


Regulation of dendritic cell function improves survival in experimental sepsis through immune chaperone

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Abstract

Dendritic cells (DCs) are professional Ag-presenting cells that play a critical role in both innate and adaptive immune responses. DCs recognize and respond to bacteria through multiple PRRs, including TLRs. Heat shock protein gp96/grp94 is a master essential chaperone for TLRs in the endoplasmic reticulum. We generated DC-specific gp96-knockout (KO) mice and showed that gp96 KO DCs were unable to respond to multiple TLR ligands. TLR-mediated hyper-inflammatory response can lead to sepsis. However, the roles of neither DCs nor the DC-intrinsic gp96 in the process are completely understood. In a LPS-induced sepsis model, we hereby found that deletion of gp96 in DCs significantly reduced serum TNF- α levels and improved survival. Furthermore, using the well-defined polymicrobial sepsis model of cecal ligation and puncture, we found that DC-specific ablation of gp96 improved survival with significantly attenuated liver and renal injuries, decreased circulating inflammatory cytokines, altered DC maturation and activation, and increased serum Ig. Collectively, we demonstrate that deletion of gp96 in DCs is beneficial in protecting mice against sepsis induced by both endotoxemia and polymicrobial infections. We conclude that targeting gp96 in DCs may provide a potential novel approach for reducing the morbidity and mortality of sepsis.

Keywords

Dendritic cell, chaperone, gp96/grp94, sepsis, TLR

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Introduction

Sepsis is a life-threatening condition caused by a dys-regulated host response to microbial infections or bacterial products, such as LPS. The cellular components of the innate immune system serve as the first line of defense against invading pathogens; however, excessive activation of various immune cells, including B cells, T cells, macrophages, and dendritic cells (DCs), which leads to release of inflammatory cytokines including TNF- α and IL-12, is a hallmark of sepsis.¹

Among professional Ag-presenting cells, DCs serve as an essential interface between innate and adaptive immune responses.^{2–4} DCs recognize and respond to microbes such as bacteria and virus through multiple PRRs, including TLRs, NLRs, RIG I-like receptors, C-type lectins, and mannose receptors.^{5–7} During sepsis, DCs can be activated by TLRs and this activation contributes to sepsis-associated

immunosuppression, organ injury and mortality.^{8–10} Activation of adenosine monophosphate kinase (AMPK), which suppresses the activation of DCs, has been reported to prevent the inflammation and organ damage during sepsis.^{9,11,12} Targeting DCs was therefore thought to be promising for the management of sepsis via both an immunotherapeutic and an immunomodulatory approach.⁹ Thus, it becomes very important

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to understand the function and related mechanisms of DCs in the immunopathogenesis of sepsis.

Heat shock protein (HSP) gp96,¹³ also known as grp94,¹⁴ endoplasmic, ERp99,¹⁶ and HSP90b1,¹⁷ is an endoplasmic reticulum (ER) paralogue of HSP90. As a molecular chaperone, gp96 is the most abundant and ubiquitous protein in the ER lumen and is induced by the accumulation of misfolded proteins.¹⁸ Genetic studies have demonstrated that gp96 is an essential master molecular chaperone for most of the TLRs,^{19–24} multiple integrins,^{20,23} GARP,^{25–27} and Wnt co-receptor LRP6.^{28,29} gp96 can bind to and act as a master chaperone for TLRs on macrophages and DCs, while it itself might also stimulate pro-inflammatory cytokines (TNF- α and IL-12) secretion.^{20,30} Our recent study demonstrated that macrophage-specific gp96-knockout mice have significantly less inflammation in the colon and lower percentages of Th17 and Th1 cells in colonic lamina propria (cLP) compared with their wild type (WT) littermates.³¹ Furthermore, we generated a unique CD11c⁺ cell-specific gp96-deficient mouse model (abbreviated as KO mice hereafter) with selective deletion of gp96 in DCs and demonstrated that gp96-deficient DCs were unable to respond to TLR ligands,³² suggesting a critical role of gp96 in DC activation and in exacerbating inflammation. This constellation of findings led us to explore the role of DC-intrinsic gp96 in sepsis.

