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ARTICLE

CCR2-deficient mice are protected to sepsis by the disruption of the inflammatory monocytes emigration from the bone marrow

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Abstract

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Inflammatory monocytes are recruited to both the infection site and vital organs during sepsis; however, the mechanisms that orchestrate their migration, as well as the participation of these cells in systemic inflammation and vital organ damage, are still not fully elucidated. In this context, we described that CCR2-deficient mice had diminished migration of inflammatory monocytes from bone marrow to the circulation and subsequently to the site of infection and vital organs during cecal ligation and puncture (CLP)-induced polymicrobial sepsis. The reduction in the migration of inflammatory monocytes to the infection site was accompanied by a significant increase in the number of neutrophils in the same compartment, which seemed to counterbalance the absence of inflammatory monocytes in controlling microbial growth. Indeed, wild-type (WT) and CCR2-deficient mice under CLP presented similar control of infection. However, the CCR2-deficient mice were more resistant to sepsis, which was associated with a decrease in inflammatory mediators and organ damage biomarkers. Furthermore, the systemic adoptive transfer of CCR2-WT or CCR2-deficient inflammatory monocytes into CCR2-deficient mice equally increased the susceptibility to sepsis, demonstrating the deleterious role of these cells in the periphery even when CCR2 is absent. Thus, despite the host-protective role of inflammatory monocytes in controlling infection, our results demonstrated that the mechanism by which CCR2 deficiency shows protection to CLP-induced sepsis is due to a decrease of inflammatory monocytes emigration from bone marrow to the circulation and vital organs, resulting in the reduction of organ damage and systemic cytokine production.

KEYWORDS

CCR2, inflammatory monocytes, organ damage, sepsis, systemic inflammation

1 | INTRODUCTION

Sepsis is caused by a dysregulated host response to infection with life-threatening organ dysfunction.[1] It is estimated that there is an annual worldwide incidence of 31.5 million cases of sepsis, with 19.4 million cases considered severe and 5.3 million cases progressing to death.[2] Therefore, the World Health Organization (WHO) classified sepsis as a global health priority and adopted a resolution to improve its prevention, diagnosis, and management (resolution WHA70.7).

Sepsis outcome relies on a complex immunologic balance between controlling pathogen growth and avoiding excess systemic inflammation, which causes dangerous organ dysfunction.[3] Neutrophils are the first immune cell population recruited to the infection

Abbreviations: AST, aspartate aminotransferase; CK-MB, creatine kinase-MB; CLP, cecal ligation and puncture; WT, wild-type.

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site and have an essential role in bacterial control.[4] However, neutrophils are also related to tissue injury due to their accumulation in vital organs and the release of potent oxidant mediators, toxic proteases, and neutrophil extracellular traps.[5–8] Our group demonstrated that the key events associated with sepsis exacerbation are, on the one hand, due to the reduction in neutrophil migration to the infection site and, on the other hand, due to neutrophil accumulation in vital organs. Indeed, during the onset of an experimental severe sepsis episode with 100% of mortality, the accumulation of neutrophils in the infection site decreases, which is associated with G protein-coupled receptor kinase 2 mediated CXCR2 internalization.[9,10] Concurrently, there is an increase in the infiltration of these cells into vital organs due to a significant increase in the expression of CCR2.[11] The consequence is the spread of the infection and worsening of systemic inflammation, which is characterized by an increase in the serum concentration of inflammatory cytokines and an increase in vital organ damage.[8]

Beyond the role of neutrophils during sepsis, it is also known that inflammatory monocytes, which are the main population of monocytes, are also recruited to the infection site and vital organs during sepsis.[12–15] In an attempt to understand the role of chemokine receptors in the recruitment of these cells, our group recently demonstrated that CCR5 mediates the migration of inflammatory monocytes to the infection site, which was important for bacterial clearance and host survival.[16] However, the participation of inflammatory monocytes in vital organ lesions during sepsis is still not fully elucidated. In addition to CCR5, other receptors are involved in the recruitment of inflammatory monocytes, such as CCR2, that mediate the migration of these cells from the bone marrow to the bloodstream.[17] In this context, we aimed to investigate the role of CCR2 in the trafficking of inflammatory monocytes from the bone marrow to the bloodstream and its role in the infection control and in the systemic inflammation during cecal ligation and puncture (CLP)-induced sepsis.

