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Original Article

Role of biphasic changes in splenic dendritic cell activity in a mouse model of multiple organ dysfunction syndrome

Yi Lv*, Qian Liu*, Min Zhao, Yiduo Jin, Jiangyang Lu

*Department of Pathology, The First Affiliated Hospital of General Hospital of PLA, Beijing 100048, China. *Equal contributors.*

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Abstract: To analyze the changes in splenic dendritic cell (DC) activity and serum cytokine levels during the progression of multiple organ dysfunction syndrome (MODS). A C57BL/6 mouse model of MODS was established by intraperitoneal injection of zymosan. Immunohistochemistry and flow cytometry were used to detect expression of I-A^b (MHC-II molecules of mice) as well as co-stimulatory and co-inhibitory molecules in spleen and DC surface. The levels of various cytokines in serum and spleen tissue were analyzed 6 h, 12 h, 24 h, 48 h, 5 d and 12 d after injury. Death occurred at 24-48 h and 10-12 d after injury. The expression of I-A^b and CD86 in spleen tissue and on DCs increased 6-12 h after injury, followed by gradual reduction and at 12 d. The inhibitory molecule, PD-L1, was expressed on normal DCs, but expression of PD-1 was undetectable. PD-L1 and PD-1 expression increased and remained high at 5 d and 12 d after injury. In addition, TNF and IL-1 levels increased 6-12 h after injury; HMGB1 and IL-10 levels increased 24 h and 5 d after injury, respectively. In contrast, IL-2 and IL-12 decreased with disease progression. At 12 d after injury, proinflammatory and anti-inflammatory cytokine levels remained high, while IL-2 and IL-12 were significantly reduced. IL-10 and IL-12 changes in spleen were consistent with those in serum. MODS progression was characterized by changes in splenic DC activity as well as altered serum pro-inflammatory and anti-inflammatory cytokine levels, suggesting early immune activation and predominant immune tolerance at the late stage.

Keywords: Splenic dendritic cell, MODS, immunohistochemical staining

Introduction

Multiple organ dysfunction syndrome (MODS) is an important cause of death in late-stage sepsis, and its mortality rate ranks first in the intensive care unit (ICU) [1, 2]. Interrupting the occurrence and development of sepsis as soon as possible is crucial for preventing the incidence of MODS in clinical practice. Although the effects of dozens of pro-inflammatory cytokine antagonists have been examined in clinical trials in the past 20 years, their efficacy in humans is far inferior to that observed in animal studies [3-6], suggesting a deviation in the target or the timing of use [7-9].

In the early stages of sepsis, patients are characterized by an excessive inflammatory response or "cytokine cascade" [10, 11]. Advancements in clinical treatment enable the majority of patients to pass through this stage and survive [12, 13], but subsequent nosocomial infections of non-pathogenic bacteria and activation of potential viruses often ensue, resulting in death [12, 14, 15]. Animal experiments showed that sepsis/MODS progression is characterized by an excessive inflammatory reaction in the early phases followed by gradual development into subsequent significant immune suppression [10, 16-19]. Clinical observations also confirmed that some patients, who died due to sepsis and MODS, experienced a transformation from an early excessive inflammatory response to immunosuppression [20]. During the progression of sepsis, the changing balance between inducing and inhibiting injury may result in a pathological condition, which may cause mismatch between the antagonistic and preventive measures employed for the early excessive inflammatory response and subsequent MODS, failure of anti-inflammatory treatment, and even aggravate injury. Therefore,

determining the appropriate measures for early excessive inflammatory response and late immune paralysis are critical for the prevention and treatment of sepsis. In addition, understanding the mechanism by which immune activation changes to immunosuppression in sepsis and intervention prior to progression to immune paralysis will represent effective strategies for reducing the mortality of MODS.

The spleen plays an important role in the innate and acquired immune responses [21, 22]; therefore, changes in splenic function will affect the systemic immune status. In a mouse model of cecal ligation and puncture (CLP) sepsis, splenectomy reduced mortality and serum levels of high-mobility group protein B1 (HMGB1) [23]. In addition, analysis of the cholinergic anti-inflammatory pathway revealed that parasympathetic (vagal) activity attenuated systemic inflammation through regulating the activity of splenic immune cells [24], significantly reducing the death of septic animals [25, 26]. However, no anti-inflammatory effects were observed with vagus nerve excitation or cholinergic receptor activation in animals with splenectomy or when the splenic nerve was cut [26, 27], indicating that splenic function has an important impact on the prognosis of sepsis.

Immunomodulation by the spleen requires the synergistic action of splenic dendritic cells (DCs) and various immune cells, including T cells, B cells, and Treg cells. DCs are the most important professional antigen-presenting cells (APCs), bridging the innate and adaptive immunity [28]. It was previously thought that DCs only present foreign antigens and activate T cells, thereby triggering immune responses. Recent studies have found that under certain conditions, DCs can produce immune tolerance and even negatively regulate immune function. Therefore, DCs have bidirectional immunomodulatory effects [29-31], which are achieved through interactions between co-stimulatory and co-inhibitory molecules located on the cell surface and the corresponding ligands on the lymphocytes [32, 33]. Understanding the functional changes of splenic DCs in MODS progression and their impact may help develop new and more effective prevention and treatment strategies.

Our previous studies described an increase in the number and size of splenic DCs as well as their activation in the early stage zymosan-induced MODS in mice [34]. Additionally, the activity of splenic DCs was reduced with increased DC and lymphocyte apoptosis and lytic necrosis in the late stage [34]. We speculate that during the development of sepsis, changes in immune function of splenic DC could activity and therefore immune function mediate at least in part the early immune activation and late immunosuppression through affacting regulating different T cell subsets. Thus, analyzing related changes in serological immune parameters (pro-inflammatory and anti-inflammatory cytokines and cytokines from different cell sources) may provide guidance for predicting disease progression and prognosis. This study used intraperitoneal injection of zymosan to replicate the MODS model and analyzed the changes in the splenic DC phenotype and various serum cytokines at different stages during MODS progression. We found that changes in splenic DC immune activity and sequential changes in serum immunological indicators were closely related to disease progression.

