ORIGINAL RESEARCH PAPER

CD14 signaling mediates lung immunopathology and mice mortality induced by *Achromobacter xylosoxidans*

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Abstract

Objective and design Our research aimed to investigate the role of CD14 in pulmonary infection by *Achromobacter xylosoxidans* in an experimental murine model.

Methods C57Bl/6 or CD14-deficient mice were infected intratracheally with non-lethal inoculum of *A. xylosoxidans*. At times 1, 3 and 7 days after infection, lungs, bronchoalveolar lavage and blood were collected. CD14 gene expression was determined by RT-PCR. The bacterial load in the lungs was assessed by counting colony forming units (CFU). Cytokines, chemokines, lipocalin-2 and sCD14 were quantified by the ELISA method. Inflammatory infiltrate was observed on histological sections stained with HE, and leukocyte subtypes were assessed by flow cytometry. In another set of experiments, C57Bl/6 or CD14-deficient mice were inoculated with lethal inoculum and the survival rate determined.

Results CD14-deficient mice are protected from *A. xylosoxidans*-induced death, which is unrelated to bacterial load. The lungs of CD14-deficient mice presented a smaller area of tissue damage, less neutrophil and macrophage infiltration, less pulmonary edema, and a lower concentration of IL-6, TNF-α, CXCL1, CCL2 and CCL3 when compared with lungs of C57Bl/6 mice. We also observed that *A. xylosoxidans* infection increases the number of leukocytes expressing mCD14 and the levels of sCD14 in BALF and serum of C57Bl/6-infected mice.

Conclusions In summary, our data show that in *A. xylosoxidans* infection, the activation of CD14 induces intense pulmonary inflammatory response resulting in mice death.

Keywords *Achromobacter xylosoxidans* · CD14 · Soluble CD14 · Pneumonia

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Introduction

A. xylosoxidans is a Gram-negative, mobile, biofilm-forming bacillus and resistant to various antibiotics which cause recurrent pulmonary infections in patients with cystic fibrosis (CF), contributing to disease progression [1, 2]. CF patients infected with *A. xylosoxidans* present high amounts of cytokines in serum and sputum in comparison with health individuals [3]. Furthermore, other authors have shown that in vitro exposure of human lung lineage cells with cytotoxins free of lipopolysaccharide (LPS) extracted from *A. xylosoxidans*, can induce the production of cytokines, vacuolization and lung cell death [4]. Recently, our group showed that leukotriene B$_4$ (LTB$_4$) has a protective role in a mice model of *A. xylosoxidans* infection by inducing the production of α-defensin-1, an antimicrobial peptide responsible for reduction of bacterial load in the lungs, and

control of inflammation [5]. Together, these data seem to point to the importance of the inflammatory process in the outcome of *A. xylosoxidans* infection.

The recognition of LPS, the main component of the wall of Gram-negative bacteria, is well described [6, 7]. Soluble Lipopolysaccharide-Binding Protein (LPB) binds to bacterial membrane sites rich in LPS, forming aggregates, which are recognized by CD14 and then presented to the MD2-TLR4 complex, resulting in the activation of transcription factors, such as NF-κB, and induction of pro-inflammatory genes [8, 9]. CD14 is a myeloid differentiating glycoprotein, expressed mainly in immune cells, such as macrophages, monocytes and neutrophils [10, 11], although it is also expressed in non-immune cells, such as epithelial cells [12] and hepatocytes [13]. CD14 can be anchored to the membrane (mCD14) by a tail of glycosylphosphatidylinositol (GPI) or soluble (sCD14) in plasma and biological fluids [14]. In addition to being considered a co-receptor in the recognition of LPS, studies have shown that CD14 is important in the clearance of apoptotic cells [15], potentiates cardiovascular and metabolic complications in obesity [16] and mediate lung inflammation induced by scorpion venom as previously reported by our group [17, 18].

CD14 signaling has divergent impacts, depending on stimulus and tissue location [19]. Previously, we showed that CD14 controls macrophage metabolism, which varies according to the stimulus and origin of these cells [20]. In addition, the blockade or deficiency of CD14 has been shown to confer resistance to mortality induced by systemic infection of *E. coli* and endotoxemic shock induced by LPS, with reduction in bacteremia and levels of TNF-α and IL-6 in serum [21–23]. In contrast, the role of CD14 in the outcome of lung infections caused by Gram-negative bacteria still controversial [24]. Despite advances in understanding some aspects of *A. xylosoxidans* infection in recent years, the mechanisms involved in the recognition of this bacillus and the receptors involved, remain poorly understood. Therefore, in this work, we evaluated the role of CD14 in a mice model of pneumonia induced by *A. xylosoxidans*.

Materials and methods

Mice

C57Bl/6 (wild-type) and *Cd14*$^{-/-}$ mice aged 8–9 weeks and weighing 24–26 g were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed in ventilated shelf with free access to water and food at the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (FCFRP-USP). All procedures performed were

approved by the FCFRP Animal Use Ethics Committee (CEUA) under protocol number 17.1.820.60.0.

A. xylosoxidans culture

The LMG1836 strain of *A. xylosoxidans* from the Belgian Co-ordinated Collections of Micro-organisms BCCM/LMG was used. For each experiment, the strain was grown in brain and heart infusion broth (BHI) with agar (Difco, Detroit, MI, USA) and maintained at 37 ºC for 18 h. The colonies were resuspended in sterile PBS and the number of bacteria was quantified by spectrophotometer (600 nm) for later infection [5].

Intratracheal infection, collection of bronchoalveolar lavage fluid (BALF), blood collection and experimental design

C57Bl/6 and *Cd14*$^{-/-}$ mice were anesthetized via intraperitoneal (i.p.) administration of ketamine (75 mg/kg body weight) and xylazine (10 mg/kg) and infected intratracheally (i.t.) with 100 μL of bacterial inoculum containing 2.0×10^7 bacilli (non-lethal inoculum) in the kinetic experiments, for assessment of lung pathology, cell recruitment and production of inflammatory mediators in lungs and blood. On days 1, 3 and 7 d.p.i., the mice were anesthetized and euthanized by cervical dislocation to collect BALF ($n = 3$–7 mice/group). In another set of experiments ($n = 3$–6 mice/group), blood and lungs were collected without performed the BALF. The control groups received i.t. 100 μL of sterile PBS. The blood was collected in a tube without anticoagulant and centrifuged at 1500*g* for 12 min, at 4 ºC, to obtain the serum. To determine the survival curve, a separate group of C57Bl/6 and *Cd14*$^{-/-}$ mice ($n = 8$/group) were infected with 6.3×10^7 bacilli and the mortality monitored daily.