2 | METHODS

2.1 | Mice

C57Bl/6J mice (wild-type, WT) and CCR2-deficient mice on a C57Bl/6 background (number: 027619) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in the facility of the University of São Paulo in São Paulo, Brazil. All experiments were carried out with 7-wk-old male mice according to the guidelines of the Care and Use of Laboratory Animals.[18] The study was approved by the Ethics Committee on the Use of Animals of the University of São Paulo (register number: 080/2018).

2.2 | Model of sepsis and experimental protocols

Sepsis was induced using the CLP method as previously described.[19] Briefly, the animals were anesthetized with inhalation of isoflurane (1.5% isoflurane), and two punctures were made through the ligated caecum with a 26-gauge needle to induce nonsevere CLP sepsis (mortality rate of ~50%). The animals were euthanized after sepsis induction at different time points, depending on the experiment. The survival rate of the mice was analyzed for 7 d.

2.3 | Flow cytometry

Cells from blood, peritoneal lavage fluid, bone marrow, lung, and kidney were stained with the following antibodies and viability markers: fixable live/dead BV421 (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Ly6G FITC, Ly6C PerCP, CD45 APC, CD11b APC-Cy7, and FcBlock (BD Biosciences, San Jose, California, USA). Fluorescence signal acquisition was carried out by using a FACS Verse cytometer (BD Biosciences, San Jose, California, USA) and was analyzed with FlowJo software (BD Bioscience, San Jose, California, USA). Inflammatory monocytes were determined to be negative for the viability dye and Ly6G, positive for CD11b, and expressed high levels of Ly6C. Neutrophils were determined to be negative for the viability dye and positive for Ly6G. For cell sorting of inflammatory monocytes, bone marrow was collected from C57Bl/6-WT mice, and the $Ly6G^{-}$ $Ly6C^{high}$ and $CD11b^{+}$ cells were sorted by a FACS Aria III sorter (BD Bioscience, San Jose, California, USA). For tissue cytometry, we collected tissue samples and dissociated the cells using 0.5 mg ml^{-1} collagenase type 2 for 45 min at 37°C (Sigma Aldrich, San Luis, Missouri, USA). For peritoneal cell cytometry, cells were obtained by perineal lavage with 1.5 ml of saline.

2.4 | Transfer of inflammatory monocytes to CCR2-deficient mice

Sorted inflammatory monocytes from WT-mice or CCR2-deficient mice (0.5×10^6), or PBS (control) were injected i.v. through the retro-orbital plexus 1 h after CLP induction in CCR2-deficient mice. The survival rate of the mice was analyzed for 7 d, or the plasma levels of IL-10 and IL-6 were assessed 24 h after sepsis induction.

2.5 | Measurement of organ damage biomarkers

Animals were euthanized 3 or 12 h after CLP induction, and aspartate aminotransferase (AST) and creatine kinase-MB (CK-MB) activity in the plasma were determined using a commercial kit (Labtest, São Paulo, Brazil) according to the manufacturer's instructions.

2.6 | Bacteria quantification

Blood and peritoneal lavage samples were plated in Difco Mueller Hinton Agar plates (BD Biosciences, San Jose, California, USA). The plates were incubated for 18 h at 37°C, and then the bacteria were quantified by determining the CFUs.

2.7 | Cytokine assays

The plasma, peritoneal lavage, or tissue levels of TNF, CXCL2, CCL2, IL-6, and IL-10 were measured by ELISA (R&D Systems, Mineapolis,

