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Research article

Hemoperfusion with polymyxin B-immobilized fibers reduced the number of $CD16^+CD14^+$ monocytes in patients with septic shock

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Background: $CD16^+CD14^+$ monocytes dramatically increase in number in patients with severe infection. Hemoperfusion with PMX-F (direct hemoperfusion with polymyxin B immobilized fibers) has been reported to be a safe and effective treatment for patients with septic shock, although the molecular mechanism that accounts for its effectiveness is still unclear. The purpose of this study was to quantify the number of $CD16^+CD14^+$ monocytes in patients with an intra-abdominal infection and to evaluate the effects of PMX-F treatment on clinical parameters and leukocyte surface antigen expression in these patients.

Materials and Methods: Seventeen septic patients who had an intra-abdominal infection were enrolled in this study; 7 of these patients received PMX-F treatment. Peripheral blood samples were obtained immediately after admission, and were also collected from the above 7 patients before, during, and immediately after their PMX-F treatment. The expression of CD14, CD16, and Toll-like receptor (TLR)-4 on these patients' monocytes was evaluated using flow cytometry. In addition, lipopolysaccharide (LPS)-induced production of TNF-α and IL-1β by these cells was measured by ELISA.

Results: Monocytic expression of CD16 and TLR-4 was significantly greater in septic patients than in healthy controls, and their proportion of $CD16^+CD14^+$ monocytes was similarly elevated. LPS-induced production of TNF-α and IL-1β by peripheral blood mononuclear cells (PBMCs) of septic patients was significantly reduced compared to controls. Furthermore, there was a reduction in the proportion of $CD16^+CD14^+$ monocytes during PMX-F treatment, and in the expression of TLR-4 on monocytes after PMX-F treatment.

Conclusions: These results showed that the number of peripheral blood $CD16^+CD14^+$ monocytes and monocytic TLR-4 expression were markedly increased, and the production of pro-inflammatory cytokines in response to LPS significantly reduced in patients with sepsis. PMX-F treatment was found to be effective in reducing the number of $CD16^+CD14^+$ monocytes and in decreasing the monocytic expression of TLR-4 in patients with septic shock.

Keywords: Polymyxin B-immobilized fiber (PMX), sepsis, $CD16^+CD14^+$ monocytes

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INTRODUCTION

Peripheral blood monocytes are constituents of the mononuclear phagocytic system and play a central role in immunoregulation and the host defense against pathogenic organisms.[1] Monocytes are activated by the binding of their receptors by bacteria and inflammatory mediators, and were shown to exhibit varying morphologies[2] and functions.[3]

CD14 has the endotoxin receptor. The endotoxin receptor is probably a trimeric complex of CD14, TLR-4 and MD2, and CD14 is a pattern recognition molecule that recognizes multiple other microbial ligands in addition to endotoxin.[4] Among the different subpopulations of monocytes, those which express CD16 (Fc receptor III) have been most extensively characterized. $CD16^+$ monocytes that also express CD14 were shown to be increased in patients with sepsis and AIDS.[5] Blumenstein *et al.*[6] reported that $CD16^+CD14^+$ monocytes were increased in patients with sepsis. Belge *et al.*[7] reported that $CD16^+CD14^+$ monocytes from normal individuals expressed higher levels of intracellular TNF-α following exposure to LPS than did $CD16^-CD14^+$ monocytes. On the other hand, Horelt *et al.*[8] reported that $CD16^+CD14^+$ monocytes in patients with erysipelas showed significantly less expression of intracellular TNF-α following LPS stimulation than did those isolated from healthy volunteers. We hypothesized that an increase in the number of activated $CD16^+CD14^+$ monocytes in patients with severe infection contributes to the pathogenesis of sepsis and/or septic shock.

The Critical Network Group in Japan reported that direct hemoperfusion with polymyxin B immobilized fibers (PMX-F) was safe and effective for the treatment of sepsis.[9,10] Polymyxin B has long been known to be able to neutralize endotoxin.[11] It was recently also suggested that the absorption of anandamide by polymyxin B reduces anandamide-induced hypotension, immunosuppression, and cytotoxicity.[12] Further reports also suggested that PMX-F therapy could result in a reduction in serum cytokine levels[13] and monocyte mRNA expression.[14] However, the molecular mechanism by which these effects were mediated is still unclear.

In this study, we examined whether there was a correlation between the number of circulating $CD16^+CD14^+$ monocytes and the production of pro-inflammatory cytokines from LPS-stimulated monocytes in septic patients. In addition, we evaluated the effects of PMX-F treatment on the number of $CD16^+CD14^+$ monocytes and on the clinical features of patients with septic shock.

PATIENTS AND METHODS

Patients

Seventeen septic patients who were admitted under emergency conditions to the National Defense Medical College Hospital with a diagnosis of an intra-abdominal infection and who met the systemic inflammatory response syndrome (SIRS) criteria were included in this study (8 men and 9 women; mean age ± SD = 59.2 ± 11.2 years). Thirteen healthy control volunteers were also enrolled into this study, which included 10 men and 3 women with a mean ± SD age of 44.8 ± 7.2 years.

