Electronic supplementary material

Additional details on methods
Subjects
We consecutively recruited 195 patients with clinically suspected NAFLD who had undergone liver biopsies at Kanazawa University Hospital from 1997 through 2008. Fatty liver was clinically diagnosed based on ultrasound examination showing an increase in hepatorenal contrast. Hepatorenal contrast, also known as "bright liver" (1), is defined as the ratio of hepatic to kidney echo levels of over 1.0. In each patient, all other liver disorders were excluded, including viral hepatitis B and C, primary biliary cirrhosis, autoimmune hepatitis, sclerosing cholangitis, hemochromatosis, Wilson's disease, drug-induced liver injury, and biliary obstruction. All patients reported drinking less than 20 g/day of ethanol. Liver biopsies were performed during hospitalization. Serum elevations in transaminase (>40 IU/L) were examined and metabolic disorders such as diabetes mellitus and obesity were treated. None of the patients were on medication (e.g., vitamin E or ursodeoxycholic acid), which could influence histological changes in the liver throughout the follow-up period.

Data collection
A diagnosis of diabetes mellitus was based on the American Diabetes Association criteria (2). Hypertension and dyslipidemia were defined according to metabolic syndrome definitions provided by the National Cholesterol Education Program–Adult Treatment Panel III (3).
Metabolic syndrome was defined as the presence of abdominal obesity (given as waist circumference: $\geq$85 cm for men, $\geq$90 cm for women) and included at least two of the following components: hypertriglyceridemia [$\geq$150 mg/dl ($\geq$1.69 mmol/l)] and/or low HDL-cholesterolemia [<40 mg/dl (<1.03 mmol/l)]; blood pressure (systolic $\geq$130 and/or diastolic $\geq$85 mmHg), and fasting plasma glucose (FPG) [$\geq$110 mg/dl ($\geq$6.11 mmol/l)] according to the Japanese diagnostic criteria for metabolic syndrome (4). Laboratory tests included liver enzymes, blood counts, fasting lipid profile, A1C, glucose, insulin, high-sensitivity C-reactive protein (hs-CRP), ferritin, liver fibrosis markers such as type IV collagen domain 7S, hyaluronic acid (HA), and procollagen III peptide (p-III-p) . All tests were conducted and analyzed at the central clinical laboratory in our hospital. Insulin resistance indicators such as homeostasis model assessment of insulin resistance (HOMA-IR), quantitative insulin-sensitivity check index (QUICKI), and indices for muscle and hepatic insulin resistance were calculated based on the results of the 75-g oral glucose tolerance tests, using previously reported formulas: HOMA-IR = [fasting insulin (μU/ml) $\times$ fasting plasma glucose (μmol/l)]/22.5 (5), QUICKI = 1/[log (fasting insulin expressed in μU/ml) + log (fasting plasma glucose expressed in mg/dl)] (6). Index for muscle insulin resistance = 10,000/log [fasting glucose (mg/dl) $\times$ fasting insulin (μU/ml)] $\times$ [mean glucose (mg/dl) $\times$ mean insulin during the oral glucose tolerance test (μU/ml)], and Index for hepatic insulin resistance = glucose$_{0-30}$ area under the curve (AUC) $\times$ insulin$_{0-30}$ AUC, respectively (7).

Liver biopsies
Sections were cut from a paraffin block and stained with hematoxylin and eosin, and Azan–Mallory, and silver reticulin impregnation. The biopsied tissues were scored for steatosis (from 0 to 3), stage (from 1 to 4), and grade (from 1 to 3) as described (8), according to the standard criteria for grading and staging of NASH proposed by Brunt et al. (9). All cases showed variable degrees of steatosis that histologically corresponded to NAFLD. NASH was

histologically diagnosed based on the presence of ballooned hepatocytes with lobular hepatitis. In contrast, livers without ballooned hepatocytes were diagnosed as fatty liver. The scoring system according to Brunt classification (9) applies to NASH, but not fatty liver. Therefore, for fatty liver without any features of steatohepatitis, we assigned a score of stage 0 and grade 0. In addition, when histological activity or fibrosis varied within a biopsy, cases were scored as an average (i.e., 0.5 or 1.5) so that we could examine histological changes in detail.