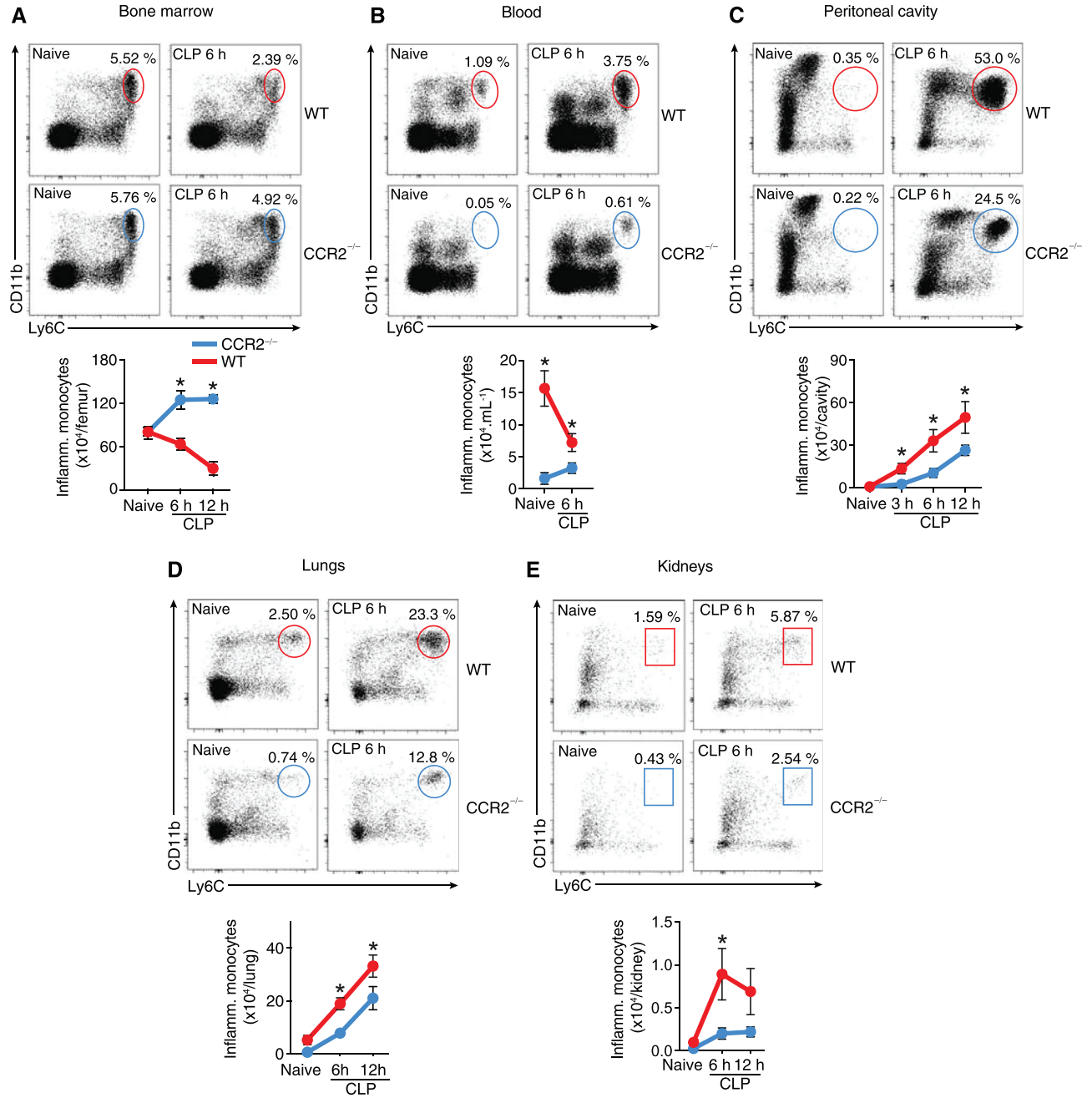

FIGURE 1 **CCR2-deficient mice have diminished recruitment of inflammatory monocytes to the circulation, infection site, and vital organs during experimental sepsis.** Wild-type (WT; red) and CCR2-deficient mice (blue) under naïve ($n = 3$) or cecal ligation and puncture (CLP) conditions ($n = 6$) were euthanized at 3, 6, or 12 h after sepsis induction. The absolute number of inflammatory monocytes (defined as Ly6G$^-$ CD11b$^+$, and Ly6C^{high}) in the bone marrow (**A**), blood (**B**), peritoneal cavity lavage fluid (**C**), lung and kidney, and (**D** and **E**, perspectively) were analyzed. The panels show the results of an individual experiment that is representative of two independent experiments, and the results are presented as the mean ± SEM. * indicates differences ($P < 0.05$) between WT and CCR2-deficient mice analyzed by ANOVA followed by Bonferroni correction each time. The dot plots are pregated on live cells, CD45$^+$ cells, and Ly6G$^-$ cells. The gates represent the percentage of inflammatory monocytes

Minnesota, USA) according to the manufacturer's instructions. The optical density of the individual samples was measured at 450 nm using a spectrophotometer (Spectra Max-250, Molecular Devices, Sunnyvale, CA, USA). Kidneys and lungs were collected after perfusion with PBS. The tissues were macerated, and the supernatant was used for cytokine determination. The total protein of the tissue samples was determined using a bicinchoninic acid assay to normalize the data. The concentration of cytokines in the peritoneal cavity was measured in the peritoneal lavage fluid (lavage with 1.5 ml of saline).

