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Effect of *Hochuekkito* on Alveolar Macrophage Inflammatory Responses in Hyperglycemic Mice

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Abstract—Diabetes mellitus reduces immunological activity and increases susceptibility to various infections. *Hochuekkito* (TJ-41) has been reported to improve the weakened physical condition of various chronic diseases. BALB/c mice were divided into three groups; groups A and B were fed a standard diet, and group C, a TJ-41 diet. Two weeks after starting these diets, hyperglycemia was induced in groups B and C by injection with streptozotocin. Two weeks later, bronchoalveolar lavage was performed. Toll-like receptor (TLR) ligands (TLR2: peptidoglycan, PGN; TLR4: lipopolysaccharide, LPS; TLR5: flagellin, FLG) were used to stimulate alveolar macrophages (AMs), and TNF-α production was measured. Under hyperglycemic conditions and PGN or FLG stimulation, TNF-α production from AMs was significantly reduced in group B compared with group A. However, treatment with TJ-41 (group C) significantly improved the impaired production of TNF-α. These results suggest that, under hyperglycemic conditions, TJ-41 can improve the inflammatory responses of AMs with stimulation of TLR ligands.

KEY WORDS: alveolar macrophage; Toll-like receptor; diabetes mellitus; streptozotocin; inflammatory response.

INTRODUCTION

It is well known that patients with diabetes mellitus have decreased immunological activity and have an increased susceptibility to various infections, including lower respiratory tract infections. Several previous studies of patients with community-acquired pneumonia have reported that hyperglycemia was associated with an increased risk of pneumonia-related hospitalization [1].

Alveolar macrophages (AMs) are first-line defense cells targeting invading pathogens and therefore play a central role in innate respiratory host defense [2]. Few reports have investigated the effects of hyperglycemic conditions on the function of AMs, such as decreased phagocytosis and bactericidal activity [3], and depressed respiratory burst [4]. Recent studies focusing on macrophage inflammatory responses under hyperglycemic conditions have mainly been performed using peritoneal macrophages [5–7] and bone marrow-derived macrophages [8, 9], while data on AMs are limited. Lipopolysaccharide (LPS)-induced macrophage inflammatory protein-2 gene expression in diabetic mice [10] and production of tumor necrosis factor alpha (TNF-α), interleukin (IL)-12, and nitric oxide (NO) in diabetic rats infected with *Mycobacterium tuberculosis* were reported to be decreased [11].

Toll-like receptors (TLRs) are cellular receptors that recognize molecular signatures of pathogens and initiate an inflammatory signaling cascade that is critical to the innate immune response. In humans, ten TLRs have been identified which recognize pathogen-specific ligands. TLR2, TLR4, and TLR5 play important roles in bacterial infection: TLR4 recognizes LPS, a major cell wall component of Gram-negative bacteria, whereas TLR2 and TLR5 recognize peptidoglycan (PGN), another bacterial wall component, and flagellin (FLG), respectively. All three TLRs are expressed and functionally active on AMs [12, 13]. When stimulated with a ligand, TLRs induce the production of inflammatory

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cytokines and provoke natural immune responses. Our preliminary data showed that hyperglycemic conditions cause an impaired responsiveness of AMs to selective TLR ligands by inhibiting the production of pro-inflammatory cytokines [14].

Bu-Zhong-Yi-Qi-Tang (*Hochuekkito*; TJ-41) is a *kampo* (Japanese and Chinese traditional) herbal medicine and has been used to improve the weakened physical condition of patients with various chronic diseases. TJ-41 was prepared as a spray-dried powder of a hot-water extract obtained from ten medical plants, including *Astragali radix*, *Atractylodis Lanceae rhizoma*, *Ginseng radix*, *Angelicae radix*, *Bupleuri radix*, *Zizyphi fructus*, *Auranti Nobilis pericarpium*, *Glycyrhizae radix*, *Cimicifugae rhizoma*, and *Gingiberis rhizoma* [15]. TJ-41 has been reported to exhibit a pharmacological immunopotentiating activity [15] and enhance the suppressed reactive oxygen-producing activity of neutrophils in diabetic rats [16]. Additionally, treatment of human monocytic cells (THP-1 cell line) with TJ-41 has been reported to cause slightly increased expression of TLR4 [17].

In the present study, we evaluated the immune-activating effects of TJ-41 by studying its effects on inflammatory responses of AMs from hyperglycemic mice.

MATERIALS AND METHODS

Reagents

TJ-41 was provided by Tsumura Co. (Tokyo, Japan). Mouse food was produced by CLEA Japan (Tokyo, Japan) and was supplemented with 2 mg/5 g (0.04%) TJ-41. Streptozotocin (STZ), a known diabetogen, was purchased from Sigma-Aldrich (St. Louis, MO). *Escherichia coli* LPS was purchased from Sigma. *Staphylococcus aureus* PGN and *Salmonella typhimurium* FLG were purchased from Invitrogen (San Diego, CA). PE-labeled anti-murine TLR2 antibody (Ab) and TLR4 Ab were purchased from eBioscience (San Diego, CA). PE-labeled anti-murine TLR5 Ab was purchased from Imgenex (San Diego, CA). Culture media and supplements were purchased from Sigma.

Animals

Specific pathogen-free male Balb/c mice at 6–8 weeks of age were purchased from Japan SLC (Tochigi, Japan). Animals were housed in standard cages with carefully controlled ambient temperature (25°C) and artificial light (12 h of light from 8:00 am to 8:00 pm) and were fed with standard laboratory chow with or without TJ-41 and tap water at the animal facility of Jichi Medical University. All experiments described in this study were approved by the Institutional Animal Care and Use Committee of Jichi Medical University.

Administration of TJ-41 and Injection of STZ

The experimental setup of this study is outlined in Fig. 1. TJ-41 was administered orally with a composite of 2 mg/5 g (0.04%) per day. Mice were divided into three groups: groups A and B were given standard food, and group C was given food containing TJ-41.

Two weeks after the initiation of TJ-41 treatment, STZ, in 0.01 M citrate buffer (pH 4.5), was injected intraperitoneally at a dose of 250 μg/g body weight into groups B and C. Two weeks later (4 weeks after the beginning of TJ-41 treatment), blood glucose levels were measured using Glutest Ace (Sanwa Chemical Co.,

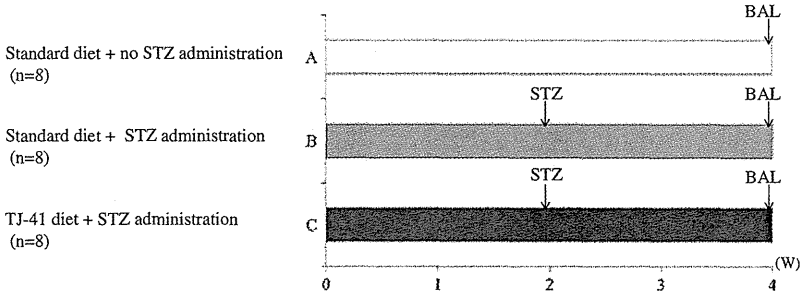


Fig. 1. Experimental protocol. Oral administration of TJ-41 or standard diet by gavage for 4 weeks. Two weeks after the beginning of feeding, STZ was injected intraperitoneally to groups B and C. One week after injection, blood glucose levels were measured, and only the mice with blood glucose levels exceeding 200 mg/dl were used in the experiments. Four weeks after the beginning of feeding, mice were sacrificed, before bronchoalveolar lavage (BAL) was performed and blood glucose levels measured.

Nagoya, Japan) and Glutest sensor (Sanwa Chemical Co.). Only mice with a fasting blood glucose level of at least 200 mg/dl were considered diabetic and used in the following experiments.

AM Isolation and Culture

Two weeks after STZ injection, tracheas were cannulated with an 18-gauge catheter, and bronchoalveolar lavage (BAL) was performed under deep anesthesia with pentobarbital, by instilling 1 ml of phosphate-buffered saline (PBS) into both lungs through the trachea four times [18]. After each instilment, fluid was collected and pooled. Cells were counted by trypan blue dye exclusion. Cytospins for differential cell counts were prepared and stained with modified Wright-Giemsa (Diff-Quick; International Reagents Co., Kobe, Japan). Cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Harlan, Indianapolis, IN), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Based on trypan blue dye exclusion and differential cell counts, equal numbers of AMs were plated at the indicated cell density. After 2 h of incubation at 37°C in a 5% CO_2 incubator, nonadherent cells were removed by washing twice with PBS. Adherent cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and were either left unstimulated or were stimulated with PGN (10 μg/ml), LPS (100 ng/ml), or FLG (1 μg/ml).

TNF-α Measurement

AMs plated in 96-well plates at 5×10^4 cells per well were cultured in the medium alone or stimulated with specified reagents at indicated concentrations for 18 h. Cell supernatants were harvested, and levels of TNF-α were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Analysis of Cell-Surface TLR Expression

AMs from control and diabetic mice were obtained by BAL as described above. AMs were resuspended at 1×10^5 cells per 100 μl in staining buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide). Nonspecific staining was blocked by incubation with anti-murine CD16/32 Ab (eBioscience) for 15 min. After blocking, cells were incubated for 30 min with PE-labeled anti-murine TLR2, TLR4, and TLR5 Abs. AMs were washed twice with staining buffer and fixed with staining buffer containing 1% formalin. Flow cytometry was performed using a FACS LSR flow cytometer (BD Bioscience, San Jose, CA). Using forward scatter and side scatter parameters to define macrophage populations, 1×10^4 events were acquired. Flow cytometry data were analyzed using the CellQuest Pro software.