The progression of liver histology was defined as an increased stage score in the final biopsy with respect to the baseline; a decreased stage score was considered an improvement.
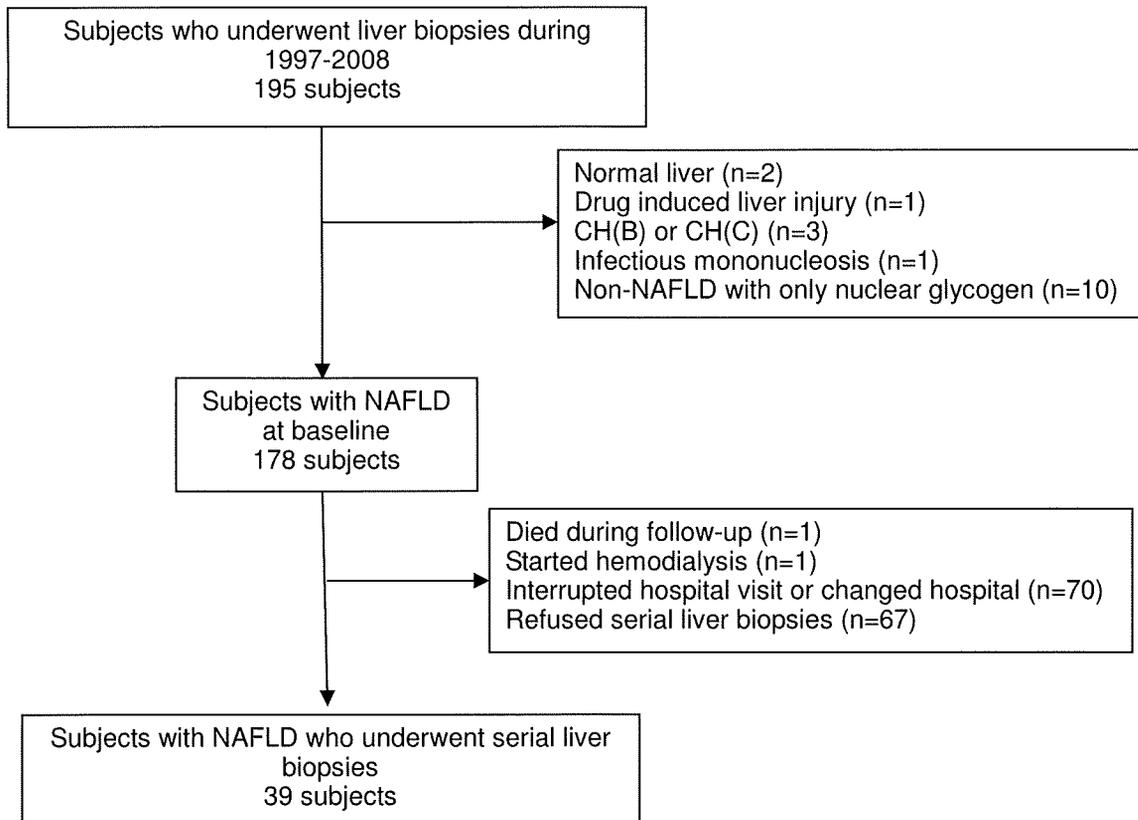
Statistical analyses

Continuous data are presented as the medians (ranges) and categorical data are presented as a number (%). A Kruskal–Wallis test and a χ^2 test were applied for the analysis of differences in continuous and categorical variables. Cox proportional hazard model was used to calculate multivariate (age, gender and body mass index) adjusted hazard ratio for improving liver fibrosis, and chi-square were used to compare the strength of relationship.

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Supplementary Figure 1 – Description of study subjects and exclusion criteria

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┌─────────────────────────────────────┐
│ Subjects who underwent liver biopsies during │
│              1997-2008               │
│            195 subjects              │
└─────────────────────────────────────┘
          │
          │        ┌──────────────────────────────────────────┐
          │        │ Normal liver (n=2)                       │
          ├───────▶│ Drug induced liver injury (n=1)          │
          │        │ CH(B) or CH(C) (n=3)                     │
          │        │ Infectious mononucleosis (n=1)           │
          │        │ Non-NAFLD with only nuclear glycogen (n=10) │
          │        └──────────────────────────────────────────┘
          ▼
┌─────────────────────┐
│  Subjects with NAFLD │
│     at baseline      │
│    178 subjects      │
└─────────────────────┘
          │
          │        ┌──────────────────────────────────────────────┐
          │        │ Died during follow-up (n=1)                  │
          ├───────▶│ Started hemodialysis (n=1)                   │
          │        │ Interrupted hospital visit or changed hospital (n=70) │
          │        │ Refused serial liver biopsies (n=67)         │
          │        └──────────────────────────────────────────────┘
          ▼
┌─────────────────────────────────────┐
│ Subjects with NAFLD who underwent serial liver │
│              biopsies                │
│             39 subjects              │
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Supplementary Table1 – Baseline clinical and biochemical data of 39 patients with NAFLD

		normal range
Age (years)	47 (20-79)	
Gender (M:F)	22 : 17	
Body mass index (kg/m^2)	27.8 (22.5-44.4)	18-25
AST (IU/L)	40 (11-106)	10-48
ALT (IU/L)	54 (10-209)	3-50
Fasting plasma glucose (mg/dl)	128 (76-414)	70-110
A1C(%)	6.6 (4.7-13.6)	4.3-5.8
HOMA-IR	3.9 (0.7-11.1)	<2.0
Total cholesterol (mg/dl)	199 (128-276)	132-220
Triglycerides (mg/dl)	112 (28-521)	32-150
HDL-cholesterol (mg/dl)	48 (28-82)	40-97
Platelets (x10^4/μl)	22.8 (7.1-41.2)	13-35
Ferritin (μg/dl)	185 (13.5-64.0)	6.2-138
hsCRP	0.17 (0.00-7.53)	<0.2
Type IV collagen 7S (ng/dl)	4.1 (2.7-10.0)	<6.0
HA	24.7 (0-299)	<50
P-III-P	0.6 (0.4-45.0)	0.3-0.8
Diabetes (%)	30 (76.9)	
Dyslipidemia (%)	25 (64.1)	
Hypertension (%)	14 (35.8)	
Metabolic syndrome (%)	15 (38.4)	

Data are median (range) or numbers (%) of patients; n=39.
AST, aspartate aminotransferase, ALT, alanine aminotransferase; hs-CRP, high-sensitivity C-reactive protein; HA, hyaluronic acid; P-III-P, procollagen III peptide.