2.8 | Statistical analysis

The statistical analyses were performed using GraphPad Prism 7 (San Diego, California, USA). Bacterial counts are expressed as the medians,

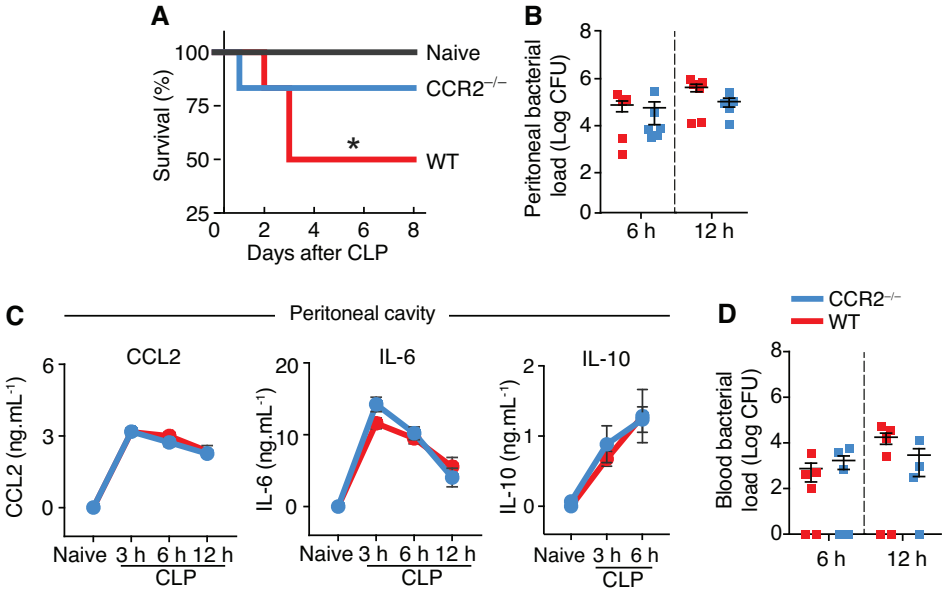

FIGURE 2 **CCR2-deficient mice are more resistant to sepsis than wild-type (WT) mice.** WT (red) and CCR2-deficient (blue) mice under naïve ($n = 3$) or cecal ligation and puncture (CLP) conditions ($n = 6$) were euthanized at 3, 6, or 12 h after sepsis induction. (**A**) Survival rates of WT and CCR2-deficient mice ($n = 6$ per group) under CLP-induced sepsis. The results represent the combination of three independent experiments. The data are presented as the percentage of survival and were analyzed by Mantel-Cox log-rank test; (**B** and **D**) Bacterial load is presented as the log of CFUs. Horizontal bars represent the mean value ± SEM, and the data were analyzed by *t*-test; (**C**) Concentrations of CCL2, IL-6, and IL-10 were determined by ELISA in the peritoneal cavity lavage fluid. The data are a combination of two independent experiments, and the results are presented as the mean ± SEM and were analyzed by ANOVA followed by Bonferroni correction (* indicates significant differences; $P < 0.05$)

and all other data are expressed as the mean ± SEM. The survival rate was analyzed by the Mantel-Cox log-rank test, bacterial counts by Student's *t*-test, and the other data by ANOVA followed by Bonferroni correction. A value of $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | CCR2-deficient mice have less inflammatory monocyte in the circulation, infection site, and vital organs during experimental sepsis

To understand the role of CCR2 in mediating the recruitment of inflammatory monocytes during sepsis, we performed CLP on CCR2-deficient mice (CCR2$^{-/-}$). As reported for local infection,[17,20] CCR2$^{-/-}$ mice showed reduced inflammatory monocyte recruitment from the bone marrow to the bloodstream and to the infection site after CLP-induced sepsis (Fig. 1a–c). Additionally, CCR2-deficient mice also presented less of these cells in vital organs (lungs and kidneys) in comparison to that of WT animals (Fig. 1d,e). Thus, these findings demonstrate that CCR2 plays an important role in the emigration of inflammatory monocyte from the bone marrow to the bloodstream, and, as a consequence, CCR2-deficient mice have less inflammatory monocytes in the blood, infection site, and vital organs following CLP-induced sepsis.

Previously, we demonstrated that CCR5 is induced on WT inflammatory monocytes during sepsis, mediating their recruitment to the infection site.[16] To understand whether CCR5 also plays a role in the recruitment of inflammatory monocytes in CCR2-deficient mice, we evaluated the expression of CCR5 in these animals after sepsis induction. Surprisingly, we observed that inflammatory monocytes of septic CCR2-deficient mice did not express CCR5 (Supporting Information Fig. S1a).