In order to assess the severity of their illness, patients were evaluated with the Acute Physiology and Chronic Health Evaluation (APACHE) II test. The criteria of the American College of Chest Physicians Consensus were used to confirm a diagnosis of sepsis and septic shock.[15]

Peripheral blood samples were obtained from septic patients immediately after admission. Informed consent was obtained from each patient or family prior to initiation of this study.

PMX-F treatment

Polymyxin B-immobilized fibers (PMX) were produced by covalently immobilizing polymyxin B onto polystyrene fibers. The direct hemoperfusion column contained 53 g of PMB-immobilized fibers, which were supplied by Toray Industries (Toraymyxin, Toray Medical Co., Tokyo, Japan). The PMX-F procedures have been described previously.[8,9] Briefly, access to the blood for direct hemoperfusion with PMX-F was obtained via a double-lumen catheter that was inserted into the femoral vein using Seldinger's method. Direct hemoperfusion was carried out for 2 h at a flow rate of 80–100 ml/min through a venovenous catheter. PMX-F treatment was performed in 7 patients who met the criteria for septic shock. Blood samples were collected from these patients before, 1 h after the start (during PMX), and immediately after the completion, of the PMX-F treatment (after PMX) and leukocytes were harvested from each blood sample in order to evaluate their surface antigen expression. Clinical parameters such as mean blood pressure, heart rate, urine volume, and catecholamine index (CAI = dopamine + dobutamine + [noradrenaline + adrenaline] × 100 mg/kg/min) were also evaluated before and after PMX-F treatment. Patients in this study continued to receive conventional therapy for the treatment of septic shock.

Isolation of peripheral blood mononuclear cells (PBMCs)

Aliquots of heparinized peripheral blood (6 ml) obtained from septic patients and healthy volunteers were overlaid onto 4 ml of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) in 15 ml tubes, which were then centrifuged at 1800 rpm for 15 min. The cells that collected at the interface were harvested and washed twice in serum-free RPMI 1640 (Gibco, Grand Island, NY, USA). The cells were then adjusted to a volume of 1×10^6 ml^{-1} in RPMI 1640 supplemented with 10% human serum and were either cultured or analyzed by flow cytometry.

Table 1. Clinical characteristics of septic patients with intra-abdominal infections

Patient	Diagnosis	Sex	APACHE score	Shock	PMX-F (treatment)	Outcome	Relevant	Bacteria isolates (site)
1	Colon perforation	M	7	No	No	R	Abdominal cavity	*E. faecalis*, Bacteroides spp.
2	Colon perforation	F	10	No	No	R	Abdominal cavity	NI
3	Colon perforation	F	3	No	No	R	Abdominal cavity	*E. faecalis*, *E. coli*
4	Colon perforation	F	5	No	No	R	Abdominal cavity	NI
5	Cholangitis	F	7	No	No	R	Bile	NI
6	Cholangitis	M	3	No	No	R	Bile	*E. faecalis*
7	Cholangitis	F	10	No	No	R	Bile	*K. pneumoniae*
8	Strangulation	M	20	Yes	Yes	R	Abdominal cavity	NI
9	Strangulation	M	21	Yes	Yes	R	Abdominal cavity	*E. faecalis*
10	Strangulation	F	17	No	No	R	Abdominal cavity	*E. coli*, *E. faecalis*
11	SMAO	F	29	Yes	No	D	Abdominal cavity	Bacteroides spp.
12	SMAO	M	14	Yes	Yes	R	Abdominal cavity	NI
13	Pseudomembrane colitis	M	26	Yes	Yes	D	Feces	*Ps. aeruginosa*
14	Endometritis	F	16	Yes	Yes	R	Discharge in uterus	*Staph. aureus*
15	Cholecystitis	F	13	No	No	R	Blood	*K. pneumoniae*
16	Colon perforation	M	21	Yes	Yes	R	Abdominal cavity	*E. faecalis*, *E. coli*
17	Cholangitis	M	18	Yes	Yes	R	Bile	*K. pneumoniae*

APACHE II score, Acute Physiology And Chronic Health Evaluation II score; strangulation, strangulation obstruction of small intestine; SMAO, superior mesenteric arteric occlusion; NI, not isolated; D, died; R, recovered.

Flow cytometric analysis

Adjusted PBMCs (100 µl; 1×10^6 ml^{-1}) were incubated with 0.25 µg/10 µl of anti-human Toll-like receptor (TLR)-4 antibody (Medical & Biological Laboratories, Nagoya, Japan) for 30 min at 4°C, after which they were washed twice with cold calcium- and magnesium-free phosphate buffer saline (PBS) that was supplemented with 2% fetal bovine serum (FBS) and 0.1% sodium azide. The cells were then incubated with 1 µl of biotinylated anti-mouse IgG (DAKO, A/S, Denmark) for 30 min at 4°C, after which they were washed twice, and then incubated with 1 µl of R-phycoerythrin (PE) conjugated streptavidin (Beckman Coulter, Marseilles, France), 1 µl of fluorescein-conjugated isothiocyanate (FITC), 1 µl of anti-human CD16 antibody (Beckman Coulter), and 1 µl of allophycocyanin (APC)-conjugated anti-human CD14 antibody (Beckman Coulter) for 30 min at 4°C. After being washed twice, the cells were analyzed within 6 h using a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) and a specialized software package (CELLQuest, Becton Dickinson). Isotype controls included FITC, PE, and APC-conjugated mouse IgG1 and IgG2a. Expression was evaluated using the arithmetic mean fluorescence intensity (MFI).