In this study, we investigated whether DC-intrinsic gp96 contributes to the pathogenesis of sepsis. First, we challenged WT and KO mice with a lethal dose of LPS, and we found that WT mice produced significantly more serum TNF- α than did KO mice. Consistent with the detrimental effect of this cytokine in sepsis, we found that the WT mice were more susceptible to LPS-induced endotoxemia than the KO mice. Furthermore, using a well-defined sepsis model of cecal ligation and puncture (CLP),^{33–35} we found that KO mice had significantly attenuated liver and renal injury, decreased circulation inflammatory cytokines, and improved survival. Interestingly, we found that KO mice had reduced DC maturation but increased serum level of Ig after CLP, which may contribute to the protection against sepsis. Our results demonstrate that deletion of gp96 in DCs is protective against sepsis induced by both endotoxemia and polymicrobial infection. This work supports the notion of targeting gp96 in DCs as a potential novel approach for the treatment of sepsis.

Materials and methods

Mice

DC-specific gp96-deficient mice (CD11cCre⁺ *Hsp90b1*^{fllox/fllox}) and control littermates

(CD11cCre⁻ *Hsp90b1*^{fllox/fllox}) were generated by crossing *Hsp90b1*^{fllox/fllox} mice with CD11c-Cre transgenic mice.³² All animal experimental protocols were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (IACUC). All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD, USA) as well as established institutional guidelines and regulations.

Reagents

Abs used for flow cytometry were obtained from BD Biosciences (Mountain View, CA) and eBioscience (San Diego, CA). LPS (055: B5) was purchased from Sigma-Aldrich (St Louis, MO). All other chemicals were obtained from Sigma-Aldrich (St Louis, MO) and Fisher Scientific (Pittsburgh, PA).

LPS-induced endotoxemia and survival study

Endotoxemia was induced in DC-specific gp96 KO mice and their WT littermates (8–12 wk old) by i.p. injection of LPS (25 mg/kg body mass; *Escherichia coli* LPS 055: B5; Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline, as described previously.^{20,33} The sera were collected at 1.5 h after LPS administration for cytokine analyses. For the survival study, mouse survival was monitored every 12 h for a total of 3 d.

CLP-Induced sepsis and survival study

DC-specific gp96 KO mice and their WT littermates (12–16 wk old) were housed in a specific pathogen-free environment. All surgery was performed under anesthesia. CLP was performed as described previously.³³ Briefly, the cecum was ligated at the colon juncture and punctured once with a 22-gauge needle. All animals were fluid-resuscitated subcutaneously with sterile normal saline. Sham operations were performed in the same way as CLP, but without ligation and puncture of the cecum. Mice were sacrificed at 24 h after CLP, and serum was collected for biochemical analyses. The spleen was isolated for DC maturation analysis by flow cytometry. For the survival study, mice were monitored every 24 h for a total of 7 d.

ELISA

IL-12p40 and TNF- α levels in the serum were measured with ELISA kits from BD Biosciences (San Diego, CA) according to the manufacturer's protocol. IL-10 levels in the serum were measured using a mouse IL-10

ELISA kit (eBioscience, San Diego, CA) according to the manufacturer's protocol. The serum alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine levels were measured using appropriate mouse ELISA kits (BioAssay Systems, Atlanta, GA). Ig levels in the sera were determined by a sandwich ELISA kit from Southern Biotechnology Associates (Birmingham, AL).

Flow cytometry

Surface staining of cells and flow cytometry were done as described previously.^{20,36} Briefly, splenocytes were isolated and RBCs were lysed. After washing with FACS buffer (PBS with 2% FBS and 0.09% NaN₃), cells were pelleted and blocked with FcR Ab for 10 min, followed by incubation with fluorochrome-labeled Abs at the appropriate dilution for 30 min at 4°C. After staining, cells were then washed with FACS buffer and acquired on FACSVerse (Becton Dickinson, Franklin Lakes, NJ). Dead cells were always gated out by 7AAD exclusion. The results were analyzed with the FlowJo software (Tree Star, Ashland, OR).

Culture of bone marrow-derived DCs

WT and KO bone marrow (BM) cells were isolated from femurs and tibias. BM cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL of penicillin, 100 mg/mL of streptomycin, 20 ng/mL GM-CSF, and 10 ng/mL IL-4 for 6 d. The floating cells were harvested, which were mostly BM-derived DCs (BMDCs) by phenotypic analysis.