Supplementary Table2 – Changes of pathological score and the treatment for diabetes and hypertension in 39 patients with NAFLD

Case	Initial diagnosis	Final diagnosis	Stage	Grade	Steatosis	DM	Treatment for DM	HT	ARB
Improved group									
1	NASH	FL	3 → 1	3 → 1	2 → 1	+	diet → diet	-	-
2	FL	FL	2 → 1	0 → 0	3 → 3	-		-	-
3	NASH	FL	2 → 1	1 → 1	2 → 1	+	diet → insulin	+	+
4	NASH	FL	2 → 1	2 → 0	2 → 2	+	diet → insulin	-	-
5	NASH	FL	1 → 0.5	1 → 1	3 → 2	+	SU → insulin	-	-
6	FL	FL	2 → 1	0 → 0	2 → 1	+	diet → insulin	-	-
7	NASH	FL	3 → 1	2 → 0	2 → 1	+	diet → diet	+	+
8	NASH	NASH	1 → 0.5	2 → 1	2 → 2	+	diet → insulin	+	+
9	NASH	FL	3 → 2	2 → 1	3 → 2	-		+	-
10	NASH	FL	3 → 2	2 → 0.5	3 → 2	+	diet → diet	-	-
11	FL	FL	1 → 0	0 → 0	1 → 1	+	diet → insulin	-	-
12	NASH	NASH	4 → 3	2 → 0.5	2 → 1	+	diet → insulin	+	-
Progressed group									
13	FL	FL	1 → 2	0 → 0	1 → 1	+	diet → insulin	+	+
14	FL	NASH	1 → 3	0 → 2	3 → 3	+ → IGT	diet → diet	-	-
15	FL	FL	1 → 2	0 → 1	1 → 1	+	diet → diet	+	+
16	FL	NASH	1 → 2	0 → 1	1 → 1	+	α-GI → SU, Met	+	+
17	FL	NASH	1 → 2	0 → 1	1 → 2	+	diet → α-GI	-	-
18	FL	FL	1 → 1.5	0 → 0.5	1 → 2	-		-	-
19	FL	NASH	1 → 2	0 → 3	3 → 2	+	diet → insulin	+	+
20	FL	FL	1 → 2	0 → 0	1 → 2	+	Met → Met, α-GI	+	-
21	FL	FL	1 → 1.5	0 → 0.5	3 → 2	+	diet → insulin	+	+
22	FL	FL	1 → 1.5	0 → 0.5	2 → 2	-		+	-
23	NASH	NASH	1 → 1.5	1 → 1.5	3 → 2	IGT	diet → diet	-	-
Stable group									
24	NASH	NASH	3 → 3	3 → 3	1 → 1	+	Met, SU → Met, SU	-	-
25	FL	FL	1 → 1	0 → 0	2 → 1	+	α-GI → insulin	+	+
26	FL	FL	1 → 1	0 → 0	2 → 1	+	diet → diet	-	-
27	FL	FL	1 → 1	0 → 0	2 → 1	+	Met, SU → insulin	-	-
28	FL	FL	1 → 1	0 → 0	1 → 1	+	diet → insulin	+	+
29	FL	FL	1 → 1	0 → 0	2 → 1	- → IGT	diet	-	-
30	FL	FL	1 → 1	0 → 0	1 → 2	- → IGT	diet	-	-
31	NASH	NASH	4 → 4	2 → 2	2 → 1	+	diet → insulin	-	-
32	FL	FL	1 → 1	0 → 0	3 → 3	+	diet → insulin	-	-
33	FL	FL	1 → 1	0 → 0	2 → 3	+	diet → diet	+	-
34	NASH	NASH	1 → 1	1 → 1	1 → 1	+	SU, Pio → insulin	-	-
35	NASH	NASH	1 → 1	1 → 1	3 → 3	+	diet → diet	-	-
36	FL	FL	2 → 2	0 → 0	3 → 3	+	diet → diet	-	-
37	NASH	NASH	2 → 2	1 → 1	2 → 2	+	diet → insulin	-	-
38	NASH	NASH	4 → 4	3 → 3	1 → 1	IGT	diet → diet	-	-
39	NASH	NASH	4 → 4	3 → 2	3 → 1	- → DM	diet	-	-

Patients with histological improvement (Cases 1–12), progression (Cases 13–23), or stability (Cases 24–39) are indicated in the Improved, Progressed, and Stable groups, respectively. Presence of diabetes and hypertension, and the treatment with ARB are indicated by +.
α-GI, α-glucosidase inhibitor; ARB, angiotensin II receptor blocker; FL, fatty liver; IGT, impaired glucose torelance; Met, metformin; Pio, pioglitazone; SU, sulfonylurea.

Supplementary Table 3 – Factors associated with improvement of hepatic fibrosis

	χ^2	p	Hazard Ratio	(95%CI)		
Age (1 year)	0.07	0.74	1.01	(0.97	-	1.05)
Gender (men)	0.29	0.60	0.72	(0.22	-	2.36)
BMI (higher 1 kg/m2)	1.41	0.24	0.92	(0.81	-	1.05)
Diabetes mellitus	0.84	0.36	2.24	(0.40	-	12.60)
Hypertension	3.35	0.07	3.52	(0.91	-	13.57)
Dyslipidemia	0.52	0.47	0.62	(0.16	-	2.30)
Treatment with insulin	5.53	0.02	6.00	(1.35	-	26.74)
A1C (higher 1%)	0.38	0.54	1.10	(0.81	-	1.50)
Treatment with ARB	0.02	0.89	0.89	(0.19	-	4.28)
ΔBW (higher 1 kg)	9.68	0.00	0.72	(0.59	-	0.89)
ΔA1C (higher 1%) *	7.75	0.01	0.16	(0.05	-	0.58)
ΔA1C (higher 1%)* †	7.97	0.01	0.18	(0.05	-	0.59)
Treatment with insulin* †	4.58	0.03	8.59	(1.20	-	61.59)

A Cox proportional hazard model was used to calculate multivariate (age, gender and body mass index) adjusted hazard ratio for improving liver fibrosis.

* Adjusted for age, gender, body mass index and baseline A1C levels.

†Two variables were included in the same model and adjusted for each other.