Real-Time PCR

AMs were plated in 12-well plates at 4×10^5 cells per well. Cells were cultured in the medium alone or stimulated with specified reagents, and RNA was isolated at the indicated time points using the RNAqueous Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Reverse transcription was performed on similar amounts of RNA per group using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Specific primers for murine TNF-α, TLR2, 4, and 5 were designed, and PCR was performed in triplicate with Fast Universal PCR Master Mix in the ABI 7500 Fast Real-Time PCR System (Applied Biosystems).

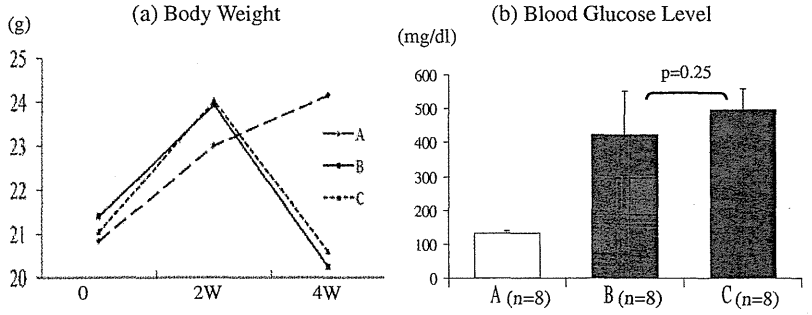
Statistical Analysis

All data are shown as mean±SEM of each group. The statistical significance of any difference in each parameter among the groups was evaluated by a Student's t test (Statview, SAS Institute, Cary, NC). A value of $p<0.05$ was considered statistically significant.

RESULTS

To test the immune-activating potential of TJ-41 in the context of diabetic conditions, the drug was administered to mice under both basal conditions and in conjunction with the diabetogen STZ. As shown in Fig. 2, significant body weight loss in animals was observed after 2 weeks in groups B and C, to which STZ was administered. Furthermore, blood glucose levels at week 4 of the study were significantly higher in groups B and C, compared with the untreated control group, but did not differ between groups B and C.

No difference was observed in the cell fractions in the bronchoalveolar lavage fluid among the three groups, and they were mostly macrophages (Fig. 3).

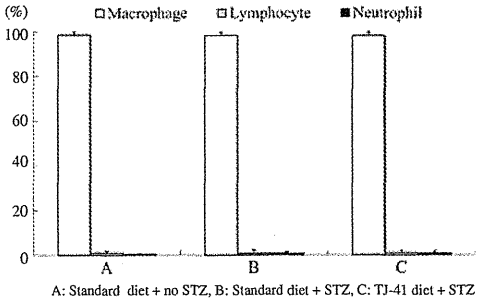


A: Standard diet + no STZ, B: Standard diet + STZ, C: TJ-41 diet + STZ

Fig. 2. Change in body weight and blood glucose levels in mice after STZ injection. **a** Mouse body weight was measured at 0, 2, and 4 weeks. Body weight of groups B and C (STZ injected) were significantly decreased compared with group A (no STZ). **b** Blood glucose levels of mice after 4 weeks. Data are expressed as mean±SEM.

We initially tested TNF-α production under PGN or FLG stimulation in the three treatment groups. As shown in Fig. 4, production of TNF-α was lower in diabetic (STZ-treated) animals, compared with untreated animals. Interestingly, treatment with TJ-41 significantly increased TNF-α production following PGN and FLG stimulation in diabetic animals (PGN, group B 1,742± 80 pg/ml versus group C 2,091±166 pg/ml, and FLG, group B 5,626±288 pg/ml versus group C 7,143± 315 pg/ml, $p<0.05$, respectively) but not following LPS stimulation (Fig. 4). These results suggested that suppressed TNF-α production under diabetic conditions with PGN or FLG stimulation improved with intake of TJ-41.

We next examined whether the above-described modulation of cytokine production by TJ-41 treatment (Fig. 4) was associated with changes in TLR expression. The expression of TLR2, TLR4, or TLR5 on the cell surface was evaluated by flow cytometry. As shown in Fig. 5, no difference in TLR expression was observed between the three groups. In addition to protein levels, we also tested whether TLR mRNA levels were altered in response to TJ-41 treatment. RT-PCR using specific primers for TLR2, 4, and 5 was performed on cDNA generated from AMs, but no differences were observed in the three treatment groups (data not shown).



A: Standard diet + no STZ, B: Standard diet + STZ, C: TJ-41 diet + STZ

Fig. 3. Cell differentiation in bronchoalveolar lavage fluid. There was no difference in cell differentiation in bronchoalveolar lavage between the three groups.

Initially, we divided mice into four groups: groups A–C as previously described and one further group which was given food containing TJ-41 but was not injected with STZ. However, in this group, we found entirely no difference comparing with the data of control group A (standard diet+no STZ administration) (data not shown).

To summarize the above results, in diabetic mice, TNF-α production from AMs was significantly reduced following stimulation with TLR2 or TLR5 ligands, compared with the control group. However, TNF-α production in diabetic mice was increased upon treatment with TJ-41. Interestingly, no difference was noted in the expression of TLR mRNA in macrophages or TLR expression on the cell surface between the three groups.

DISCUSSION

Japanese herbal medicine is a mixture of many plant materials, and the mechanism of action mediating the effect can appear to be complicated. There have been numerous reports describing the beneficial effects of *Bu-Zhong-Yi-Qi-Tang* (*Hochuekkito;* TJ-41) on immune functions. Harada *et al.* reported an enhancement of immune function, particularly of antitumor immunity, through the augmentation of cytostatic activity [19].

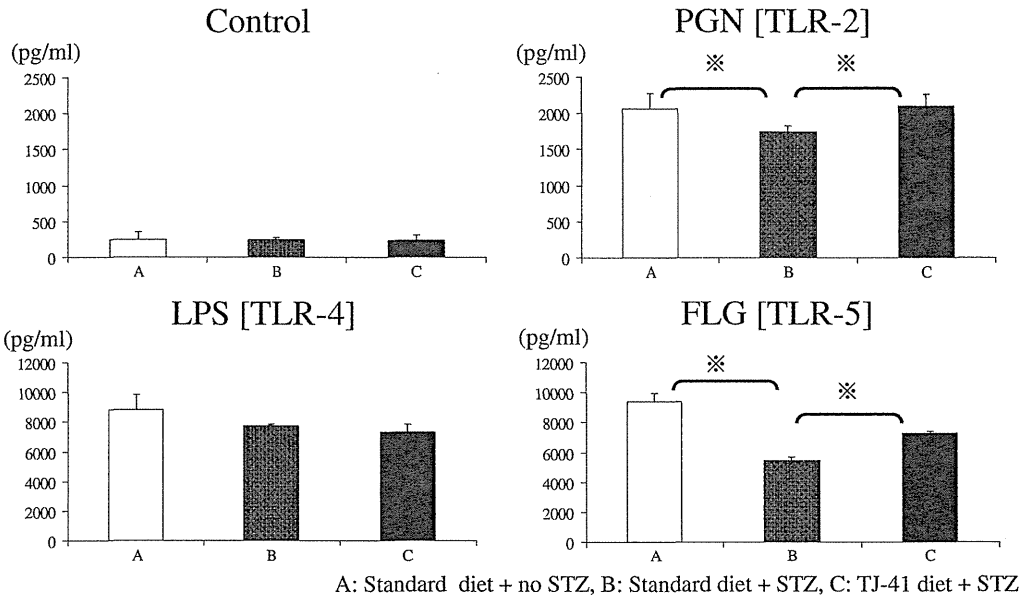


Fig. 4. Effect of TJ-41 on TNF-α production in pulmonary-alveolar macrophages. After stimulating AMs with the TLR ligands, PGN (10 μg/ml), LPS (100 ng/ml), or FLG (1 μg/ml) for 18 h, supernatants were harvested, and TNF-α levels were measured in cell supernatants by ELISA. Data are expressed as mean±SEM.

Elderly people who received doses of TJ-41 had a significant increase in their serum interferon-gamma (IFN-γ) levels, which is thought to be associated with increased NK cell activity [20]. TJ-41 combined with IFN-γ moderately enhanced the daily activity of chronic fatigue syndrome mice by increasing NK cell activity [21]. Utsuyama *et al.* showed that TJ-41 could enhance the impaired immune function of old mice by increasing the numbers of T and NK cells [22]. Mice administered with TJ-41 for 32 weeks had a significant increase in their splenic NK cell population, and the CD4/CD8 ratio in the spleen was increased [23]. TJ-41 increased IL-18-induced ICAM-1 and CD86 expression, resulting in enhanced TNF-α and IFN-γ production. This suggests that TJ-41 enhances IL-18-induced cell-mediated immunity and may enhance host defense mechanisms against pathogens [24]. Therefore TJ-41 may have beneficial effects via its ability to enhance immune system activation.

As for innate immunity in the diabetic mouse model, most of the studies regarding the impacts of a hyperglycemic state on tissue macrophage inflammatory responses to TLR ligands have been performed on cells other than AMs. Moreover, the examinations have focused on the response to LPS, a TLR4 ligand, but not other TLR ligands.