Microarray and pathway analysis

WT and KO BMDCs were stimulated with 200 ng/mL LPS (*E. coli* 055:B5) from Sigma for 6 h. Total RNA was extracted from WT and gp96 KO BMDCs using an RNeasy Mini Kit (Qiagen, Valencia, CA). Fluorescent antisense amplified RNA (aRNA) target preparation was performed using an Eberwine-based amplification method with an Amino Allyl MessageAmp II aRNA Amplification Kit. Cy5-Labeled RNA targets were hybridized to Mouse Whole Genome OneArray[®] v2 (Phalanx Biotech Group, Belmont, CA), and slides were scanned by the Axon 4000 scanner (Molecular Devices, Sunnyvale, CA). The Cy5 fluorescent intensity of each spot was analyzed by Genepix 4.1 software (Molecular Devices, Sunnyvale, CA). Genes with log₂ ratio ≥ 2.0 or log₂ ratio ≤ -2.0 and *P* value < 0.05 are further analyzed. Genes were classified using KEGG Pathway Database (<http://www.genome.jp/kegg/pathway.html>) and those involved in inflammation were highlighted. A heat map was generated to demonstrate differential

expression of genes in the WT and KO DCs. In the heat map, the given gene is presented as compared to the median value for that gene in the WT and KO DC data sets.

Statistical analysis

Statistical significance was determined by analysis of variance (ANOVA) with Fisher's probable least-squares difference test, and Student's t-test or log-rank (Mantel-Cox) test using GraphPad Prism software. Error bars represent SEM. A value of *P* < 0.05 was considered statistically significant.

Results

Attenuation of LPS-induced endotoxemia in dendritic cell-specific gp96-deficient mice

Recently, we have generated DC-specific gp96-deficient mice by crossing *Hsp90b*^{fllox/fllox} mice with CD11cCre mice.^{20,21,32,37} We found that deletion of gp96 in DCs rendered them unresponsive to stimulation by TLR2, TLR4, and TLR9 ligands.³² Thus, our DC-specific gp96-deficient mice present a DC-specific pan TLR KO mouse model. To determine the effect of DC-intrinsic gp96 in endotoxin shock, WT and KO mice were administered intraperitoneally with a lethal dose of LPS. Mouse survival was monitored every 12 h. By d 2, 100% of the WT mice succumbed to endotoxemia; in contrast, 40% of DC gp96 KO mice survived after LPS administration (*P* < 0.0001; Figure 1a). Compared to the LPS treated WT mice, deletion of gp96 markedly reduced serum levels of TNF-α (WT: 22.3 ± 1.9 ng/mL, KO: 4.3 ± 0.9 ng/mL; *P* < 0.0001; Figure 1b). This result demonstrates that DCs play a significant role during the hyperinflammatory phase of LPS-induced endotoxemia and deletion of gp96 in DCs attenuates the TLR-induced inflammatory response.

Deletion of gp96 in DCs protects against CLP-Induced polymicrobial sepsis

To further investigate whether DC-intrinsic gp96 plays a pivotal role in sepsis, we used a well-defined sepsis model of CLP. We found that KO mice exhibited significantly increased the survival rates in comparison with their WT littermates (*n* = 21, *P* < 0.01; Figure 2a). In addition, deletion of gp96 dramatically lowered CLP-Induced serum levels of IL-12p40 (WT: 903.9 ± 42.7 pg/mL, KO: 341.6 ± 55.8 pg/mL; *n* = 5, *P* < 0.001; Figure 2b) and IL-10 (WT: 5.1 ± 1.4 ng/mL, KO: 0.5 ± 0.1 ng/mL; *n* = 4, *P* < 0.05; Figure 2c). Liver function was significantly improved in KO mice after CLP surgery evidenced by reduced circulating