CD14$^+$monocytes are vulnerable and functionally impaired under ER stress in patients with type 2 diabetes

A short running title: Diabetic monocyte is attenuated under ER stress

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Objective—While patients with diabetes suffer from increased infections and a higher incidence of cancer due to impaired immune function, details on diabetes-induced decrease in immunity are lacking. We assessed how immune-mediating peripheral blood mononuclear cells (PBMCs) are affected in diabetes.

Research designs and methods—We obtained PBMCs from 33 patients with type 2 diabetes and 28 healthy volunteers, and investigated their susceptibility to apoptosis and functional alteration.

Results—In a subpopulation of PBMCs, monocytes derived from patients with diabetes were more susceptible to apoptosis than monocytes from healthy volunteers. Monocytes from patients with diabetes had decreased phagocytotic activity and were less responsive to Toll-like receptor (TLR) ligands, although the expression of TLRs did not differ significantly between the two groups. Furthermore, monocytes from patients with diabetes had a distinctly different gene expression profile compared to monocytes from normal volunteers as assessed with DNA microarray analysis. Specifically, quantitative real-time detection PCR measurements showed an elevated expression of the markers of endoplasmic reticulum (ER) stress in diabetic monocytes, and electron microscopic examination of monocytes revealed morphological alterations in the ER of cells derived from patients with diabetes. Consistently, the ER stress inducer tunicamycin increased apoptosis of otherwise healthy monocytes and attenuated the pro-inflammatory responses to TLR ligands.

Conclusions—These data suggest that monocytes comprise a substantially impaired subpopulation of PBMCs in patients with diabetes and that ER stress is involved in these pathological changes mechanistically. This implies that the affected monocytes should be investigated further to better understand diabetic immunity.

2

Type 2 diabetes is the most frequent metabolic disease and the leading cause of human morbidity and mortality (1, 2). Based on epidemiological data, patients with diabetes are immunocompromised and have an increased incidence of infections in the respiratory tract, urinary tract, and skin (3–5). The high incidence of colorectal, breast, and pancreatic malignancies in patients with diabetes is also considered to be a consequence of diabetes-associated defects in immune function (6, 7).

Although studies on immune cells and circulating cytokines have shed some light on this diabetic immunological phenomenon, conflicting results have been reported and do not adequately explain the perturbed immune function in patients with diabetes. Controversial results concerning the phagocytotic activity of polymorphonuclear neutrophils and monocytes are in part due to differences in the patients themselves, insufficient numbers in the study populations, or inconsistencies in the collection of the cell populations under investigation (8–11). Therefore, further studies are needed to explain the decreased immune function of patients with diabetes.

We previously investigated the gene expression signatures of peripheral blood mononuclear cells (PBMCs) in patients with diabetes and observed transcriptional expression features that were distinct from those of healthy volunteers (12). Apoptosis-related genes were up-regulated in the PBMCs of patients with diabetes. Based on this result, we investigated apoptotic activity and immunological function in PBMCs from patients with type 2 diabetes.

We observed that the $CD14^+$ monocyte fraction was the most affected subpopulation of PBMCs from these patients; these cells were especially vulnerable to apoptosis compared to other cell subpopulations. We also found that $CD14^+$ monocytes demonstrated attenuated phagocytotic activity and deficient Toll-like receptor (TLR) signaling, both of which are important for innate immunity (13, 14). Transcriptional analysis and electron microscopic examination of monocytes from patients with diabetes showed evidence of endoplasmic reticulum (ER) stress, which may underlie the functional defects in these cells. Collectively, the data presented herein show that $CD14^+$ monocytes are a vulnerable cell population under ER stress in these patients that could contribute to decreases in immune function in diabetes.

RESERCH DESIGN AND METHODS

Patients: Thirty-three patients with type 2 diabetes (male/female, 15/18; age, 62.0 ± 8.6 years; HbA_{1c}, 9.2 ± 2.0%) and 28 healthy volunteers (male/female, 15/13; age, 58.2 ± 10.2 years; HbA_{1c}, 5.4 ± 0.7%) were enrolled consecutively for the apoptosis assay (Table 1). The groups were not significantly different in terms of their clinical parameters, except for the fasting plasma glucose (FPG) and HbA_{1c} levels. The patients with diabetes ($n = 16$) from whom adequate numbers of monocytes were obtained were enrolled for additional experiments along with 17 other patients with diabetes (male/female, 8/9; age, 60.5 ± 7.2 years; HbA_{1c} 8.8 ± 1.8) whose clinical profiles fit the diabetic profile (Table 1). Informed consent for this study was obtained from all subjects. The experimental protocol was carried out in accordance with the Declaration of Helsinki.

Isolation of subpopulations of PBMCs and flow cytometric analysis: PBMCs were freshly isolated from heparinized venous blood using Ficoll–Hypaque (Sigma–Aldrich, St. Louis, MO) as previously described (12). $CD4^+$ T cell and $CD14^+$ monocyte subpopulations were isolated using a magnetic cell sorting

3

system in accordance with the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated cells were purified by > 90 % as measured by flow cytometric analysis using FACSCalibur ^{TM} flow cytometer (BD Biosciences, San Jose, CA). To assess the expression of TLRs on monocytes, PBMCs were incubated with phosphatidylethanolamine (PE)-labeled anti-TLR2, TLR3, or TLR4 (eBioscience, San Diego, CA) and fluorescein isothiocyanate (FITC)-labeled anti-CD14 antibodies (BD Biosciences) and analyzed by flow cytometry. Data were analyzed using CELLQuest^{TM} Software (BD Biosciences).

