CD204-positive monocytes and macrophages ameliorate septic shock by suppressing proinflammatory cytokine production in mice

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ABSTRACT

Sepsis is defined as a life-threatening multiorgan dysfunction caused by dysregulated inflammatory response to infection. It remains the primary cause of death from infection if not diagnosed and treated promptly. Therefore, a better understanding of the mechanism for resolving inflammation is needed. Monocytes and macrophages play a pivotal role not only in the induction but also in the suppression of inflammation. However, a tissue-resident macrophage subset that regulates a hyperinflammatory state during sepsis has not been explored. Here we show that CD204⁺ monocytes and/or macrophages rescued mice from endotoxin-induced septic shock. Serum and tissue proinflammatory cytokine levels were significantly upregulated in the absence of these cells. This study provided evidence that CD204⁺ monocytes and/or macrophages ameliorate septic shock by suppressing proinflammatory cytokine production.

1. Introduction

Sepsis is defined as a multiorgan dysfunction caused by a dysregulated host response to an infection [1,2]. Despite recent therapeutic innovations, it can turn into a life-threatening condition known as septic shock if not diagnosed and treated promptly. Therefore, a precise understanding of sepsis pathophysiology is needed.

Macrophages are considered to play a pivotal role not only in the induction but also in the resolution of inflammation [3–5]. This concept is based on the fact that macrophages exhibit immunologically polarized states when stimulated in vitro [6,7], that is, classically activated or M1 macrophages produce large amounts of reactive oxygen species and proinflammatory cytokines and contribute to killing pathogens. On the other hand, alternatively activated or M2 macrophages produce anti-inflammatory cytokines that may support resolution. Much effort has been exerted to elucidate the characteristics of tissue macrophages on the basis of criteria established in vitro. In many cases, however, macrophages in the human body exhibit a mixed phenotype having features of both M1 and M2. The exploration of immunoregulatory macrophages in vivo is under way.

To examine the kinetics of immunoregulatory macrophages in vivo, we used an M2 marker CD204/Macrophage scavenger receptor 1 (Msr1)/Class A scavenger receptor (SRA). This receptor was initially described as an acetylated low-density lipoprotein receptor [8] that is predominantly expressed by subsets of macrophages including tumor-infiltrating macrophages in human and mouse. The abundance of CD204⁺ macrophages is correlated with low five-year survival rates in breast cancer [9] or oral squamous cell carcinoma [10] patients, suggesting their role in suppressing anti-tumor immunity. However, the tissue distribution and the roles of CD204⁺ macrophages have not been analyzed in vivo.

In this study, we globally analyzed the distribution of CD204⁺ macrophages among various organs in mouse. By generating CD204-diphtheria toxin receptor (DTR) knock-in mice, we revealed elevated TNFα and IL-6 levels in the absence of CD204⁺ macrophages in a mouse model of sepsis. This finding suggests that CD204⁺ macrophages contribute to the resolution of hyperinflammatory response by suppressing proinflammatory cytokine production.

2. Materials and methods

2.1. Mice

C57BL/6J (7- to 12-week-old) mice were purchased from CLEA Japan, Inc. All mice were housed under specific-pathogen-free conditions in the Tokyo University of Pharmacy and Life Sciences animal facility. All experiments using the mice described herein were approved by Tokyo University of Pharmacy and Life Sciences Animal Use Committee.

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(L17-23, 25, L18-22, 23 and L19-20) and performed in accordance with applicable guidelines and regulations.