Culture of PBMCs

Cytokine production by PBMCs was determined using cells that were cultured (37°C and humidified 5% CO_2) in 24-well plates in the presence or absence of 1 µg/ml of LPS (*Escherichia coli* O55:B5; Sigma Chemical Company, St Louis, MO, USA). After 24 h, the culture media were harvested and centrifuged at 3000 rpm for 3 min, and the supernatants collected and stored at –80°C until analysis.

Cytokine assays

TNF-α and IL-1β concentrations were measured in culture supernatants using commercially available enzyme-linked immunoabsorbent assays (ELISAs; Medical & Biological Laboratories). The optical density of each sample was determined at an absorbance of 405 nm using a microplate reader (Well reader SK-601, Seikagaku Corporation, Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SD. Statistical analyses were performed using the StatView 5.0 statistical software package (Abacus Concepts, Inc., Berkeley, CA, USA). Differences between groups were analyzed using the Mann Whitney U-test, with a Bonferroni correction for multiple comparisons. Correlations between cytokine production and cell surface antigen expression were analyzed using the Spearman's rank correlation test. Probabilities of less than 0.05 were considered to be significant.

Table 2. Expression of TLR-4, CD14, and CD16 on peripheral blood monocytes and the relative proportion of $CD16^+CD14^+$ monocytes in septic patients and healthy controls

	Septic patients		Controls
	With shock	Without shock	
TLR-4 expression (MFI)	97.5 ± 55.9	105.6 ± 79.9	67.5 ± 26.7
CD14 expression (MFI)	113.2 ± 34.2	150.6 ± 76.0	160.0 ± 60.0
CD16 expression (MFI)	87.4 ± 57.2[a]	52.9 ± 52.1[a]	12.6 ± 9.0
Relative proportion of $CD16^+CD14^+$ monocytes (%)	41.1 ± 16.4[a,b]	22.1 ± 14.8[a]	10.7 ± 3.9

MFI, mean fluorescence intensity.
[a]$P < 0.05$ versus control; [b]$P < 0.05$ versus without shock.

RESULTS

Clinical characteristics of the patients

The clinical features of the 17 septic patients with intra-abdominal infections who were enrolled in this study are shown in Table 1. Infections in these patients included colon perforation ($n = 5$), acute cholangitis ($n = 4$), small bowel strangulation obstruction ($n = 3$), superior mesenteric arteric occlusion ($n = 2$), pseudomembrane colitis ($n = 1$), endometritis ($n = 1$), and cholecystitis ($n = 1$). Two of the patients died from multiple organ dysfunction syndrome, while the others survived for at least the 28 days of this study. Causative pathogenic agents included Gram-negative bacilli in 5 cases, Gram-positive cocci in 6 cases, and anaerobic bacteria in 1 case. The average APACHE II score of the patients at the time of admission was 14.1 ± 6.8.

Comparison of the number of $CD16^+CD14^+$ monocytes and TLR-4 and CD14 expression on PBMCs between septic patients and healthy controls

TLR-4 expression was significantly elevated on monocytes obtained from septic patients as opposed to healthy individuals (116.2 ± 70.8 versus 67.5 ± 26.7, respectively; $P < 0.05$), but there was no significant difference between the septic patients with shock and without shock (Table 2). In addition, the number of $CD16^+CD14^+$ monocytes was significantly increased in septic patients compared to controls (32.4 ± 20.4% versus 10.7 ± 3.9%, respectively; $P < 0.01$). Moreover, the number of $CD16^+CD14^+$ monocytes in septic patients with shock was significantly increased compared to septic patients without shock. Furthermore, the MFI of CD16 on monocytes obtained from septic patients was significantly up-regulated compared to controls (67.7 ± 55.1 versus 12.6 ± 9.0, respectively; $P < 0.01$), but there was no significant difference between the septic patients with shock and without shock.

Production of pro-inflammatory cytokines by LPS-stimulated PBMCs

The production of both TNF-α and IL-1β by LPS-stimulated PBMCs derived from both septic patients and controls was significantly greater than that produced by PBMCs without LPS-stimulation (Table 3). TNF-α production by PBMCs stimulated with LPS was significantly lower in septic patients with shock than that in controls; however, there was no statistically significant difference between the septic patients with shock and without shock. IL-1β production by PBMCs from septic patients with shock was significantly reduced compared to healthy controls.

