present than in specimens from simple fatty liver associated with metabolic syndrome. These findings indicate that LPS plays a significant role in the occurrence of NAFLD after PD, even from an early stage.

Previous reports have hypothesized that the overgrowth of small intestinal bacteria might play a contributory role in NASH pathogenesis, particularly via a component of the Gram-negative bacterial population, through the

production of LPS[30-34]. Among those patients who underwent PD, bacterial overgrowth may have occurred due to dissection around the SMA leading to intestinal motor dysfunction and stasis, decreased secretion of gastric juices, or blind loops.

The fatty liver is vulnerable to additional inflammatory insults such as oxidative stress and gut-derived bacterial endotoxins, both of which can trigger hepatocellular inflammation and fibrosis[35,36]. Accumulation of FFA in the liver due to malabsorption of essential amino acids such as choline and gut-derived LPS perhaps from intestinal bacterial overgrowth are important in the pathogenesis of NAFLD after PD.

In conclusion, NAFLD after PD is characterized not only by malnutrition, but also by up-regulation of CD14 on Kupffer cells with hepatic steatosis. Our results suggest that gut-derived endotoxin contributes to the development of NAFLD after PD.

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TABLES

Table 1. Comparison between pre- and intraoperative data between patients with and without NAFLD after PD

	non-NAFLD (n=52)	NAFLD (n=30)	*P* #
BMI (kg/m^2)	21.7±3.3	21.4±2.6	0.622
Age (years)	63.6±10.9	63.4±11.9	0.94
Pancreatic head cancer (yes, %)	31	53	<0.001$
Sex (male, %)	69	43	0.035$
Diameter of MPD (mm)	3.9±3.2	5.6±3.2	0.025
Total cholesterol (mg/dl) *	188.2±53.3	202.0±39.9	0.238
Triglycerides (mg/dl) *	119.8±69.2	139.9±74.8	0.363
Total protein (g/dl) *	8.0±0.9	8.3±0.5	0.322
Albumin (g/dl) *	4.0±0.4	4.0±0.5	0.761
Cholinesterase (U/L) *	230.6±88.2	244.5±104.9	0.531
Insulin (μU/ml) *	5.5±2.3	7.7±3.5	0.5
HOMA-IR*	1.6±1.0	3.2±3.4	0.324
HbA1c (%)*	5.5±0.8	6.2±1.8	0.06
ALT*	65.6±0.8	79.9±107.3	0.506
Operation (PPPD, %)	15	10	0.738$
Operation time (min)	400.9±49.0	394.6±37.8	0.544
Intraoperative blood loss (ml)	425.2±171.9	419.3±155.6	0.878
Patients with nerve plexus dissection (%)	15	93	<0.001$
Pancreatic resection line (SMA, %)	19	37	0.081$

P values calculated using the χ^2 test.

$P values calculated using Fisher's exact test

Values represent mean ±standard deviation unless otherwise indicated.

BMI body mass index, MPD main pancreatic duct, HOMA-IR homeostasis model assessment for insulin resistance, HbA1c hemoglobin A1c, ALT alanine aminotransferase.

Table 2. Comparison of postoperative data between patients with and without NAFLD after PD

	non-NAFLD (n=52)	NAFLD (n=30)	*P* #
BMI (kg/m^2)*	20.1±3.5	17.4±2.8	0.004
Total cholesterol (mg/dl)*	159.2±40.4	133.3±36.4	0.011
Triglycerides (mg/dl)*	102.2±36.4	84.9±49.0	0.169
Total protein (g/dl)*	7.0±0.7	6.1±0.7	<0.001
Albumin (g/dl) *	4.0±0.5	3.1±0.7	<0.001
Cholinesterase (U/L) *	215.5±78.5	156.9±88.1	0.006
Insulin (μU/ml) *	9.7±9.8	4.1±3.8	0.009
HOMA-IR*	3.1±3.3	1.3±1.2	0.117
HbA1c (%)*	5.5±0.9	5.6±0.9	0.589
ALT*	31.6±18.5	52.0±45.1	0.033
Insulin treatment (%)	12	27	0.126

P values calculated using the χ^2 test.

