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Novel Sca-1$^+$ macrophages modulate the pathogenic progress of endotoxemia

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ABSTRACT

Macrophages are important innate immune cells that play crucial roles in inflammatory responses. Accumulating evidence has demonstrated macrophage heterogeneity based on biomarkers, functions, and localization. Here, we report a novel stem cell antigen-1 (Sca-1)-positive macrophage population induced in the pathological conditions caused by lipopolysaccharide (LPS). Sca-1 is only upregulated in macrophages but not in monocytes and neutrophils upon LPS injection. Sca-1$^+$ macrophages develop from resident peritoneal macrophages. LPS-induced Sca-1$^+$ macrophage generation was partly blocked by anti−IFN−γ antibody, suggesting a role of IFN-γ in the process. LPS-stimulated production of IL-6, TNF-α, and CCL2 is significantly lower in Sca-1$^+$ macrophages compared to their counterpart Sca-1$^-$ macrophages. Depletion of Sca-1$^+$ macrophages using anti-Sca-1 antibody significantly increased survival rate and reduced lung and kidney damage in an LPS-induced sepsis model. Taken together, we discovered a novel population of Sca-1$^+$ macrophages in LPS-induced septic conditions.

1. Introduction

Macrophages, large phagocytes located in every tissue, are essential for immune surveillance and regulation of the homeostatic environment [1]. Tissue resident macrophages develop from the yolk sac and basally exist in homeostatic conditions [2]. In the mononuclear phagocytic system, macrophage precursors are released from the bone marrow as monocytes and circulate to various tissues where differentiated macrophages show defense activities against encroaching microbes [3]. Macrophages mediate innate defense activity by their phagocytic, antigen presentation and cytokine secretion activity. Peritoneal macrophage subsets are divided by their morphology and origin. Large peritoneal macrophages (LPMs) express high levels of F4/80 and CD11b, but small peritoneal macrophages (SPMs) have low levels of F4/80 and CD11b [4]. Each subset is derived from different origins. LPMs are originated from progenitors of the yolk sac [5] and SPMs are developed from hematopoietic stem cells (HSCs) in the bone marrow [6,7].

Macrophages are dynamic and heterogeneous cells. In specific conditions, macrophages are polarized to the M1 or M2 type. The characteristics of M1 and M2 are very different, including surface receptors, profiles of cytokines and chemokines, and immune responses [8,9]. M1 macrophages have pro-inflammatory characteristics but M2 macrophages have anti-inflammatory characteristics [10,11]. Some subtypes exist for M2 macrophages, but the subtypes of M1 macrophages have not been fully studied yet. In addition to M1 and M2, many reports have demonstrated macrophage heterogeneity in different tissues by focusing on phenotypic diversity and functions. Splenic CD68$^+$ macrophages transport antigens to B and T cells [12], and alveolar macrophages which express CD11b^{low}F4/80^{low} provide defense to mycobacterial infection in the lungs [13]. Previous reports demonstrated that extreme stress such as severe infection or cancer can generate new populations of leukocytes that are not observed under healthy normal conditions [14,15]. Therefore, it would be important to reveal unidentified macrophage populations that play roles in sepsis.

Stem cell antigen-1 (Sca-1) is a marker of mouse hematopoietic stem cells (HSCs) [16]. Previous studies showed that some mature immune cells such as plasmacytoid dendritic cells (pDCs) [17], B lymphocytes [18], monocytes [19], and Gr-1$^+$ myeloid cells [20] also express Sca-1 on their surface in response to bacterial infection. Unlike Sca-1$^-$ pDCs, Sca-1$^+$ pDCs are defective in IFN-α production in response to TLR9 stimulation [17]. Sca-1$^+$ B cells are a major source of antibody production compared to Sca-1$^-$ B cells,

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suggesting an important role for Sca-1 in B cell differentiation [18]. Previously, we demonstrated that Sca-1$^+$ Gr-1$^+$ myeloid cells drive mortality in a bacterial infection model [20]. However, no reports have demonstrated the expression of Sca-1 in macrophages. In this study, we identified and characterized Sca-1$^+$ macrophages that are generated in an LPS-induced experimental sepsis model.

2. Materials and methods

2.1. Animals and LPS-induced sepsis model

Wild type (WT) C57BL/6 N male, 8-week-old mice, purchased from Orient Bio Inc. (Seongnam, Korea), were given an intraperitoneal injection of LPS (2 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) for immune cell analysis. After 12 h, peritoneal exudate cells (PECs) were collected and analyzed with flow cytometry. To monitor the survival rate and septic pathology, WT mice were intraperitoneally injected with 30 mg/kg of LPS. Survival was monitored daily for 7 days and tissues were sampled after 12 h. All animal experiments were performed in accordance with the Korea Food and Drug Administration guidelines. Protocols were approved by the Institutional Review Committee for Animal Care and Use at Sungkyunkwan University (Suwon, Korea).

2.2. Antibodies and flow cytometry

Antibodies to CD11b (M1/70), F4/80 (BM8), Ly-6g (1A8), Ly-6c (HK1.4), Sca-1 (D7), CD80 (B7-1), CD86 (B7-2) and MHC II (IA/IE) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Receptor expression was analyzed using a BD FACS Canto II flow cytometer and FlowJo software program (BD Biosciences, San Jose, CA, USA).