Correlation between the proportion of $CD16^+CD14^+$ monocytes and TNF-α and IL-1β production from LPS-stimulated PBMCs in septic patients and healthy controls

A statistically significant correlation was seen between TNF-α production by LPS-stimulated PBMCs and the proportion of $CD16^+CD14^+$ monocytes in healthy controls ($r = 0.688$; $P < 0.05$), though no significant correlation between LPS-induced IL-1β production and the proportion of $CD16^+CD14^+$ monocytes was observed in healthy controls ($r = 0.257$; $P = 0.45$; Fig. 1). On the other hand, there was significant correlation between LPS-induced IL-1β production and the proportion of $CD16^+CD14^+$ monocytes in septic patients ($r = –0.582$; $P < 0.05$), and a trend but not significant correlation between LPS-induced TNF-α production and the proportion of $CD16^+CD14^+$ cells was observed monocytes in septic patients ($r = –0.507$, $P < 0.1$).

Effects of PMX-F treatment on patients' clinical parameters

PMX-F treatment was performed on 7 patients with septic shock (Table 1). The mean arterial blood pressure in these

Table 3. TNF-α and IL-1β production by PBMCs, cultured in the presence or absence of LPS, derived from septic patients and healthy controls

	Septic patients		Controls
	With shock	Without shock	
TNF-α			
Without LPS	17.9 ± 6.7[a]	20.3 ± 4.0[a]	461.8 ± 798.9
With LPS	1089.3 ± 237.4[a]	1825.9 ± 1910.3	2468.6 ± 1080.5
IL-1β			
Without LPS	5.2 ± 4.9[a]	19.1 ± 23.2[a]	275.4 ± 314.9
With LPS	1300.3 ± 1644.9	2768.4 ± 731.2	4154.0 ± 2123.2

[a]$P < 0.05$ versus control.

patients, which was 67.5 ± 7.8 mmHg before PMX-F treatment, rose significantly to 95.9 ± 13.9 mmHg after treatment ($P < 0.05$), although there were no remarkable changes in the heart rate of these patients (Fig. 2). Urine volume increased significantly in these patients from 62.9 ± 54.5 to 201.0 ± 126.8 ml/h after PMX-F treatment ($P < 0.05$), while their CAI diminished significantly to 6.5 ± 3.1 from 9.9 ± 4.6 points ($P < 0.05$).

Changes in leukocyte surface markers in response to PMX-F treatment

The MFI of leukocyte surface markers obtained during and after PMX-F treatment were expressed as a percentage of their initial value (Fig. 3). The mean TLR-4 MFI obtained during and after PMX-F treatment decreased to 82% (NS) and 60.1% ($P < 0.05$), respectively. The corresponding