Quantitative real-time detection PCR (RTD-PCR): RTD-PCR was performed as previously described (15). Briefly, total RNA obtained from cells using a MicroRNA isolation kit (Stratagene, La Jolla, CA) was reverse-transcribed using 1 μg oligo (dT) primer and Super Script II Reverse transcriptase (Invitrogen, Carlsbad, CA). The relative quantities of mRNA expression were analyzed by RTD-PCR using ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). All primer pairs and probes were obtained from the TaqMan assay reagents library. Expression levels of genes were calculated with the $2^{-\Delta\Delta Ct}$ method using either β-actin or GAPDH as internal control genes.

Apoptotic cell detection assay: Freshly isolated PBMCs were incubated with AIM-V (Invitrogen) serum-free culture media containing 5 or 30 mM glucose at 37°C with 5% CO_2 for up to 24 h. The cells were incubated with FITC-labeled anti-CD4, CD14, or CD56 antibodies (BD Biosciences) and with PE-labeled Annexin-V and 7-amino-actinomycin D (7-AAD) (BD Biosciences) in PBS containing 2% BSA (Sigma–Aldrich). Apoptotic cells were determined by flow cytometry as the fraction of cells labeled with Annexin-V that were

7-AAD negative. At least 10,000 cells per sample were analyzed.

Phagocytosis assay: Phagocytotic activity was assessed using Phagotest Kit (Orpegen Pharma, Heidelberg, Germany) and FITC-labeled opsonized *E. coli* in accordance with manufacturer's protocol. Briefly, heparinized whole blood obtained from the 33 patients with diabetes and 28 healthy volunteers was incubated with FITC-labeled *E. coli* for 10 min at 37°C. After removing the erythrocytes, the remaining cells were incubated with propidium iodide to detect viable leukocytes by flow cytometry. Monocyte populations were assessed based on cellular granularity and size as side scatter and forward scatter, respectively, and FITC-positive cells were assessed as monocytes with phagocytosed FITC-labeled *E. coli*.

TLR ligand stimuli and expression of pro-inflammatory cytokine genes: Peptidoglycan (PGN) (1 μg/ml) from *Streptomyces sp.* (Sigma–Aldrich), Poly (I:C) (5 μg/ml) (Sigma–Aldrich), and Lipopolysaccharide (LPS) (2 μg/ml) from *E. coli* (Sigma–Aldrich), which are TLR2, TLR3, and TLR4 ligands, respectively, were added to monocytes (3 × 10^{5}cells) freshly isolated from the 33 patients and 28 healthy volunteers in AIM-V media. Before and 3 h after incubation, the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) was analyzed by RTD-PCR.

Analysis of gene expression by DNA microarray: Total RNA was obtained from CD14$^+$ monocytes using MicroRNA isolation kit (Stratagene, La Jolla, CA), and the mRNA was amplified twice using the Amino-allyl MessageAmp aRNA Kit (Ambion, Austin, TX). The reference RNA sample was isolated from CD14$^+$ monocytes from a 30-year-old healthy male volunteer and amplified in the same manner. Amplified mRNA was labeled with Cyanine (Cy) 5 or Cy3 (Amersham, Buckinghamshire, UK).

4

Equal amounts of the amplified mRNAs were hybridized to an oligo-DNA chip (AceGene®Human Oligo Chip 30K; Hitachi Software Engineering Co., Yokohama, Japan) overnight and washed prior to image scanning.

The fluorescence intensity of each spot on the oligo-DNA chip was obtained using cDNA Microarray Scan Array G (PerkinElmer, Wellesley, MA). The obtained images were quantified using DNAsis array v2.6 software (Hitachi Software Engineering Co.). For normalization, the intensity of each spot with oligo DNA was subtracted from that of spots without oligo DNA in the same block. The spot was validated when the intensity was within the intensity plus or minus a 2-fold range of standard deviation within each block. By calibrating the median as the base value, the intensities of all spots were adjusted for normalization between Cy5 and Cy3. Hierarchical clustering of gene expression was calibrated using the method described above using BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.htm l). The non-filtered data were log-transformed and applied to the average linkage clustering with centered correlation. For the functional analysis of the 813 upregulated genes, we used GenMAPP (http://www.genmapp.org), a computer program designed for viewing and analyzing genome-scale data on MAPPs representing biological pathways and any other groups of genes.

Electron microscopy: Monocytes obtained from 3 healthy volunteers and 3 patients with diabetes were fixed with 2.5% glutaraldehyde, and then post-fixed in 1% (v/v) cacodylate-buffered osmium tetroxide. Samples were dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon-Araldite. Ultrathin sections were obtained and observed under a Hitachi H-7500 electron microscope (Hitachi High-Technologies Co., Hitachinaka, Japan).

Caspase-3 assay and enzyme-linked immunosorbent assay (ELISA) of cytokines: Monocytes from a healthy volunteer were harvested and treated with tunicamycin (1 or 5 µg/ml) in AIM-V media. Every 3 h up to 12 h after tunicamycin treatment, we assessed apoptosis by flow cytometry as described above. After 12 h of incubation, the expression levels of BCL-2, C/EBP homologous protein (CHOP) and (immunoglobulin heavy chain binding protein) BiP were assessed by RTD-PCR. The DEVD-cleaving activity of active caspase-3 was measured using labeled Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNA) as the substrate and the Caspase-3 Colorimetric Assay Kit (Promega, Madison, WI) in accordance with manufacturer's protocol. The pNA light emission was quantified using a microtiter plate reader at a wavelength of 405 nm. In addition, we measured the production of pro-inflammatory cytokines by RTD-PCR 6 h after treatment of monocytes (3 × 10^5 cells) with tunicamycin (1 or 5 µg/ml) or the TLR ligands PGN (1 µg/ml), Poly (I:C) (5 µg/ml), and LPS (2 µg/ml). The concentrations of TNF-α, IL-1β, and IL-6 in the culture supernatants were measured using ELISA kit (eBioscience).