2.3. Sorting of mouse macrophages from LPS-injected model

WT C57BL/6 N male, 8-week-old mice, were injected with 2 mg/kg of LPS. PECs were collected after 12 h and stained with anti-CD11b, anti-F4/80, and anti-Sca-1. Sca-1$^-$ and Sca-1$^+$ macrophages were sorted using a BD FACSaria III (BD Biosciences).

2.4. In vitro stimulation

Mouse peritoneal cells (1×10^6 cells) were stimulated with LPS (100 ng/ml) or IFN-γ (40 ng/ml) (R&D system Inc., Minneapolis, MN, USA), for 12 h at 37 °C in 5% CO_2 in DMEM medium (Thermo Fisher Scientific) including 5% FBS. αIFN-γ (10 μg/ml) (Bio X Cell, Lebanon, NH, USA) was pre-treated for 2 h prior to LPS or IFN-γ. Cells were stained with antibodies to CD11b, F4/80, and Sca-1.

2.5. Phagocytosis assay

Sorted Sca-1$^-$ and Sca-1$^+$ macrophages were incubated with dextran sulfate (Sigma-Aldrich), PE-latex beads (Cayman Chemical, Ann Arbor, MI, USA), or fluorescein isothiocyanate-*S. aureus* (Thermo Fisher Scientific) for 2 h at 37 °C in 5% CO_2 in serum-free DMEM medium. Control samples were incubated at 4 °C in the same conditions.

2.6. Bactericidal assay

Sorted Sca-1$^-$ and Sca-1$^+$ macrophages (1×10^6 cells) were incubated with *Escherichia coli* (*E. coli*) (1×10^7 cells) at for 1 h at 37 °C in 5% CO_2 in RPMI 1640 medium (Thermo Fisher Scientific) including 5% FBS. After incubation, 40 μg/ml of gentamicin (Sigma-Aldrich) was treated for 4 h to remove the remained pathogens. Whole cells were centrifuged at 15,000 RPM for 5 min and supernatant was eliminated. Cells were lysed with autoclaved distilled water and lysates were plated on LB agar dishes for 24 h at 37 °C.

2.7. Measurement of cytokines and chemokines

Sorted Sca-1$^-$ and Sca-1$^+$ macrophages (2×10^6 cells) were incubated with or without 100 ng/ml of LPS (Sigma-Aldrich) in DMEM medium including 5% FBS at 37 °C in 5% CO_2 incubator. After 24 h, supernatant was collected and applied to measure the levels of cytokines and chemokines by enzyme-linked immunosorbent assay (ELISA) kits, purchased from Thermo Fisher Scientific, according to the manufacturer's instructions.

2.8. Quantitative polymerase chain reaction (qPCR) analysis

The levels of gene expression were measured by qPCR using the Rotor-Gene Q (2 plex on PC) instrument from QIAGEN (Hilden, Germany) with SYBR Green qPCR Mix purchased from Biofact (Daejeon, Korea) according to a previous report [20]. Total RNA was extracted from sorted Sca-1$^-$ and Sca-1$^+$ macrophages using TRIzol Reagent from Life Technology (Carlsbad, CA, USA). cDNA was generated using Maxime RT Premix purchased from iNtRON Biotechnology (Seongnam, Korea). The primers used for qPCR are as follow: *P22 phox*-forward, 5′-GTGCTCATCTGTCTGCTGGA-3'; *P22 phox*-reverse, 5′-TAGGCTCAATGGGAGTCCAC-3'; *P67 phox*-forward, 5′-GACCTTAAAGAGGGCCTTGACG-3'; *P67 phox*-reverse, 5′-ATGCCAACTGCTCTTCTGCT-3'; *Gp91 phox*-forward, 5′-CAAGATGGAGGTGGGACAGT-3'; *Gp91 phox*-reverse, 5′-GCTTATCACAGCCACAAGCA-3'; *Cxcr1*-forward, 5′-AATCTGTTGTGGCTTCACCCA-3′; *Cxcr1*-reverse, 5′-GCTATCTTCCGCCAGGCATAT-3′; *Cxcr2*-forward, 5′-AGCAAACACCTCTACTACCCTCTA-3′; *Cxcr2*-reverse, 5′-GGGCTGCATCAATTCAAATACCA-3′; *Tlr2*-forward, 5′-GCTCCAGGTCTTTCACCTCTATTC-3'; *Tlr2*-reverse, 5′-TCCAGCAGGAAAGCAGACTCGCTTA-3'; *Tlr4*-forward, 5′-ATGGCATGGCTTACACCACC-3'; *Tlr4*-reverse, 5′-GAGGCCAATTTTGTCTCCACA-3'; *Marco*-forward, 5′-CGAATCTTTCCAACGCGTCC-3'; *Marco*-reverse, 5′-TCTCTGTGCCCCGACAATTC-3'; *Fizz1*-forward, 5′-CCCTTCTCATCTGCATCTCCC-3'; *Fizz1*-reverse, 5′-AAGCACACCCAGTAGCAGTC-3'; *Gapdh*-forward, 5′-TCCACCACCCTGTTGCTGTA-3′; and *Gapdh*-reverse, 5′-AATGTGTCCGTCGTGGATCT-3′. For qPCR analysis, 50 PCR cycles were performed at 94 °C (denaturation, 30 s), 60 °C (annealing, 30 s), and 72 °C (extension, 1 min). The data were normalized by using the $2^{-(\Delta\Delta Ct)}$ method against *Gapdh* expression as an internal control for the calculation of ΔCt values for each gene.