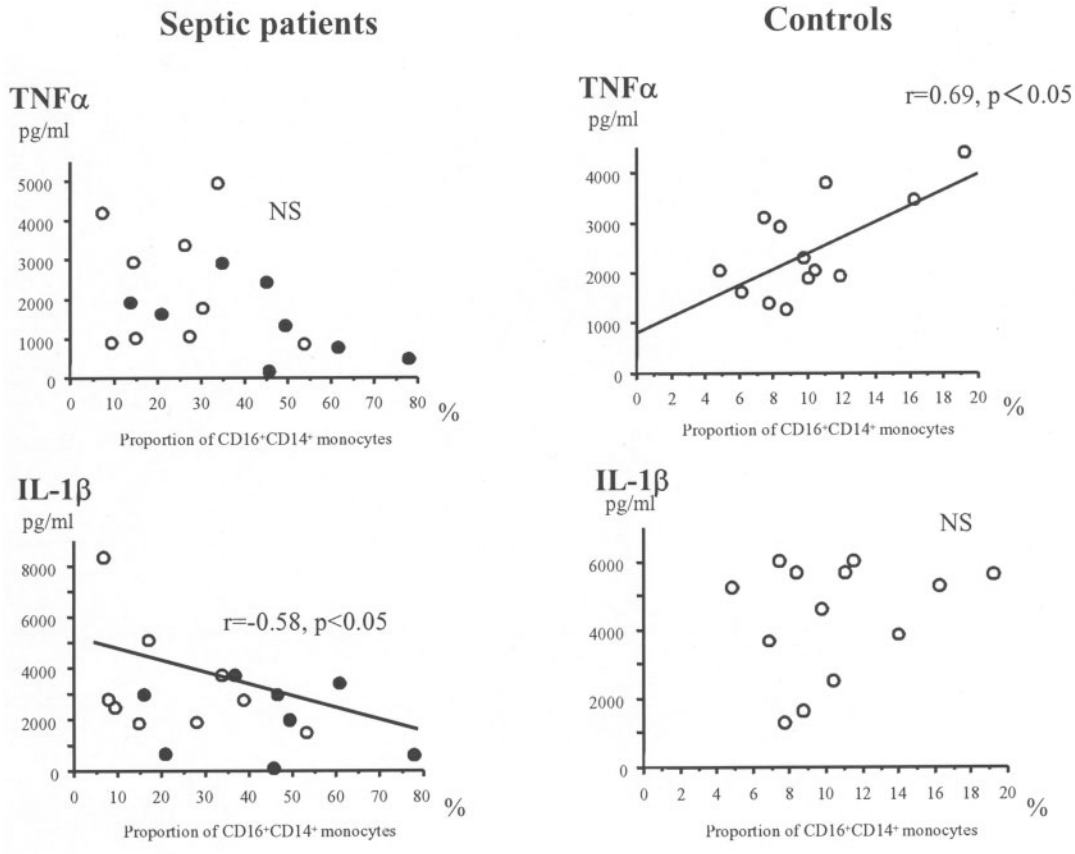

Fig. 1. Correlation between the proportion of $CD16^+CD14^+$ monocytes and TNF-α and IL-1β production from LPS-stimulated PBMCs in septic patients and healthy controls. LPS-stimulated PBMCs and the proportion of $CD16^+CD14^+$ monocytes in healthy controls ($r = 0.688$; $P < 0.05$), though no significant correlation between LPS-induced IL-1β production and the proportion of $CD16^+CD14^+$ monocytes was observed in healthy controls ($r = 0.257$; $P = 0.45$). On the other hand, there was a significant correlation between LPS-induced IL-1β production and the proportion of $CD16^+CD14^+$ monocytes in septic patients ($r = –0.582$; $P < 0.05$), and a trend but not significant correlation between LPS-induced TNF-α production and the proportion of $CD16^+CD14^+$ monocytes was observed in septic patients ($r = –0.507$; $P < 0.1$). Closed circles indicate the septic patients with shock and open circles septic patients without shock.

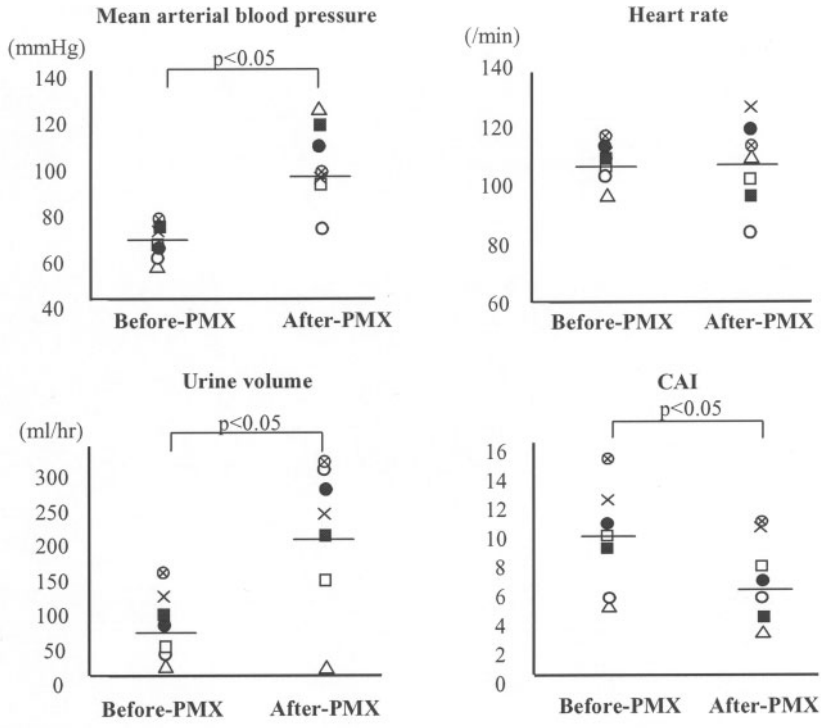

Fig. 2. Effects of PMX-F treatment on patients' clinical parameters. PMX-F treatment was performed in 7 patients with septic shock. PMX-F treatment significantly improved mean arterial blood pressure, urine volume, and CAI (catecholamine index). Horizontal bars represent their median values.