Histopathological analysis of the lungs and immunohistochemistry

The middle right lobe of the lungs of the mice from the different groups were removed and fixed in 10% buffered formaldehyde for 7 days and then prepared in paraffin blocks. Histological sections of 5 μM thick were stained with hematoxylin and eosin (HE). Seven random photos were taken of each lung (400× magnification) to assess the area of tissue injury using ImageJ software (U.S. NIH, Bethesda, MD, USA) as described [25]. Immunohistochemistry was performed to mark CD14 in the lungs of C57Bl/6 mice. The sections were dewaxed and incubated with peroxidase blocking reagent. The sections were treated with 1% bovine

serum albumin (BSA) to block nonspecific reactions. Clone [1H5D8] anti-CD14 antibody (Abcam, Burlingame, CA, USA) was applied to the cuts followed by improved horseradish peroxidase (HRP) based on improved luminol (ECL) (GE Healthcare, Chicago, IL, USA). The reaction was developed with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) and the sections were counterstained with HE.

Bacterial burden and flow cytometry of lungs cells

The left upper pulmonary lobe was digested at 37ºC for 45 min in 1 mL per lung of digestion buffer [RPMI 1640, 0.05 mg/mL liberase (Roche, Basel, Switzerland) and DNAse 0.5 mg/mL (Sigma–Aldrich, St. Louis, Missouri, USA)]. 50 μL of these suspensions were diluted and cultivated on BHI agar and maintained at 37 ºC for 36 h to count the CFU. The bacterial burden was expressed as an absolute number of CFU/gram of lung. The rest of the suspensions were filtered through a cell filter with 100 μm pore (BD Biosciences, Franklin Lakes, New Jersey, USA) to characterize the lung cells. The resulting suspension was centrifuged at 300g at 4 ºC for 10 min. The red cells present were lysed with ammonium potassium chloride (ACK). The number of viable cells was determined to 2×10^6 using Trypan blue and Neubauer chamber [26]. Following fixation and blocking, the antibodies used were: CD11b (clone: M1/70), CD11c (clone: N418), F4/80 (clone: BM8), MHC-II (clone: M5/114.15.2), CD14 (clone: rmC5-3), Ly6G (clone: 1A8), CD45 (clone: 30F11), CX3CR1 (clone: SA011F11), Ly6C (clone: HK1.4), all purchased from eBioscience (San Diego, CA) or BD Biosciences (Franklin Lakes, New Jersey, USA). After labeling, the cells were resuspended in Cytofix fixation buffer (BD Biosciences Franklin Lakes, New Jersey, USA). 2×10^6 events were acquired on the LSR Fortessa cytometer (BD Biosciences, Franklin Lakes, New Jersey, USA) and analyzed using FlowJo software v.10.0.7 (BD Biosciences, Franklin Lakes, New Jersey, USA).

Quantification of sCD14

The BALF was centrifuged at 500 g for 10 min at 4ºC and the supernatant was frozen at − 80 ºC. To quantify sCD14 in the BALF supernatant and animal serum, the CD14 quantikine ELISA kit mouse (range 62.5–4000 pg/mL; R&D Systems, Minnesota, MN, USA) was used and the optical density was read at 450 nm in a spectrophotometer, according to the manufacturer's specifications.

Quantification of cytokine and total proteins

The upper and lower right lung lobes were homogenized in 2 mL RPMI-I with Ultra-Turrax (IKA, Labortechnik,

Staufen, Germany) and the suspensions obtained were centrifuged. The supernatants of the lung homogenates and the serum were used for quantification of cytokines (IL-6, TNF-α, IL-1α, IL-1β, IL-17, IFN-γ) and chemokines (CXCL1, CCL2, CCL3) by ELISA (R&D Systems, Minnesota, MN, USA), according to the manufacturer's instructions. Total proteins were quantified in the lungs and BALF supernatants, using the Bradford colorimetric method (Coomassie reagent, Pierce Chemical, Rockford, IL, USA), according to the manufacturer's instructions.

Nitrite quantification

For indirect quantification of nitric oxide, the supernatants of the BALF and lung homogenates were used to quantify nitrite (NO_2^-) using the Griess method as described [27].

RNA extraction from the lungs and qRT-PCR

To evaluate the CD14 gene expression in C57Bl/6 mice, the lower left lobe of the lungs was removed and homogenized for mRNA extraction with Purelink RNA MiniKit kit (Life Technologies, Carlsbad, California, USA) according to the manufacturer's recommendations. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, MA, USA). Real-time quantitative PCR was performed using the TaqMan system in Step One Plus (Applied Biosystems, Waltham, MA, USA). The *Cd14* expression values (primer: Mm00438094_g1; Life Technologies—CA, EUA) were normalized by the expression values of the constitutive *Gapdh* gene (primer: Mm99999915_g1; Life Technologies—CA, EUA). For representation of the results, the gene expression of the uninfected mice was considered as reference samples, with $2^{-\Delta\Delta CT}$ equal to 1 [28].