3.2 | CCR2-deficient mice have an increased survival rate under CLP-induced sepsis

Our data thus far demonstrated that CCR2 is involved in the recruitment of inflammatory monocytes from bone marrow to the bloodstream, and as a consequence of it, CCR2-deficient mice have less inflammatory monocytes in the infection site and vital organs. To understand whether the lack of this receptor has a protective or deleterious role during sepsis, we next assessed the survival rate of WT and CCR2-deficient mice during CLP-induced sepsis pathogenesis. Interestingly, CCR2-deficient mice presented a higher survival rate to CLP-induced sepsis compared to that of their WT counterparts (Fig. 2a). Notably, these results were not explained by differences in controlling the primary site of infection, because the bacterial loads in the peritoneal cavities of WT and CCR2-deficient mice were not significantly different (Fig. 2b). Similarly, the levels of CCL2, IL-6, and IL-10 in the peritoneal cavities were also similar between the CCR2-deficient mice and their WT counterparts (Fig. 2c). Furthermore, the spread of infection, herein characterized by the number of bacteria in the bloodstream, was also comparable between CCR2-deficient and WT mice (Fig. 2d).

After determining that the improved sepsis survival rate of the CCR2-deficient mice was not correlated with the control of the

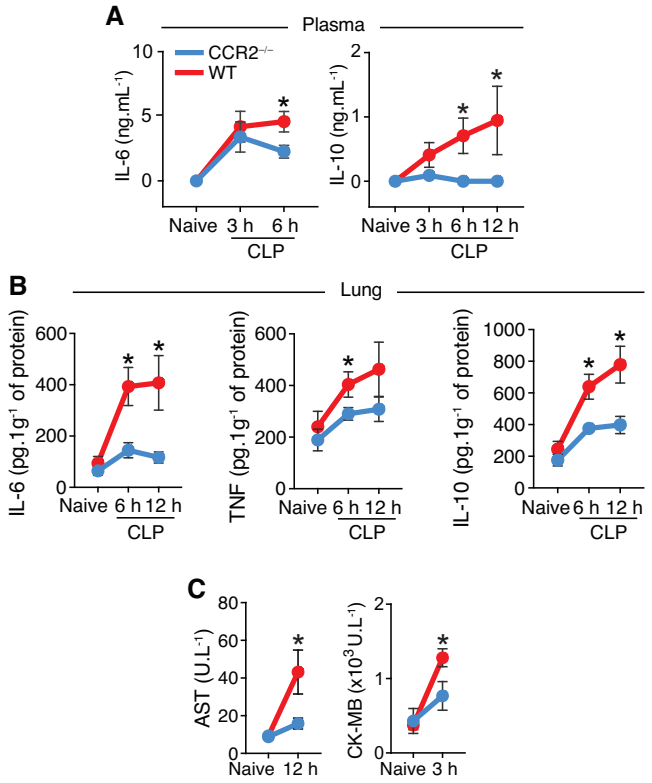

FIGURE 3 **CCR2-deficient mice showed reduced systemic inflammation**. WT (red) and CCR2-deficient mice (blue) under naïve ($n = 3$) or cecal ligation and puncture (CLP) conditions ($n = 6$) were euthanized at 3, 6, or 12 h after sepsis induction. (**A**) The concentrations of IL-6 and IL-10 in the plasma were determined by ELISA. The results are presented as the mean ± SEM and were analyzed by ANOVA followed by Bonferroni correction. (**B**) The concentrations of IL-6, TNF, and IL-10 in the lung homogenate were determined by ELISA, and the values were normalized to the total protein concentration. The results are presented as described in item (**A**). (**C**) The activities of aspartate aminotransferase (AST) and creatine kinase-MB (CK-MB) were determined, and the results are presented as the mean ± SEM and were analyzed by *t*-test. The results are a representative experiment of two independent experiments, and * indicates significant differences ($P < 0.05$)

primary site of infection or with the spread of the infection, our next step was to investigate whether the recruitment of inflammatory monocytes to the bloodstream and to vital organs resulting in systemic inflammation and tissue damage, explaining the differences observed in the survival rates of the analyzed groups. Consistently, the septic CCR2-deficient mice, which had reduced numbers of inflammatory monocytes in the bloodstream and in vital organs, showed lower levels of cytokines in the blood (IL-6 and IL-10) (Fig. 3a), lungs (IL-6, TNF, and IL-10) (Fig. 3b), and kidneys (IL-6) (Supporting Information Fig. S1b) compared to those of the WT group. Additionally, septic CCR2-deficient mice also had lower levels of biochemical markers of the liver (AST) and heart (CK-MB) tissue damage compared to those of the WT group (Fig. 3c). Next, we assessed the recruitment of neutrophils into the lung and kidney tissues of septic mice, and we observed no difference in the accumulation of this cell subtype in septic CCR2-deficient and WT mice (Supporting Information Fig. S1c).