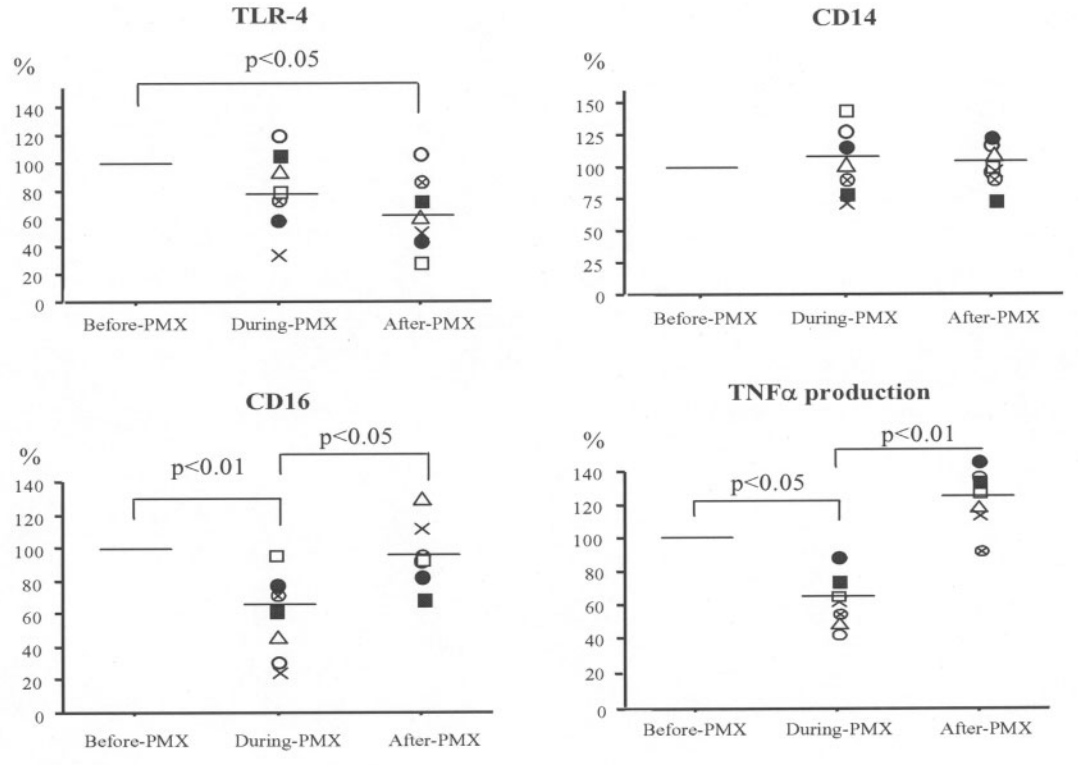

Fig. 3. Changes in leukocyte surface markers in response to PMX-F treatment. The mean TLR-4 MFI after PMX-F treatment significantly decreased compared to before PMX-F. The corresponding mean frequencies of $CD16^{+}CD14^{+}$ monocytes during PMX-F significantly decreased compared to before PMX-F, but significantly increased after the PMX-F treatment There were no significant changes in CD14 MFI during and after PMX-F treatment. TNF-α production by LPS-stimulated PBMCs during PMX-F significantly decreased compared to before PMX-F, but significantly increased after the PMX-F treatment Horizontal bars represent their median values.

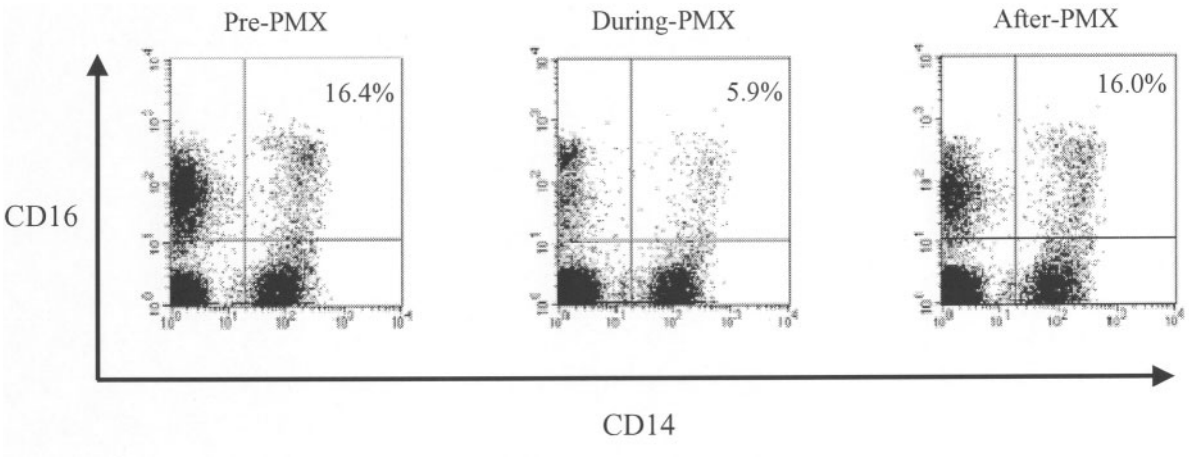

Fig. 4. A representative dot plot from a septic patient showing a reduced proportion of $CD16^+CD14^+$ monocytes during PMX-F treatment. The proportion of $CD16^+CD14^+$ monocytes was 16.4% before the PMX-F therapy (left panel), and reduced to 5.9% during (middle panel), and increased to 16.0% after the PMX-F treatment (right panel).

mean proportions of $CD16^+CD14^+$ monocytes at these same time points were 64.9% ($P < 0.01$) and 97.2% (NS), respectively. There were no significant changes in CD14 MFI during and after PMX-F treatment (110.5% and 99.2%, respectively; Fig. 3). A representative dot plot from a septic patient showing a reduced proportion of $CD16^+CD14^+$ monocytes during PMX-F treatment is shown in Figure 4. All patients responded to PMX-F treatment in a similar manner vis-à-vis their regulation of $CD16^+CD14^+$ monocytes.