Statistical analysis: Data are expressed as means ± SEM. The Mann–Whitney U test was applied to assess the significant differences between the two groups. Statistical significance was determined as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Increased apoptosis of CD14$^+$ monocytes from patients with diabetes: We first assessed the frequency of apoptosis in the PBMC fractions from 33 patients with diabetes and 28 non-diabetic, healthy volunteers. Apoptosis of the isolated cells was assessed after 3 h incubation in AIM-V

5

serum-free media containing 5 mM glucose (physiological concentration in blood). As shown in Fig. 1A, a significant difference in the frequency of apoptosis was observed in the PBMCs isolated from patients with diabetes and healthy volunteers. Adding serum to AIM-V serum-free media did not affect the difference in apoptosis (data not shown). The numbers of whole PBMCs, $CD4^+$, $CD14^+$, and $CD56^+$ cells were similar in both diabetic and healthy subjects (data not shown). $CD14^+$ monocytes were observed to be the major contributor to the increased apoptosis measured in the PBMCs. In contrast, apoptosis of $CD4^+$ T cells and $CD56^+$ NK cells were not significantly different between the two groups (Fig. 1A). When the incubation period in culture media with or without serum was extended to 24 h, about 20% of the $CD56^+$ NK cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis. When incubation period was extended to 5 days, about 5% of $CD4^+$ T cells of both patients with diabetes and healthy volunteers were induced to undergo apoptosis; there was no significant difference in cell viability of $CD56^+$ NK cells and $CD4^+$ T cells between the two groups (data not shown). BCL-2 expression of $CD4^+$ T cells was not different between the two groups (data not shown). Apoptosis of PBMC subpopulations incubated in culture media containing 30 mM glucose was not different from cells incubated in 5 mM glucose-containing media (data not shown). Moreover, the susceptibility of PBMCs from patients with diabetes to apoptosis was not related to clinical features such as vascular complications, insulin treatment, and FPG concentrations (data not shown).

However, among the 33 patients with diabetes, the frequency of apoptotic $CD14^+$ monocytes from those with poor glycemic control ($HbA_{1c} \geq 9.0$) was elevated compared to patients with fair glycemic control (HbA_{1c} < 9.0) (Fig. 1B). Furthermore, after 3 h incubation, the increased ratio of the expression of the anti-apoptotic gene, BCL-2, was substantially lower in monocytes from the 15 patients with $HbA_{1c} \geq 9.0$ compared to the 18 patients having HbA_{1c} < 9.0, as assessed by RTD-PCR (Fig. 1C). These data suggest that the monocytes of patients with diabetes are susceptible to apoptosis, especially under conditions of poor glycemic control.

Attenuated function of monocytes from patients with diabetes: To determine if functional alterations exist in monocytes isolated from the 33 patients with diabetes, we cocultured the monocytes with FITC-labeled *E. coli* and counted the number of fluorescent monocytes indigeting the labeled *E. coli* by flow cytometry. The ratio of monocytes that phagocytosed *E. coli* to all monocytes in patients with diabetes was higher than in the healthy volunteers (Fig. 2A and B). No significant correlation was observed between the ratio of phagocytosed *E. coli* and HbA_{1c} levels among the patients (data not shown).

Next, we assessed the responsiveness of monocytes to external pathogenic stimuli *in vitro*. Monocytes typically express pattern-recognition molecules such as the TLRs that are important for innate immunity against various pathogens (13, 14). The expression levels of TLR2, TLR3, and TLR4 were not significantly different between monocytes from patients with diabetes and those from healthy volunteers, as assessed by RTD-PCR (Fig. 3A) and flow cytometry (data not shown). We also found that transcriptional expression of TLR signal molecules (MyD88, IRAK1, and TRAF6 for TLR2 and TLR4 signaling and TRIF for TLR3 signaling) was not altered in diabetic monocytes compared to non-diabetic monocytes (data not shown). Next, we exposed the monocytes from the patients with diabetes and healthy volunteers to the TLR ligands, PGN (a TLR2 ligand),

6

Poly (I:C) (a TLR3 ligand), and LPS (a TLR4 ligand) and measured the expression of the pro-inflammatory cytokine genes, TNF-α and IL-1β. After incubation, the expression of the cytokines was not significantly different between the groups (Fig. 3B), but the responsiveness to PGN, Poly (I:C), and LPS was significantly attenuated in monocytes from patients with diabetes compared to those from healthy volunteers as assessed by RTD-PCR (Fig. 3C and D). These results demonstrate that the monocytes of patients with diabetes are functionally impaired, which implies that they could contribute to immune deficiency in diabetes.

ER stress is a molecular feature of impaired monocytes: To elucidate the molecular features of the diabetic monocytes that were distinctly susceptible to apoptosis, DNA microarray analysis was performed on CD14$^+$ cells isolated from five randomly selected patients with diabetes and five healthy volunteers. These subjects demonstrated clinical features near the median of all study subjects. Unsupervised hierarchical clustering analysis was performed to assess the gene expression profiles of monocytes obtained from patients with diabetes and healthy volunteers; 17,184 filtered genes were evaluated after excluding genes that were not expressed or those with low expression levels that prevented their analysis in 50 % of the cases. As shown in Fig. 4A, two completely discernible clusters formed between the patients with diabetes and the healthy volunteers.