Taken together, these results suggest that the reduced numbers of inflammatory monocytes in the bloodstream and vital organs of CCR2-deficient mice is associated with the reduced systemic inflammation characterized by less plasmatic and tissue cytokines and also less tissue damage, culminating in the increase of the survival rate in these animals.

3.3 | CCR2-deficient mice have enhanced migration of neutrophils to the infection site

Despite the recognized role of inflammatory monocytes in pathogen control during sepsis in WT mice,[16] we demonstrated that the reduced numbers of inflammatory monocytes in the infection site that were observed in the CCR2-deficient mice were not accompanied by an uncontrolled bacterial load. To understand which immune cells are mediating the control of infection in CCR2-deficient mice during CLP-induced sepsis, we next investigated the migration of neutrophils to the peritoneal cavity in these animals. Interestingly, septic CCR2-deficient mice had enhanced neutrophil migration compared to that of the WT group (Fig. 4a). Curiously, the enhanced neutrophil migration in septic CCR2-deficient mice was not associated with an increase in the level of CXCL2, a neutrophil chemoattractant (Fig. 4a). However, the CCR2-deficient septic mice had reduced CXCR2 internalization in peripheral blood neutrophils (Fig. 4b), which may explain the lack of a reduction in sepsis-induced neutrophil migration to the infection site, as described in previous studies.[8,10,21]

3.4 | Systemic adoptive transfer of inflammatory monocytes exacerbates sepsis pathogenesis

To confirm that CCR2-deficient mice were more resistant to sepsis due to a reduction in the numbers of circulating inflammatory monocytes, we sorted inflammatory monocytes from the bone marrow of WT mice and transferred them to CCR2-deficient animals. Consistently, inflammatory monocytes transfer resulted in an increase in the mortality rate after CLP-induced sepsis (Fig. 5a), together with an increase in the plasma levels of IL-6 and IL-10 (Fig. 5b). Similarly, the systemic transference of CCR2-deficient inflammatory monocytes to the CCR2-deficient mice also promotes an increase of the mortality, similar to that observed with the transference of WT-CCR2 inflammatory monocytes (Fig. 5a). Taken together, our data demonstrate that inflammatory monocytes play an important role in exacerbating the systemic inflammatory response during sepsis pathogenesis and that once the monocytes are in the periphery, CCR2 is not involved in the trigger of the systemic inflammatory response by these cells.

4 | DISCUSSION

The role of the CCR2 receptor in mediating the migration of inflammatory monocytes from the bone marrow to the bloodstream is well established for local restricted infections with bacteria, fungi, protozoa, and viruses.[17,20,22,23] Extending this knowledge to sepsis, we