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (Gran Pad Software Inc., San Diego, CA, USA) by ANOVA one-way with multiple Tukey's comparisons. For analysis of the survival curves, the Log-Rank test and Mantel-Cox test were performed in the post-test. Differences were considered significant when p values < 0.05. The correlation analysis between sCD14 and total proteins was performed using Pearson r statistical test.
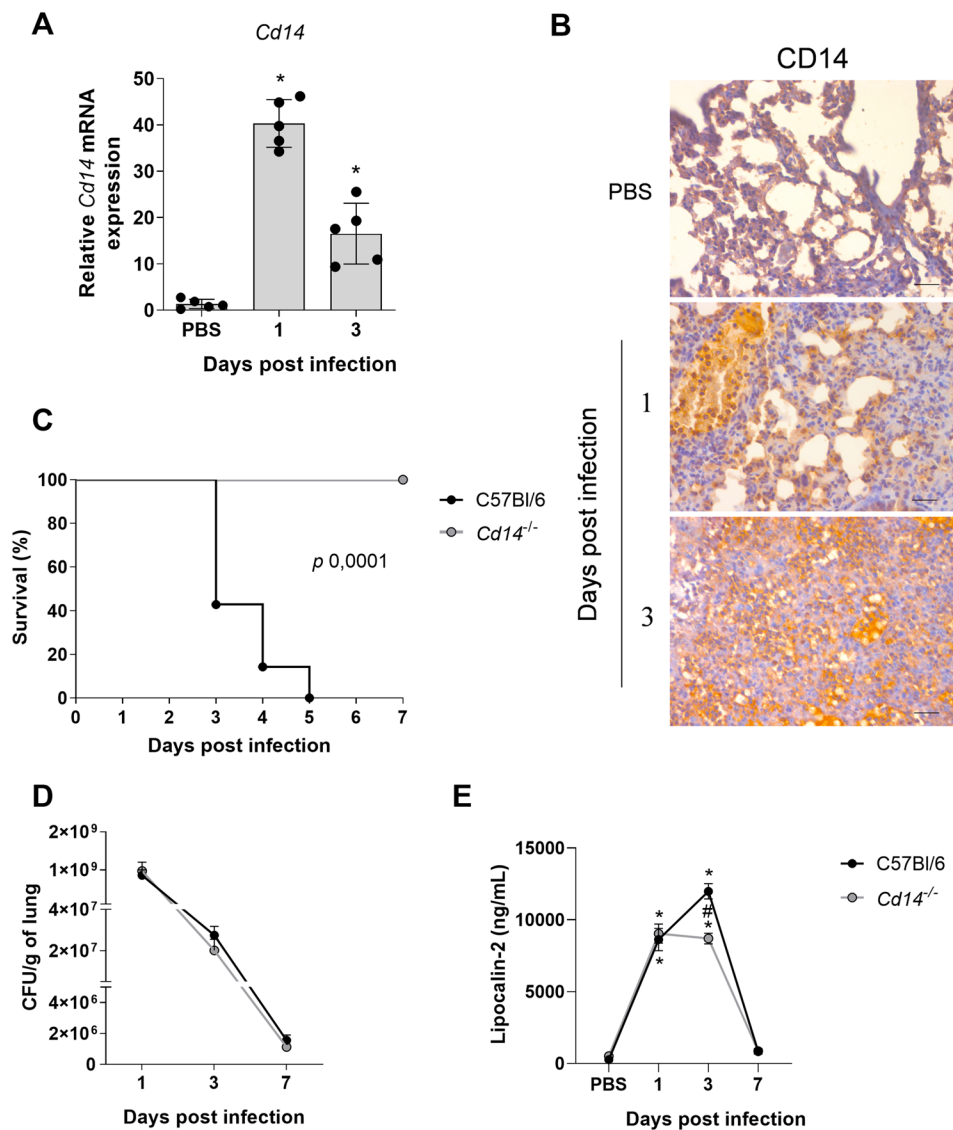
Fig. 1 CD14 is increased in the lungs after infection and mediates *A. xylosoxidans*-induced mortality, but does not change the bacterial burden. **A**. *Cd14* gene expression in the lungs of C57Bl/6 mice infected with 2.0×10^7 CFU (non-lethal inoculum) evaluated by RT-PCR. The values were normalized by the values of *Gapdh* expression. **B**. Representative photomicrographs showing the expression of CD14 in the lungs of C57Bl/6 mice at 1 and 3 d.p.i., stained with anti-CD14 by immunohistochemistry ($400 \times$ magnification, scale bar: 50 μm). **C**. The survival rate of C57Bl/6 and *Cd14*$^{-/-}$-infected mice with 6.3×10^7 CFU (lethal inoculum) was monitored during 7 days post-infection. **D**. Bacterial burden determined by *A. xylosoxidans* CFU counts in the lungs of C57Bl/6 or *Cd14*$^{-/-}$ mice infected with 2.0×10^7 bacilli (non-lethal inoculum). **E**. Concentrations of lipocalin-2 measured by ELISA in lungs of infected mice. **A**, **B**, **D** and **E** are representative of two independent experiments ($n = 4$–5 animals), data were analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test, and are expressed as mean ± s.e.m. *$p < 0.05$. **C** is representative of three independent experiments ($n = 8$/experiment) analyzed by Log-rank test and Mantel-Cox post-test. *infected *vs* PBS, #*Cd14.*$^{-/-}$ *vs* C57Bl/6

Results

The absence of CD14 protects against *A. xylosoxidans*-induced mortality without changing the bacterial load

To investigate whether *A. xylosoxidans* infection increases CD14 in the lungs, we infected C57Bl/6 mice intratracheally with 2.0×10^7 *A. xylosoxidans* bacilli (non-lethal inoculum) and evaluated CD14 expression. As shown in Fig. 1A, the *Cd14* gene was up-regulated in the lungs on days 1 and 3 after infection when compared to the uninfected group (PBS control group). Similarly, CD14 protein labeling in the lungs was increased after infection (Fig. 1B). The

role of CD14 in pneumonia induced by Gram-negative bacteria is controversial, in some models it is harmful, as it induces intense pulmonary inflammation, in others it is essential, as it induces microbicidal mechanisms and control of bacterial load [24, 29]. Therefore, to assess the role of CD14 in our pneumonia model, we used CD14-deficient mice. To evaluate the survival curve, mice were infected with 6.3×10^7 *A. xylosoxidans* bacilli (lethal inoculum), and as shown in Fig. 1C, on the 5th day after infection 100% of C57Bl/6 mice died, whereas all mice deficient for CD14 survived 7 days after infection. Surprisingly, we did not detect differences in bacterial load in the lungs (Fig. 1D) and in the production of nitric oxide (data not shown), but we observed reduced production of lipocalin-2 in the lungs of CD14-deficient mice on the 3 d.p.i. when compared to the C57Bl/6 animals (Fig. 1E). Taken together, our data show that *A. xylosoxidans* infection increases CD14 expression, and that the absence of this molecule protects mice from pneumonia-induced lung injury and mortality.

CD14 deficiency protects mice from lung injury induced by *A. xylosoxidans* infection.