Materials and methods

Establishment of an in vivo model of MODS

Male 6-8 week-old C57BL/6 mice (n = 165) weighing 20-25 g were purchased from the Experimental Animal Center of the Military Medical Academy of Sciences. Animals were acclimated for one week under 12 h light/12 h dark cycles. Mice were fed standard diet and had free access to drinking water. Prior to induction of MODS, animals were fasted for 12 h. Mice were randomly divided into a control group (n = 10) or experimental group (n = 155), and the experimental group was further divided into the following six subgroups based on time points post zymosan injury: 6 h group (n = 15), 12 h group (n = 15), 24 h group (n = 15), 48 h group (n = 30), 5 d group (n = 30), and 12 d group (n = 50).

The zymosan-induced MODS model was established as reported by Jansen et al. [35]. Briefly 1 g of zymosan powder (Sigma, USA) and 40 mL paraffin oil were mixed to form a 25 g/L zymosan suspension, which was sterilized in a water bath at 100°C for 80 min and cooled to room temperature. After abdominal skin disinfection, mice received an intraperitoneal injec-

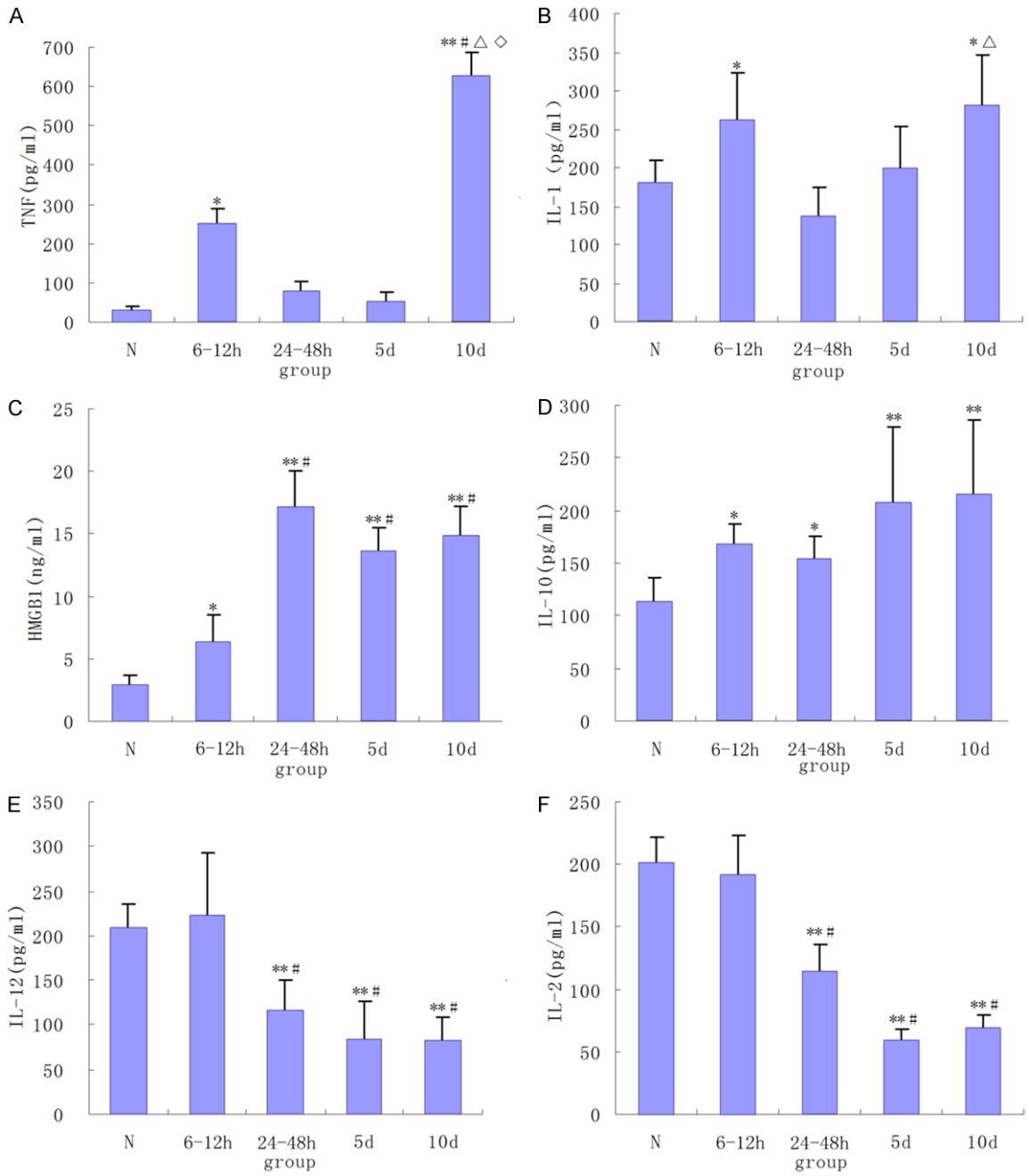

Figure 1. Serum concentrations of cytokines in mice with zymosan-induced injury. Blood samples were collected at the indicated time points after zymosan-induced injury (n = 8). Serum (A) TNF, (B) IL-1, (C) HMGB1, (D) IL-10, (E) IL-12 and (F) IL-2 concentrations were detected by ELISA. Data were analyzed using one-way ANOVA, and the LSD test was used for between-group comparisons. *P < 0.05 versus the normal control group, **P < 0.01 versus the normal control group; #P < 0.01 versus the 6-12 h group. ΔP < 0.01 versus 24-48 h group, ◊P < 0.01 versus 5 d group.

tion of the zymosan suspension (800 mg/kgBW). Then, the animals were fed conventionally. In addition to monitoring for death, blood and spleen specimens were collected at corresponding time points for subsequent analysis.