2.9. In vivo administration of anti-Sca-1 antibody

Purified anti-Sca-1 antibody (clone: E13) purchased from Bio X cell was used to eliminate the Sca-1$^+$ macrophages. Isotype control (rat IgG2a) or anti-Sca-1 antibody (4.55 mg/kg) was intraperitoneally injected into mice at 2 h after LPS injection in LPS-induced sepsis models.

2.10. Hematoxylin and eosin (H&E) staining

The lungs and kidneys from mice were harvested at 16 h after LPS injection (30 mg/kg) for paraffin sectioning. The tissues were fixed in 10% formalin for 24 h at room temperature, embedded into paraffin and cut into 4-μm-thick slices, deparaffinized, and rehydrated using xylene, ethanol, and water by standard methods. H&E staining was conducted as described previously [20].

2.11. Statistical analysis

All results are expressed as the means ± s.e.m. Statistical analysis was performed using an unpaired two-tailed Student's *t*-test. Survival data was analyzed using the log-rank test. The *P* values $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant.

3. Results

3.1. Generation of Sca-1$^+$ macrophages in an LPS-induced sepsis model

To examine the effects of LPS on the generation of Sca-1$^+$ myeloid leukocytes *in vivo*, we intraperitoneally injected 2 mg/kg of LPS into WT mice, and PECs were collected after 12 h for FACS analysis (Fig. 1A). While there were no Sca-1$^+$ macrophages in the PECs collected from WT mice, Sca-1$^+$ macrophages were detected from LPS-injected mice (Fig. 1B). LPS injection did not elicit the generation of Sca-1$^+$ neutrophils (Ly6g$^+$) or Sca-1$^+$ monocytes (Ly6c$^+$) (Fig. 1B). Sca-1$^+$ macrophage generation was significantly induced by 1–2 mg/kg LPS, and the population was apparent at 6 h, peaked at 12 h and then decreased (Fig. 1C). These results show that previously unidentified Sca-1$^+$ macrophages are generated from peritoneal fluid in response to LPS administration in mice.

3.2. The Sca-1$^+$ macrophages induced by LPS are derived from LPMs, and not SPMs

We found novel Sca-1$^+$ macrophages in the LPS-injected peritoneum. Peritoneal macrophages of a pathogenic model are composed of resident LPMs and recruited SPMs [4]. Therefore, we examined two types of cells, resident peritoneal macrophages and bone marrow progenitors, to determine which ones are the progenitors of the Sca-1$^+$ macrophages. Resident peritoneal macrophages and total bone marrow cells were isolated from WT mice with phosphate buffered saline (PBS). Stimulation of resident peritoneal cells with 100 ng/ml of LPS for several lengths of time (30 min, 3 h, 16 h, and 24 h) results in Sca-1$^+$ macrophage generation, in a time-dependent manner. LPS significantly elicited Sca-1$^+$ macrophage generation at 16 and 24 h after LPS stimulation (Fig. 2A). However, stimulation of total bone marrow cells with 100 ng/ml of LPS did not generate Sca-1$^+$ macrophages (Fig. 2B). These results suggest that Sca-1$^+$ macrophages can be derived from the resident peritoneal macrophages, LPMs.

3.3. IFN-γ is required to generate Sca-1$^+$ macrophages

Previous reports demonstrated that Sca-1 level is strongly upregulated by type I and type II IFN in HSCs [21]. We also reported that IFN-γ signaling is essential in the upregulation of Sca-1 in Gr-1$^+$ myeloid cells in a *Staphylococcus aureus* (*S. aureus*) infection model [20]. Based on these previous reports [20,21], we tested the effects of IFN-γ on the generation of Sca-1$^+$ macrophages in an LPS-induced sepsis model. Stimulation of resident peritoneal macrophages, LPMs, with 100 ng/ml of LPS for 16 h generated Sca-1$^+$ macrophages, which was significantly decreased by treatment with anti-IFN-γ antibody (Fig. 2C). We also found that stimulation of resident peritoneal macrophages with 40 ng/ml of recombinant IFN-γ for 16 h also generated Sca-1$^+$ macrophages, which was also blocked by anti-IFN-γ antibody (Fig. 2C). These results suggest that IFN-γ is necessary to generate Sca-1$^+$ macrophages in the LPS-induced sepsis model. Stimulation of bone marrow progenitors, with 100 ng/ml of LPS or 40 ng/ml of recombinant IFN-γ for 16 h failed to generate Sca-1$^+$ macrophages (Fig. 2D).