For example, several studies have shown impaired inflammatory responses in peritoneal macrophages, such as reduced LPS-induced TNF-α and IL-6 production in type 1 diabetic rat models [5, 6] and reduced TNF-α and IL-1β production by LPS plus IFN-γ stimulation in type 2 diabetic (db/db) mice [25]. Contrary to these reports, peritoneal macrophages from diabetic mice produced more IL-1β in response to LPS, resulting in increased peritoneal levels of IL-1β induced by LPS [26]. Also, exposure of bone marrow-derived macrophages derived from non-obese diabetic mice to a hyperglycemic environment resulted in elevated levels of TLR2, TLR4, and TNF-α [9]. Thus, immunomodulatory effects of a hyperglycemic state on macrophage cytokine reactions are complex. Although these data were different in part from our present study, the discrepancy was probably due to the different experimental models, origin of macrophages, and additional stimuli.

Our preliminary data suggested that the hyperglycemic state impairs the reactivity of alveolar macrophages to selective TLR ligands, TLR2 ligand (PGN), and TLR5 ligand (FLG), by inhibiting the production of TNF-α [14]. Additionally, in the present study, the suppressed TNF-α production from alveolar macrophages in mice stimulated with PGN or FLG was alleviated by TJ-41. TNF-α activates T cells, B cells,

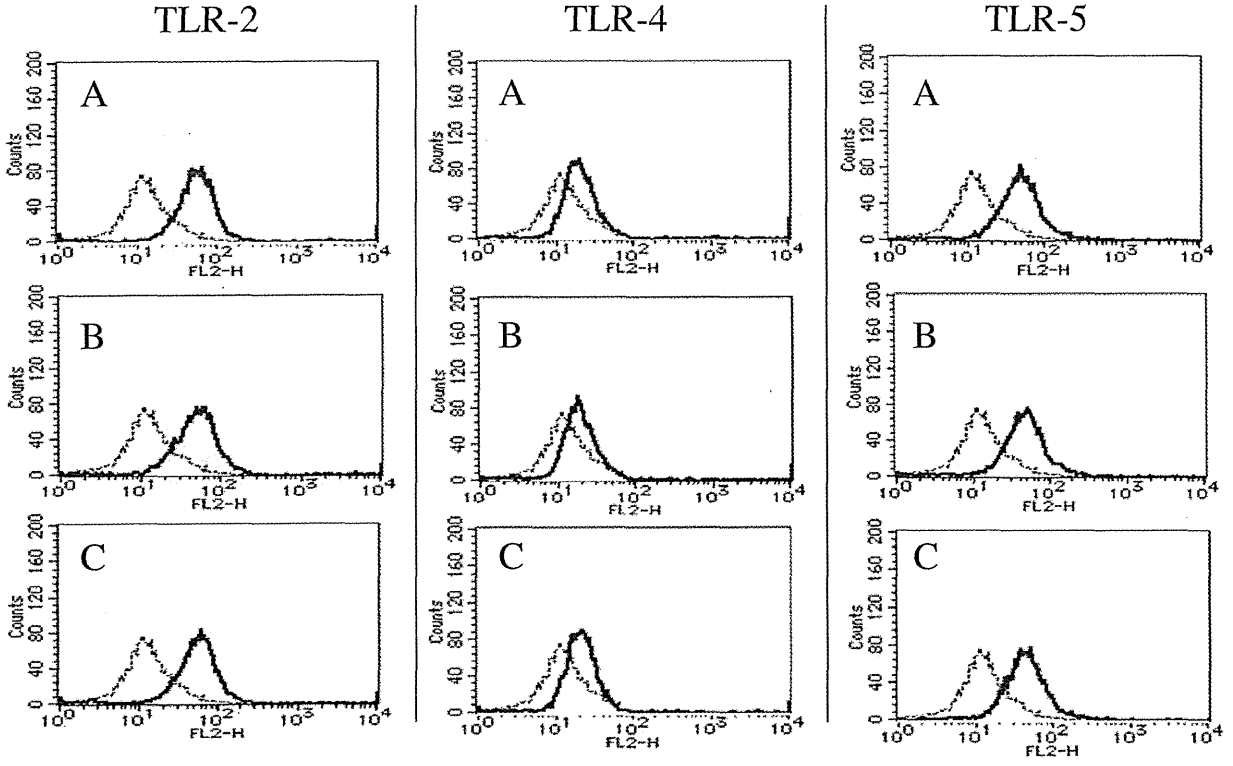


Fig. 5. Effect of TJ-41 on TLR surface expression. Flow cytometric analysis of AM surface phenotype was performed by direct immunofluorescence using a BD LSR cell analyzer. AMs were resuspended in staining buffer (1×10^5 cells/100 μl) and nonspecific Fc receptor staining blocked using Fc block. PE-labeled TLR2, 4, and 5 Ab were added to stain the cells before analysis using a FACScan flow cytometer.

and macrophages [27]. TNF-α induces the synthesis and release of immunostimulatory polypeptides such as IL-1 and IL-6 from various cells. Together with IL-1, TNF-α acts on a variety of cells like T cells, B cells, fibroblasts, and macrophages to secrete various cytokines which are essential for the development of an effective immune response. These TLRs are involved in responses to Gram-positive bacteria, mycoplasma, and bacteria with flagellar filaments. In contrast, no effect was observed on the level of cell surface receptor expression in this study. Therefore, TJ-41 is likely to have an effect on as yet unidentified intracellular signal pathways.

Diabetes may predispose to increased morbidity and mortality of certain pulmonary infections, such as those caused by *S. aureus*, *Streptococcus pneumoniae*, *M. tuberculosis*, and *Legionella pneumophila* [28]. TLR2 or TLR5 is indeed important in the recognition of these microorganisms. Moreover, recent clinical studies have shown the association of TLR2 and TLR5 gene polymorphisms with susceptibility to infection with *M. tuberculosis* [29] and *L. pneumophila* [30], respectively. In the present study, TNF-α production from alveolar macrophages stimulated with TLR2 or TLR5 ligands during diabetic conditions was decreased, suggesting that inflammatory responses to certain bacteria may be impaired in diabetic patients. Alternatively, TJ-41 recovered suppressed TNF-α production from alveolar macrophages in a hyperglycemic state. This suggests that this drug normalizes the dysregulated inflammatory response in hyperglycemic states and, furthermore, is useful for host biological responses to bacterial infections.

There are several reports which have investigated TLR signaling in hyperglycemic conditions. LPS-dependent TNF-α production in mice increased due to an augmentation of LPS-induced p38 MAPK activity. The extracellular signal-regulated kinase (ERK) MAPK pathway is thought to be critical in TLR3 and TLR7

activation signaling in non-obese diabetic mice [31]. However, several reports have addressed the effects of TJ-41 on TLR signaling. Chino *et al.* reported the effect of *Shi-Quan-Da-Bu-Tang* (*Juzentaihoto*; TJ-48) on TLR4-mediated cellular responses in peritoneal exudate macrophages [32]. Although FACS analysis revealed that TJ-48 had no effect on TLR4 surface expression, it activated the NF-κB and p38 pathways while inhibiting the JNK and ERK pathways. In contrast, Mita *et al.* suggested that TJ-48 increased the expression of TLR4 and might enhance defense against Gram-negative bacteria *in vitro* [17]. Despite the contrasting data in these two reports, the present study and the work by Chino *et al.* suggest that TJ-41/TJ-48 specifically influences the downstream signaling pathways of TLR without affecting its surface expression [32]. Furthermore, the results presented here suggest that TJ-41 modulates MAPK signaling pathways to enhance TNF-α production.

Our study had limitations. We examined only one inflammatory cytokine, TNF-α. There are many inflammatory cytokines involving the site of inflammation. To clarify the exact mechanism of *Hochuekkito*, more examinations will be needed. Furthermore, the differences of the responses of TLR-2 and 4 to hyperglycemic environment between alveolar and other macrophages should be clarified.

CONCLUSION

Our results suggest that TJ-41 alleviates the suppressed inflammatory responses elicited from AMs following stimulation with TLR ligands in a hyperglycemic state, but the mechanism of this effect requires further investigation.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Prevalence of Pulmonary Arteriovenous Malformations as Estimated by Low-Dose Thoracic CT Screening

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Abstract

Background Pulmonary arteriovenous malformations (PAVMs) are rarely encountered in clinical practice. The prevalence of PAVMs associated with hereditary hemorrhagic telangiectasia (HHT) has been estimated based on the rate in the family members of HHT patients, but the prevalence of PAVMs in the general population remains unknown.

Methods We retrospectively examined the prevalence and clinical characteristics of PAVMs as detected by a low-dose thoracic CT screening program for lung cancer at the Hitachi Medical Center and the Hitachi General Health Care Center in the northern part of Ibaraki Prefecture, Japan.

Results From 2001 to 2007, we identified eight patients (seven females and one male) with PAVMs among 21,235 initial screening participants (the mean age of the patients with PAVMs and that of the screening participants was 60.6 years). The prevalence of PAVMs was estimated at 38 per 100,000 individuals [95% confidence interval (CI)=18-76]. The diameter of the PAVMs was a mean of 6.6 mm, and none of the lesions could be detected by chest X-ray. Females older than 60 years tended to have larger PAVMs than younger women did (p=0.06). Two patients (25%) were diagnosed with HHT. One patient had previously undergone surgery for a brain abscess.