TNF-α production by LPS-stimulated PBMCs during PMX-F treatment significantly decreased compared to before PMX-F treatment, but TNF-α production by LPS-stimulated PBMCs after the PMX-F treatment significantly

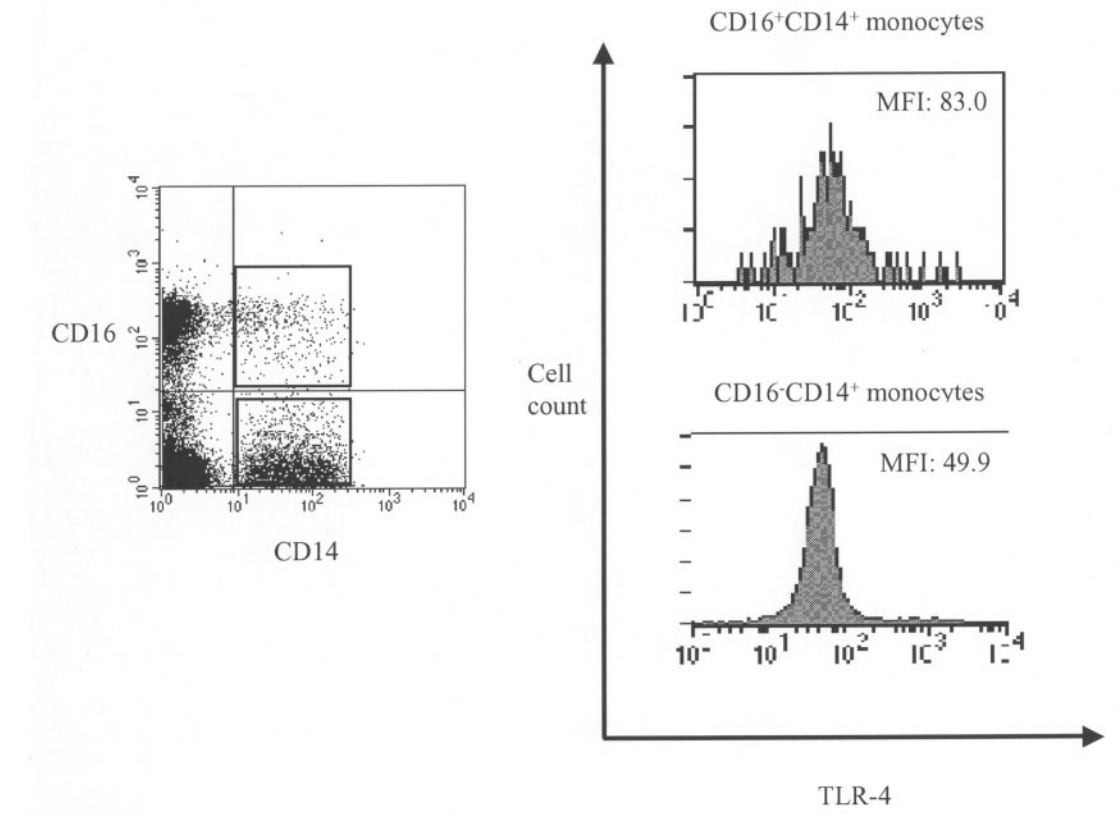

Fig. 5. Enhanced TLR-4 expression on $CD16^+CD14^+$ monocytes compared with that on $CD16^-CD14^+$ monocytes. Three-color immunofluorescence for CD16, CD14, and TLR-4 revealed that $CD16^+CD14^+$ monocytes had a strong expression of TLR-4 compared with $CD16^-CD14^+$ monocytes in both septic patients and healthy controls. A representative flow cytometry histogram showing TLR-4 expression on monocytes from one septic patient is depicted.

increased compared to during PMX-F treatment ($P < 0.01$), and there was a tendency to increase compared to before PMX-F treatment ($P = 0.07$; Fig. 3).

Enhanced TLR-4 expression on $CD16^+CD14^+$ compared to $CD16^-CD14^+$ monocytes

Three-color immunofluorescence staining for CD16, CD14, and TLR-4 revealed stronger TLR-4 expression on $CD16^+CD14^+$ than $CD16^-CD14^+$ monocytes in both septic patients and healthy controls. A representative flow cytometry histogram showing TLR-4 expression on monocytes from one septic patient is shown in Figure 5.

DISCUSSION

In this study, we found that peripheral blood $CD16^+CD14^+$ monocytes were significantly increased in patients with sepsis compared to healthy controls, and that they strongly expressed TLR-4. Furthermore, the number of $CD16^+CD14^+$ monocytes was reduced during PMX-F treatment, and there was a significant reduction in monocyte TLR-4 expression following PMX-F therapy.