3.4. Sca-1$^+$ macrophages secrete less amounts of cytokines and CCL2 compared to the Sca-1$^-$ counterparts

Since we discovered a previously unidentified novel population of peritoneal macrophages in an LPS-induced sepsis model, we compared the functional activities of Sca-1$^+$ macrophages and the Sca-1$^-$ counterparts. Regulation of the production of cytokines and chemokines in macrophages is very important to modulate inflammatory responses caused by infection or injury [9]. After sorting the Sca-1$^+$ macrophages and Sca-1$^-$ counterparts from LPS-

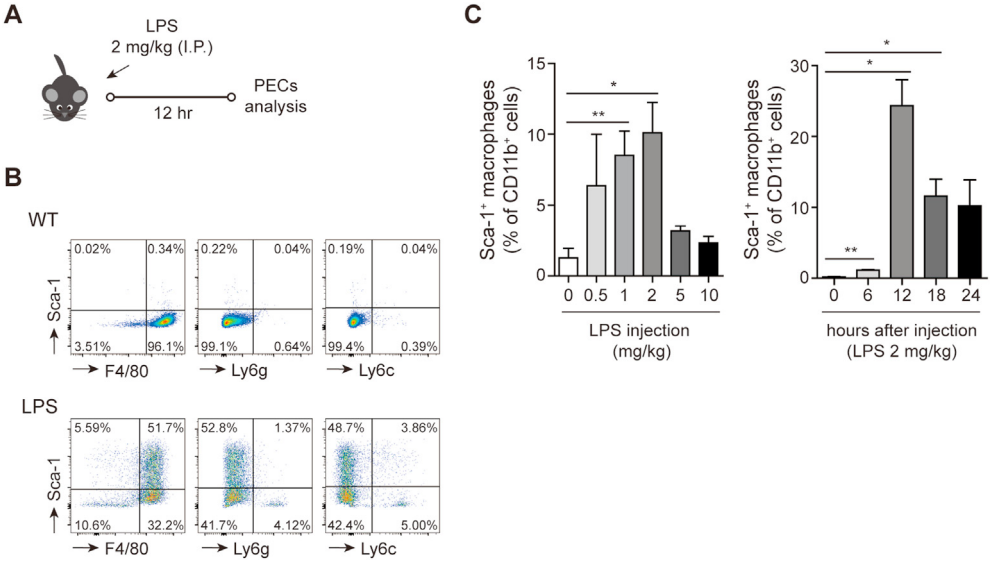

Fig. 1. Sca-1$^+$ macrophages are generated in an LPS-induced sepsis model. (A) A graphical protocol of the LPS-induced sepsis model for PECs analysis. (B) CD11b$^+$ cells of PECs collected from WT or LPS-injected mice at 12 h post-injection were stained with anti-F4/80, anti-Ly-6g, anti-Ly-6c, and anti-Sca-1. (C) Percentages of Sca-1$^+$ macrophages at 12 h post-injection from several different dosages (0, 0.5, 1, 2, 5, 10 mg/kg) of LPS-injected mice (left) and at different time points (0, 6, 12, 18, 24 h) from 2 mg/kg of LPS-injected mice (right). The data are representative of three independent experiments (B). Data are expressed as the mean ± s.e.m. (n = 3) (C). $*P < 0.05$ and $**P < 0.01$ by Student's *t*-test.