Detection of cytokine levels in the serum and spleen tissue

Blood samples were collected from the retinal artery. After remaining in a stationary position

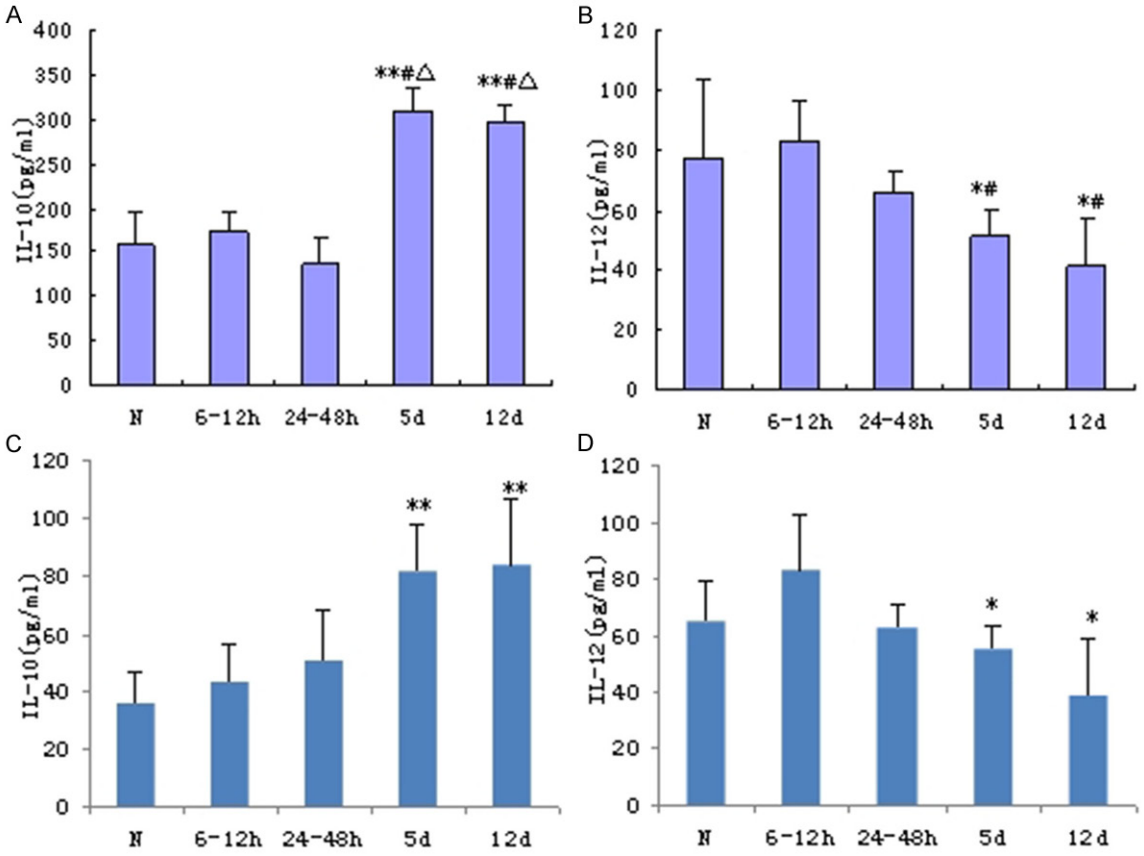

Figure 2. IL-10 and IL-12 expression in the spleen and secretion by DC cultures. Spleen tissues were collected at the indicated time points after intraperitoneal injection of zymosan. IL-10 and IL-12 levels in spleen tissue homogenates (A and B, respectively; n = 6) or DC culture media after 24 h (C and D, respectively n = 4) were determined by ELISA. Data were analyzed using one-way ANOVA and t-test was used for between-group comparisons. $*P < 0.05$ versus the normal control group, $**P < 0.01$ versus the normal control group; $\#P < 0.01$ versus the 6-12 h group; $\Delta P < 0.01$ versus the 24-48 h group.

for 30 min, the serum was isolated after centrifugation at 3000 rpm for 15 min and stored at -80°C. The spleen tissue was frozen in liquid nitrogen, and 100 mg was collected and homogenized in 1 mL PBS in an ice bath. After centrifugation at 3000 rpm for 15 min at 4°C, the supernatant was collected. Enzyme-linked immunosorbent assays (ELISAs) were used to detect serum concentrations of tumor necrosis factor (TNF) α, interleukin (IL)-1β, high-mobility group protein B1 (HMGB1), IL-10, IL-12 and IL-2 and levels of IL-10 and IL-12 in splenic tissue homogenate using commercial kits, following manufacturer's instructions.

Immunohistochemical staining and immunofluorescence labeling of spleen tissue

After the spleen tissues were collected, a portion of the spleen tissue was fixed with 10% neutral formalin, paraffin embedded, and sliced into sections of 5-μm thickness. Conventional immunohistochemical staining methods were used, and horseradish peroxidase (HRP)-peroxide-diaminobenzidine (DAB) was used to label I-A^b, CD86, and TGFβ. The samples were counterstained with hematoxylin, and imaged using an Olympus BX40F microscope (Olympus, Melville, NY, USA). The other portion of spleen tissue was embedded in optimal cutting temperature (OCT) compound, and 4-μm frozen sections were prepared and attached onto the APES film. The samples were dried at room temperature and fixed in acetone at 4°C for 10 min. The immunofluorescence labeling of programmed cell death ligand 1 (PD-L1) was performed, and samples were observed under a fluorescence microscope and photographed using a Nikon Eclipse 50i fluorescence microscope.