Conclusion PAVMs are more prevalent than previously reported, especially among females.

Key words: pulmonary arteriovenous malformations (PAVMs), prevalence, low-dose CT screening, hereditary hemorrhagic telangiectasia (HHT)

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Introduction

Pulmonary arteriovenous malformations (PAVMs) are abnormal communications between pulmonary arteries and veins. Frequently reported complications associated with PAVMs are stroke, transient ischemic attack, brain abscess, massive hemoptysis, and spontaneous hemothorax. PAVMs have been reported to be sporadic or to occur in association with hereditary hemorrhagic telangiectasia (HHT) (1). The prevalence of PAVMs associated with HHT has been estimated based on the rate in the family members of HHT patients (1-3), but the prevalence of PAVMs in the general population remains unknown.

Since 2001, we have been providing annual lung cancer screening using low-dose spiral thoracic CT to residents of the northern part of Ibaraki Prefecture, Japan (4, 5). Through this program, we have found that PAVMs can be

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Table 1. Characteristics of the Participants*

Characteristics	Male Subjects		Female Subjects	
	Initial Screening	Repeat Screening	Initial Screening	Repeat Screening
Age, yr				
-39	170	42	67	10
40-49	409	175	173	60
50-59	2,510	1,263	3,790	1,568
60-69	4,991	2,950	5,344	3,042
70-79	1,775	1,418	1,711	1,491
80-	156	159	139	76
total	10,011	6,007	11,224	6,247

*Data are presented as number of participants unless otherwise indicated.

suspected by low-dose CT. Therefore, we planned the present study as a sub-analysis of this program, and estimated the prevalence of PAVMs in Japan.

Materials and Methods

Participants

In the northern part of Ibaraki Prefecture, Japan, the Hitachi Medical Center (HMC) has, since 2001, been providing low-dose spiral thoracic CT screening to local inhabitants aged 50 years or above. In addition, the Hitachi General Health Care Center (HGHCC) has, since 2001, provided similar screening to company employees and their spouses aged 50 years or above as part of a general health examination, for the detection of primary lung cancer. At the HGHCC, applicants in their 30s and 40s underwent CT screening as an optional health check-up. The participants underwent the screening voluntarily. We obtained approval from the respective ethics committees of our institutions. All participants provided written informed consent before CT screening.

CT scanners and scan prescriptions

In the HMC group, a mobile multi-slice spiral CT scanner (Asteion; Toshiba Medical Systems, Tokyo, Japan) was used; the scanning parameters were 120 kV peak, 25 mA tube current, 8-mm collimation, and 5.5:1 pitch. In the HGHCC group, a single-slice spiral CT scanner (Pronto; Hitachi Medical, Tokyo, Japan) was used, with 120 kV peak, 50 mA tube current, 10-mm collimation, and 2:1 pitch.

Outline of lung cancer screening at HMC and HGHCC

From 2001 to 2007, 33,489 CT screens were performed across both institutions, including 21,235 initial and 12,254 repeat screenings (Table 1). The number of initial screening participants remained approximately constant each year, with 6,612 males and 9,684 females at HMC, and 3,399 males and 1,540 females at HGHCC, for a total of 10,011 male and 11,224 female participants. The mean age of the initial screening participants was 64.8 years (range 50 to 85 years) at HMC, and 57.4 years (range 35 to 80 years) at HGHCC; the overall mean was 60.6 years. The detection rate of primary lung cancer during this period was 0.90%, and 85.4% was stage IA.

Diagnosis of PAVMs

Using low-dose CT, we suspected that the well-defined, homogeneous, round or lobular, nodule-like lesions, with vessels running towards them, were PAVMs. We further evaluated the lesions by high-resolution CT and confirmed the diagnosis of PAVMs by contrast-enhanced CT angiography and/or pulmonary angiography.

Diagnosis of HHT

We consulted patients with PAVMs at Akita University Hospital, which is the specialist HHT center in Japan. Patients were examined on the basis of the Curaçao Criteria (6).

Staging of lung cancer

Postoperative staging of lung cancer was conducted according to the TNM classification from the Union for International Cancer Control (UICC), 5th edition (7).

Statistical analysis

We retrospectively examined the prevalence and clinical characteristics of PAVMs detected by low-dose CT as a sub-analysis of our lung cancer screening program. Quantitative data with a normal distribution were described using mean ± SD. The 95% confidence interval (CI) of PAVM prevalence was estimated with the Adjusted Wald Formula. Differences in PAVM prevalence between males and females were assessed with Fisher's exact test. The relationship between PAVM diameter and age in females was analyzed using the Student's *t*-test.

Results

Of the 21,235 initial screening participants between 2001 and 2007, after low-dose CT we suspected eight participants to have PAVMs. After further evaluation, we confirmed the diagnosis of PAVMs in all eight patients, with a female predominance (87.5%). All patients had PAVMs confirmed for the first time in this screening. The detection rate of PAVMs (the number of patients with PAVMs / the number of initial screening participants, for seven years) was 0.038% overall, with 0.037% at HMC and 0.040% at HGHCC. We assumed the detection rate of PAVMs in CT screening participants to be close to the frequency in the general population. The prevalence of PAVMs was therefore estimated at 38 per 100,000 individuals (95% CI=18-76). There was a tendency for PAVMs to be more prevalent in females than in males (p=0.07).

The characteristics of the eight PAVMs patients are sum-

Table 2. Characteristics of the PAVM Patients Detected by Thoracic CT Screening

Patient	Year	Age	Sex	Related Disease	Number of PAVMs	Location of PAVMs	Maximal Diameter (mm)
1	2002	44	female	none	single	Rt. S^9	5.0
2	2003	60	female	none	single	Lt. S^3	8.0
3	2004	56	female	none	single	Lt. S^3	4.0
4	2004	68	female	brain abscess	single	Rt. S^9	5.5
5	2005	67	male	none	single	Rt. S^{10}	12.0
6	2006	65	female	none	single	Lt. S^9	6.0
7	2007	62	female	none	single	Rt. S^8	6.2
8	2007	63	female	none	multiple	Rt. S^3, Rt. S^9	5.0, 8.0

PAVMs: Pulmonary Arteriovenous Malformations

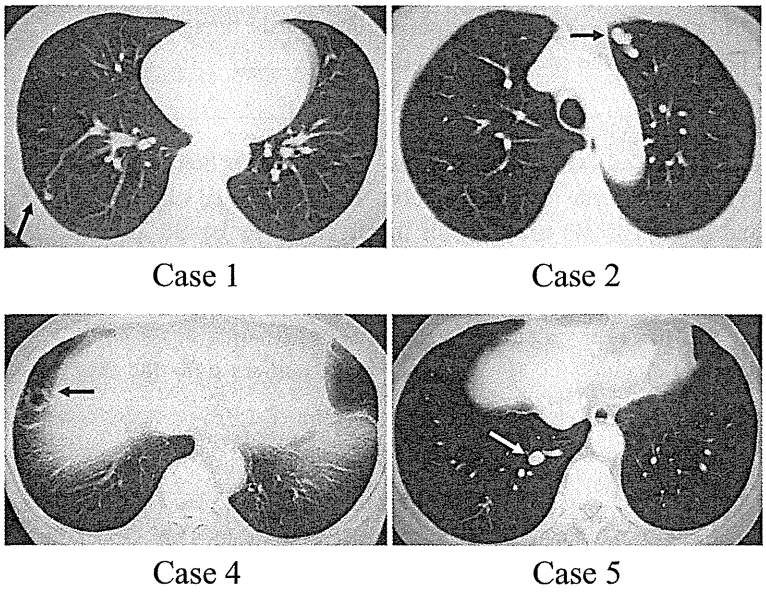

Figure 1. Low-dose CT images of PAVMs: 4 representative cases. PAVMs are indicated by arrows.

marized in Table 2. The mean age was 60.6 years. Seven patients had a single lesion, and one had two lesions. All nine PAVMs were simple lesions. Six out of the nine lesions were found in the lower lobes. The diameter of the lesions was a mean of 6.6 mm (range 4.0-12.0 mm). Low-dose CT images of the four patients are shown in Fig. 1; the PAVMs were recognized as subpleural nodular-like lesions with feeding vessels. There was no significant relationship between PAVM diameter and age in females, but there was a tendency for older females to have larger PAVMs (younger than 60 years, 4.5±0.8 mm vs. older than 60 years, 6.7±0.5 mm; p=0.06). None of the PAVM lesions could be recognized on a chest X-ray. Seven patients did not have severe complications associated with PAVMs; however, one patient had a history of surgery for a brain abscess five years before undergoing CT screening.

The eight patients were examined for an association with HHT at Akita University Hospital. Two patients, patient 4 who had had a brain abscess, and patient 8 who had multiple PAVMs, were diagnosed with possible HHT based on Curacao criteria, as both patients had visceral lesions (PAVMs) and episodes of repeated epistaxis. The detection rate of PAVMs not associated with HHT was 0.028%, and that associated with HHT was 0.009%. The prevalence of PAVMs not associated with HHT was estimated as 28 per 100,000 (95% CI=11-63), and that associated with HHT was 9 per 100,000 (95% CI=0.2-37).