We observed that CD14-deficient mice presented less lung injury and edema and are protected from mortality, despite showing no difference in bacterial load when compared to C57Bl/6 mice. To investigate a putative mechanism that induces mortality in C57Bl/6 mice, we evaluated the inflammation in the lungs of infected mice. Macroscopically, the lungs of infected C57Bl/6 mice showed hemorrhagic foci and more intense lung damage (Fig. 2A) and more pronounced lung edema (Fig. 2B) when compared to CD14-deficient mice. We confirmed the reduction in lung inflammation in the absence of CD14 through histological analysis. As we reported before [5], we observed that *A. xylosoxidans* infection induces intense recruitment of leukocytes to the lungs of C57Bl/6 mice, especially at 3 d.p.i.; however, CD14-deficient animals presented significant reduction in cellular recruitment (Fig. 2C), resulting in reduced damage in the architecture of the lung (Fig. 2D). In addition, $Cd14^{-/-}$ mice exhibited lower BALF total protein

Fig. 2 The absence of CD14 restrains pulmonary inflammation induced by *A. xylosoxidans*. **A**. Macroscopic aspect of the lungs of mice infected with 2.0×10^7 *A. xylosoxidans* at 3 d.p.i. showing the intensity of lung damage. **B**. Weight of the lungs of mice infected with *A. xylosoxidans*. **C**. Representative photomicrographs of the lung pneumonia of mice infected with *A. xylosoxidans*, stained with HE, at 100× (scale bar:100 μm) and 400× (scale bar: 50 μm, in inset) magnification. **D**. Percentage of inflamed area in the lungs of mice infected with *A. xylosoxidans*, analysis performed by ImageJ software. **E**. Total proteins in BALF of infected mice. **A**, **B**, **E** and **F** are representative of two independent experiments (n = 4–6), **C**, **D** are representative of one experiment (n = 4–6). Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test, and are expressed as mean ± s.e.m. *$p < 0.05$.* infected *vs* PBS, # $Cd14.^{-/-}$ *vs* C57Bl/6
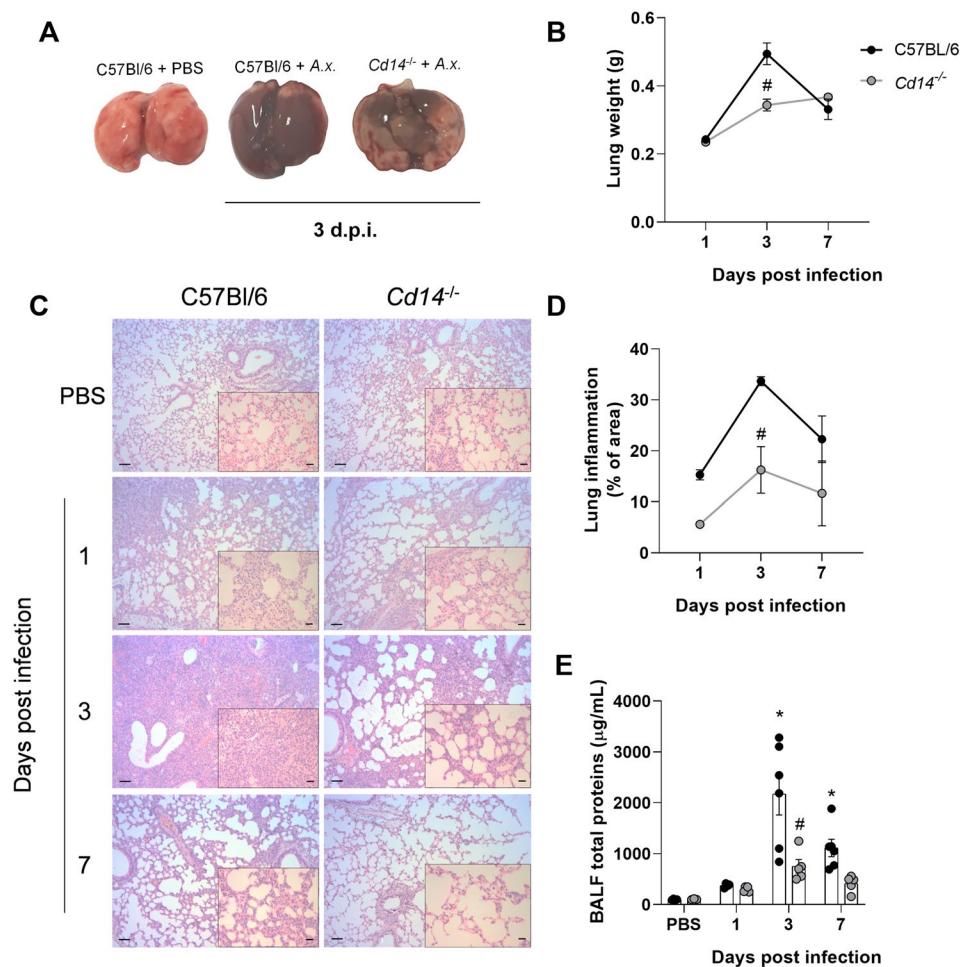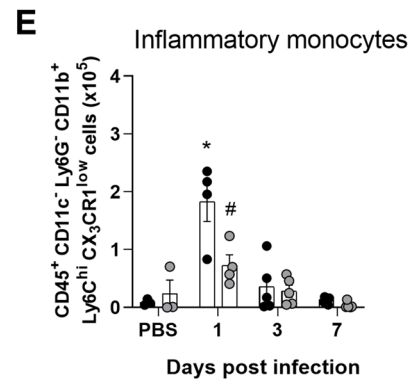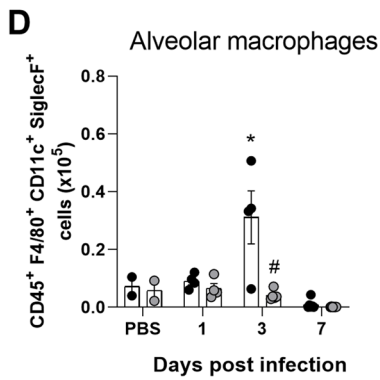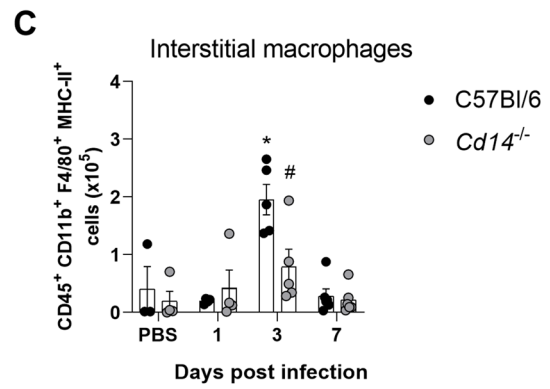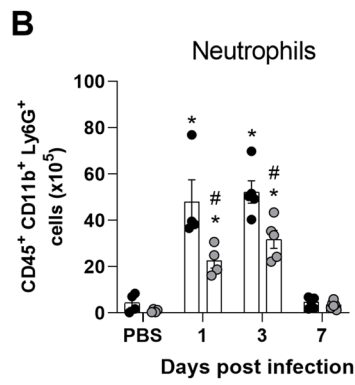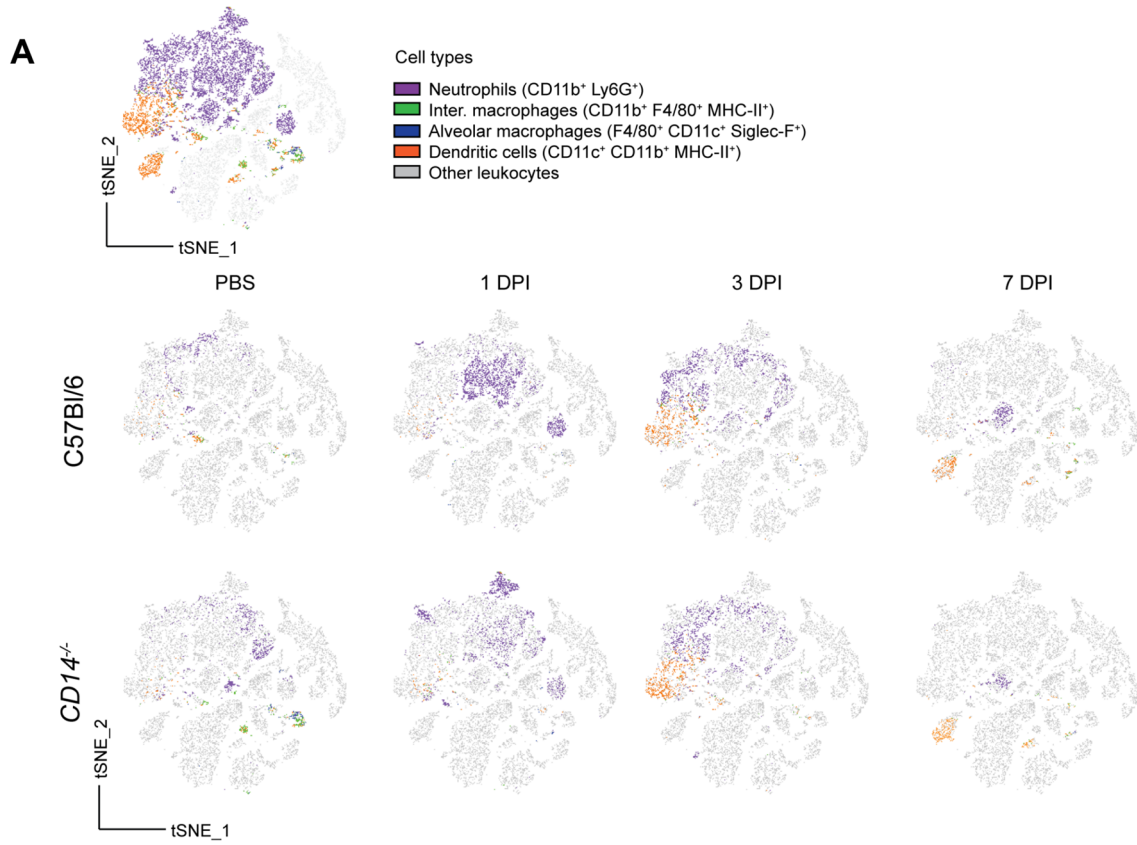
◄**Fig. 3** CD14 modulates the recruitment of neutrophils, macrophages and DCs to the *A. xylosoxidans*-infected lungs. **A**. t-SNE map show the representative neutrophils (CD45$^+$ CD11b$^+$ Ly6G$^+$), interstitial macrophages (CD45$^+$ CD11b$^+$ F4/80$^+$MHC-II$^+$), alveolar macrophages (CD45$^+$ F4/80$^+$ CD11c$^+$ Siglec-F$^+$) and DCs (CD45$^+$ CD11c$^+$ CD11b$^+$ MHC-II$^+$). Absolute number of neutrophils **B**, interstitial macrophages **C**, alveolar macrophages **D** and inflammatory monocytes **E** in lungs. Data are representative of two independent experiments (*n* = 4–6), analyzed by one-way ANOVA with Tukey's multiple comparisons test, and are expressed as mean ± s.e.m. *p < 0.05.* infected *vs* PBS, # *Cd14.$^{-/-}$ vs* C57Bl/6