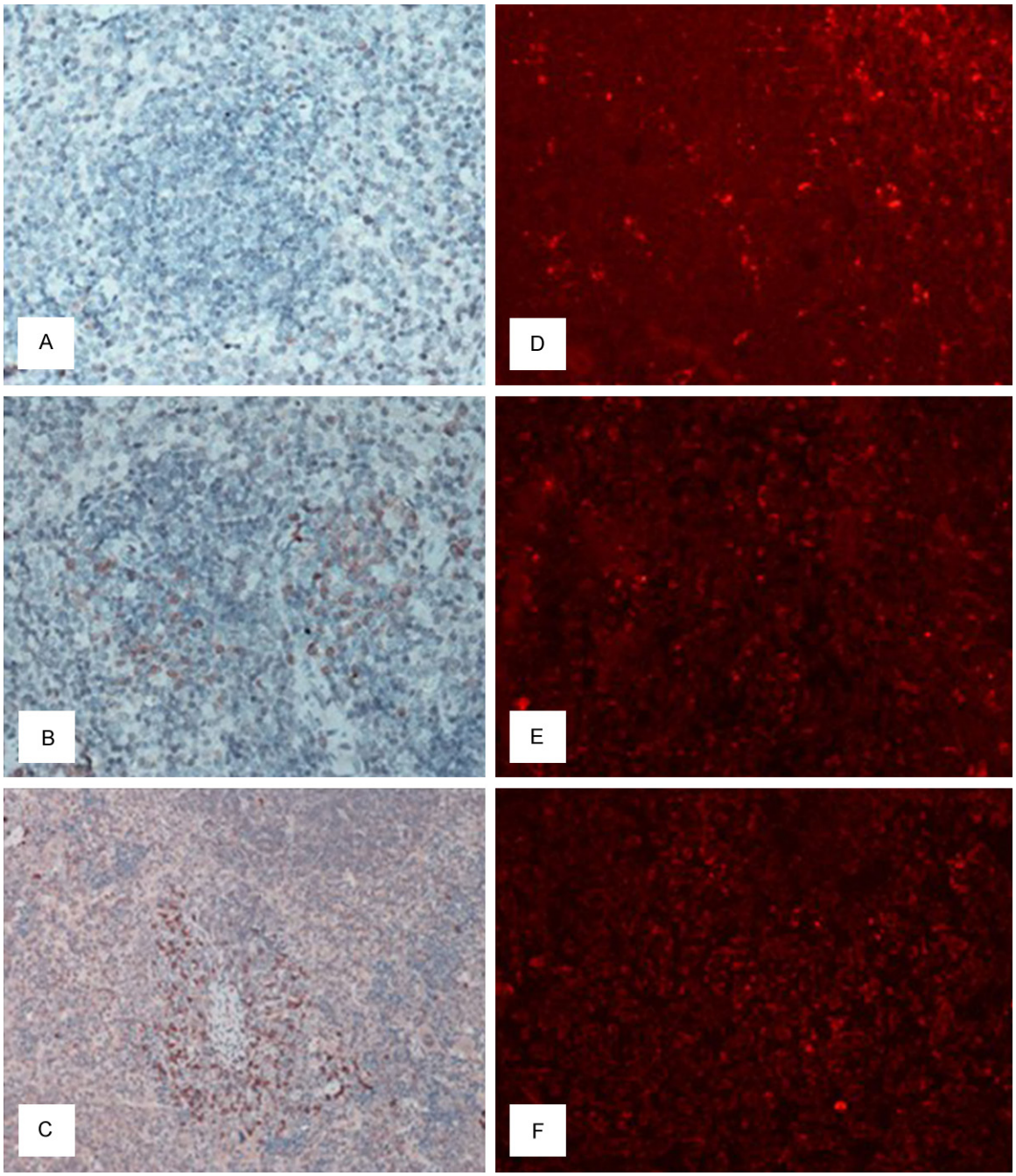

Figure 3. Immunolabeling of TGF-β and PD-L1 in spleen tissues. (A-C) Immunohistochemical labeling of TGF-β using the SABC method in (A) the normal control group and at (B) 6 h and (C) 12 d after zymosan injection. (D-F) Immunofluorescence labeling of spleen PD-L1 using PE-anti mouse-CD274 in (D) the normal control group and at (E) 48 h and (F) 12 d after zymosan injection.

Splenic DC isolation and culture and detection of cytokines in cell culture supernatant

The spleen tissue was collected at corresponding time points and placed on a petri dish. The capsule was trimmed off, and the sample was immersed in 1.25 mL collagenase IV (1 mg/mL) (Sigma, USA). A 1 mL syringe and 25 G needle were used to inject 500 μL collagenase IV into the spleen, and the tissue was shredded and incubated at 37°C for 25 min. After addition of 10 mM EDTA, the sample was incubated for 5 min. The digested spleen tissue was grinded on a 400 mesh metal net and washed with PBS.