* Measured 6 months postoperatively.

Values represent mean ± standard deviation unless otherwise indicated.

BMI body mass index, HOMA-IR homeostasis model assessment for insulin resistance, HbA1c hemoglobin A1c, ALT alanine aminotransferase.

FIGURES

FIGURE LEGENDS

Figure 1: **Immunohistochemical staining of liver biopsy specimens for CD68 and CD14 (×60)**. Kupffer cells (CD68+) and LPS-sensitized Kupffer cells (CD14+) were compared between samples from conventional NAFLD and NAFLD after PD. Black arrows represent CD68+ Kupffer cells. White arrows represent CD68+ and CD14+ Kupffer cells.

a) Conventional NAFLD.

b) NAFLD after PD.

Figure 2: **Number of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells)**.

Conventional NAFLD and NAFLD after PD. conventional NAFLD and NAFLD after PD specimens showed mean cell counts of 50.6±4.0 and 104.3±13.3 CD68+ Kupffer cells per individual, respectively (*P<0.05 conventional NAFLD *vs* NAFLD after PD).

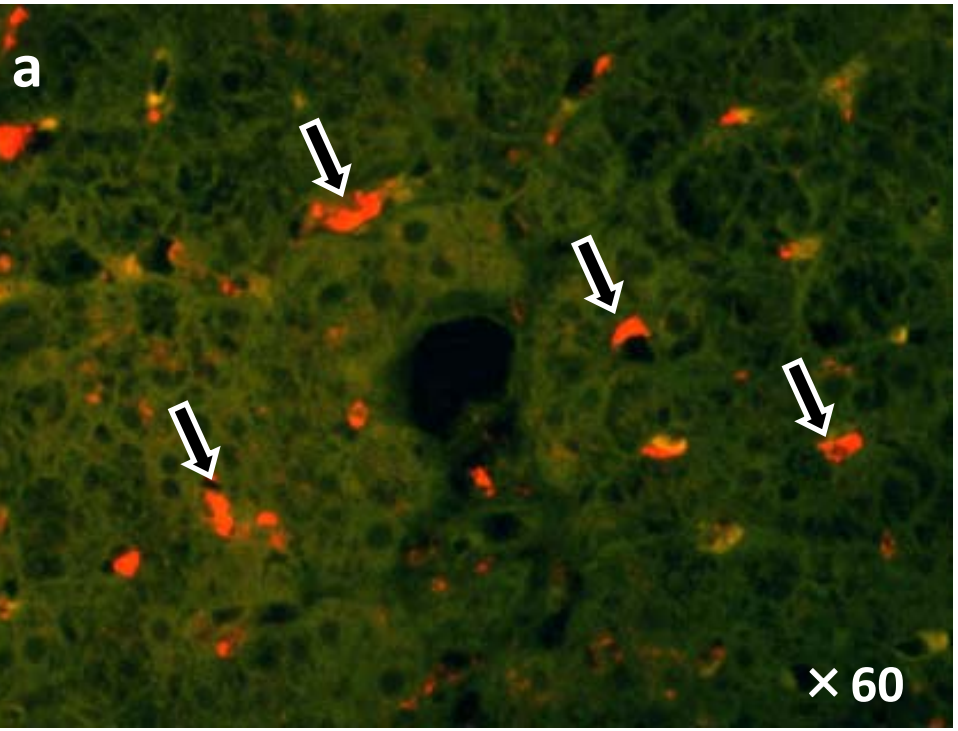
Cell counts for CD14+ Kupffer cells were 0.6±0.3 and 13.5±4.2 cells per individual, respectively ($^{\#}$P<0.001 conventional NAFLD *vs* NAFLD after PD).