Discussion

In our study, we detected PAVMs in eight patients out of 21,235 thoracic CT initial screening participants over a seven-year period, which represents a detection rate of 0.038%. The two institutions, HMC and HGHCC, used a different type of CT scanner and different scanning parameters. However, the observed prevalence of PAVMs was similar between the two institutions. This suggests that the different techniques exerted almost no influence on the detec-

tion of small PAVMs.

PAVMs are reported to occur twice as often in females as in males (8). However, in our study, seven out of the eight patients were female, even though there was a slight bias in the sex ratio of the participants. From the recent questionnaire regarding PAVMs, performed in many Japanese institutions, approximately 80% of patients not associated with HHT were female (9). Compared to previous reports, this suggests that the female predominance of PAVMs is greater in Japan than in Western countries. Furthermore, in the present study, the female patients were all multiparae, and there was a tendency for older females to have larger PAVMs. During pregnancy, increases in systemic circulation, cardiac output, and the secretion of progesterone can cause the diameter of PAVMs to expand (10, 11). Approximately 25% of PAVMs can enlarge gradually in size between 0.3-2.0 mm each year in about 25% of cases (12). This may explain the increased prevalence in females and the increased size in older patients.

The prevalence of PAVMs in the general population has not been previously reported for the following reasons: it is a very uncommon disorder; asymptomatic patients rarely visit medical institutions; and small PAVMs are difficult to detect on chest X-rays. The prevalence of HHT is estimated to be between 13 and 20 per 100,000 among European, U. S., and Japanese populations; this has been calculated based on the family members of HHT patients (1-3). Approximately 70% of patients with PAVMs have HHT (1), and 30-50% of patients with HHT have PAVMs (3, 13, 14). Based on these data, the prevalence of PAVMs associated, or not associated, with HHT is estimated as 5 and 14 per 100,000 individuals, respectively. In some geographical areas, there is a high prevalence of HHT; HHT is estimated to be present in 61 per 100,000 in the County of Fyn, Denmark (15), and in 76 per 100,000 in the Afro-Caribbean population of the Netherlands Antilles (16). In these areas, the prevalence of PAVMs is estimated to be between 26 and 56 per 100,000. In the area where we performed CT screening, the estimated prevalence of PAVMs was 38 per 100,000, which is much higher than that reported in previous epidemiologic studies, and is closer to the value in the high-prevalence areas. The screening test commonly used for PAVMs in HHT patients is transthoracic contrast echocardiography (TTCE), which has a high sensitivity and low risk (2). However, CT is not recommended as a screening test due to radiation exposure (2). CT screening for PAVMs has not been previously reported. Thus, our estimated prevalence of PAVMs could not be compared accurately with previous studies due to different screening tools. Considering that low-dose CT could detect small PAVMs, of approximately 4 mm in size in our study, the sensitivity of low-dose CT for detection of PAVMs would be high. However, TTCE can detect subtle PAVMs which CT cannot (17). This implies that the real prevalence of PAVMs in the area we studied may be slightly higher than our original estimation. Furthermore, as most of the participants in our study were between 50 and 80 years old, our estimated prevalence of PAVMs does not truly reflect that in the general population. However, because we performed a large-scale screening with over 20,000 participants, we believe that our data are valuable to estimate the prevalence of PAVMs in the general population.

Among the present eight patients, only two (25%) were diagnosed with HHT, which is a lower incidence than in previous reports. However, a recent Japanese questionnaire reported that 24.8% of patients with PAVMs have HHT (9), which is closer to our data. Furthermore, the prevalence of PAVMs associated with HHT in our study, 9 per 100,000, is close to the prevalence noted in the above-mentioned reports (1-3, 12, 13). Considering that CT screening could detect small PAVMs in asymptomatic patients, which could not have been detected by chest X-ray, the prevalence of PAVMs not associated with HHT is likely to be higher than previously thought.

Conclusion

The detection rate of PAVMs by CT screening was 0.038%. Based on these data, we estimate the prevalence of PAVMs to be 38 per 100,000 individuals. This is more prevalent than has been previously reported, especially among females.

The authors state that they have no Conflict of Interest (COI).

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Inflammation Research

ORIGINAL RESEARCH PAPER

Impaired inflammatory responses to multiple Toll-like receptor ligands in alveolar macrophages of streptozotocin-induced diabetic mice

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Abstract
Objective To investigate the effect of hyperglycemic state on the activation of alveolar macrophages (AMs) mediated via Toll-like receptors (TLRs) typically associated with bacterial infection.
Methods AMs obtained from normoglycemic control mice and streptozotocin-induced diabetic mice were stimulated ex vivo with the following: a TLR2 ligand, peptidoglycan (PGN); a TLR4 ligand, lipopolysaccharide (LPS); or a TLR5 ligand, flagellin (FLG). Cytokine production and mRNA expression were measured by ELISA and real-time PCR, respectively. TLR expression was assessed by real-time PCR and flow cytometry.
Results AMs from diabetic mice produced significantly less TNF-α after PGN or FLG stimulation, and less IL-6 after FLG stimulation, compared with AMs from control mice. The decrease in the production of these cytokines was associated with reduced mRNA expression of the corresponding cytokines. In contrast, production of TNF-α and IL-6 after LPS stimulation did not differ between groups. Furthermore, there was no substantial difference in the expression of TLR2, TLR4, and TLR5 in AMs between the groups. The increased JNK phosphorylation induced by PGN or FLG stimulation was downregulated in AMs from diabetic mice.
Conclusions Hyperglycemic state impairs the reactivity of AMs to multiple TLR ligands. This effect might result from hyperglycemia-induced alteration of intracellular signaling and is unlikely due to the modulation of TLR expression.

Keywords Alveolar macrophages · Toll-like receptor · Diabetes · Streptozotocin · Inflammatory response

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Introduction

Patients with diabetes mellitus are considered more susceptible to several types of infections, including lower respiratory tract infections. Diabetic patients are not only predisposed to pneumonia but are also liable to develop complications such as bacteremia, delayed resolution, and recurrent pneumonia. In fact, several previous studies of patients with community-acquired pneumonia have reported that diabetes is associated with an increased risk of pneumonia-related hospitalization [1, 2] and mortality [3–5].

Disease-associated impairment in host defensive functions, including dysfunction of the monocyte–macrophage system, is a likely mechanism underlying increased susceptibility to infection in diabetes. Numerous phenotypic abnormalities, such as altered metabolism [6, 7], phagocytosis [8, 9], antigen presentation [10], and cytokine release [11–13], have been found in peripheral blood mononuclear cells from diabetic patients. However, data on tissue-resident macrophages, which are phenotypically different from free-floating monocytes, are available only from studies on relevant animal models [14–16], but not from diabetic patients.

The lung is an organ which is connected directly to the external environment. Of the multiple resident macrophage populations, alveolar macrophages (AMs), highly specialized mononuclear phagocytes, are the first-line defense to invading pathogens and play a central role in innate

respiratory host defense [17]. To date, only a small number of reports have addressed the functions of AMs, such as depressed respiratory burst [18], impaired phagocytic and bactericidal functions [19], impaired lipopolysaccharide (LPS)-induced macrophage inflammatory protein (MIP)-2 gene expression [20], decreased capacity of cytokine (tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-12) secretion, and nitric oxide production in tuberculosis [21] in diabetic animal models. Unexpectedly, recent works concerning macrophage inflammatory response under hyperglycemic conditions or diabetes have been performed mainly on peritoneal [14–16, 22, 23] and bone marrow-derived macrophages (BMDMs) [24, 25], whereas the data on AMs remain limited. Moreover, because the behavior of macrophages differs according to their tissue of residence, this issue needs to be elucidated for AMs.

Toll-like receptors (TLRs) recognize conserved pathogen-associated molecular patterns (PAMPs) and induce innate immune responses that are essential for host defense. Among the TLRs, TLR2, TLR4, and TLR5 play an important role in bacterial infection. TLR4 was shown to be a specific receptor for lipopolysaccharide (LPS), a major cell wall component of gram-negative bacteria, whereas TLR2 is involved in the recognition of peptidoglycan (PGN), another bacterial wall component, and TLR5 can recognize bacterial flagellin (FLG). TLR2, TLR4, and TLR5 are expressed and functionally active on AMs [26, 27], and once activated by bacterial PAMPs, their respective TLR signaling is initiated, resulting in recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) [28]. Subsequently, further downstream signaling pathways including those of mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) are activated [28], leading to the production of inflammatory cytokines, such as TNF-α and IL-6, which are key players in host inflammatory response to bacterial infections.

In the present study, we investigated the effect and mechanism of hyperglycemic state on activation of AMs mediated by TLR2, TLR4, and TLR5 using a streptozotocin-induced diabetic mice model. Specifically, we focused on pro-inflammatory cytokine (TNF-α and IL-6) production and mRNA expression induced by ex vivo stimulation with ligands for TLR2, TLR4, and TLR5. In addition, we examined the expression of these TLRs and activation of MAPKs in AMs.

Materials and methods

Animals

Male Balb/c mice (8–10 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed under specific pathogen-free conditions with unlimited access to food and water at the animal facility of Jichi Medical University. All experiments were performed in strict accordance with the Jichi Medical University Guide for Laboratory Animals, based on the Helsinki convention for the use and care of animals. The study protocol was approved by the animal ethics committee of Jichi Medical University.