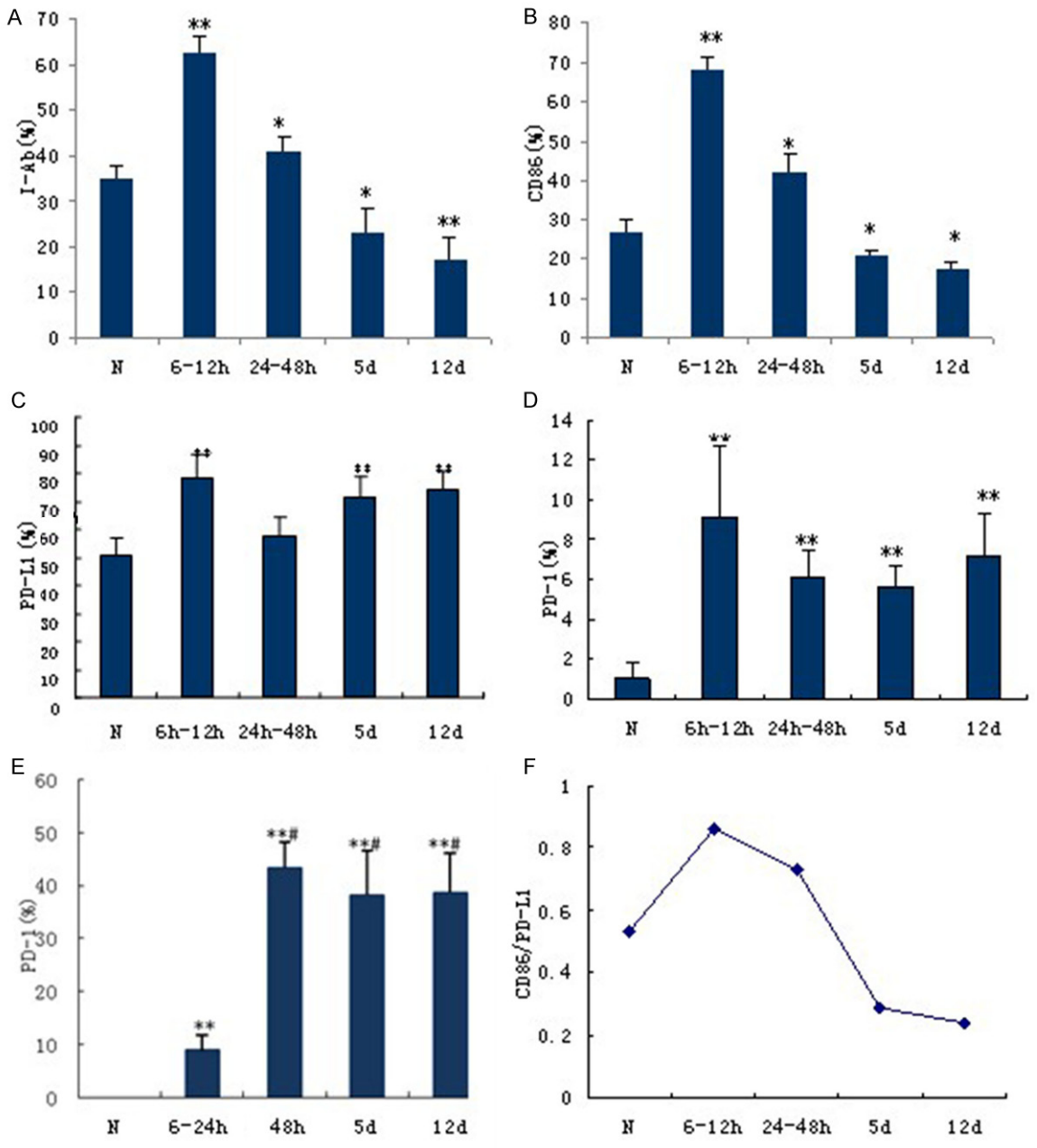

Figure 4. Immunohistochemistry analysis of I-A^b and CD86 expression in the spleen. (A-C) I-A^b; and (D-F) CD86 expression was analyzed in (A and D, respectively) normal control tissue and at (B and E, respectively) 6 h and (C and F, respectively) 12 d after zymosan injection. Tissues were counterstained with hematoxylin.

The cell suspension was collected and isolated by centrifugation at room temperature and 2000 rpm for 10 min. After the supernatant was discarded, 5 mL pre-chilled PBS was added to the cells, which were dispersed evenly using a micropipette. The sample was slowly added into a sterile conical centrifuge tube with 10 mL lymphocyte separation solution (Ficoll-Papue) and centrifuged at room temperature at 3000 rpm for 15 min. The cells in the middle layer were collected and washed twice with PBS and dispersed evenly using a micropipette in 2 mL PBS. Cell viability was detected using trypan blue staining and was greater than 97%. Anti-CD11c magnetic beads and positive selection MS+ columns were used to isolate DCs following the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The cells were labeled with a PE-CD11c fluorescent antibody to assess the purity of the DCs, which was > 99%.

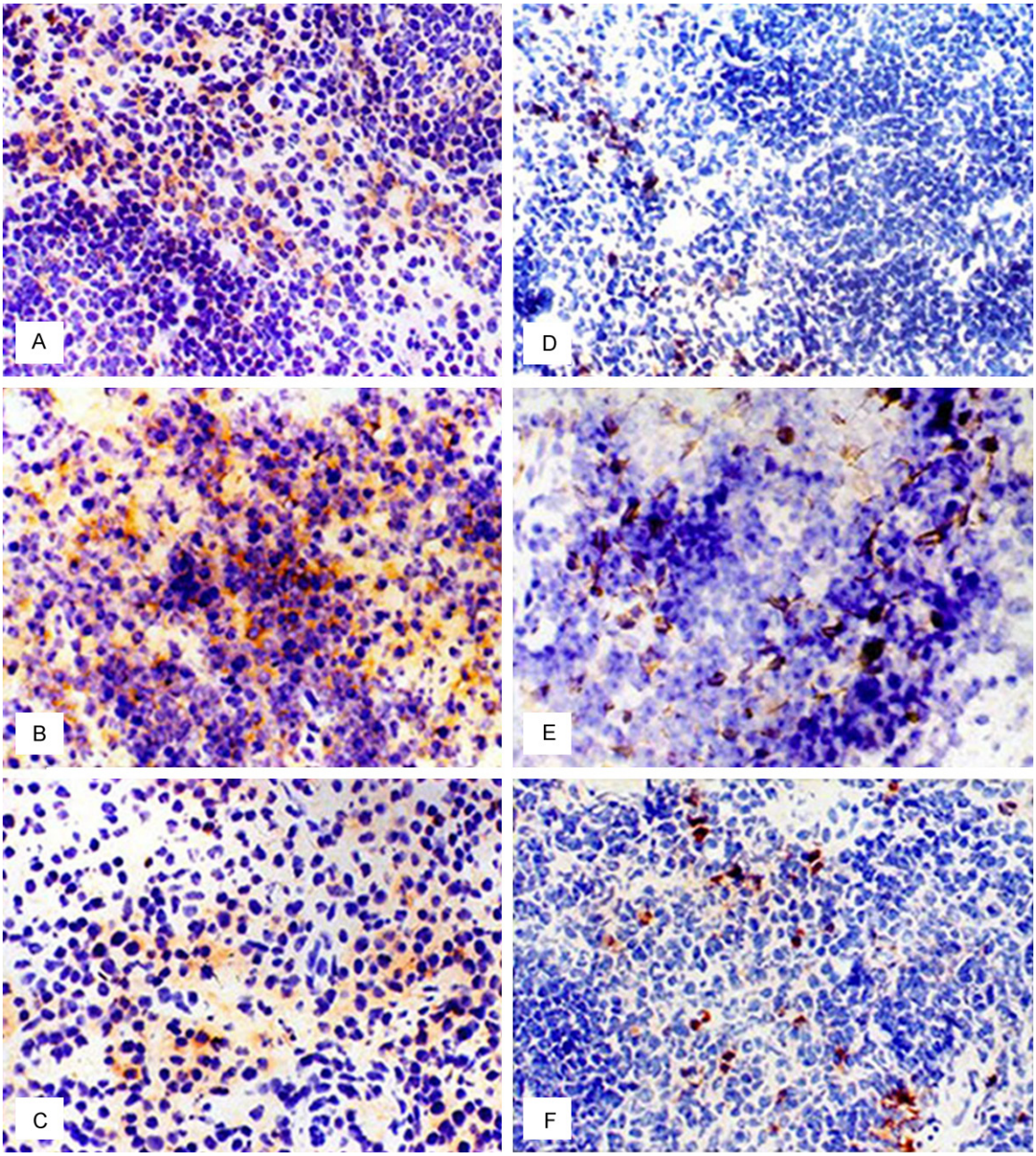

Figure 5. Splenic DC surface molecule expression after zymosan-induced injury. Spleens were collected at the indicated time points (n = 4). After DC isolation using CD11c magnetic beads, the cells underwent FACS analysis to assess (A) I-A^b, (B) CD86, (C) PL-L1, (D) PD-1 and (E) PIR-B expression. (F) The CD86/PD-L1 ratio on the surface of isolated DCs was also determined. Results are expressed as mean ± SD, and the data were analyzed using one-way ANOVA, and t-tests for between-group comparisons (n = 4). *P < 0.05 versus the normal control group, **P < 0.01 versus the normal control group, #P < 0.01 versus 6-12 h group.