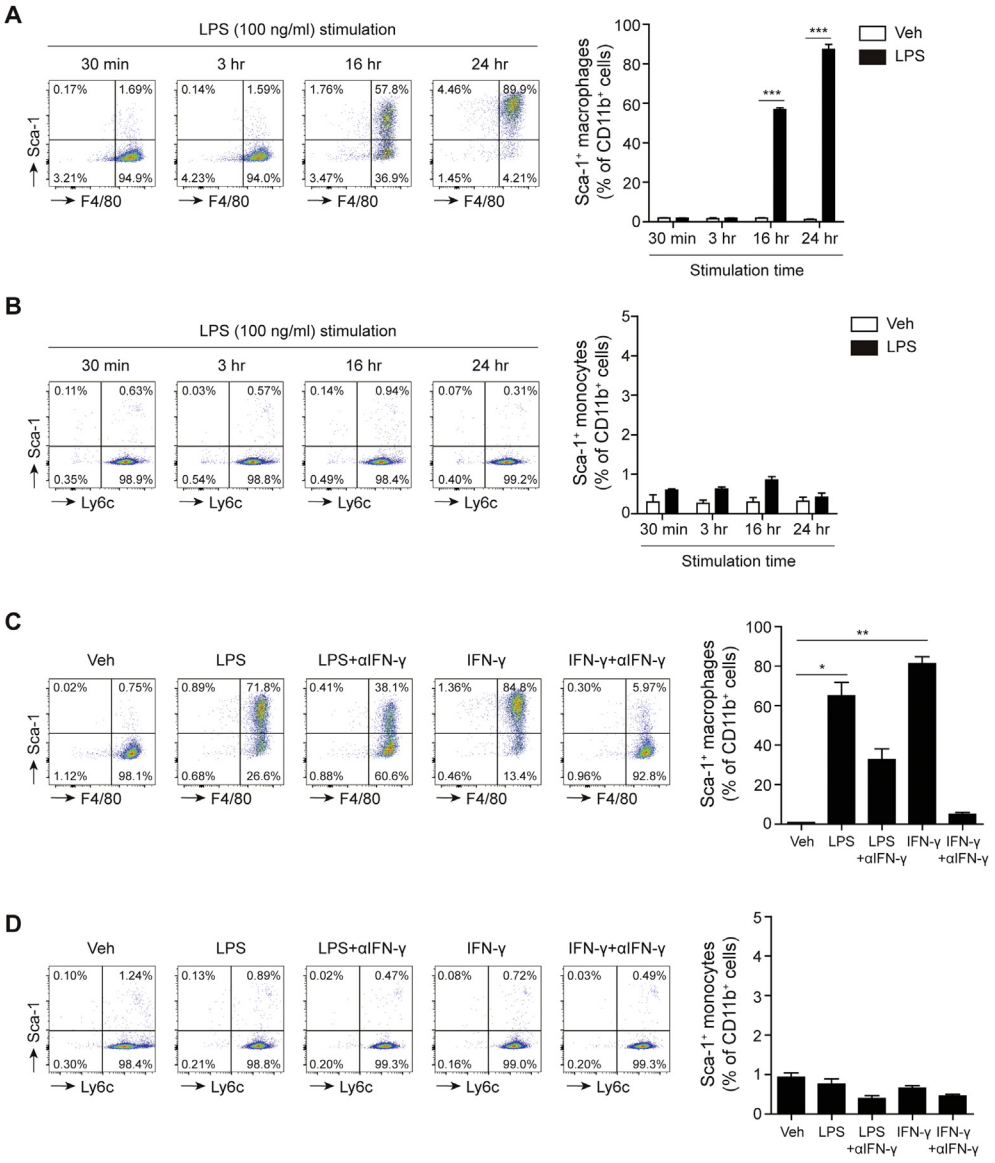

Fig. 2. Sca-1$^+$ macrophages develop from resident peritoneal macrophages with IFN-γ signaling. (A) Resident peritoneal cells or (B) bone marrow cells of WT mice were stimulated with 100 ng/ml of LPS for 30 min, 3 h, 16 h, and 24 h. After stimulation, CD11b$^+$ cells were stained with anti-F4/80, anti-Ly-6c and anti-Sca-1 antibodies (A left, B left). Percentages of Sca-1$^+$ macrophages from resident peritoneal cells (A right) and bone marrow cells (B right) after stimulation were quantified. (C) Resident peritoneal cells or (D) bone marrow cells of WT mice were stimulated by 100 ng/ml of LPS or 40 ng/ml of recombinant IFN-γ, with or without αIFN-γ. Stimulated CD11b$^+$ cells were stained with anti-F4/80, anti-Ly-6c and anti-Sca-1 antibodies (C left, D left). Percentages of Sca-1$^+$ macrophages from resident peritoneal cells (C right) and bone marrow cells (D right) 12 h after stimulation were quantified. The data are representative of three independent experiments (A left, B left, C left, D left). Data are expressed as the mean ± s.e.m. (n = 3) (A right, B right, C right, D right). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by Student's t-test.

induced sepsis model mice with a flow cytometer, we compared their capacity to produce several cytokines (IL-6, IL-1β, TNF-α, IFN-γ, IL-10) and a chemokine (CCL2). Sca-1$^+$ macrophages secrete less amounts of IL-6, TNF-α and CCL2 basally as well as in response to LPS compared to their Sca-1$^-$ counterparts (Fig. 3A).

Macrophages perform innate defense activity by phagocytosis of invading pathogens [2]. Here, we assessed phagocytic activity in Sca-1$^-$ and Sca-1$^+$ macrophages using dextran, latex beads, and *S. aureus* labeled with fluorescent dyes. Engulfment of dextran, latex bead, and *S. aureus* was similar in Sca-1$^-$ and Sca-1$^+$ macrophages (Fig. 3B). We also measured the bactericidal activity of Sca-1$^-$ and Sca-1$^+$ macrophages using co-culture of the two macrophages with *E. coli*. Colony-forming units (CFUs) analyses show that bactericidal activity of Sca-1$^+$ macrophages is slightly lower than that of Sca-1$^-$ macrophages, but there is no significant difference between the two populations (Fig. 3C). Macrophages are well-known antigen-presenting cells. We compared the expression of major histocompatibility complex class II (MHC II), and co-stimulatory molecules such as CD80 and CD86 with flow cytometer. Sca-1$^-$ and Sca-1$^+$ macrophages express very similar levels of MHCII, CD80 and CD86 (Fig. 3D).

Reactive oxygen species (ROS) are important weapons to remove invading pathogens from macrophages [22]. We compared the expression levels of components of ROS-producing machinery (*P22 phox*, *P67 phox*, and *Gp91 phox*). mRNA levels of *P22 phox*, *P67 phox*, and *Gp91 phox* are similar in Sca-1$^-$ and Sca-1$^+$ macrophages (Fig. 3E left). The expression levels of chemokine receptor (*Cxcr1* and *Cxcr2*), toll-like receptor (TLR) (*Tlr2* and *Tlr4*), and M1- or M2-related genes (*Marco* and *Fizz1* respectively [23]) are important characteristics to define macrophage sub-populations [24].