Reagents

Streptozotocin was purchased from Sigma (St. Louis, MO, USA). *Staphylococcus aureus* PGN and *Salmonella typhimurium* FLG were purchased from Invivogen (San Diego, CA, USA). *Escherichia coli* LPS was purchased from Sigma. MAPK inhibitors, SB203580 and PD98059, were purchased from Calbiochem (Darmstadt, Germany). SP600125 was purchased from Alexis Biochemicals (San Diego, CA, USA). PE-labeled anti-murine TLR2 antibody (Ab), PE-labeled anti-murine TLR4/MD-2 Ab, and PE-labeled IgG2a isotype control were purchased from eBioscience (San Diego, CA, USA). PE-labeled anti-murine TLR5 Ab was purchased from Imgenex (San Diego, CA, USA). All culture media and additives, unless specified otherwise, were purchased from Sigma.

Preparation of streptozotocin-induced diabetic mice

Experimental diabetes was induced by a single intraperitoneal injection of streptozotocin (250 mg/kg of body weight) in 0.01 M citrate buffer (pH 4.5). Control mice received an equal volume of citrate buffer without streptozotocin. Random blood glucose measurements were performed with a Glutest Ace meter (Sanwa Chemical Co., Nagoya, Japan) and Glutest sensor (Sanwa Chemical Co.) 14 days after streptozotocin injection. Only mice with a fasting blood glucose level of ≥250 mg/dl were considered diabetic and used in the experiments.

AM isolation and culture

Fourteen days after streptozotocin injection, mice were anesthetized and killed by exsanguination before excision of the lungs. Tracheas were cannulated and bronchoalveolar lavage (BAL) was collected from control and diabetic mice by instilling 1 ml of phosphate-buffered saline (PBS) into the lungs through the trachea four times. After each instilment, fluid was collected and pooled, and cells were counted by trypan blue dye exclusion. Cytospins for differential cell counts were prepared and stained with modified Wright-Giemsa (Diff-Quick; International Reagents Co., Kobe, Japan). From these stained cytospin preparations, the percentage of mononuclear cells and neutrophils was determined under a microscope by counting at least 200 cells per slide.

In each experiment, isolated cells from the BAL of control and diabetic mice had a consistent viability of >96% by trypan blue dye exclusion, and >98% of macrophages were confirmed by morphology. No difference in cellular composition in the BAL was observed between groups. Cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Halan, Indianapolis, IN, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Subsequently, on the basis of trypan blue dye exclusion and differential cell counts, equal numbers of AMs were plated at the indicated cell density. After 2 h of incubation, nonadherent cells were removed by washing twice with PBS. Adherent cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine, and left either unstimulated or stimulated with PGN (10 μg/ml), LPS (100 ng/ml), or FLG (1 μg/ml) for the indicated time intervals. In some experiments, cells were preincubated with MAPK inhibitors at indicated doses before stimulation.

ELISA measurement

Alveolar macrophages (5×10^4 cells) plated in each well of a 96-well plate were cultured in medium alone or stimulated with specified reagents at indicated doses for 3 or 18 h. Cell supernatants were collected and levels of TNF-α and IL-6 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. The respective limits of detection for the assays were 19.5 and 7.8 pg/ml.

Analysis of cell-surface TLRs expression

Alveolar macrophages from control and diabetic mice were obtained by BAL as described above and resuspended at 1×10^5 cells per 100 μl in staining buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide). Nonspecific staining was blocked with anti-murine CD16/32 (eBioscience) by incubating for 15 min. After blocking, cells were incubated for 30 min with PE-labeled anti-murine TLR2 Ab, PE-labeled anti-murine TLR4/MD-2 Ab, PE-labeled anti-murine TLR5 Ab, or a nonspecific PE-labeled IgG2a. AMs were washed twice with staining buffer and fixed with staining buffer containing 1% formalin. Flow cytometry was performed using a FACS LSR flow cytometer (BD Biosciences, San Jose, CA, USA) acquiring 1×10^4 events by using forward scatter and side scatter properties to define the macrophage population. Data analysis was done using CellQuest Pro software. The levels of surface TLRs were determined as mean fluorescence intensity (MFI), and nonspecific binding was corrected by subtracting the MFI values corresponding to the isotype controls.

Real-Time RT-PCR

AMs (4×10^5 cells) were plated in each well of 12-well plate. Cells were cultured in medium alone or stimulated with specified reagents, and RNA was isolated at indicated time points (Results and Figure Captions) with the RNAqueous Kit (Ambion, Austin, TX, USA) according to manufacturer instructions. Reverse transcription was completed on similar amounts of RNA per group using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Specific primers for murine TNF-α, murine IL-6, murine TLR2, murine TLR4, murine TLR5, and a housekeeping gene 18S as well as FAM-labeled probes were purchased from Applied Biosystems, and polymerase chain reaction (PCR) was performed in triplicate with Fast Universal PCR Master Mix in an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Analysis of relative expression levels was completed by first normalizing gene expression to levels of the housekeeping gene 18S in the same sample (ΔCT) and then compared with the control group (ΔΔCT). With this method, RNA extracted from unstimulated AMs, isolated from control mice, had a relative fold induction defined as 1. Experimental groups are expressed as fold change over this control condition.

MAPK determination

For MAPK inhibitor studies, AMs were preincubated with p38 inhibitor (SB203580, 10 μM), extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (PD98059, 50 μM), or c-jun N-terminal kinase (JNK) inhibitor (SP600125, 20 μM) for 30 min before stimulation with specified reagents, then incubated for 18 h, followed by TNF-α measurement in culture supernatants. For assessment of JNK kinase activation in AMs, cells (5×10^4 cells) plated in each well of CellBIND 96-well plate (Corning, Lowell, MA) were either unstimulated or stimulated with specified reagents for 30 min. Quantification of phospho- and total JNK-kinase in AMs was performed using specific ELISAs (CASE cell-based ELISA kit, SuperArray Bioscience, Frederick, MD, USA) according to manufacturer instructions.

Statistical analysis

Data are expressed as mean ± SEM for each group. Experimental data were analyzed by the Student's *t* test or ANOVA, followed by post hoc testing with Fisher's

protected least significant difference test, where appropriate. A value of $P < 0.05$ was considered significant.

Results

Effect of hyperglycemic state on pro-inflammatory cytokine production by AMs stimulated with specific TLR ligands

We first investigated the impact of hyperglycemic state on selective AM activation via TLR2, TLR4, or TLR5. AMs from normoglycemic control mice and streptozotocin-induced diabetic mice were cultured in medium alone or stimulated with the following: a TLR2 ligand, PGN; a TLR4 ligand, LPS; or a TLR5 ligand, FLG ex vivo. After 3 or 18 h, levels of TNF-α in cell supernatants were measured by ELISA. Stimulation with these specific TLR ligands resulted in significant induction of TNF-α protein by AMs. After stimulation with either PGN or FLG, AMs isolated from diabetic mice produced significantly less TNF-α compared with AMs from control mice at both earlier and later time points. In contrast, TNF-α production after LPS stimulation did not differ between the groups (Fig. 1a). We also measured the levels of IL-6 in supernatants from AMs culture. At the later time point (after FLG stimulation), the levels of IL-6 produced by AMs from diabetic mice had decreased compared with those from control mice. IL-6 production induced only minimal levels after PGN stimulation and did not differ between the groups after LPS stimulation (Fig. 1b).

Effect of hyperglycemic state on TNF-α and IL-6 mRNA expression in AMs stimulated with specific TLR ligands

Next, we investigated whether or not the modulation of cytokine production by AMs from diabetic mice was upstream of protein translation. AMs from control and diabetic mice were cultured in medium alone or stimulated with PGN, LPS, or FLG. After 3 h, RNA was extracted from adherent cells, and levels of TNF-α and IL-6 mRNA were measured by real-time quantitative PCR. As shown in Fig. 2, TNF-α mRNA expression in AMs from diabetic mice after stimulation with either PGN or FLG was reduced compared with the level observed in AMs from