The purified DCs were resuspended with RPMI-1640 (Hyclone, USA), and the cell concentration was adjusted to 5×10^6 cells/mL prior to seeding onto 96-well culture plates (1.25×10^6 cells/well). The cells were maintained in an incubator with 5% CO_2 at 37°C. After 24 h, the cells were centrifuged at 1500 rpm for 5 min, and the supernatant was collected for analysis of cytokine concentrations by ELISA as previously described.

Detection of DC surface molecule expression using flow cytometry

Samples of 100 μL DCs were resuspended in PBS containing 2% bovine serum albumin

(BSA), which were added into a flow cytometric tube (5×10^4 cells/tube). After 2-3 μL FITC- or PE-labeled fluorescent antibodies specific for target surface markers (or PE-IgG Isotype Control) were added, the samples were incubated at 4°C for 45 min and washed twice with PBS before centrifugation at 1500 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in 400 μL PBS. Detection and analysis were carried out using a FACS calibur (Becton Dickinson, USA). The labeled antibodies included CD11c-PE, MHC-II ($I\text{-}A^b$)-FITC, PD-L1-PE, programmed death-1 (PD-1)-PE (all from BD Biosciences, USA), CD86-PE, and paired-immunoglobulin-like receptor (PIR)-B-PE (Biolegend, USA).

Statistical analysis

Data are shown as mean ± standard deviation (SD). SPSS13.0 software was used for single factor analysis of variance (ANOVA). Between-group comparisons were undertaken using the least significant difference (LSD) test, and the statistical significance level α was set at 0.05.

Results

Symptoms, physical signs, and survival rate of MODS mice at different stages of disease progression

The experimental mice showed reduced activity and decreased dietary intake 3 h after injection of zymosan. The symptoms were aggravated at 12 h after injection as the mice were listless, shivering, refusing food intake, and had diarrhea, wet, dirty hair, closed eyes and increased secretions. The first peak of death occurred at 24-48 h post zymosan injection (mortality 30.1%). At 48 h, the status of experimental animals gradually improved, which was accompanied by gradual restoration of basic living habits. No deaths occurred during this period (mortality 0%). At 5-7 d after injection, animals showed systemic symptoms, which were aggravated at 10-12 d and were accompanied by drowsiness, lack of food intake, and dyspnea. The second peak of death occurred during that period with a mortality rate of 21.9%.

Changes in the serum cytokine levels

In mice with zymosan-induced injury, serum cytokine levels were altered with disease progression (**Figure 1**). Specifically, serum concentrations of proinflammatory cytokine, TNF-α, showed a bimodal increase that coincided with the biphasic changes in systemic symptoms and mortality (**Figure 1A**). In normal mice, serum TNF-α levels were low, rising sharply at 6 h-12 h after zymosan injection ($P < 0.05$) and returning to near normal levels at 24 h-5 d. TNF-α concentration increased again at 12 d to a greater extent than that observed at the 6-12 h time point ($P < 0.01$). A similar trend was observed with serum IL-1 levels, but to a lesser extent (**Figure 1B**; $P < 0.05$). In contrast, the anti-inflammatory cytokine, IL-10, increased slightly at 6 h-48 h after zymosan injection ($P < 0.05$) with greater levels observed at 5-12 d (**Figure 1D**; $P < 0.01$).

Serum concentrations of HMGB1, an inflammatory mediator in late-stage sepsis, increased gradually after injury, peaked at 24 h and remained at a high level for the duration of the study (**Figure 1C**; $P < 0.01$). Serum IL-12 secreted by mononuclear macrophages and antigen presenting DCs gradually decreased at 24-48 h after injury (**Figure 1E**). Its level was only 50% of that in the control group at 12 d after injury ($P < 0.05$), suggesting that the immune activity of antigen-presenting cells gradually decreased during MODS progression.

Secretion of IL-2 by activated T cells can promote T cell proliferation of T cells; therefore, IL-2 levels are reflective of the degree of T cell activation. At the early stage of zymosan-induced injury (i.e., 6-12 h), no changes in IL-2 were noted (**Figure 1F**). However, its level was significantly reduced at 24-48 h after injury, which continued for the duration of the study ($P < 0.01$). Il-2 levels at 5 and 12 d were only one-third that of the control and 6-12 h groups, indicating that the T cell immune activity was significantly reduced at the later stages of MODS progression.

IL-12, IL-10 and TGF-β expression in the spleen and DC cultures during MODS progression

Because altered IL-12 and IL-10 levels that can reflect immune activation and immunosuppression of splenic DCs, their levels were determined in spleen homogenate at both the early and late phases of MODS progression. At the early stage, IL-12 and IL-10 levels did not change significantly (**Figure 2A** and **2B**). In con-

trast, at 5 and 12 d after injury, IL-10 levels were significantly increased ($P < 0.01$) while IL-12 were reduced ($P < 0.05$) as compared to the control and 6-12 h groups. Similar changes in IL-10 and IL-12 secretion by splenic DCs were observed (**Figure 2C** and **2D**, respectively; $P < 0.05$).

As shown in **Figure 3A**, scattered expression of TGF-β, an anti-inflammatory factor, was observed in the spleen of mice in the normal control group; most of the TGF-β immunoreaction was detected in the marginal zone and red pulp area. At 6 h post induction of MODS injury, the number of TGF-β-positive cells increased slightly in the marginal zone of the white pulp (**Figure 3A**). At late-stage MODS, greater TGF-β expression was observed with a patchy distribution in the red pulp area and the area close to the marginal zone (**Figure 3C**). These results suggest that the immune activity of the spleen and splenic DCs was reduced and immunosuppression enhanced with the disease progression.

Expression of co-stimulatory molecules in splenic tissue and DCs

DCs are the most powerful APC in the body. The expression of CD84 and I-A^b DC surface markers was next determined (**Figure 4**). Analysis of CD86 expression in a normal spleen revealed that splenic DCs were mainly distributed in the marginal zone with a small number of them located in the white pulp and splenic cord (**Figure 4D**). In the early stage of zymosan-induced injury (6-12 h), the number of CD86-positive cells increased (**Figure 4E**), which was subsequently reduced at 12 d (**Figure 4F**). During the MODS progression, changes in the expressions of the key signal molecule in the spleen which mediates antigen presentation, I-A^b, coincided with those observed for the CD86 marker (**Figure 4A-C**).