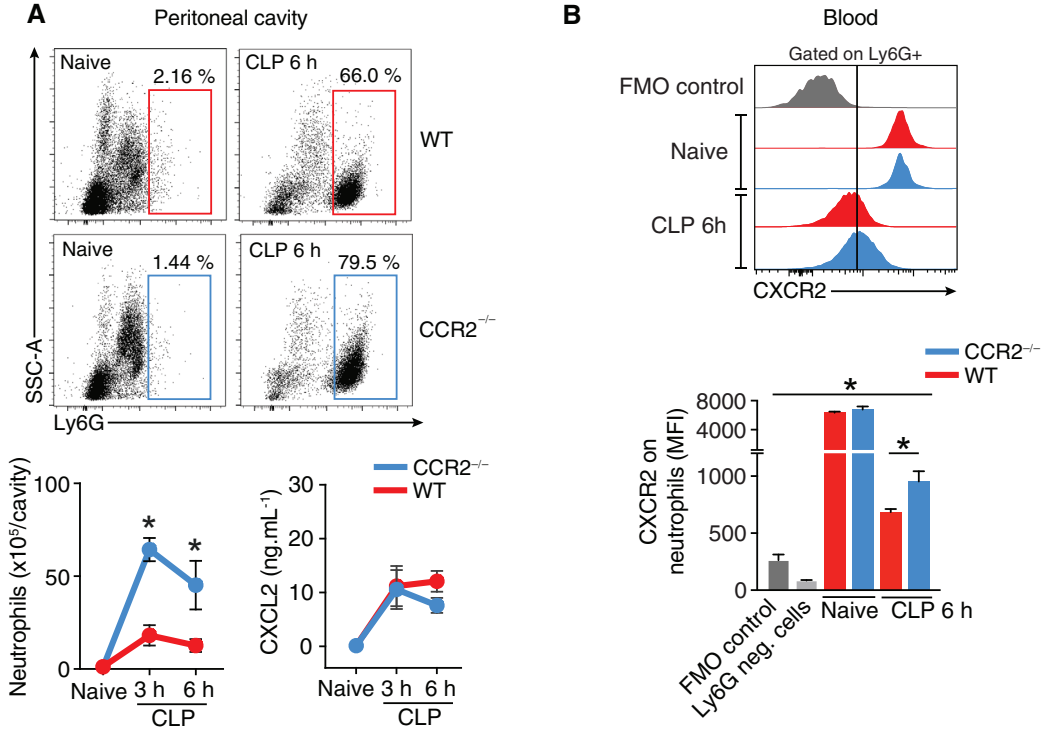

FIGURE 4 **CCR2-deficient mice have increased migration of neutrophils to the peritoneal cavity.** The blood and peritoneal cavity lavage fluid of WT (red) and CCR2-deficient (blue) mice under naïve ($n = 3$) or cecal ligation and puncture (CLP; $n = 6$) conditions were harvested 3 or 6 h after sepsis induction. (**A**) Neutrophil number (viable cells, Ly6G$^+$, and Ly6C$^-$) and the concentration of CXCL2 were determined in the peritoneal cavity lavage fluid by cytometry and ELISA, respectively. The dot plots are pregated on live cells. The gates represent the percentage of neutrophils. (**B**) Mean of fluorescence intensity (MFI) of CXCR2 on circulating neutrophils (viable cells, Ly6G$^+$, and Ly6C$^-$) was determined by cytometry. The results are the mean ± SEM from an individual representative experiment that was repeated twice, and * indicates $P < 0.05$ by ANOVA followed by Bonferroni correction

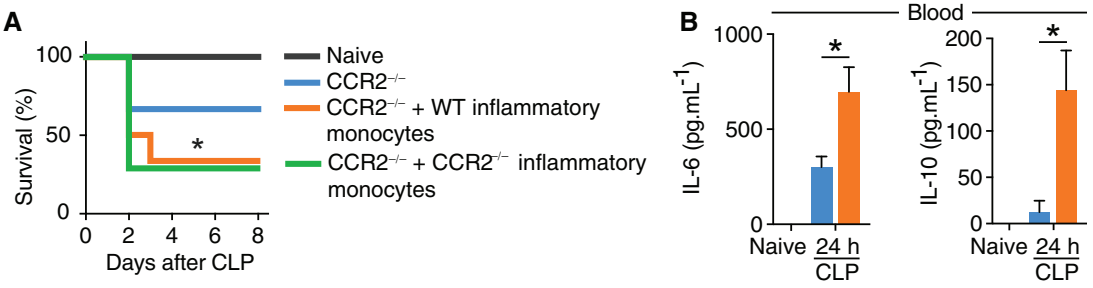

FIGURE 5 **Systemic transference of inflammatory monocytes from WT and CCR2-deficient mice to CCR2-deficient mice increases the mortality and systemic inflammation of the CCR2-deficient mice under cecal ligation and puncture (CLP)-induced sepsis.** (**A**) Survival rate of CCR2-deficient (blue) ($n = 6$), CCR2-deficient mice that received 0.5×10^6 WT inflammatory monocytes (orange) ($n = 6$) or CCR2-deficient mice that received 0.5×10^6 CCR2$^{-/-}$ inflammatory monocytes (green). The results indicate the percentage of survival and were analyzed by the Mantel-Cox log-rank test. (**B**) The concentrations of IL-6 and IL-10 were determined by ELISA in plasma harvested 24 h after CLP. The results are presented as the mean ± SEM and were analyzed by ANOVA followed by Bonferroni correction. The panels show the results from an individual experiment that was repeated twice, and * indicates significant differences ($P < 0.05$)

demonstrated that CCR2 plays an essential role in the emigration of inflammatory monocytes from bone marrow to the bloodstream, and as a consequence of it, CCR2-deficient mice have less inflammatory monocytes in the infection site and vital organs. In addition to the participation of CCR2, we recently described that inflammatory monocytes acquire CCR5 expression during CLP-induced sepsis, which is important for their migration to the infection site.[16] Herein, we observed impairment in CCR5 expression in CCR2-deficient mouse inflammatory monocytes, suggesting a possible CCR2-dependent mechanism for CCR5 expression.