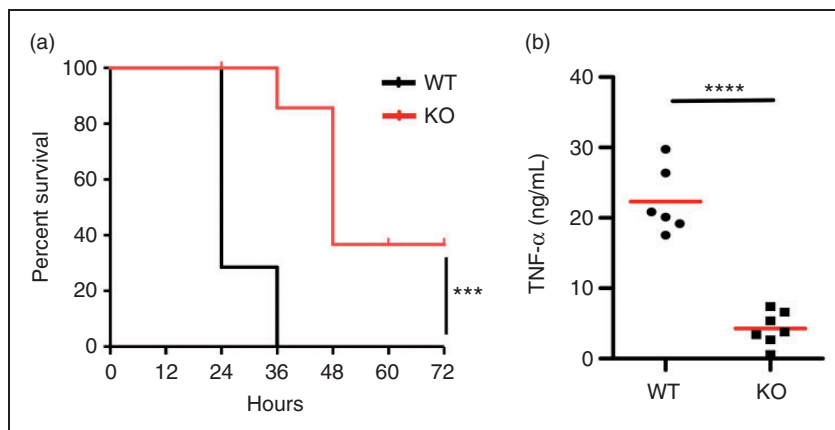


Figure 1. Attenuation of LPS-induced endotoxemia in DC-specific gp96-deficient mice. (a) 8–12-wk-old KO mice ($n = 7$) and control littermates ($n = 6$) were administered i.p. with 25 mg/kg body mass LPS, and mouse survival was monitored for 72 h. (b) Mice were bled at 1.5 h after LPS injection. Serum TNF- α was measured by ELISA. *** $P < 0.001$, **** $P < 0.0001$.

ALT (WT: 38.8 ± 3.5 U/l, KO: 24.4 ± 2.5 U/l; $n = 3-6$, $P < 0.05$; Figure 2d) and AST (WT: 65.3 ± 8.1 U/l, KO: 36.0 ± 3.9 U/l; $n = 3-6$, $P < 0.05$; Figure 2e). However, the ALT (WT: 18.3 ± 0.6 U/l, KO: 24.6 ± 4.8 U/l) and AST levels (WT: 21.7 ± 2.1 U/l, KO: 21.1 ± 5.8 U/l) were comparable between WT and KO mice after Sham operation (Figure 2d and e). Moreover, two important renal functional indicators, serum creatinine (WT: 0.7 ± 0.1 mg/dl, KO: 0.4 ± 0.02 mg/dl; $n \geq 4$, $P < 0.001$; Figure 2f) and BUN levels (WT: 134.7 ± 11.0 mg/dl, CLP KO: 59.6 ± 11.1 mg/dl; $n \geq 4$, $P < 0.01$; Figure 2g) were also significantly attenuated in KO mice after CLP surgery, in comparison with WT mice. These data indicated that deletion of gp96 in DCs protected against CLP-Induced polymicrobial sepsis.

Deletion of gp96 in DCs impairs DC maturation in CLP-Induced septic mice

We reported previously that gp96-deficient DCs were unable to respond to TLR ligands.³² We next examined whether gp96 deletion in DCs has an impact on the DC maturation and activation in CLP-Induced septic mice as well as sham mice *in vivo*. We isolated splenic cells from WT and KO mice after CLP or Sham procedure and performed comparative phenotypic analysis of DCs. Previous studies demonstrated that gp96 is an essential molecular chaperone for multiple integrins.^{20,21,23} Deletion of gp96 from DCs resulted in decreased surface expression of CD11c. Thus, we used a negative gating strategy (B220⁺MHC class II⁺) to define DC lineages (Figure 3a, left),^{32,38} and further checked surface expression of CD83 and CD86 in DCs (Figure 3a, right), which are characteristic cell surface markers for fully matured DCs and are important for providing costimulatory signals for T cell activation

and survival. We found that deletion of gp96 in DCs completely abrogated up-regulation of CD83 and CD86 in the CLP-Induced septic mice ($n = 6-7$, $P < 0.001$; Figure 3b and c). This result suggested that DC-intrinsic gp96 is essential for the maturation and activation of DCs during the CLP-Induced sepsis.

Deletion of gp96 in DCs significantly increases the systemic Ig production

A recent study reported that commensal bacteria-induced systemic IgA contributed to protecting against polymicrobial sepsis.³⁹ Previously we have demonstrated that DC-specific gp96-deficient mice developed spontaneous colitis with age. Consistent with the report that intestinal IgA coating represents ongoing inflammatory conditions, we also found that DC-specific gp96 KO mice had significantly higher levels of IgA and IgG1 in the sera compared with WT controls at baseline, and the level of fecal IgA was also significantly higher in KO mice.³² Next, we examined whether deletion of gp96 in DCs affects systemic Ig production in CLP-Induced septic mice. Indeed, we found that sham KO mice had significantly higher levels of IgA (Figure 4a), IgG1 (Figure 4b), and IgG2c (Figure 4d) but not IgG2b (Figure 4c), IgG3 (Figure 4e), and IgM (Figure 4f) compared with sham WT controls. Moreover, the levels of IgA (Figure 4a) and IgG1 (Figure 4b) were also significantly increased in the CLP KO mice compared with the CLP WT controls, while the levels of IgG2b and IgM remained the same. However, IgG2c and IgG3 levels were significantly reduced in the CLP KO mice compared with the CLP WT controls (Figure 4d and e). Our data suggest that systemic IgA and IgG1 contribute to protecting against polymicrobial sepsis. Thus,