2.2. Generation of CD204-DTR mice

A genomic clone containing the Mr1 gene (encoding CD204 molecule) was obtained from BACPAC Resources CHORI (Oakland, CA, USA). To generate the targeting vector, the region from –5.4 to +6.5 kb relative to the transcriptional start site of the Mr1 gene was subcloned into the pBluescript II SK (+) vector. Then, an 8-bp fragment including the endogenous ATG start site in exon 1 was replaced with human DTR cDNA with a poly-A tail by a BAC recombineering technique. To allow selection for homologous recombinants, an FRT-flanked Neo cassette was cloned downstream of the human DTR gene. The thymidine kinase (TK) gene was inserted upstream of the FRT arm to select against random integrants. To generate CD204-DTR mice, B6/JN/1 ES cells were transfected with the linearized targeting vector by electroporation. G-418- and ganciclovir-resistant clones were screened for homologous recombination by Southern blot analysis with a 436-bp digoxigenin (DIG)-labeled DNA probe located outside the targeting vector using a DIG DNA Labeling Kit and Detection Kit (Roche, Basel, Switzerland). Germline chimeric mice were generated by aggregation methods. Chimeric mice with high embryonic stem cell contribution were crossed with C57BL/6J mice to produce CD204+/DTR mice. The CD204+/DTR mice were backcrossed to C57BL/6J mice for more than four generations, and wild-type (+/+) and heterozygous CD204-DTR (+/DTR) littermates were used for analysis.

2.3. Depletion of CD204+ cells

For systemic CD204+ cell depletion, CD204-DTR mice were injected intraperitoneally (i.p.) or intravenously (i.v.) with 500 ng/mouse of DT (Calbiochem, CA, USA or Sigma-Aldrich, MO, USA).

2.4. Sepsis model

For sepsis induction in mice, WT and CD204-DTR mice were injected i.v. with 2.5 mg/kg of LPS (O111:B4, Sigma-Aldrich) diluted in PBS.

2.5. ELISA

Serum TNFα and IL-6 levels were measured by ELISA MAX™ Standard Kits (BioLegend, CA, USA) according to the manufacturer’s protocols.

2.6. Total RNA extraction for quantitative reverse transcription PCR

Liver, lung, colon, and spleen were dissected from mice and soaked in RNA later Stabilization Solution (Thermo Fisher Scientific, MA, USA) for 24 h at 4°C. Total RNA from these organs was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) or a FavorPrep Total RNA Extraction Column (Favorgen, Pingtung County, Taiwan) according to the manufacturers’ protocols. For quantitative reverse transcription PCR (qRT-PCR), complementary DNA (cDNA) was synthesized using ReverTra Ace Ace (Toyobo, Osaka, Japan). qRT-PCR was performed on cDNA using a THERMUS SYBR qPCR Mix (Toyobo). Expression levels were normalized to 18s ribosomal RNA. Primer sequences were as follows: IL-6 (Fwd) 5′-CTGGAGTACCATAGCTACC-3′, (Rev) 5′-CTGTATAGAGACAGTGGA-3′; TNFα (Fwd) 5′-AACCTCACACTGATGACCT-3′, (Rev) 5′-GAGTAGACAAAGTTGACACCC-3′; IL-1β (Fwd) 5′-GGATGAGGACTGACACCC-3′, (Rev) 5′-AGCTCATATGGGTCCGA-3′; and 18s RNA: (Fwd) 5′-CGGACAGGATTACAGTTG-3′, (Rev) 5′-CAAATGCTCCACCAACTAA-3′.

2.7. Antibodies for flow cytometry analysis

Anti-Ly6G (1A8), anti-Ly6C (HK1.4), anti-CD45.2 (104), anti-NK1.1 (PK136), anti-CD19 (6D5), anti-Thy1.2 (53-2.1), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-CD64 (XS4-5/7.1), and anti-CD16/32 (93) were purchased from BioLegend. Anti-CD11c (HL3) and anti-SiglecF (E50-2440) were purchased from BD Biosciences (CA, USA). Anti-CD115 (AS98) and anti-MHC class II (M5/114.15.2) were purchased from Thermo Fisher Scientific. Anti-CD204 (REA148) and REA control (REA293) were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). Dead cells were excluded by DAPI (Dojindo, Kumamoto, Japan).