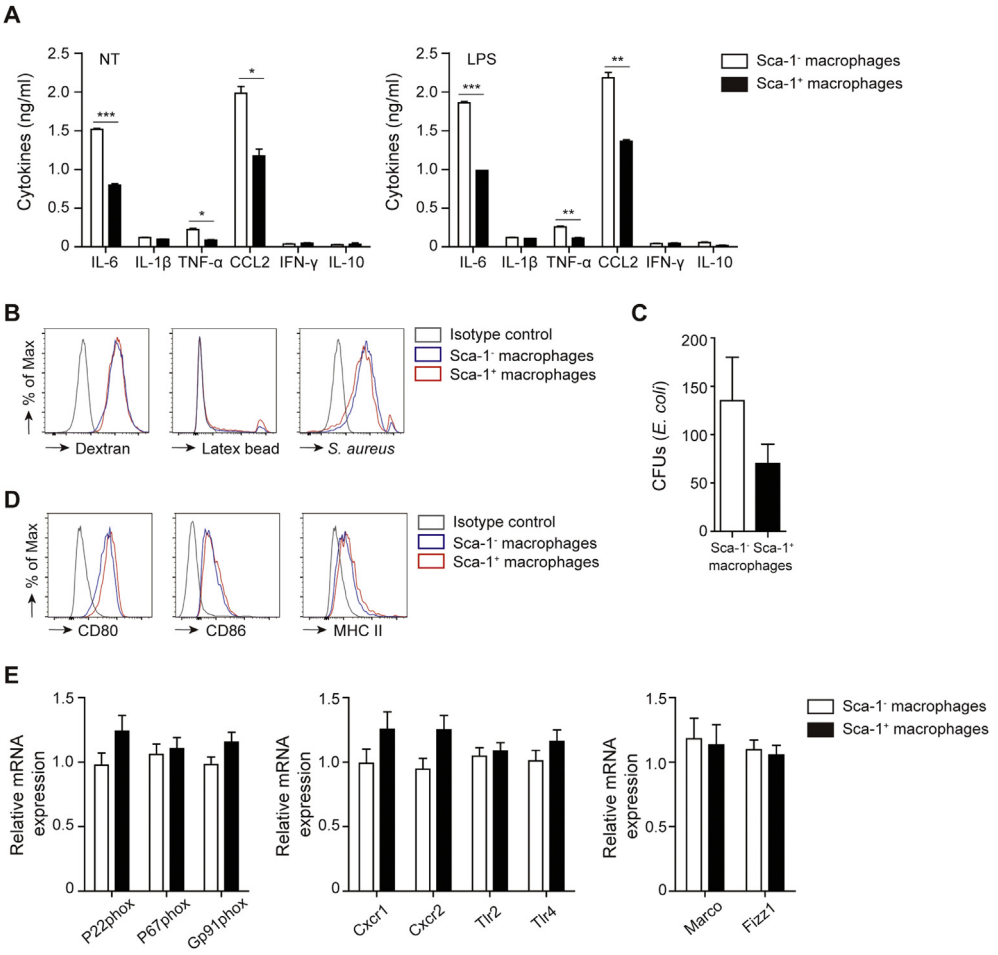

Fig. 3. Sca-1$^+$ macrophages release less cytokines and CCL2 than Sca-1$^-$ macrophages. (A) The levels of secreted IL-6, IL-1β, TNFα, CCL2, IFN-γ, and IL-10 were measured from conditioned medium of Sca-1$^-$ and Sca-1$^+$ macrophages cultured for 24 h, stimulated with (right) or without (left) 100 ng/ml of LPS. (B) Sorted Sca-1$^-$ and Sca-1$^+$ macrophages were analyzed with flow cytometry 2 h after incubation with fluorescent-labeled dextran, latex beads, or *S. aureus*. (C) CFUs from the lysate of Sca-1$^-$ and Sca-1$^+$ macrophages incubated with *E. coli* for 1 h was counted. (D) Sca-1$^-$ and Sca-1$^+$ macrophages were stained with anti-CD80, anti-CD86, and anti-MHC II antibodies, and applied to flow cytometry analysis. (E) Expression of components of ROS generating machinery (*P22phox, P67phox, Gp91phox*), surface receptors (*Cxcr1, Cxcr2, Tlr2, Tlr4*), and M1- or M2-markers (*Marco, Fizz1*) were analyzed by qPCR. The data are representative of three independent experiments (B, D). Data are expressed as the mean ± s.e.m. (n = 2) (A, C, E). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by Student's *t*-test.

Quantitative PCR analyses showed that Sca-1$^-$ and Sca-1$^+$ macrophages express similar levels of *Cxcr1*, *Cxcr2*, *Tlr2*, *Tlr4*, *Marco*, and *Fizz1* (Fig. 3E middle, 3E right). Together, these results suggest that Sca-1$^+$ macrophages secrete less amounts of some cytokines and CCL2, but possess similar activities such as phagocytosis, bactericidal activity and ROS production compared to Sca-1$^-$ macrophages.