Tissue macrophages are characterized by a broad heterogeneity in phenotype and function, but little is known about the heterogeneity of peripheral blood monocytes. While CD14 is not expressed on monoblasts, its expression increases as monocytes mature, ultimately resulting in its high degree of expression on fully developed circulating monocytes. Although the CD16 antigen is similarly not expressed on monoblasts, there is minimal CD16 expression on circulating monocytes and no expression on peritoneal macrophages; however, alveolar macrophages strongly express CD16.[15] Circulating monocytes do not form a homogenous pool of cells but can be divided into subpopulations with different phenotypes and functions. For example, they can easily be differentiated based on their surface expression of CD14 and CD16. In contrast to regular monocytes which are strongly positive for CD14, the subpopulation of CD16-expressing monocytes is characterized by reduced expression of CD14. These cells appear to represent a mature version of $CD14^+$ monocytes that exhibit features of activated cells. The role of $CD16^+CD14^+$ monocytes in patients with sepsis is still unclear.

Previous studies demonstrated that the $CD16^+CD14^+$ monocyte subpopulation was expanded in patients with sepsis.[17] It was not clear, however, whether these cells were responsible for the excessive production of cytokines seen in these patients or whether their expansion was cytokine-induced. Blumenstein *et al*.[6] reported that in cases of repeated urinary tract infection and sepsis due to intravesical and intravenous injection of feces where there was a clear beginning time point for the onset of sepsis, the expansion of the $CD16^+CD14^+$ monocyte population seemed to follow, by about 24 h, the activation of the monocyte–macrophage system, suggesting that it was due to the activity of cytokines such as TNF-α and IL-6. Thus, the presence of increased numbers of $CD16^+CD14^+$ monocytes in the peripheral blood may indicate the presence of a significant underlying infection. Mackensen *et al*.[18] reported that treatment of cancer patients with LPS resulted in an elevation in their serum cytokine levels, but that this response diminished with repeated LPS injection. These repeated LPS injections, however, led to a significant increase in the number of $CD16^+CD14^+$ monocytes.[18] Frankenberger *et al*.[19] reported that $CD16^+CD14^+$ monocytes were no longer able to synthesize anti-inflammatory cytokines such as IL-10, and might represent a pro-inflammatory effecter cell phenotype in the monocyte-macrophage system. Recently, Horelt *et al*.[8] showed that $CD16^+CD14^+$ monocytes were expanded in patients with erysipelas and that they exhibited reduced pro-inflammatory cytokine production (TNF-α). In our study, we similarly demonstrated that $CD16^+CD14^+$ monocytes from septic patients were significantly increased, in particular, in patients with septic shock, and those monocytes lost their ability to synthesize pro-inflammatory cytokines such as IL-1β (Fig. 1). These expanded $CD16^+CD14^+$ monocytes in septic shock patients might contribute to the immunosuppression associated with severe infections.

The immunofluorescence signal of TLR-2 was reported to be 2-fold higher in $CD16^+CD14^+$ than in other monocytes, and $CD16^+CD14^+$ monocytes contributed significantly to the LPS-induced production of cytokines that occurred in Gram-negative infection.[7] Assuming that the levels of TLR-4 correlated with the magnitude of cellular activation, the higher TLR-4 levels in these cells may have been responsible for their increased responsiveness to LPS. Thus, bacteria or their products that seed into the blood could stimulate these $CD16^+CD14^+$ monocytes and contribute to the development of sepsis.

Standard therapies for septic shock are not entirely effective, probably because they do not remove the bacterial toxins, or endogenous toxic mediators produced by the host in response to these toxins, that have already been released into the circulation. Hemoperfusion therapy using PMX-F columns is a newly-developed treatment for sepsis that relies on the removal of endotoxin and other toxins from the blood.[20,21] Recently, Tani *et al*.[13] compared the survival rates of patients who received PMX-F treatment to those who received conventional therapies and found a significant benefit of this treatment. Nemoto *et al*.[22] similarly reported that PMX-F treatment effectively prolonged survival time when it was applied during early sepsis. It was not clear what accounted for the increased survival rate in these patients.

We found that the proportion of $CD16^+CD14^+$ monocytes in the peripheral blood of our septic patients went down during PMX-F therapy, and also found that after such treatment, about 30% of the peripheral monocytes were absorbed to the column (data not shown). When we checked, we found that $CD16^+CD14^+$ monocytes with increased TLR-4 expression also absorbed to our columns. Therefore, PMX-F treatment was effective in reducing the population of expanded $CD16^+CD14^+$ monocytes whose TNF production was decreased and TLR-4 expression was increased. In contrast, $CD16^+CD14^+$ monocytes that were increased after the PMX-F treatment had increased TNF production and decreased TLR-4 expression. Therefore, PMX-F therapy might remove $CD16^+CD14^+TLR4^{high}$ monocyte selectively in patients with septic shock. Absorption of $CD16^+CD14^+$ monocytes might be the mechanism by which PMX-F treatment improves the clinical outcome in patients with septic shock.

Conclusions

The number of peripheral blood $CD16^+CD14^+$ monocytes and monocytic TLR-4 expression were significantly elevated, and the LPS-induced production of pro-inflammatory cytokines reduced in patients with sepsis. PMX-F treatment was effective in reducing the population of expanded $CD16^+CD14^+$ monocytes and diminishing the expression of TLR-4 on monocytes in patients with septic shock.

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