2.8. Preparation of immune cells for flow cytometry analysis

Immune cells in peripheral blood, bone marrow [11], lung [12], and colon [13] were prepared as described previously. Liver minced with scissors was digested with digestion mixture (100 μg/ml Liberase TM (Roche)/5 μg/ml DNase I (Sigma-Aldrich)/1XHBSS) for 25 min at 37°C with gentle shaking. Digested liver was passed through a 70 μm Cell Strainer (BD Biosciences). After centrifugation at 300xg for 4 min at 4°C, the supernatant was transferred to a new tube and centrifuged again at 100xg for 2 min to remove non-immune cells. This process was repeated five times. The final supernatant was centrifuged at 300xg for 4 min. Red blood cells (RBCs) within the pellet were lysed with 2 ml of lysing buffer (Pharm Lyse, BD Biosciences) and then diluted with FACS staining buffer (0.5% BSA/2 mM EDTA/PBS). The spleen was cut into four pieces and squeezed over a 70 μm Cell Strainer that was placed on a 6-cm dish. The filtrate was harvested in a 15 ml tube and centrifuged at 500g for 5 min at 4°C. RBCs within the pellet were lysed with 2 ml of lysing buffer and diluted with FACS staining buffer.

2.9. Statistical analysis

The survival rate was compared between two groups using the log-rank test. Data were analyzed either by analysis of variance (ANOVA) followed by multiple comparison or by the unpaired t-test with GraphPad Prism (GraphPad Software, CA, USA). P < 0.05 was considered statistically significant.

3. Results

3.1. Identification of CD204+ immune cell types in vivo

First, we explored CD204+ immune cell types in several major organs and peripheral blood by flow cytometry. In the steady state, CD204 was expressed by Kupffer cells (Fig. 2 A); Ly6C+ monocytes in spleen (Fig. 1 A). Both CD11c+CD11b+CD64+ alveolar macrophages and CD11c+CD11b+MHC class II+ interstitial macrophages in lung were CD204-negative (Fig. 1 A). In bone marrow and peripheral blood, Ly6C+CD115hi monocytes and Ly6C+CD115lo monocytes were CD204+ but neutrophils, eosinophils, T cells, B cells, NK cells, and NKT cells did not express CD204 (Fig. 1 A, B and C). These results indicate that CD204 expression is strictly limited to monocyte-macrophage lineage cells among leukocytes.

3.2. Generation of CD204-DTR mice

To analyze the function of CD204+ monocytes and macrophages, we generated mice that harbor hDTR gene at the CD204 gene locus (CD204-DTR mouse, Fig. 2 A). The targeting construct in CD204-DTR mouse genome was confirmed by Southern blot analysis (Fig. 2 B). In these mice, we examined the depletion of CD204+ cells by intraperitoneal diphtheria toxin (DT) administration. Twenty-four hours after DT injection, Kupffer cells (Fig. 3 A); Ly6C+CD115hi monocytes and...
Ly6C<sup>lo</sup>CD115<sup>hi</sup> monocytes in spleen (Fig. 3B); macrophages and monocytes in colon (Fig. 3C); and Ly6C<sup>hi</sup>CD115<sup>hi</sup> monocytes and Ly6C<sup>lo</sup>CD115<sup>hi</sup> monocytes in bone marrow and peripheral blood (Fig. 3D and E) were substantially decreased in DT-injected CD204-DTR mice compared with PBS-injected CD204-DTR mice. On the other hand, neutrophils, eosinophils, and lymphocytes were not depleted by DT administration in any of the organs analyzed and in peripheral blood (Fig. 3). Notably, lung macrophages that are CD204-negative did not decrease after DT injection (Fig. 3H). These results indicate that CD204<sup>+</sup> monocytes and macrophages can be specifically depleted by DT.