concentration on the 3 d.p.i. when compared to C57Bl/6 mice, indicating less vascular leakage in the lungs (Fig. 2E).

Absence of CD14 alters the population of cells recruited into the lungs

To evaluate the impact of CD14 deficiency on the population of cells recruited during *A. xylosoxidans* infection, C57BL/6 and *Cd14$^{-/-}$* mice were infected with 2×10^7 CFU and cell subpopulations were characterized in the lungs, applying the flow cytometric gating hierarchy shown in the Suppl. 1A. Initially, we analyze the complexity of lung cells in a two-dimensional way and displayed their distribution in relation to the CD45$^+$ population. The t-SNE map of the populations of some innate cells was produced according to the combination of surface markers expressed by each population (Fig. 3A). The analysis of the frequency of these cells in the lungs of mice infected with *A. xylosoxidans* showed that CD14-deficiency promotes a reduction in alveolar macrophages accompanied by a significant increase in dendritic cells at 3 d.p.i., and reduction of patrolling monocytes, at 7 d.p.i. (Suppl 2A-D). In addition, although we did not observe differences in the frequency of neutrophils between C57Bl/6 and *Cd14$^{-/-}$* mice, CD14-deficiency results in significant decrease in the absolute number of neutrophils in the lungs, at 1 and 3 d.p.i. (Fig. 3B), whereas the number of interstitial (Fig. 3C) and alveolar macrophages (Fig. 3D) only decreased at 3 d.p.i. The number of inflammatory monocytes was significantly reduced in the *Cd14$^{-/-}$* mice when compared with C57Bl/6 mice at 1 d.p.i. (Fig. 3D). We did not observe differences in the absolute number of dendritic cells between C57Bl/6 and *Cd14$^{-/-}$* mice (Suppl. 2E). Interestingly, the number of patrolling monocytes was reduced at 7 d.p.i. in the lungs of *Cd14$^{-/-}$* mice (Suppl. 2F). These data show that CD14 plays an important role for the influx of leukocytes into the lungs after *A. xylosoxidans* infection.