The immunohistochemistry analysis of I-A^b and CD86 expression was also confirmed by FACS analysis of isolated splenic DCs. Specifically, the expression levels of I-A^b by isolated splenic DCs significantly increased at 6 h-12 h after injury as compared with the normal group (**Figure 5A**; $P < 0.01$); its level dropped at 24-48 h and then continued to gradually decrease until the end of the study, resulting in a level that was only 50% of that in the control group ($P < 0.01$). Changes in the expression of CD86 on the DCs were consistent with that of I-A^b, suggesting that the immune activity of splenic APCs experienced a transformation from enhancement to reduction during MODS progression (**Figure 5B**).

Expression of PD-L1 and PD-1 in the spleen and on the surface of splenic DCs

PD-L1 and PD-1 transfer negative co-stimulatory signals. After double labeling of spleen tissue with CD11C-FITC and PD-L1-PE, a large number of splenic DCs were observed in normal mice, with only a small proportion of DCs expressing PD-L1 (red-orange color) (**Figure 3D**). In late-stage MODS, the number of PD-L1-positive cells increased in the spleen (**Figure 3F**). In contrast, PD-1 expression was not detected in the spleen of normal mice; however, its expression was increased at different time points during MODS progression.

Flow cytometry was used to also detect PD-L1 and PD-1 surface expression on splenic DCs (**Figure 5C** and **5D**, respectively). Under normal conditions, the positive expression rate of PD-L1 was 50.31%, and a biphasic increase at 6-12 h and 5 d-12 d after injury was observed as compared with the normal group (**Figure 5C**; $P < 0.05$). In contrast, PD-1 expression was not detected in the normal control group; however, its expression increased at 6 h-24 h and continued to be elevated for the duration of the study as compared with the normal group ($P < 0.05$). It is worth noting that while biphasic increases in PD-L1 expression were observed at 6-12 h and again at 5 d after injury, PD-1 expression was increased at 48 h after injury, which was before the second increase of PD-L1 expression (**Figure 5C** and **5D**). As shown in **Figure 5F**, ratio of co-stimulatory and co-inhibitory molecules on DCs at different stages during MODS progression revealed that the level of CD86 co-stimulatory molecule in the normal group was only half of that observed for the co-inhibitory molecule, PD-L1. In early MODS, the CD86/PD-L1 ratio increased at 6 h -24 h, returned to normal levels at 48 h, and was half that of the control group at 12 d. Thus, DC function changed with MODS progression.

Upregulation of PIR-B expression, a DC molecule with inhibitory effects, is one of the characteristics of DC tolerance. In the spleen of normal mice, the expression of PIR-B was high

(over 50%). While its expression remained the same at the early stage after injury, PIR-B levels increased at 5 d and 12 d after injury as compared with the normal group ($P < 0.05$), which was the same profile observed for PD-L1/PD-1 at the late stage of MODS progression (**Figure 5E**).

Discussion

In this study, intraperitoneal injection of zymosan was used to establish a mouse model of MODS [35]. The immune activation and disorder of mice were consistent with that observed in MODS progression, which is in line with the inflammatory mediator theory of MODS [36, 37]. During the disease progression, changes in the immune phenotype of splenic DCs and levels of serum pro-inflammatory and anti-inflammatory cytokines were indicative of predominant immune activation in the early stage and dominant immune tolerance in the late stage, which coincided with the two death peaks. During MODS progression, the sequential changes in serum cytokine levels are important as a reference value for the timely detection of immune status transition and prediction of disease progression.

The spleen is a core organ that regulates systemic immune balance. Its regulatory function is realized through interactions with a variety of immune cells, including macrophages, DCs, T and B lymphocytes and their subgroups [38]. DCs capture and process foreign antigens and present them to naive T cells, playing an important role in stimulating the adaptive immune response, inducing peripheral tolerance and determining the direction, intensity and duration of the immune response [38-41]. DCs occupy a dominant position in spleen-mediated immunomodulation. The interactions between DCs and T cells are mediated through the binding between the DC surface ligands and the receptors on the T cell surface (TCRs). DCs process the antigen and load it onto MHC-II molecules, forming a MHC-II-Ag complex prior to bind to TCRs and transferring the first signal. Co-stimulatory molecules, which are B7 family members and include CD86 and CD80, interact with CD28/CTLA to transfer the second signal. IL-12 released by APC is the third signal for T cell activation (Diebold 2008). Therefore, the expression levels of MHC-II, B7 family molecules and IL-12 on DCs reflect the ability of DC to activate T cells, which determines the level of T cell activation. In this study, expression of co-stimulatory molecules, such as MHC-II and CD86 in the spleen and on the splenic DCs, at the early stage of MODS was higher than those in the normal control group, indicating enhanced DC activity and activation of the immune response. In addition, increased serum concentrations of pro-inflammatory cytokines were observed at this stage. At the late stage, expression of MHC-II and CD86 on the DCs were significantly reduced, and IL-12 levels in the spleen were decreased. Also, IL-10 levels were greatly increased along with the number of TGF-β-positive cells. Isolated splenic DC cultures secreted less IL-12, but more IL-10. Thus, the results showed that the immune function of the spleen and splenic DCs was suppressed at the late stage of the disease. Moreover, the reduction in the T lymphocyte activity was related to the increased secretion of inhibitory cytokines by DCs [42].