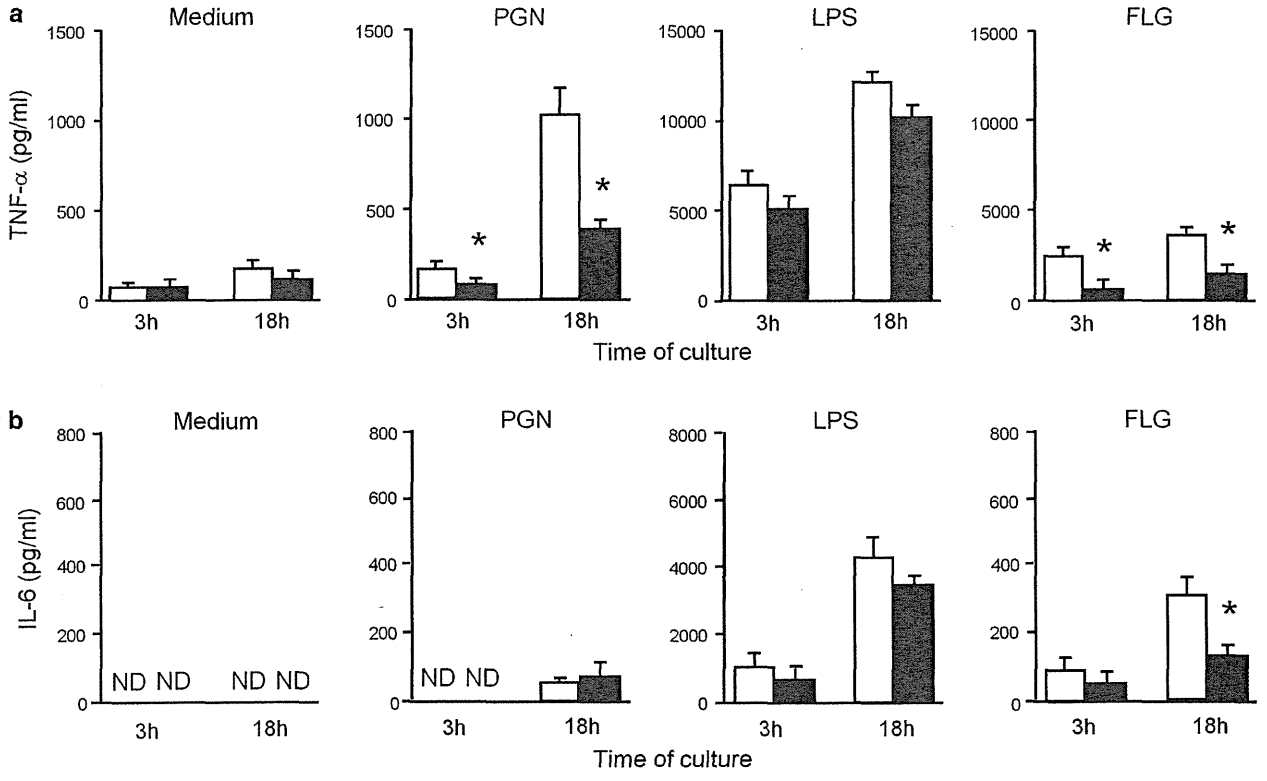

Fig. 1 Decreased TLR2 or TLR5 ligand-induced pro-inflammatory cytokine production by AMs from diabetic mice. AMs from pools of control mice (*open bars*) and diabetic mice (*solid bars*) were cultured in medium alone or stimulated with PGN (10 μg/ml), LPS (100 ng/ml) or FLG (1 μg/ml) for 3 or 18 h. Levels of TNF-α (**a**) and IL-6 (**b**) were measured in cell supernatants by ELISA. Data are expressed as mean ± SEM of four separate experiments performed in triplicate. *$P < 0.05$ compared with respective control group; *ND* not detected

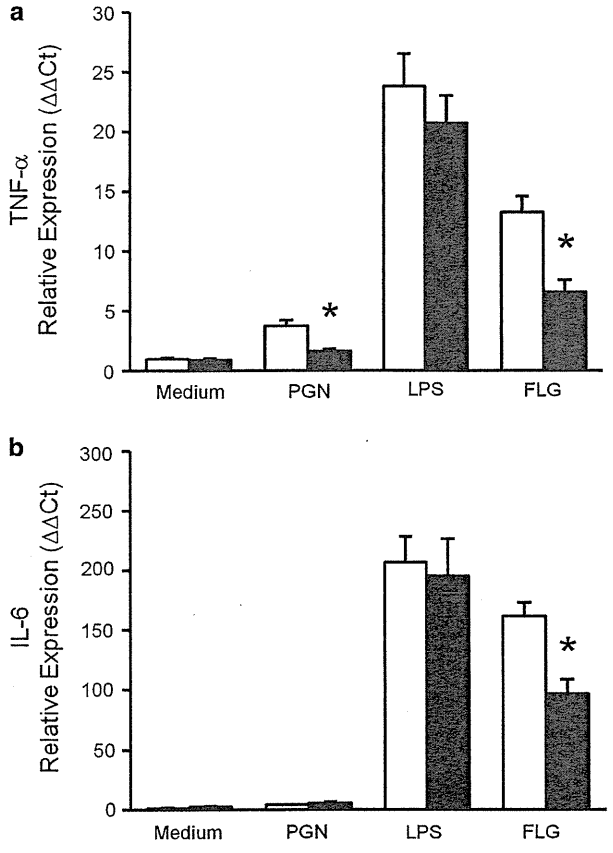

Fig. 2 Decreased TLR2 or TLR5 ligands-induced TNF-α and IL-6 mRNA expression in AMs from diabetic mice. AMs from pools of control mice (*open bars*) and diabetic mice (*solid bars*) were cultured in medium alone or stimulated with PGN (10 μg/ml), LPS (100 ng/ml) or FLG (1 μg/ml) for 3 h. RNA was isolated and levels of TNF-α (**a**) and IL-6 (**b**) mRNA expression were measured by real-time quantitative PCR analysis using the 18S housekeeping gene as an endogenous control. Data are expressed as mean ± SEM of four separate experiments. $*P < 0.05$ compared with respective control group

control mice. IL-6 mRNA expression in AMs from diabetic mice was also reduced after stimulation with FLG. In contrast, no significant differences were observed for the levels of mRNA of these cytokines after LPS stimulation. These findings are indicative that AMs from diabetic mice have a decreased ability to upregulate expression of TNF-α and IL-6 mRNA after stimulation with certain TLR ligands.

Effect of hyperglycemic state on AM expression of TLRs

Next, we investigated whether the hyperglycemic state-induced effect on TLR reactivity involved modulation of TLR expression. Therefore, we examined basal levels of TLRs expression in AMs prior to ex vivo stimulation with specific ligands. AMs were obtained from control and diabetic mice and RNA was extracted after 2 h of adherence in culture. Levels of TLR2, TLR4, and TLR5 mRNA expression were measured by real-time PCR. We observed similar levels of TLR2, TLR4, and TLR5 mRNA in AMs from both groups of mice (Fig. 3). In further experiments, we examined cell-surface TLRs expression on AMs using flow cytometry. Similar to the results of mRNA, there was no difference between control and diabetic mice in the MFI of TLR2, TLR4, and TLR5 expression on AMs (Fig. 4a, b). These findings suggest that marked changes of TLR2 or TLR5 expression do not explain the observed hyperglycemic state-induced inhibition of pro-inflammatory cytokine production by AMs stimulated with either PGN or FLG.

Effect of MAPKs inhibitors on TNF-α production by AMs stimulated with TLR2 or TLR5 ligands

Interactions of TLRs with their ligands trigger intracellular signaling events, which consequently involve activation of MAPKs. To investigate the role of the three major MAPKs in PGN or FLG-induced TNF-α production by AMs, we examined the effects of specific inhibitors including SB203580, a p38 inhibitor, PD98059, an ERK1/2 inhibitor, and SP600125, a JNK inhibitor. PGN-induced TNF-α production by AMs from both groups of mice were overall decreased in the presence of SB203580 or PD98059. Inhibition of JNK signaling by SP600125 resulted in a dramatic decrease of PGN-induced TNF production (Fig. 5a). Similarly, TNF-α production by FLG-stimulated AMs were overall decreased in the presence of SB203580 or PD98059 in both groups of cells. The presence of SP600125 led to a pronounced decrease of FLG-induced

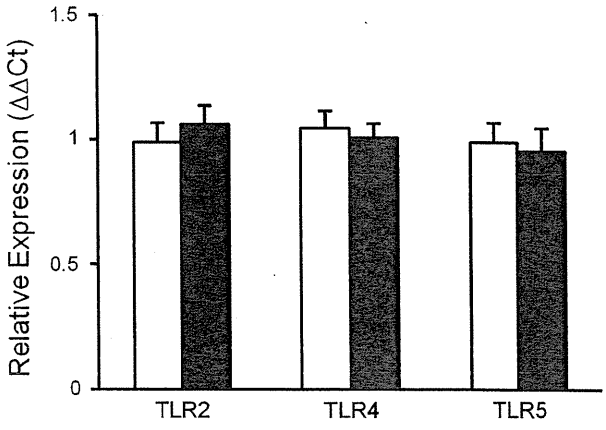

Fig. 3 TLRs mRNA expression in AMs. AMs were obtained from control mice (*open bars*) and diabetic mice (*solid bars*) and pooled. After 2 h of adherence in culture, RNA was isolated and levels of TLR2, TLR4, and TLR5 mRNA expression was measured by real-time quantitative PCR analysis using the 18S as endogenous control. Data are expressed as mean ± SEM of four separate experiments

TNF-α production by AMs from both groups (Fig. 5b). The inclusion of these three inhibitors led to almost complete inhibition of TNF-α production by all tested AM groups. The attenuation of PGN or FLG-stimulated TNF-α production by AMs from diabetic mice compared with cells from control mice were prevented in the presence of the JNK inhibitor.

Effect of hyperglycemic state on JNK kinase phosphorylation in AMs stimulated with TLR2 or TLR5 ligands

As shown above, PGN or FLG-induced TNF-α production was preferentially inhibited by SP600125, indicating a significant role of JNK signaling in AMs from both groups. We therefore determined phosphorylation of JNK kinase using specific cell-based ELISA. AMs stimulated with PGN or FLG resulted in increased JNK kinase phosphorylation. In AMs from diabetic mice, these increases in JNK kinase phosphorylation were significantly downregulated compared with AMs from control mice (Fig. 6). These findings suggest that downregulation of JNK signaling is involved in hyperglycemic state-induced inhibition of TLR2 and TLR5-mediated inflammatory responses.