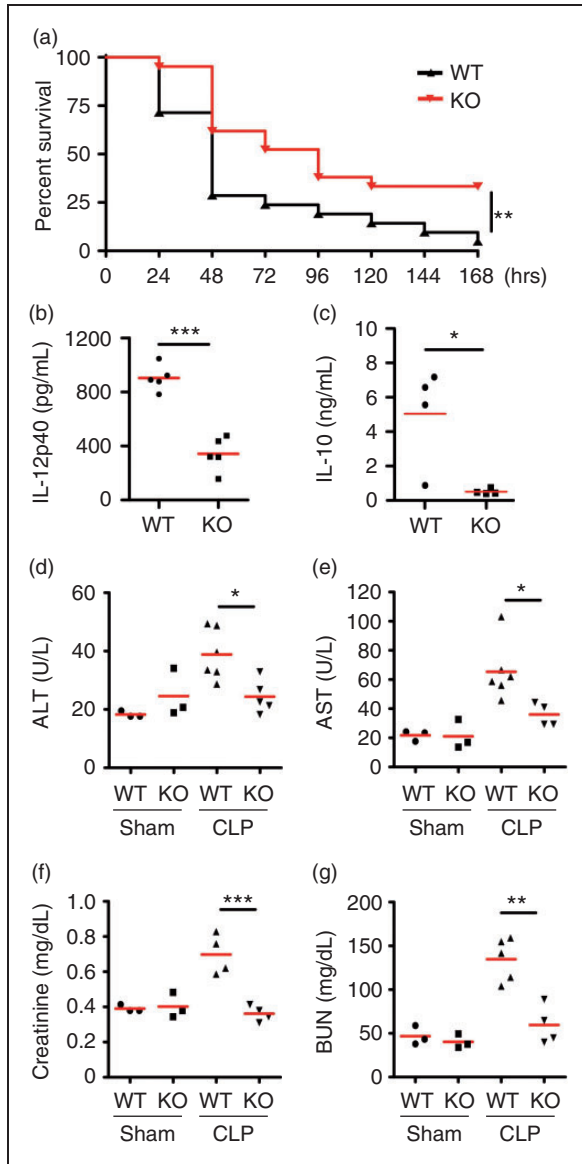


Figure 2. Deletion of gp96 in DCs protects mice against CLP-Induced polymicrobial sepsis. (a) KO mice ($n = 21$) and WT littermates ($n = 21$) were subjected to CLP and monitored for survival. (b, c) Sera were collected at 24 h after CLP from KO and WT littermates ($n = 5$), and cytokine IL-12p40 (b) and IL-10 (c) were measured by ELISA. (d–g) Sera were collected at 24 h from KO and WT littermates after a sham operation ($n = 3$) or CLP ($n \geq 4$), and ALT (d), AST (e), creatinine (f), and BUN (g) levels were measured by ELISA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

targeting gp96 in DCs will provide a potential therapeutic for the treatment of sepsis.

Microarray analysis of LPS-induced inflammatory pathways in BMDCs

We so far have shown that deletion of gp96 in DCs in mice significantly reduced the level of systemic

inflammatory cytokines, improved their survival in response to LPS-induced endotoxemia and CLP-Induced polymicrobial sepsis (Figures 1 and 2). Also, deletion of gp96 attenuated DC maturation and activation after CLP. By using a gene expression microarray analysis, we next investigated what were the LPS-inducible genes in DCs that were altered by gp96 deletion. We treated WT and KO BMDCs with or without LPS for 6 h, and then quantified the levels of mRNA encoded by genes involved in inflammatory pathways. We found that multiple inflammatory cytokine levels were significantly decreased in the KO BMDCs including *Il1a*, *Il1f6*, *Il1rn*, *Il6*, *Il12b*, and *Tnfsf4* along with inflammation-related genes (*Cxcl1*, *Cxcl5*, *Cxcl9*, *Cxcl12*, *Nr1h3*) (Figure 5) after LPS treatment. However, the levels of inflammatory cytokines in unstimulated DCs are very low or undetectable compared with LPS stimulated DCs. Also, the expression levels of these genes are comparable between unstimulated WT and KO DCs (data not shown). This result is consistent with that gp96 chaperone TLRs.^{20,21,23,32} This data suggests that gp96 deletion offers a unique advantage in treating sepsis over existing approaches by down-regulating multiple inflammatory pathways.^{40–43}