Figure 3: **Number of cells positive for CD68 (a marker of Kupffer cells) and CD14 (a marker of LPS-sensitized Kupffer cells)**.

Conventional fatty liver and fatty liver after PD, in conventional NASH and in NASH after PD. For simple steatosis, mean counts of CD68+ and CD14+ cells were 51.0±6.8 and 0.4±0.2 cells per individual in conventional fatty liver specimens, respectively, and 116.0±10.7 and 20.5±2.5 cells per individual, respectively, in fatty liver after PD specimens. This indicates more Kupffer cells and more LPS-sensitized Kupffer cells in simple fatty liver after PD specimens than in conventional simple fatty liver specimens (*P<0.001, CD68+ cells; $^{\#}$P<0.001, CD14+ cells). Regarding NASH, mean cell counts for CD68+ cells and CD14+ cells were 50.2±7.4 and 0.8±0.6 cells per individual, respectively, in

conventional NASH specimens, and 92.5±19.5 and 6.5±0.5 cells per individual, respectively, in NASH after PD specimens. This showed that more Kupffer cells and more LPS-sensitized Kupffer cells were present in NASH after PD specimens than in conventional NASH specimens (**P<0.05, CD68+ cells; ##P<0.05, CD14+ cells).

Fig.1



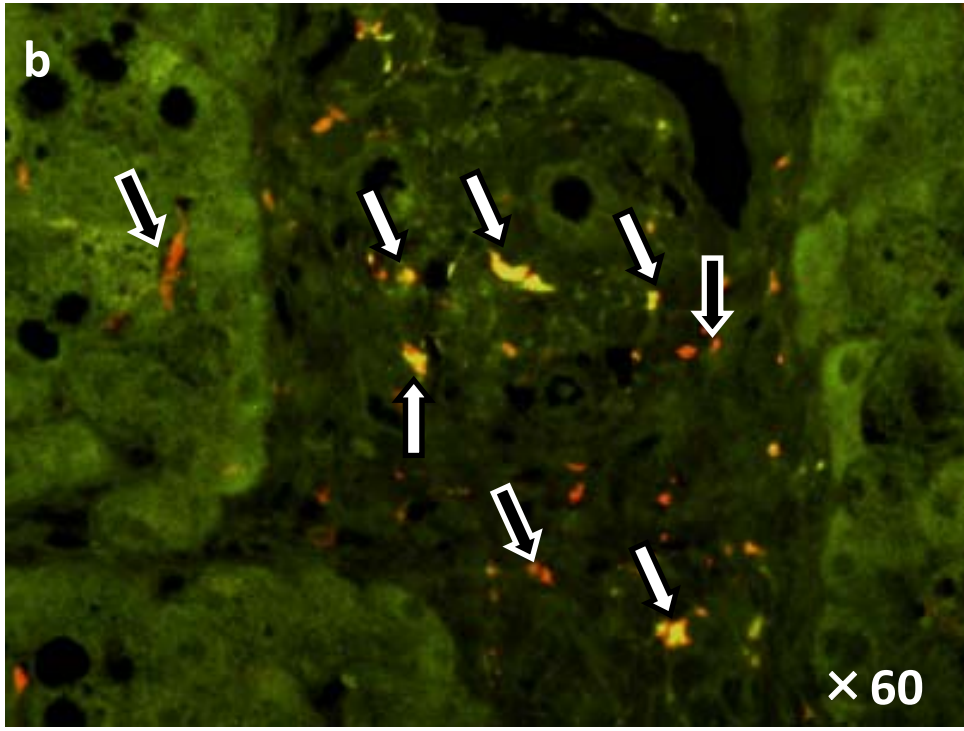


Fig.2

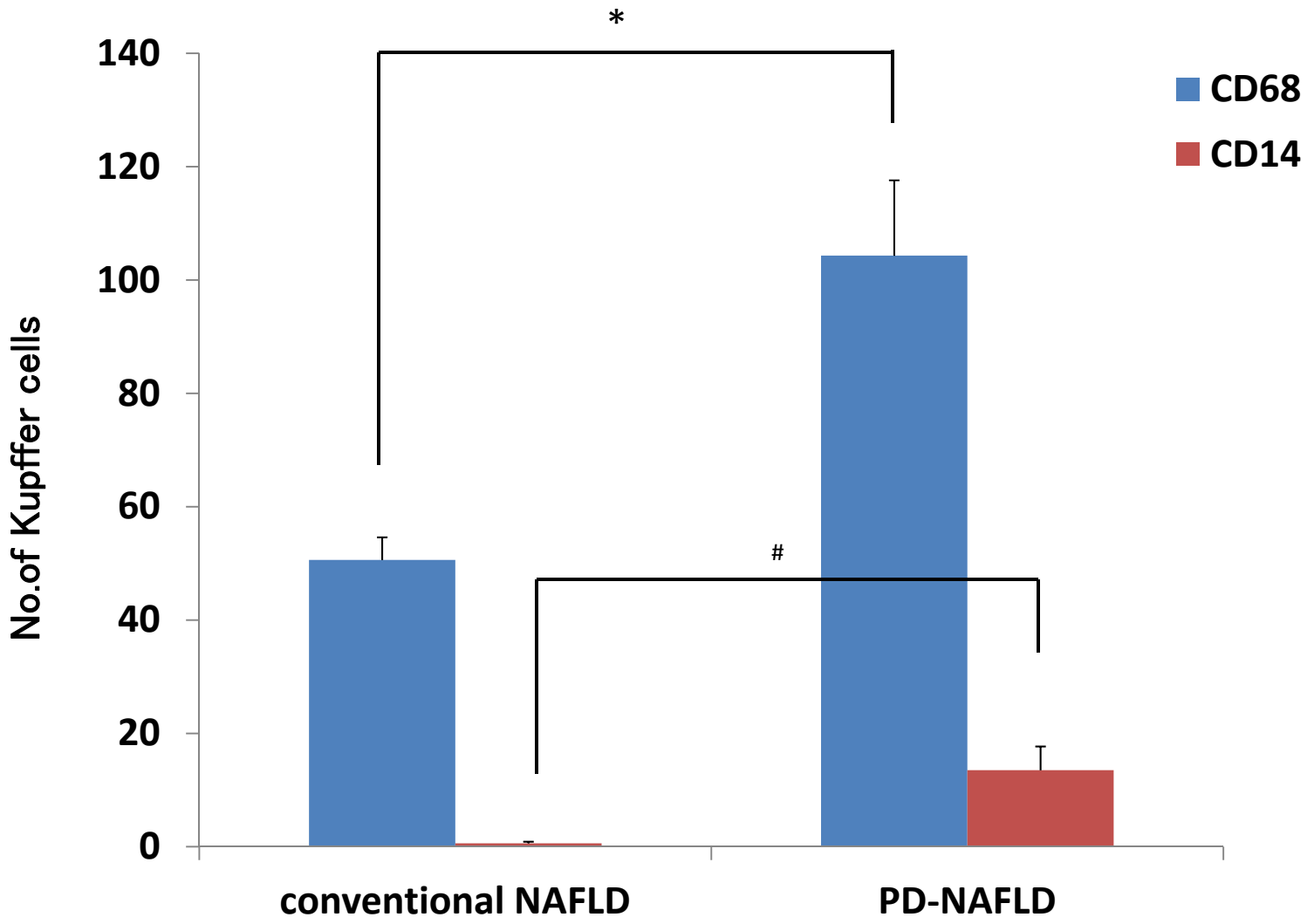
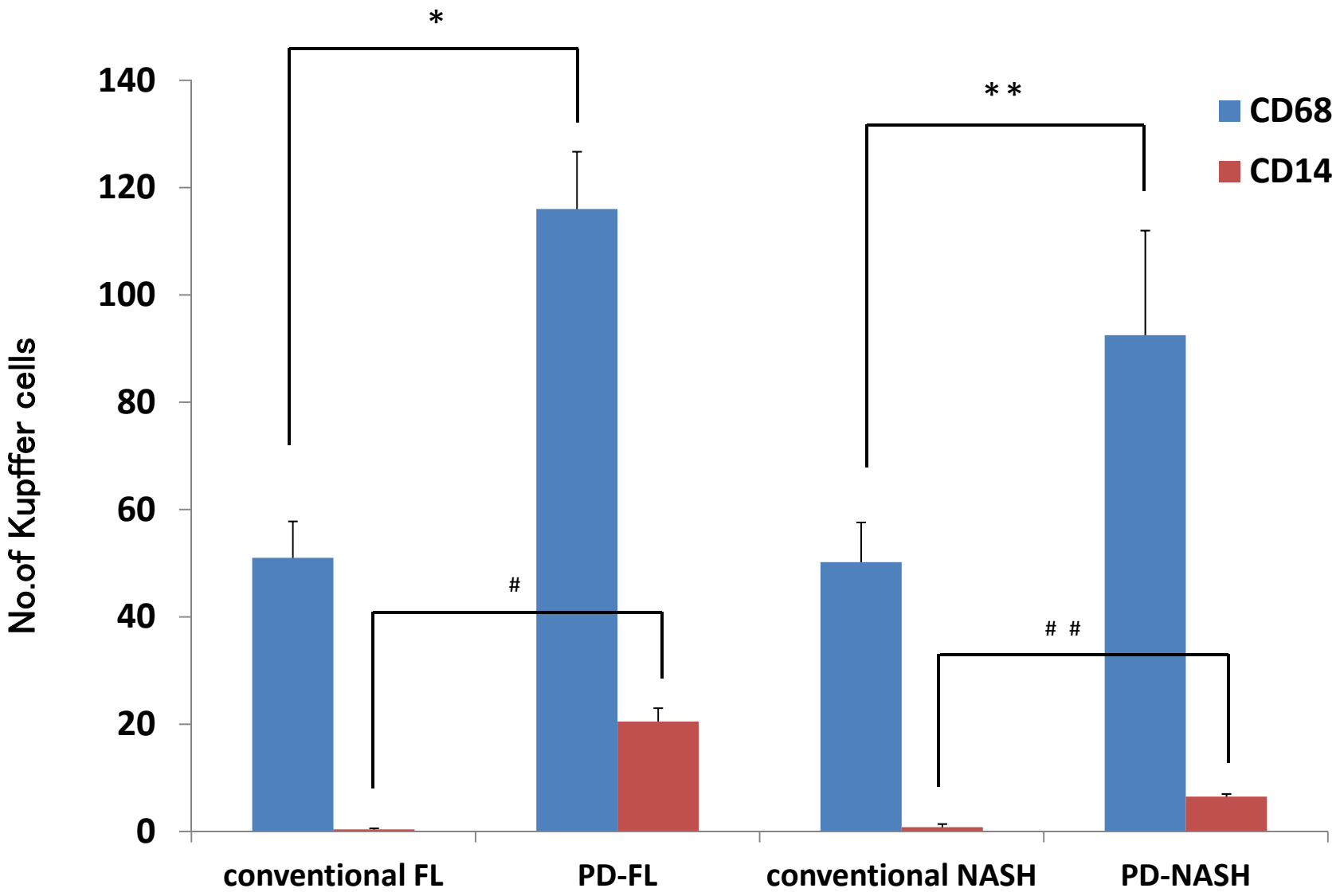

*
#
140
120
100
80
60
40
20
0
No.of Kupffer cells
CD68
CD14
conventional NAFLD
PD-NAFLD

Fig.3


*
**
#
# #
CD68
CD14
No.of Kupffer cells
140
120
100
80
60
40
20
0
conventional FL
PD-FL
conventional NASH
PD-NASH