3.5. Depletion of Sca-1$^+$ macrophages with anti-Sca-1 antibody elicits beneficial effects against an LPS-induced sepsis model

To investigate the *in vivo* functional roles of novel Sca-1$^+$ macrophages in the LPS-induced sepsis model, we administrated purified anti-Sca-1 antibody to deplete the Sca-1$^+$ macrophages in the model mice and analyzed some key pathophysiological parameters of septic conditions. WT mice were intraperitoneally administered with 2 mg/kg of LPS, and isotype control (rat IgG2a) or anti-Sca-1 antibody was intraperitoneally injected 2 h after LPS administration. Bone marrow cells and PECs were collected 10 h after antibody injection (Fig. 4A top). Sca-1$^+$ cells were analyzed by flow cytometry. The results show that intraperitoneal anti-Sca-1 antibody injection depleted all Sca-1$^+$ macrophages but had no effect on the other Sca-1$^+$ cells in the bone marrow progenitors (Fig. 4A bottom). LPS administration did not generate Sca-1$^+$ neutrophil or monocytes (Fig. 1B). In a separate experiment, we also found that LPS failed to generate CD11b$^+$Gr-1$^+$Sca-1$^+$ cells (data not shown). Taken together these results, purified anti-Sca-1 antibody would be useful to study the *in vivo* functional roles of Sca-1$^+$ macrophages in the peritoneum in LPS-induced experimental endotoxemia. Depletion of Sca-1$^+$ macrophages in the peritoneum significantly increased the survival rate of the LPS-induced sepsis model (Fig. 4B). LPS, the endotoxin of Gram-negative bacteria, is a critical factor that causes systemic inflammation and acute tissue injury in organs such as the lung, kidney, and liver during sepsis [25]. We collected the lungs and kidneys from the LPS-induced sepsis model to compare tissue damage by H&E staining. The LPS-induced sepsis model showed alveolar region thickening and lumen narrowing of the kidneys (Fig. 4C and D). Sca-1$^+$ macrophage depletion by anti-Sca-1 antibody administration reduced lung injury and recruited immune cells to alveolar regions (Fig. 4C), and reduced lumen narrowing of kidney (Fig. 4D). These results suggest that specific depletion of Sca-1$^+$ macrophages can block the pathological progress of LPS-induced sepsis, resulting in increased survival rate and protection of organ damage.