We identified 813 genes that were up-regulated in the monocytes from patients with diabetes compared to those of healthy volunteers (P < 0.05, student's t test). Analysis of the biological processes concerning these genes was performed using GenMAPP. The identified genes were shown to be involved in posttranslational protein modification systems occurring in the Golgi-apparatus or were involved in ER stress

(Table 2 and Supplemental Table 1 which can be found in an online appendix at http://diabetes.diabetesjournals.org). The elevated expression of genes related to ER stress, such as CHOP and BiP, was confirmed using RTD-PCR; the expression of these genes was significantly higher in the monocytes from the 33 patients with diabetes than in those from the 28 healthy volunteers (Fig. 4B). In contrast, no significant difference in the expression of these genes was observed in CD4$^+$ T cells from patients with diabetes and healthy volunteers (Fig. 4C).

Electron microscopy further confirmed ER stress in the monocytes derived from patients with diabetes. As shown in Fig. 4D, morphological alterations of the ER such as corruption of concentric, continuous and regular layer structure and a decreased number of ribosomes on the ER membrane were evident from the electron photomicrographic images.

ER stress-induced apoptosis and attenuation of TLR signaling in human monocytes: The results described above indicated that the monocytes from patients with diabetes have compromised immunological function and that ER stress is a distinct feature in these cells. To determine whether ER stress could be a mechanism underlying the observed increase in apoptosis and decreased responsiveness to TLR ligands, CD14$^+$ cells isolated from a healthy volunteer were treated with the ER stress inducer, tunicamycin (1 μg/ml), in AIM-V media. As shown in Fig. 5A and B an increased number of apoptotic cells was observed among monocytes treated with tunicamycin compared to untreated monocytes after more than 6 h incubation. Treatment of monocytes with a higher concentration of tunicamycin (5 μg/ml) induced more apoptosis (Fig. 5A and B), and when monocytes were treated with tunicamycin for 12 h, the activity of the pro-apoptotic protease, caspase-3,

7

significantly increased (Fig. 5C). Treatment with tunicamycin coordinately decreased the expression of BCL-2 (Fig. 5D) and increased the expression of the ER stress markers, CHOP and BiP (Fig. 5E). These results suggest that ER stress promotes apoptosis of human monocytes.

Next, we investigated how tunicamycin-induced ER stress affected the responsiveness of human monocytes to TLR ligands. Treatment of monocytes with tunicamycin for 6 h did not affect the transcriptional and translational expression of TLR2 and TLR4 (data not shown). As shown in Fig. 6A–C, however, the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 were down-regulated after stimulation with TLR2 and TLR4 ligands. Furthermore, the production of TNF-α, IL-1β, and IL-6 in media was measured by ELISA and found to decrease after treatment of human monocytes with tunicamycin and after stimulation with TLR2 or TLR4 ligands (Fig. 6D–F). However, tunicamycin-induced ER stress did not affect these expression after treatment of monocytes with the TLR3 ligand, Poly (I:C) (data not shown).

DISCUSSION

In the present study, we observed that PBMCs from patients with diabetes were more susceptible to apoptosis compared to PBMCs from healthy volunteers and that $CD14^+$ monocytes comprised the primary PBMC subpopulation undergoing apoptosis. We also found that $CD14^+$ monocytes from patients with diabetes were hypo-responsive to TLR ligands and that they had attenuated phagocytotic activity. Transcriptional analysis and electron microscopy revealed the presence of ER stress in the affected diabetic monocytes. Consistently, monocytes isolated from non-diabetic patients showed a similar increase in apoptosis and a weakened response to TLR ligands, when they were treated with tunicamycin, indicating that ER

stress may be a pivotal mechanism underlying the decreased immunological function observed in patients with diabetes.

As innate immune-defense mediators, monocytes are capable of ingesting exogenous pathogens to protect the host from infectious diseases. Previous studies have shown that phagocytosis in diabetic neutrophils and monocytes is attenuated (10, 11). Similarly, in our study population, monocytes from patients with diabetes were less capable of phagocytosing *E. coli* pathogens compared to monocytes derived from healthy volunteers. This novel finding might explain, at least in part, the decrease in immune function characteristic of patients with diabetes (16). Nevertheless, the detailed mechanisms underlying diabetes-induced decreases in phagocytotic activity remain unclear, since simple high glucose concentration did neither affect the phagocytotic activity and TLR expression, nor induce ER stress in non-diabetic monocytes *in vitro* (data not shown).

The TLRs are pattern recognition receptors that are important for recognizing pathogens, inducing pro-inflammatory responses, and preventing the host from acquiring infectious diseases (17–20). The expression of TLR2, TLR3, and TLR4 in $CD14^+$ monocytes was similar between patients with diabetes and healthy volunteers. The administration of a high dose of insulin downregulates TLRs expression (21). Transformed monocyte-lineage blastoma cells showed increased TLR expression under hyperglycemic conditions in vitro (22). Type 2 diabetes is characterized as a state of inadequately controlled glycemia associated with hyperinsulinemia due to peripheral insulin resistance (1). Taken together, the TLRs expression may be affected by hyperglycemia and hyperinsulinemia in a complex manner. In contrast to the previous finding that monocytes from patients with diabetes were hypersensitive to the TLR

8

ligand, LPS (23, 24), we observed that the TNF-α and IL-1β expression from monocytes derived from patients with type 2 diabetes diminished after exposure to PGN, Poly I:C, and LPS—ligands of the TLR2, TLR3, and TLR4 receptors, respectively. These data suggest that diabetes perturbs signaling downstream of the TLRs. In this study, we collected CD14$^+$ monocytes from PBMCs via enrichment using magnetic beads; this protocol was used to remove T cells, NK cells, B cells, dendritic cells, and basophils from the PBMC mixture. This is in contrast to the methodology used to isolate these cells in many other studies, in which monocytes were obtained as adherent cells in the culture dish or by a rosetting technique (25, 26). CD14$^+$ cells have been shown to be composed of multiple subtypes of activated states; the classical monocyte-isolation methods used in the other studies might unknowingly remove the fraction of monocytes that are susceptible to apoptosis (27). Over half of the CD14$^+$ diabetic monocytes isolated in this study were dead for 12 h culture even in media containing physiological concentration of glucose (data not shown). Our current data showing attenuation of TLR responsiveness to ligands in diabetic monocytes suggests that initial immune responses that are normally triggered by viruses, bacteria, and parasites could be impaired in diabetes, which is consistent with epidemiological data showing a high incidence of infection in patients with diabetes (3–5).