CD14 signaling induces chemokines and pro-inflammatory cytokines during *A. xylosoxidans* infection

The rapid recruitment of leukocytes to the lungs after an infection is coordinated by the production of chemokines by the resident cells [30]. In agreement with the decrease in immune cells in the lungs of CD14-deficient mice, the amount of CXCL1, CCL2 and CCL3 chemokines was also reduced at 3 d.p.i. (Fig. 4A-–C). CD14 signaling regulates the production of cytokines and others mediators of inflammation [31, 32]. The leukocytes recruited during an infection, such as neutrophils, macrophages and DCs, are important sources of pro-inflammatory cytokines, which potentiate the inflammatory response at the site of infection and can also reach the systemic circulation [33]. Based on this, we evaluated the impaction of the absence of CD14 on the production of cytokines in the lungs and the serum after *A. xylosoxidans* infection. Compared to C57Bl/6 mice, IL-6 was reduced at 3 d.p.i. in the lungs of *Cd14$^{-/-}$* mice (Fig. 4A); on the other hand, TNF-α modulation varied with time, at 1 d.p.i. there was less production of this cytokine in the lungs of *Cd14$^{-/-}$* mice, but at 3 d.p.i. TNF-α increased in the absence of CD14 (Fig. 4B). Although the infection induced production of IL-1α (Fig. 4C), IL-1β (Fig. 4D), IL-17 (data not shown) and IFN-γ (data not shown) in the lungs of C57Bl/6 mice, we did not observe differences when compared to *Cd14$^{-/-}$* animals. In serum, we observed the reduction of IL-6 at 1 d.p.i. in CD14-deficient mice (Fig. 4E), whereas TNF-α was reduced at 3 d.p.i when compared to C57Bl/6 mice (Fig. 4F). Taken together, these data show that CD14 is a strong inducer of pro-inflammatory mediators in response to *A. xylosoxidans* infection.

A. xylosoxidans infection increases CD14$^+$ leukocytes in the lung and sCD14 in BALF and serum

So far, our data show that infection by *A. xylosoxidans* increases the expression of CD14 in the lung parenchyma and that this receptor is essential to induces inflammation and injury of the lungs. Our next step was to investigate the main source of CD14 during the infection, and for that, we infected C57Bl/6 mice with 2.0×10^7 CFU of *A. xylosoxidans* and evaluate of CD14 in distinct forms. When we evaluated the absolute number of membrane-bound CD14$^+$ leukocytes (mCD14) recruited to the lung, we observed that the infection induces an increase in neutrophils (Fig. 5A), interstitial (Fig. 5B) and alveolar macrophages (Fig. 5C) expressing CD14 at 3 d.p.i. when compared to the PBS group. On the other hand, we did not observe variations in the median fluorescence intensity
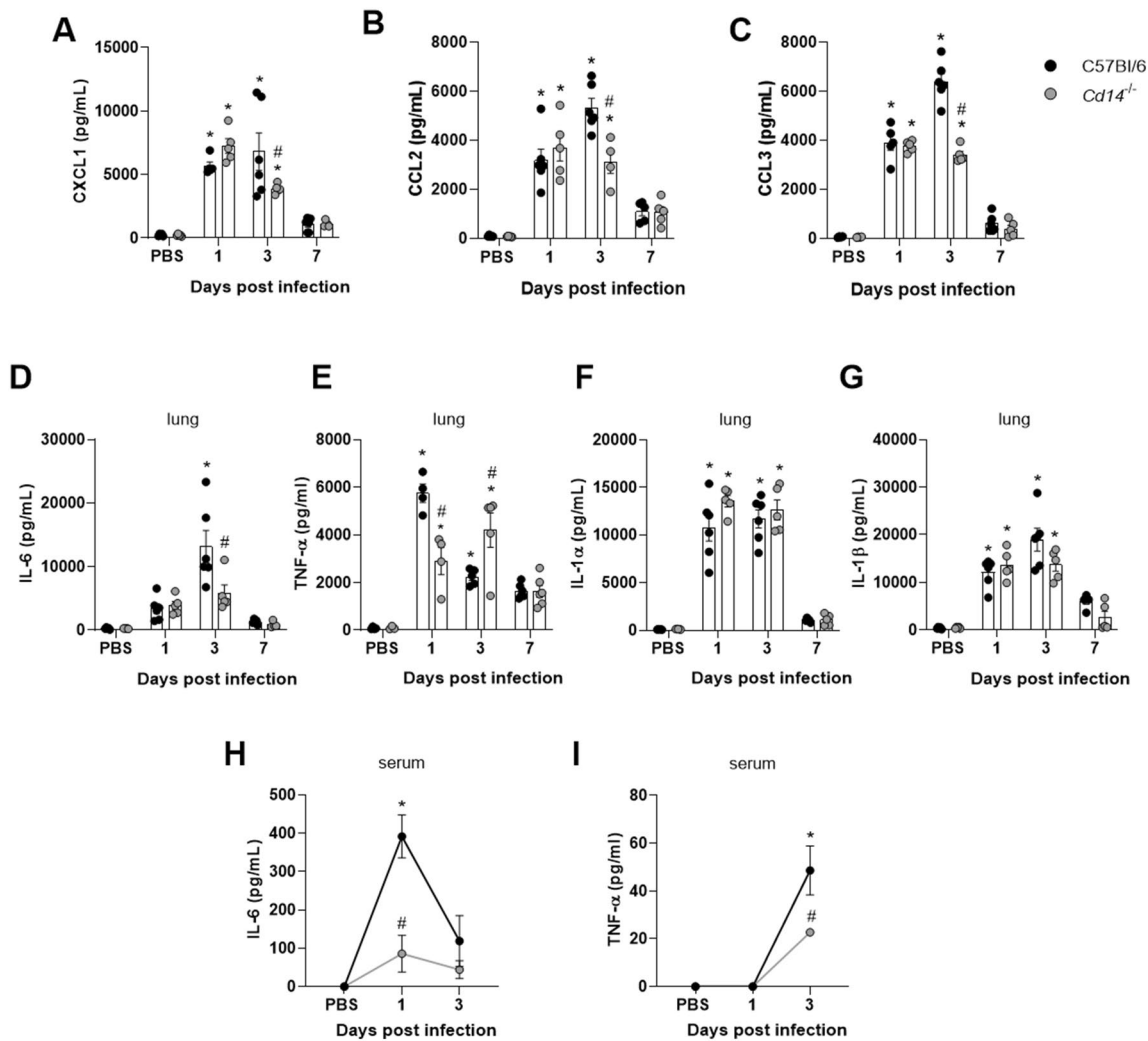
Fig. 4 The absence of CD14 impacts in the production of inflammatory mediators of mice infected with *A. xylosoxidans*. Concentration of chemokines CXCL1 **A**, CCL2 **B** and CCL3 **C**, and cytokines in the lungs, IL-6 **D**, TNF-α **E**, IL-1α **F**, IL-1β **G**, and IL-6 **H** and TNF-α **I** in the serum, determined by ELISA. **A–G** are representative of two independent experiments ($n = 4$–6), **H-I** are representative of one experiment ($n = 3$–5). Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test, and are expressed as mean $\pm$ s.e.m. *$p < 0.05$.* infected *vs* PBS, #*Cd14.$^{-/-}$ vs* C57Bl/6