Fig. 1. Identification of CD204-expressing cells in various tissues. Flow cytometry analysis of various tissues (A), bone marrow (B), and peripheral blood (C). Stained with CD204 antibody is the shaded area in red. Stained with isotype control antibody is the shaded area in blue. Mφ, macrophage; KC, Kupffer cell; Mo, monocyte; Neu, neutrophil; Eos, eosinophil; AM, alveolar macrophage; IM, interstitial macrophage; B, B cell; T, T cell; NKT, natural killer T cell; NK, natural killer cell; MHCII, major histocompatibility complex II; SSC-A, side scatter. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

Fig. 2. Generation of CD204-DTR mice. (A) Schematic diagram of the CD204-DTR targeting construct. Exons of Msr1 gene (encoding CD204 molecule) are indicated by solid boxes. Human DTR (hDTR) cDNA, FRT-flanked neomycin resistance gene (Neo), and thymidine kinase gene (TK) are indicated by open boxes. The probe used for Southern blot analysis is indicated by a solid line together with the predicted hybridizing fragments. (B) Southern blot analysis of wild-type (+/+ ) and CD204-DTR (+/CD204<sup>DTR</sup>) mouse genome using BamHI digest in combination with the indicated probe.

Ly6C<sup>lo</sup>CD115<sup>+</sup> monocytes in spleen (Fig. 3B); macrophages and monocytes in colon (Fig. 3C); and Ly6C<sup>lo</sup>CD115<sup>+</sup> monocytes and Ly6C<sup>lo</sup>CD115<sup>+</sup> monocytes in bone marrow and peripheral blood (Fig. 3D and E) were substantially decreased in DT-injected CD204-DTR mice compared with PBS-injected CD204-DTR mice. On the other hand,
administration in CD204-DTR mice.

3.3. CD204+ cells protect mice from sepsis by suppressing proinflammatory cytokine production

Having established an in vivo CD204+ cell depletion system, we aimed to reveal the role of CD204+ cells under an inflammatory condition. To this end, we injected DT into WT and CD204-DTR mice 24 h before systemic LPS injection. The injection of LPS caused hypotension and hypothermia characteristic of murine septic shock [14,15]. To our surprise, the survival rates of DT-injected CD204-DTR mice significantly decreased compared with those of DT-injected WT mice (Fig. 4A) or PBS-injected CD204-DTR mice (Fig. 4B). To investigate the reason for the increased mortality in CD204-DTR mice, we measured serum cytokine concentrations at different time points after LPS injection. Serum TNFα and IL-6 concentrations rapidly increased in the first 4 h and returned to basal levels as late as 16 h after LPS injection. We found that serum levels of TNFα at 2 h and IL-6 at 2, 4 h and 16 h were upregulated in CD204-DTR mice compared with WT mice (Fig. 4C). These results suggest that CD204+ monocytes and/or macrophages suppress acute inflammatory response induced by LPS injection.

We further explored organs that produce increased levels of proinflammatory cytokines in CD204-DTR mice. qRT-PCR of tissues revealed that TNFα and IL-6 mRNA levels in spleen and TNFα mRNA level in lung were significantly upregulated in CD204-DTR mice compared with WT mice (Fig. 4D). IL-1β mRNA level was also upregulated in the lung and spleen although the difference was not statistically significant. Collectively, these results suggest that CD204+ cells rescued mice from sepsis by regulating hyperinflammatory response.

4. Discussion

We were able to clarify two things in this study. Firstly, we revealed that CD204 expression is restricted to macrophages and monocytes in peripheral blood and some organs. Secondly, by depleting CD204+ cells in vivo in CD204-DTR mice, we demonstrated that CD204+ monocytes and/or macrophages protect mice from septic shock by suppressing TNFα and IL-6 production.

Drugs focusing on the regulation of hyperinflammatory immune response are being investigated for the treatment of septic shock [16]. Monocytes and macrophages are known to play important roles in resolving inflammation. We have recently reported that Ym1+ Ly6C+ monocytes expand during the late phase of inflammation in bone marrow. Ym1+ monocytes migrate to peripheral tissue where they promote tissue regeneration in a mouse colitis model [11]. Similarly, in an acute mucosal Toxoplasma gondii infection model, inflammatory...
monocytes acquire a tissue-specific immunoregulatory phenotype that is associated with the increased production of lipid mediator prostaglandin E2 [17]. These findings suggest that some monocytes can suppress inflammatory response during inflammation. However, a subset of tissue resident macrophages that regulate immune response during sepsis was not identified.