The migration of inflammatory monocytes to the infection site during sepsis and local restricted infections is important for the control of pathogen growth.[16,17,20] Although septic CCR2-deficient mice presented less inflammatory monocytes in the infection site, the

bacterial load was not different between the two groups. In addition to inflammatory monocytes, neutrophils also play an important role in microorganism control during sepsis.[8,10] In the present study, we demonstrated that CCR2-deficient mice showed higher migration of neutrophils to the site of infection compared to that of septic WT mice. This increased neutrophil migration could counterbalance the possible deficiency in the control of microorganism growth due to low inflammatory monocyte numbers in septic CCR2-deficient mice. Although the levels of the CXCL2 chemokine (an important chemokine involved in neutrophil recruitment) were similar in the peritoneal cavity between WT and CCR2$^{-/-}$ mice, the enhanced neutrophil migration in septic CCR2-deficient mice could be explained by the partial prevention of CXCR2 internalization on the circulating neutrophils of these animals. In fact, the phenomenon of CXCR2 internalization during sepsis was previously associated with impaired neutrophil migration to the infection site in severe sepsis.[8,10,21] It is also important to mention that other neutrophils chemokines nonevaluated here could also be acting in this differential recruitment of neutrophils. However, in this study, we are focusing on the mechanisms by which inflammatory monocytes contribute to organ damage during sepsis.

Clinical studies have demonstrated that monocytes acquire a proinflammatory phenotype in the bloodstream during sepsis, an event that contributes to the exacerbation of systemic inflammation.[15] In this context, it was reported that the migration of inflammatory monocytes to the CNS through a CCR2-dependent mechanism contributes to long-term cognitive impairment in experimental sepsis.[12] Herein, we suggest that despite the similar bacterial load, CCR2-deficient mice presented a higher survival rate than their WT counterparts due to reduced plasmatic and tissue cytokines production and due to a reduced accumulation of inflammatory monocytes in vital organs, leading to less organ damage. In fact, the transfer of WT inflammatory monocytes to septic CCR2-deficient mice led to an increase in systemic inflammation (IL-6 and IL-10), culminating in a reduction in the survival rate. Similarly, the same reduction of the survival rate was observed when CCR2-deficient inflammatory monocytes were transferred to CCR2-deficient mice. Together, these results suggest that once the monocytes are in the periphery, CCR2 is not involved in the activation of these cells, triggering the systemic inflammatory response. Therefore, our results indicate that the protective effect observed in septic CCR2-deficient mice is due to the inhibition of inflammatory monocyte recruitment to bloodstream, which is consistent with the literature. According to our data, it was described that the CCR2-deficient mice are more resistant to CLP sepsis or endotoxemia.[24,11] On the other hand, in an apparent contradiction, it was described that the genetic deletion or antibody blockade of CCL2 (CCR2 agonist) increased the susceptibility to CLP sepsis.[25,26] The simple explanation for this difference is the use of different experimental models. CCL2 and CCR2 are promiscuous agonists and receptors, respectively,[27] implicating that the CCL2 blockage does not promote the same effect of the CCR2 deficiency. In fact, the CCL2 deficiency decreases IL-10 production in the infection focus, and as a consequence, the host presents an excessive inflammation and is less susceptible to sepsis.[25] Furthermore, the antibody blockade of CCL2 culminates in the reduction of Leukotriene B4 (LTB4) induction, decreased neutrophil recruitment to the peritoneal cavity, and uncontrolled infection dissemination.[26] Contrary, in our model (CCR2 deficiency) we did not observe the reduction of IL-10 and CCL2 in the infection focus (Fig. 2c).

In summary, our results indicate that inflammatory monocytes, similar to neutrophils, play a dual role during sepsis pathogenesis, mediating host resistance by controlling infection and reducing host tolerance by promoting systemic inflammation and organ damage. Furthermore, the mechanism by which CCR2-deficient inflammatory monocytes are more resistant to CLP sepsis is due to a decrease in the emigration of these cells from bone marrow to the circulation, and, as a consequence of it, CCR2-deficient mice have less accumulation of inflammatory monocytes in vital organs and less systemic inflammation and organ damage.

AUTHORSHIP

G.C.M.C. designed and performed most of the experiments, analyzed the data, and wrote the manuscript. K.A.d.L., F.V.S.C., C.H.H., V.V.S.M., M.H.F.L., and D.C.B.N. designed and performed the experiments. J.C.A.F. and T.M.C. provided some experimental design and contributed to expert discussions of the project. F.Q.C. conceived the study, supervised the overall project, designed the experiments, and wrote the manuscript. K.A.d.L. and F.V.S.C. contributed equally to this study.

KAdL and FVSC contributed equally to this study.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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