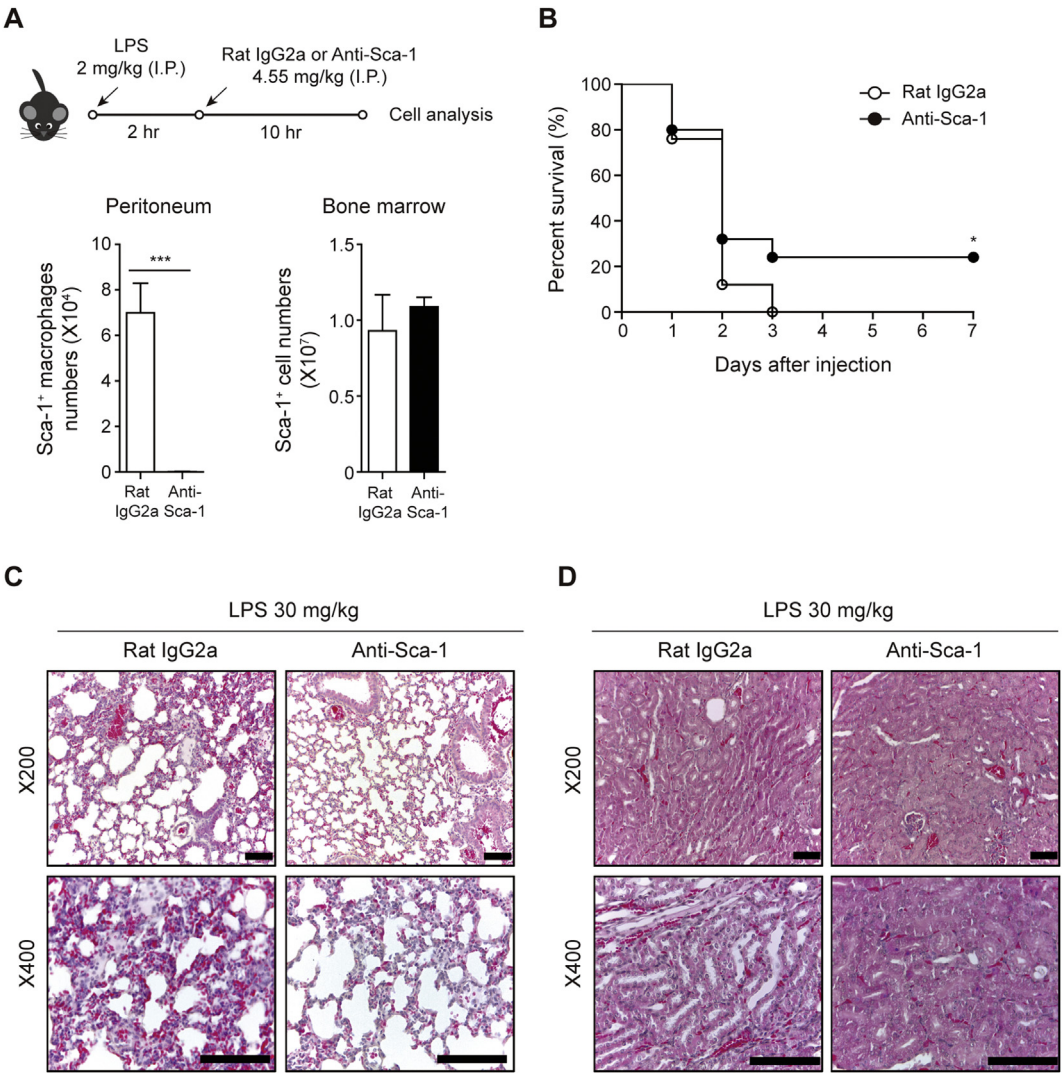

Fig. 4. Depletion of Sca-1$^+$ macrophages has therapeutic effects in the LPS-induced sepsis model. (A) Schematic of depletion protocol to examine the role of Sca-1$^+$ macrophages in the LPS-induced sepsis model (top). The numbers of Sca-1$^+$ macrophages or Sca-1$^+$ cells in the peritoneum or bone marrow of antibody-injected mice (bottom). (B) Mice were injected with 30 mg/kg of LPS, and an isotype control (rat IgG2a) or anti-Sca-1 antibody was administered to LPS-injected mice 2 h, 14 h, and 26 h after LPS injection. The survival rate was monitored for 7 days. The H&E staining data of the lungs (C) and kidneys (D) were sampled from the isotype control- or anti-Sca-1-injected LPS-induced sepsis model after 16 h. The data are representative of five mice per group from three independent experiments (C, D). Scale bar, 100 μm. Data are expressed as the mean ± s.e.m. (n = 5) (A bottom). Statistical significance was determined by the log-rank test (n = 30). *$P < 0.05$ (B). ***$P < 0.001$ by Student's t-test.

4. Discussion

Macrophages play important roles in immune responses in diverse infectious and inflammatory disorders [26]. The heterogeneity of macrophages is well established in both homeostatic conditions and pathological conditions in different tissues [27], and they show distinct expression of some markers including secreted molecules and subset-related genes [1]. Sepsis is a life-threatening inflammatory response syndrome caused by the infection of diverse pathogens [28]. Because the pathogenesis of sepsis is extremely complex and accumulating evidence demonstrates that unrevealed leukocyte populations mediate the progress of sepsis contributing to increased mortality [29], we attempted to identify previously unknown macrophage populations generated in an LPS-induced sepsis model. By focusing on Sca-1, a marker of HSCs, we successfully identified Sca-1$^+$ macrophages in the LPS-induced sepsis model (Fig. 1). Previously, it was known that an LPS-induced septic environment generates M1 polarized macrophages [30]. Here, we found that the M1 macrophages generated from the LPS-induced sepsis model can be divided into at least two different populations, Sca-1$^-$ and Sca-1$^+$ macrophages. The newly identified Sca-1$^+$ macrophages produce less amounts of inflammatory cytokines (IL-6, TNF-α) and a chemokine (CCL2) compared to their Sca-1$^-$ counterparts (Fig. 3A). The well-known M1-polarizing cytokine IFN-γ is necessary for the generation of Sca-1$^+$ macrophages by LPS. Moreover, IFN-γ itself generates Sca-1$^+$ macrophages (Fig. 2C). The results support our notion that Sca-1$^+$ macrophages are a subpopulation of M1 macrophages. On the origin of the Sca-1$^+$ macrophages, we demonstrated that isolated peritoneal macrophages, but not bone marrow cells, can be converted into Sca-1$^+$ macrophages by LPS or IFN-γ treatment (Fig. 2), suggesting that Sca-1$^+$ macrophages are derived from peritoneal macrophages. Collectively, LPS administration into the peritoneum elicits M1 polarizing conditions in mice where the produced IFN-γ may convert peritoneal macrophages into Sca-1$^+$ macrophages.

On the functional roles of Sca-1$^+$ macrophages in the LPS-induced sepsis model, we found that depletion of Sca-1$^+$ macrophages using anti-Sca-1 antibody ameliorates mortality and organ

damage to the lungs and kidneys (Fig. 4). These results suggest that Sca-1$^+$ macrophages may play a role in mediating the pathogenic events of the LPS-induced sepsis model. *In vitro* experiments with isolated Sca-1$^-$ and Sca-1$^+$ macrophages showed that Sca-1$^+$ macrophages produce less amounts of some inflammatory cytokines compared to their Sca-1$^-$ counterparts (Fig. 3A). However, no significant difference was observed in several features including phagocytosis (Fig. 3B and C), expression of antigen presenting molecules (Fig. 3D), and gene expression (Fig. 3E) between the two macrophage populations. Previously, we demonstrated that CD11b$^+$Gr-1$^+$Sca-1$^+$ myeloid cells generated in an *S. aureus* infection model have pathogenic roles by producing increased levels of several cytokines [20]. Unlike the CD11b$^+$Gr-1$^+$Sca-1$^+$ myeloid cells, Sca-1$^+$ macrophages produce decreased levels of some inflammatory cytokines. Detailed mechanisms involved in the pathogenic roles of Sca-1$^+$ macrophages in LPS-induced sepsis should be clarified in the near future.

In conclusion, in this study we found that an LPS-induced septic environment generates a novel macrophage population that expresses Sca-1 from resident peritoneal macrophages by IFN-γ. This newly identified population is found in the LPS injection site and produces less amounts of inflammatory cytokines compared to their counterparts. Our findings provide insight into a novel macrophage population that plays a role in inflammation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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