Discussion

DCs are professional Ag-presenting cells that play an important role in both innate and adaptive immunity. DCs have been shown to be significantly involved in all phases of the pathological development of sepsis from the initial inflammatory response to the immune suppressive stage; however, the extent of their contributions has not been fully elucidated. DCs recognize microbes and pathogens through PRRs, including TLRs and NLRs.^{5–7} We previously reported that heat shock protein gp96 is an essential immune chaperone for TLRs including TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9, integrins, and other vital innate receptors.^{20,21,23,44} Consistent with gp96 chaperone function, gp96 KO DCs failed to respond to stimulation by TLR2, TLR4, and TLR9 ligands *in vitro*.³² In this study, we found that specific deletion of gp96 in DCs protected against both LPS-induced endotoxemia and CLP-Induced polymicrobial sepsis, evidenced by decreased circulating inflammatory cytokines (TNF- α and IL-12), attenuated liver and renal injury, and reduced mortality. Moreover, deletion of gp96 in DCs completely abrogated up-regulation of CD83 and CD86 in the CLP mice (Figure 3b and c), which suggested that DC-intrinsic gp96 is essential for the maturation and activation of DCs during multi-microbial sepsis. Our study suggested that DCs are a major source of inflammatory cytokines during sepsis

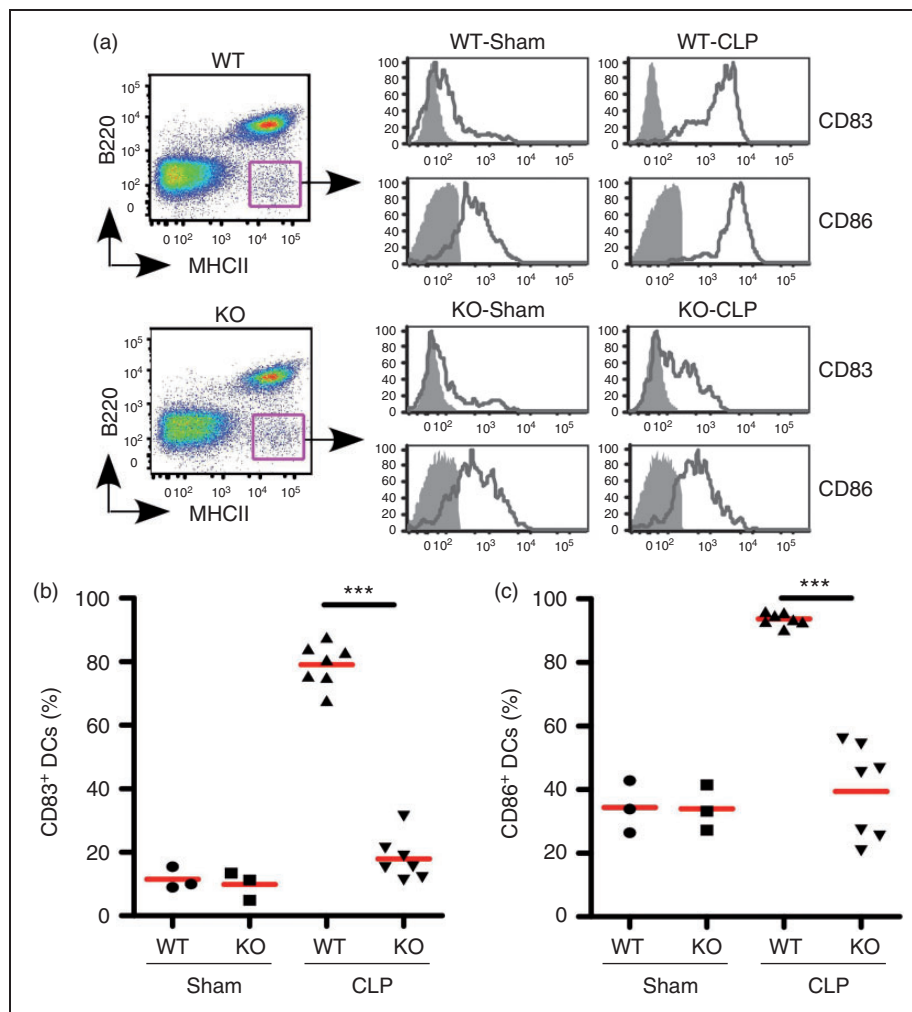


Figure 3. Deletion of gp96 in DCs impairs DC maturation in CLP-induced septic mice. (a) Flow cytometry analysis of splenic DCs by negative gating strategy to define DCs (B220⁻ MHCII⁺, left panel), followed by examining cell surface maturation markers CD83 and CD86 (right panel, open histogram with solid line). Gray-shaded histograms represent isotype controls. (b, c) Quantification of CD83⁺ (b) and CD86⁺ (c) splenic DCs as defined in (a) in WT and KO mice after Sham treatment ($n = 3$) or CLP ($n = 7$). *** $P < 0.001$.

and that DC-intrinsic TLR signaling including TLR2 and TLR4 may contribute to mortality associated with sepsis.