DC immune regulation is also affected by the expression of inhibitory receptors and negative co-stimulatory molecules [43, 44]. Mouse PIR-B protein is a type of inhibitory receptor that is expressed on the surface of DCs. It binds to its natural ligand, MHC-I, affecting the activation of NF-κB by regulating IκB phosphorylation and dephosphorylation, leading to down-regulation of expression of its downstream genes, including *CD80, CD86, IL-12p70* and *MHC-II*, and inhibiting immune cell function [43]. Therefore, upregulation of PIR-B expression in DCs indicates the formation of DC tolerance. In addition, the PD-L1/PD-1 pathway is an important inhibitory pathway through which DCs exert their immunosuppression function [44, 45]. The negative co-stimulatory molecule, PD-L1 (B7-H1, CD274), is a new member of the B7 family [46], and its receptor is PD-1 [47]. PD-L1 is mainly expressed in DCs, B cells and macrophages, and its expression increases after cell activation. PD-1 is expressed on the surface of activated CD4+ T cells, CD8+ T cells, NKT, B cells and monocytes. DC PD-L1 binds to PD-1 on the surface of immune cells, such as T lymphocytes, and then PD-1 binds to TCR, resulting in rapid phosphorylation of SHP-2 and inhibition of the TCR signal. Negative stimulatory signals are further passed to T cells, resulting in the inhibition of T cell proliferation and

alterations in the antigen presentation second signaling pathway (CD86/CD28), which is the main pathway through which DCs mediate immune tolerance [48]. Our study found that in the late stage of the disease, expression of the co-inhibitory molecules, PD-L1 and PD-1, and the inhibitory receptor, PIR-B, on splenic DCs increased greatly, suggesting decreased DC activity and reduced antigen-presenting function in the late stage of MODS. In addition, immune phenotype characteristics of DC tolerance were shown [49]. In response to DC tolerance, immune function is reduced, which can result in endogenous bacterial translocation or opportunistic pathogen infection. Upon induction of immune cell synthesis and secretion of a large number of pro-inflammatory cytokines, disease aggravation ensues. Thus, the changing pattern of co-stimulatory and co-inhibitory molecules by splenic DCs indicates that their immune phenotype experienced a transition from activation to tolerance during MODS progression.

In mice of the normal control group, MHC-II and CD86 expression was low, and PD-L1 and PIR-B expression was high in DCs, indicating immune tolerance. It should be noted that normal DCs do not express PD-1; however, PD-1 expression increased gradually during the disease progression. Therefore, we speculate that the co-expression of PD-L1 and PD-1 on DCs represent a self-regulating mechanism by DCs. Under normal circumstances and in the early stage of disease, PD-L1 was increased, but PD-1 expression is low or absent. The mismatch between their expressions will not lead to the formation of immune tolerance. However, in the late stage, expression of both PD-L1 and PD-1 increased, inducing formation of immune tolerance. It was also noted that PD-L1/PD-1 expressions on DCs increased at 6h after injury, and IL-10 concentrations in the spleen and serum were also increased at this point, suggesting that the body has triggered a negative immunoregulatory mechanism at the early stage of the inflammatory response. If anti-inflammatory treatment is given to alleviate the excessive inflammatory response at that stage, immune suppression may be promoted, and the disease may be aggravated. This might partially explain the undesired effects of anti-inflammatory treatment given at the stage of excessive inflammatory response in early sepsis; however, it needs to be confirmed by further studies.

In the past, many treatments focusing on the inflammatory mediator “cascade” in sepsis and early MODS failed [3-5, 7-9], which suggests that selecting key targets according to the characteristics of immune disorder at different stages is the premise for identifying effective prevention and treatment of MODS. Therefore, we analyzed the expression of a variety of cytokines, including early-stage proinflammatory cytokines (TNF and IL-1), a late-stage inflammatory mediator (HMGB1) [50], an inhibitory cytokine (IL-10), a T-cell activator (IL-2) [51] and a cytokine related to APC activity (IL-12) [52]. During the progression of MODS, the serum concentrations of all of these cytokines were altered. For example, the early-stage inflammatory cytokines, TNF and IL-1, increased sharply in the acute injury phase at 6-12 h after the zymosan-induced injury and subsequently fell. Then, HMGB1 levels increased at 24 h, followed by a substantial increase in IL-10 levels at 5 d. The levels of IL-2 and IL-12 decreased gradually at 24-48 h after injury. Thus, changes in the serum inflammatory mediators characterized by an increase in pro-inflammatory cytokines coincided with the first death peak at the early stage of disease. During the second period of high mortality (10-12 d), the levels of serum pro-inflammatory cytokines (TNF and IL-1), a late-stage inflammatory cytokine (HMGB1), and an inhibitory cytokine (IL-10) all significantly increased, while the T cells- and APC-derived cytokines (IL-2 and IL-12) were significantly reduced. The changes in serum inflammatory mediators clearly reflect the transition of the immune function from an excessive inflammatory response (immune activation) to the coexistence of immunosuppression and immune activation, which is consistent with the changes of splenic DC activity observed with disease progression.

It should be noted that in the stage of remission during which no deaths occurred, serum HMGB1 and IL-10 concentrations were markedly increased, while IL-2 and IL-12 concentrations decreased. HMGB1 has immunosuppressive effects, and IL-10 can inhibit the expression of co-stimulatory molecules on APCs [53], thereby inhibiting the cellular immune response. This coincides with reduced serum IL-2 and

IL-12 levels indicative of the typical serological characteristics of immunosuppression. Thus, the alleviation of the inflammatory response in remission did not result in disease alleviation, and may represent a potential signal of transition to immunosuppression. Under the immunosuppressive conditions in the late stage of disease, the pro-inflammatory cytokines, TNF and IL-1, significantly increased again, suggesting the incidence of endogenous bacterial translocation or opportunistic pathogen infection. This may be the reason that survivors of an early excessive inflammatory response ultimately died [54]. Therefore, paying close attention to changes in the serum pro-inflammatory and anti-inflammatory cytokines may help detect the signs of immune suppression early during disease progression, permitting timely intervention to prevent disease progression. The late-stage serological markers indicated the coexistence of immune activation and immune tolerance, which suggests that strategies to induce immune suppression or immune activation should not be adopted. Instead, an immunomodulatory strategy should be taken.

In summary, during the progression of MODS induced by intraperitoneal injection of zymosan, changes in the co-stimulatory and co-inhibitory molecules on the splenic DCs and in serum concentrations of various pro-inflammatory and anti-inflammatory cytokines were characterized by early immune activation and predominant immune tolerance in the late stage, which is closely related to disease progression. Dynamic monitoring of serum cytokine levels can provide early warning signs of the transition from excessive immune activation to immune tolerance, which will provide an important basis for timely and effective intervention.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jiangyang Lu, Department of Pathology, The First Affiliated Hospital of General Hospital of PLA, 51 Fu-Cheng Road, Beijing 100048, China. Tel: +86-10-68689159; Fax: +86-10-68689159; E-mail: Lujiangyang@yeab.net

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