Discussion

In this study, we reported that hyperglycemic state resulted in a selective inhibition of macrophage inflammatory responses, as exemplified by reduced production of TNF-α and IL-6 by TLR2 or TLR5 ligand-stimulated AMs of STZ-induced diabetic mice. To our knowledge, this is the first study to demonstrate the hyperglycemic state-induced modulation of macrophage inflammatory responses to multiple TLR ligands in AMs. Additionally, we showed that the reduction of pro-inflammatory cytokine production was accompanied by an impairment of intracellular signaling, including a downregulation of JNK kinase phosphorylation in AMs, but not modulation of respective TLRs expression.

To date, most studies regarding the impacts of hyperglycemic state on tissue macrophage inflammatory responses to TLR ligands have been performed on cells other than AMs. Moreover, examinations have focused on the response to LPS, a TLR4 ligand, but not other TLR ligands. For example, several studies have shown impaired inflammatory responses in peritoneal macrophages, such as reduced LPS-induced TNF-α and IL-6 production in type 1 diabetic rat models [14, 15], reduced TNF-α and IL-1β production by LPS plus interferon (IFN)-γ stimulation in type 2 diabetic (*db/db*) mice [22]. A recent report by Morran et al. [24] showed reduced expression of TNF-α mRNA and protein by BMDMs from non-obese diabetic (NOD) mice, when stimulated with a TLR2 ligand (*P. gingivalis* LPS) under hyperglycemic conditions. Contrary to these, other investigators reported augmentation of LPS-induced pro-inflammatory cytokine production by peritoneal macrophages [23] or BMDMs [25] from similar diabetic mouse models. Thus, the immunomodulatory effects of hyperglycemic state on macrophage cytokine reaction are complex. Such conflicting results might be explained by the different experimental conditions, such as origin of macrophages, duration of hyperglycemic state, and characteristics of additional stimuli.

On the other hand, there have been only limited reports on the modulation of TLR-mediated inflammatory responses under hyperglycemic state for AMs. Consistent with the study by Morran et al. using BMDMs, our results showed that TLR2 ligand (PGN)-induced TNF-α production was reduced in AMs from diabetic mice compared with control mice. The reduction of cytokine production was also observed in response to TLR5 ligand (FLG) stimulation. In contrast, pro-inflammatory cytokine production in response to LPS, unlike the above-mentioned previous reports, tended to be reduced but the difference between the groups did not reach significance. Our results suggest the possibility that the impact of hyperglycemic state on AM inflammatory responses varies among different types of TLR ligand stimulation. In contrast to the TLR2 and TLR5 signaling that occurs exclusively through the MyD88-dependent pathway [29, 30], TLR4 is unique in that it activates both the MyD88-dependent and MyD88-independent pathways. The MyD88-independent pathway signals through Toll/IL-1R domain-containing adaptor-inducing IFN-β leading to activation of IRF-3 (IFN-regulatory factor 3) and production of IFN-β [31]. This pathway is also activated through NF-κB in a delayed manner, leading to production of TNF-α and other pro-inflammatory cytokines [32]. Although the precise mechanisms were not specifically investigated in the present study, there is a possibility that the MyD88-dependent pathway is more susceptible to the hyperglycemic state than the MyD88-independent pathway, and therefore selectively impaired in AMs, which leads to a pronounced reduction of cytokine production in response to PGN or FLG but not LPS.

In the present study, the observed differences in PGN or FLG-induced pro-inflammatory cytokine production comparing AMs from diabetic and control mice were not related to differences in macrophage expression of the respective TLRs because the basal levels TLR2 or TLR5 expression in both groups of AMs were similar. Additionally, AMs from diabetic mice exhibited equivalent expression of TLR4 compared with those from control mice. There is a paucity of data examining the modulation of the investigated TLRs in tissue macrophages under

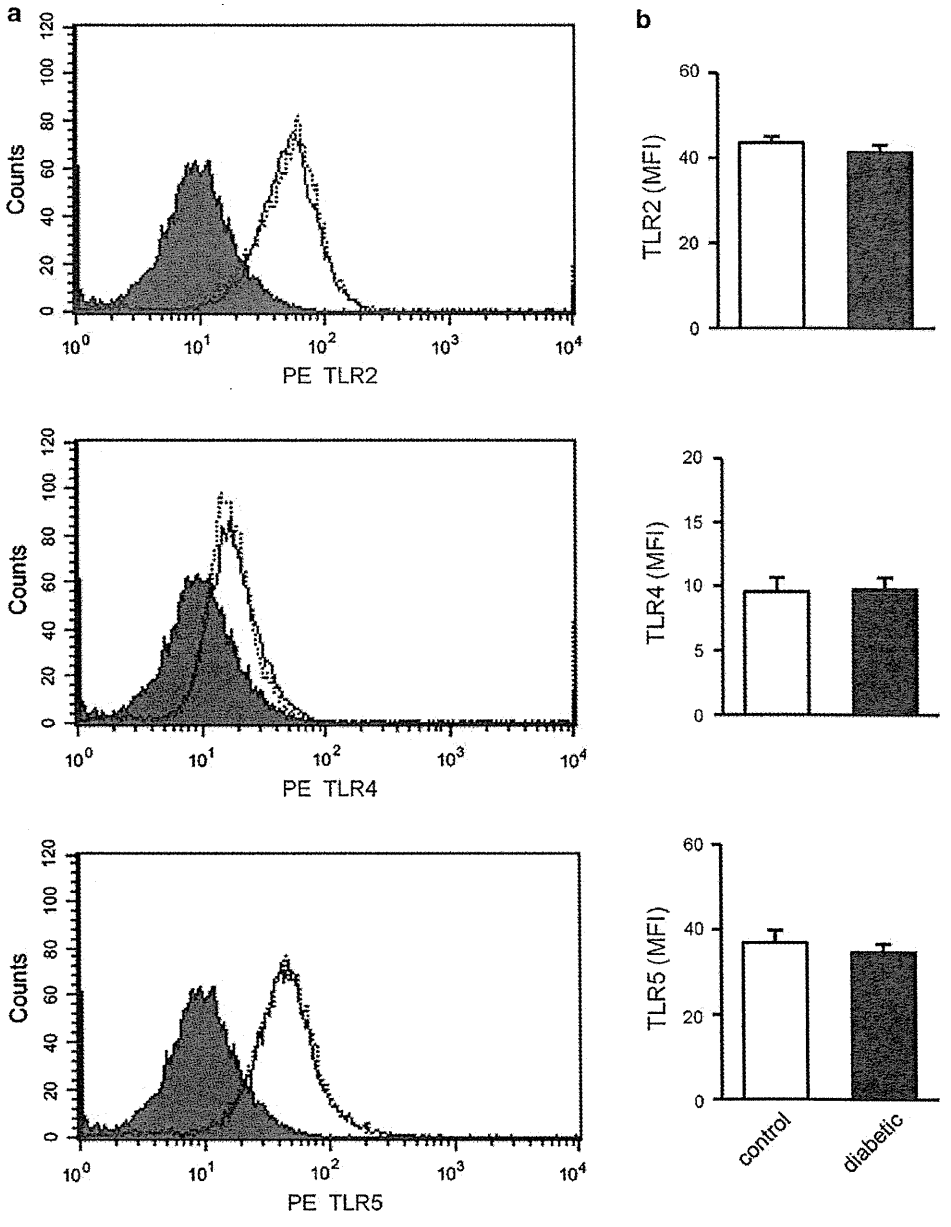

Fig. 4 Cell-surface TLR expression on AMs. AMs were obtained from control mice ($n = 5$) and diabetic mice ($n = 5$). Cells were incubated with PE-labeled anti-TLR2 Ab, PE-labeled anti-TLR4/MD-2 Ab, or PE-labeled anti-TLR5 Ab, and surface expression of each TLRs was assessed by flow cytometry. **a** Representative histogram overlay of the fluorescence intensity of TLR2, TLR4, and TLR5 expression on AMs from control mice (*dashed line*) and diabetic mice (*solid line*). The *gray shaded curves* show AMs stained with the isotype control. Control staining was identical for AMs from control and diabetic mice. **b** Quantification of TLR2, TLR4, and TLR5 expression on AMs from control mice (*open bars*) and diabetic mice (*solid bars*), as determined by mean fluorescence intensity. Data are expressed as mean ± SEM

hyperglycemic conditions and diabetes, and furthermore, there is no data available on these TLRs for AMs. Mohammad et al. [25] reported that NOD mice had equivalent basal expression levels of TLR2 and TLR4 mRNA as well as increased expression levels of TLR5 mRNA in BMDMs compared with control NOR mice. These results were different in part from our data, and the discrepancy is probably due to the different experimental models. On the other hand, the level of TLRs expression could change after exposure to specific ligands [33]. An association between diabetes-induced dysregulation of TLRs expression after ligand binding and the modulation of inflammatory response has been shown [24, 25]. Although not examined in the present study, the TLRs expression level of post-ligand binding might also affect the consequence of cytokine production in AMs.

Similar basal levels of TLR2 or TLR5 expression in AMs from diabetic and control mice suggest the possibility that reduction of PGN or FLG-induced pro-inflammatory cytokine production by diabetic AMs represents events downstream of the respective TLRs. In the present study, we focused on the effect of hyperglycemic state on MAPKs, especially JNK kinase activity, because of the preferential suppression of TNF-α production by the JNK inhibitor in both groups of AMs. We also showed that the PGN or FLG-induced JNK activation was downregulated