Recent studies demonstrate that DCs can adopt tolerogenic functions in the presence of Wnt3a and Wnt5a, and that these DCs produce high levels of IL-10, TGF- β , retinoic acid (RA), IL-27, and vascular endothelial growth factor in response to TLR ligands.⁴⁵⁻⁴⁷ In addition, DC-intrinsic TLR2 signaling activated β -catenin and induced IL-10 and RA production.⁴⁸ We have demonstrated that gp96 is an essential molecular chaperone for Wnt co-receptor LRP6. Consistent with the loss of LRP6 and Wnt signaling, gp96 KO cells failed to up-regulate *Axin2* mRNA in response to Wnt-3a.²⁸ In the present study, we found that gp96 KO mice produced a low level of IL-10 during CLP-Induced polymicrobial

sepsis (Figure 2c), which may be due to loss of both TLR and Wnt signaling. Further studies are needed to clarify the relative contribution by each of the two pathways in sepsis.

Low levels of circulating IgA, IgG1, and IgM have been associated with low survival rates in patients with severe sepsis or septic shock.^{49,50} Treatment with combined i.v. IgG/IgA/IgM decreased the risk of death after 28 d in patients with severe sepsis and/or septic shock.⁵¹ In addition, a recent study reported that commensal bacteria-induced systemic IgA contributed to protecting against polymicrobial sepsis.³⁹ We found that CLP KO mice had significantly higher levels of IgA and IgG1 (Figure 4) compared with the CLP WT controls; while the levels of IgG2b and IgM remained the same. However, IgG2c and IgG3 levels

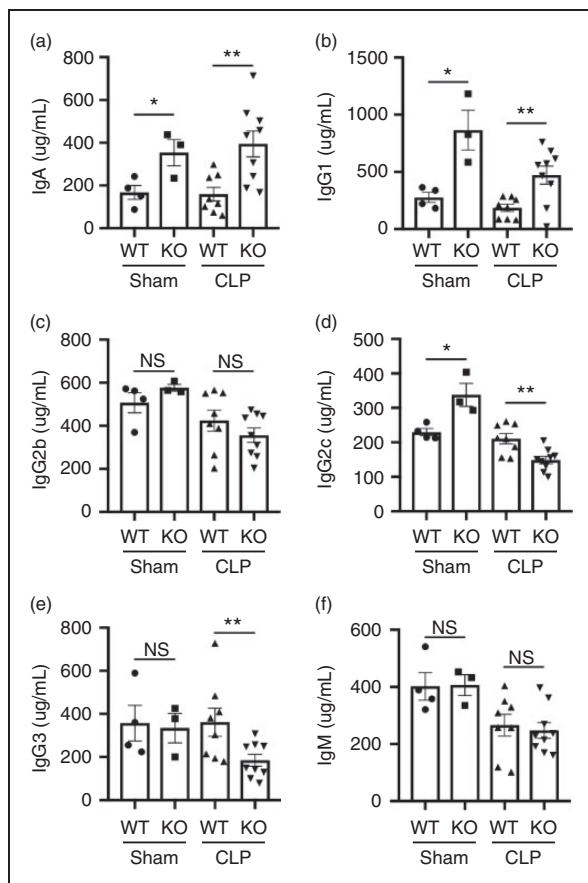


Figure 4. Deletion of gp96 in DCs results in altered the systemic Ig production. (a–f) KO mice and WT littermates were subjected to Sham ($n = 3-4$) or CLP ($n = 8$). Serum was collected at 24 h after the surgery, and then the Ig levels of IgA (a), IgG1 (b), IgG2b (c), IgG2c (d), IgG3 (e), and IgM (f) in the sera were measured by ELISA. Error bars indicate SEM. * $P < 0.05$; ** $P < 0.01$.

were significantly reduced in the CLP KO mice compared with the CLP WT controls (Figure 4d and e). Our data indicate that systemic IgA and IgG1 contribute to protecting against polymicrobial sepsis.