in the evaluated macrophages, but only a decrease in neutrophils at 7 d.p.i. when compared to the uninfected group (Suppl. 3). As CD14 is also secreted in the soluble form (sCD14) after an inflammatory stimulus [34], we also evaluated sCD14. Interestingly, in C57Bl/6 BALF infected with *A. xylosoxidans* there is an increase in sCD14 at all times evaluated, peaking at 3 d.p.i. (Fig. 5D). We also detected increase in sCD14 at 3 d.p.i. in the serum (Fig. 5E), indicating that the intense inflammatory process that occurs in the lungs may reflect on the blood circulation. Interesting, the increase in sCD14 after infection positively correlates with the augment of total proteins in BALF at 3 d.p.i., indicating pulmonary edema (Fig. 5F), and thus, impaired lung function, which leads to mouse mortality.

Discussion

Recognition of PAMPs by immune cells is the first step to start the immune response, whose objective is to eliminate the pathogen and to restore the homeostasis [35, 36]. Innate immunity receptors, such as TLRs, are sensors that detect molecules expressed by pathogens, such as LPS. This molecule is the main component of the outer membrane of Gram-negative bacteria, and is classically recognized by TLR4 and the CD14 co-receptor [6, 37]. The role of CD14 in the outcome of infections by Gram-negative bacteria is controversial and may be related to the induction of a protective immune response or exacerbation of inflammation [19, 24]. In this study, we characterized for the first time the deleterious role of CD14 in pulmonary infection induced by
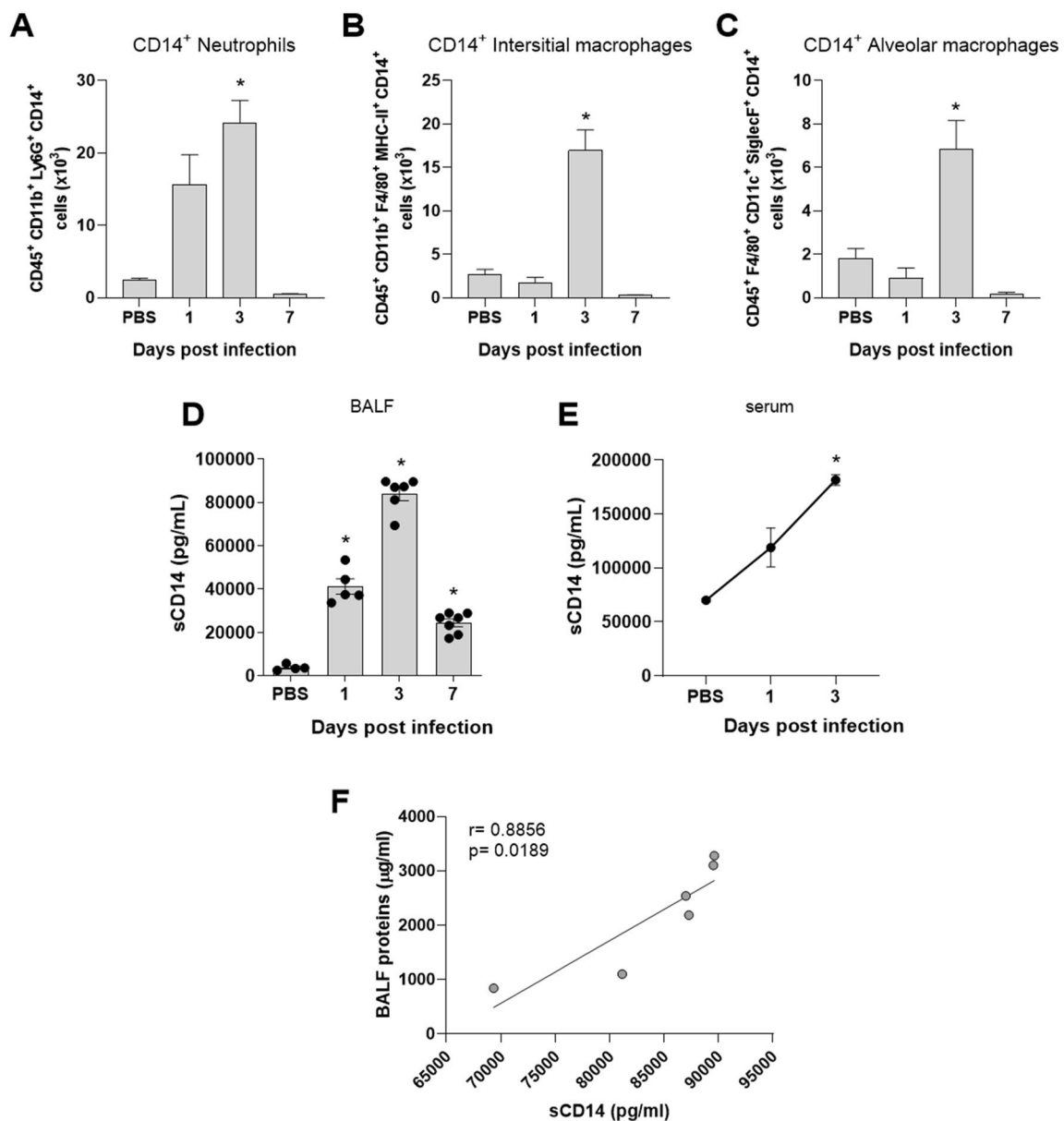
Fig. 5 *A. xylosoxidans* infection increases mCD14 in leukocytes and sCD14 in lung and serum. Absolute number of neutrophils **A**, interstitial **B** and alveolar macrophages **C** expressing CD14 in lungs evaluated by flow cytometry. sCD14 levels in BALF **D** and serum **E** detected by ELISA. **F**. Correlation study between sCD14 in lung and total proteins in BALF at 3 d.p.i. **A–E** are representative of two independent experiments ($n = 3$–7), data were analyzed by one-way ANOVA with Tukey's multiple comparisons test, and are expressed as mean $\pm$ s.e.m. *$p < 0.05$. *infected *vs* PBS. **F** correlation was performed using Pearson r statistical test. Each spot is representative of each mouse ($n = 6$) and the values of r and p are specified in the graph