We have shown that the transient and selective depletion of CD204+ cells resulted in increased serum TNFα and IL-6 concentrations in LPS-induced septic shock. Moreover, the survival rate of septic mice was significantly decreased in the absence of CD204+ cells. It was reported

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Fig. 4. Phenotype of CD204-DTR mice in septic condition. WT and CD204-DTR mice were intravenously injected with DT (500 ng/mouse) and LPS (2.5 mg/kg). (A) Survival rate of DT-injected WT and CD204-DTR mouse (n = 6), log-rank test. (B) Survival of PBS- or DT-injected CD204-DTR mice. (C) Measurement of serum concentration of inflammatory cytokines. Peripheral blood obtained from these mice at the indicated hours after LPS injection (n = 4), two-way ANOVA with multiple comparisons. (D) Measurement of gene expression levels in various tissues by qRT-PCR. Liver, lung, spleen, and colon were collected from these mice 2 h after LPS injection (n = 4), Mann-Whitney t-test. Average values are shown with SD. *P < 0.05, ****P < 0.0001.
that serum IL-6 concentration positively correlates with the severity of sepsis in human [18,19]. In addition, Marino’s group demonstrated that TNFα-deficient mice are more resistant to LPS-induced sepsis than WT counterparts [20]. However, cells responsible for dysregulated production of pro-inflammatory cytokines in sepsis were up to now not identified. These reports and our findings indicate that CD204+ monocytes and macrophages regulate host immune response by suppressing proinflammatory cytokine production. Tissue mRNA levels of TNFα and IL-6 were enhanced in spleen and lung of CD204+ cell-depleted mice (Fig. 4D). However, we were unable to identify the cellular sources of the proinflammatory cytokines during sepsis. A recent study reported that marginal zone B cells in spleen produce IL-6 in the septic condition [21]. In addition, pulmonary endothelial cells, myocardial cells, and fibroblast cells can also produce TNFs and IL-6 [22]. These findings suggest that non-immune cells as well as B cells are responsible for the upregulated proinflammatory cytokine levels during sepsis. It remains to be determined whether CD204+ macrophages produce larger amount of cytokines in the absence of CD204+ macrophages. Exploration of the cellular source of TNFs and IL-6 during sepsis may yield novel therapeutic targets for septic shock. Although CD204 is predominantly expressed on macrophages and monocytes, in vitro observations suggested ectopic CD204 expression on endothelial cells [23], lung epithelial cells [24], and fibroblasts [25]. Therefore, we cannot exclude the possibility that CD204+ non-parenchymal cells also contribute to the resolution of septic shock.

In conclusion, our study highlights the possibility that CD204+ monocytes and/or macrophages ameliorate septic shock by directing the TNFα production of pro-inflammatory cytokines in sepsis were up to now not identified. These reports and our findings indicate that CD204+ monocytes and macrophages regulate host immune response by suppressing proinflammatory cytokine production. Tissue mRNA levels of TNFα and IL-6 were enhanced in spleen and lung of CD204+ cell-depleted mice (Fig. 4D). However, we were unable to identify the cellular sources of the proinflammatory cytokines during sepsis. A recent study reported that marginal zone B cells in spleen produce IL-6 in the septic condition [21]. In addition, pulmonary endothelial cells, myocardial cells, and fibroblast cells can also produce TNFs and IL-6 [22]. These findings suggest that non-immune cells as well as B cells are responsible for the upregulated proinflammatory cytokine levels during sepsis. It remains to be determined whether CD204+ macrophages produce larger amount of cytokines in the absence of CD204+ macrophages. Exploration of the cellular source of TNFs and IL-6 during sepsis may yield novel therapeutic targets for septic shock. Although CD204 is predominantly expressed on macrophages and monocytes, in vitro observations suggested ectopic CD204 expression on endothelial cells [23], lung epithelial cells [24], and fibroblasts [25]. Therefore, we cannot exclude the possibility that CD204+ non-parenchymal cells also contribute to the resolution of septic shock.

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Declaration of competing interest

The authors declare that no competing interests exist.

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