In summary, our results demonstrate that DCs are one of the major cellular sources of inflammatory cytokines during sepsis. Deletion of gp96 in DCs is beneficial in both LPS-induced endotoxemia and CLP-induced polymicrobial sepsis through a variety of mechanisms including blocking TLR signaling, inhibiting Wnt signaling, and increasing systemic IgA and IgG1. Therefore, targeting gp96 in DCs either genetically or pharmacologically^{29,52-54} may provide a potential novel approach for the sepsis treatment.

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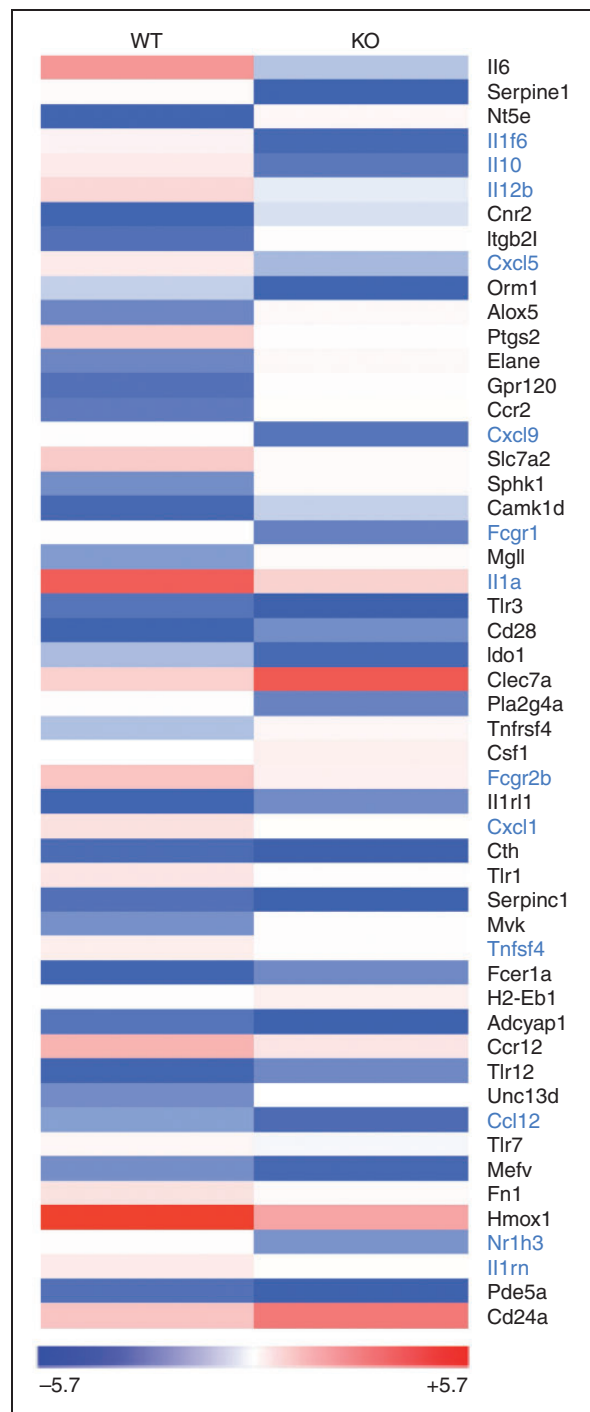


Figure 5. Microarray analysis of mRNAs levels in WT and gp96 KO BMDCs in response to LPS. WT or KO BMDCs were stimulated with LPS (200 ng/mL) for 6 h followed by mRNA extraction, reverse transcription, and cDNA microarray analysis using the whole mouse cDNA array. The heat map shows the genes involved in the inflammatory pathway using the KEGG Pathway Database analysis. Comparing with WT BMDCs, KO cells expressed significantly less inflammatory cytokines in response to LPS.

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Declaration of conflicting interests

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