Gene expression and electron microscopic analysis of monocytes derived from patients with diabetes showed active signatures of ER stress; this is important because ER is an organelle essential for the proper folding and glycosylation of proteins after protein synthesis (28). When cells are under ER stress, protein kinase R-like ER kinase, inositol requiring enzyme 1, and activating transcription factor 6 are activated and function in the adaptation to stress, proper folding of proteins, and removal of harmful unfolded proteins, respectively (29, 30). However, prolonged ER stress leads to apoptotic cell death, which is mediated by CHOP (31). CHOP is a crucial and specific molecule for ER stress-induced apoptosis and alters the transcription of the BCL-2 gene family members (32). The current study showed that diabetic monocytes had increased levels of ER stress-related apoptotic molecules. Moreover, non-diabetic monocytes treated with tunicamycin, an ER stress inducer, underwent apoptosis in a manner similar to monocytes derived from patients with diabetes. From these data, we conclude that ER stress contributes to the susceptibility of diabetic monocytes to apoptosis.

We also observed that tunicamycin-induced ER stress diminished TLR2 and TLR4 signaling without altering expression of TLRs. Tunicamycin induces ER stress by disturbing N-linked glycosylation (33), and previous reports suggest that perturbations in this glycosylation attenuates TLR2 and TLR4 signaling *in vitro* (34, 35). Hence, these data collectively indicate that ER stress may underlie decreases in TLR2 and TLR4 signaling and affect immune function in patients with diabetes.

TLR3 signaling is different from the other TLR signaling pathway; for example, it is independent of MyD88. TLR2 and TLR4 are expressed on the cell surface, whereas TLR3 is expressed in intracellular compartments such as endosomes (13), and its ligands require internalization before signaling occurs. This suggests that disturbances in TLR3 signaling in diabetic monocytes may be due to reasons other than ER stress. Further investigations are needed to elucidate the detailed mechanisms of attenuated TLR signaling in monocytes from patients with diabetes.

ER stress has been shown to be a mainstay of the diabetic condition. Its pathological importance in diabetes is

9

especially important in pancreatic β-cells, in which glucose toxicity results in ER stress and insufficient insulin secretion (36–38). The current study suggests that monocytes are yet another population of cells vulnerable to hyperglycemia-induced ER stress and dysfunction. Nevertheless, the mechanisms that render pancreatic β-cells and monocytes vulnerable to ER stress in patients with diabetes remain uncertain.

Diabetes is considered a chronic inflammatory disease. Activated macrophages that produce pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are thought to contribute to insulin resistance in muscle and adipose tissues (39, 40). Furthermore, the atherosclerotic complications in patients with diabetes have a basis in inflammation; local inflammatory foci in atherosclerotic lesions are commonly composed of foam cells derived from activated macrophages (41, 42). Further studies are needed to determine whether different subpopulations of monocyte-derived cells, for example, systemically circulating and locally residing inflammatory cells, are susceptible to hyperglycemia-induced ER stress and dysfunction.

In conclusion, our findings show that CD14$^+$ monocytes are susceptible to ER stress-induced alterations in inflammatory signaling and apoptosis, which may play a role in the decreased immune function observed in patients with diabetes. Further investigations are needed to discern the mechanisms of diabetes-induced ER stress and perturbations in inflammatory signaling in CD14$^+$ monocytes.

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10

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11

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12

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13

Table 1   Characteristics of the study subjects

	Diabetic patients (n=33)	Healthy volunteers (n=28)	P value
Age (years)	62.0±8.6	58.2±10.2	N.S
Gender (male/female)	15/18	15/13	N.S
Body mass index	23.5±4.2	23.6±4.8	N.S
White blood cell counts (/ml)	4800±1700	5600±1900	N.S
Lymphocytes (%)	23.5±3.5	22.7±2.5	N.S
Monocytes (%)	5.2±1.6	6.1±2.3	N.S
Hemoglobin (g/dl)	14.1±1.3	13.6±1.6	N.S
Total cholesterol (mg/dl)	182±24	180±35	N.S
Triglyceride (mg/dl)	138±37	163±33	N.S
FPG (mg/dl)	185±38	86±7.4	<0.001
HbA$_{1c}$ (%)	9.2±2.0	5.4±0.7	<0.001
Diabetic complications (+/-) *	19/14	N.A	
Insulin treatment (+/-)	10/23	N.A	

Data are expressed as means±SD.
* Diabetic complications: nephropathy, neuropathy, retinopathy, macroangiopathy

Table 2   Biological processes for up-regulated genes in monocytes of diabetic patients

MAPP Name	Z Score	Permute P
Golgi-apparatus	3.383	0.000
Ribosomal Proteins	3.691	0.002
Unfold protein binding	2.471	0.026
Intracellular protein transport	2.310	0.029
Enzyme liked receptor protein signaling pathway	2.175	0.042
Nuclear Receptor	2.316	0.043
Gametogenesis	-1.998	0.049

14