A. xylosoxidans, an emerging nosocomial bacillus whose host responses are not yet to be elucidated.

Previous studies have shown that in pulmonary diseases induced by infection there is an increase in membrane and soluble CD14 expression [38–40]. However, there are conflicting results, reinforcing the fact that CD14 expression and its role varies according to the stimulus and site of infection [19, 24]. Some studies show that CD14 has a protective role in the intestine, by increasing the intestinal barrier function in a colitis model [41], and by controlling the bacterial burden of *Shigella flexneri* [42]. In contrast, in sepsis models, in LPS-induced endotoxemia or lung infections, CD14 activation appears to have a deleterious role [21, 22, 43, 44], despite a study showing that in cecal ligation and puncture (CLP)-induced sepsis the P2X7 purinergic receptor maintains high levels of CD14 in serum, which contributes to the control of bacterial dissemination [45]. Here, we show that beside *A. xylosoxidans* inducing

a significant increase in the expression of CD14 in the lung parenchyma (at 1 and 3 d.p.i.), its absence attenuates inflammation, as observed by the decreases in leukocyte influx, production of inflammatory mediators, lung damage and lipocalin-2. Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is a protein normally present in neutrophil granules, but can also be expressed in macrophages, and it is associated with antibacterial activity and intense injury and inflammation [46]. Interesting, CD14 absence did not interfered with the bacterial load, suggesting that CD14 is more related to the induction of the inflammatory process than to bacterial clearance in this model. Similarly, CD14-deficient mice with pneumonia caused by *Acinetobacter baumannii* also show a reduction in polymorphonuclear cells (PMNs) and TNF-α in the BALF, but, contrary to our findings, there is an increase in the bacterial load in the lungs in the first hours after infection, indicating that CD14 may be relevant only in the first hours to control bacterial burden [47]. Interesting, CD14-deficient neutrophils infected with *Burkholderia pseudomallei* have the same phagocytic capacity as neutrophils from non-deficient animals, suggesting that the CD14-dependent bacterial load reduction mechanism does not occur via phagocytosis and killing capacity [44], but it is associated with increased release of trappin-2 in the lungs, an essential antimicrobial peptide in the elimination of *P. aeruginosa* [48].

The influx of neutrophils into the lungs is important for the control of an infection, but if exacerbated, it can contribute to lung damage through the release of NETs and enzymes with proteolytic activity, such as neutrophil elastase [49], or the release of lipocalin-2 and other molecules that increase chemokine production by PMNs [50]. After *A. xylosoxidans* infection, we observed that CD14-deficient mice show a reduction in lipocalin-2, as well as a lower number of neutrophils in the lungs, in addition to less pulmonary damage, resulting in longer survival rate than wild-type animals. In agreement with our results, the lung parenchyma of CD14-deficient animals is more preserved during *S. pneumoniae* infection when compared to WT mice, in addition to having a lower concentration of total proteins in the lungs, and a reduction in IL-1β, IL-6 and CXCL1 in lungs and plasma of infected mice [51]. In tuberculosis, CD14 also mediates PMN recruitment and release of pro-inflammatory cytokines in the lungs, culminating in lung injury and higher animal mortality [52].

CD14 was first described as anchored to the membrane of macrophages and monocytes, but after inflammatory stimuli such as LPS, CD14 can be released in soluble form via the action of proteases or even after escaping from binding to GPI in post-translational modifications [29, 53]. Defining a pattern of CD14 expression in leukocytes from pneumonia patients or from infected mice is a challenge. Patients with tuberculosis have lower CD14 expression on monocytes when compared to healthy individuals [54], and similarly, mCD14 expression is reduced in alveolar macrophages from mice infected with *E. coli* when compared to cells from wild-type mice [55]. In contrast, the expression of mCD14 on monocytes and macrophages of children with pneumonia is higher when compared to the control group [56]. In our model of pulmonary infection by *A. xylosoxidans*, although we did not observe an increase in CD14 MFI on the surface of the evaluated leukocytes, we found an increase in the absolute number of neutrophils and macrophages expressing CD14 that are recruited to the lung, suggesting that the increase in cells capable of signaling via mCD14 contribute to the exacerbation of pulmonary inflammation. sCD14 is considered a potential marker of pneumonia in children [56] and a predictor of severity of coronavirus disease 2019 (COVID-19) [57]. Furthermore, sCD14 is considered an acute phase marker [34], and it was even shown that this molecule is capable of inducing pro-inflammatory cytokines independent of LPS in CF patients, contributing to the persistence of inflammation in these patients [58, 59]. Corroborating these findings, we observed that *A. xylosoxidans* induces sCD14 release in the BALF and serum of infected animals, suggesting that part of mCD14 of leukocytes or others cells, such epithelial cells, is shedding to the soluble profile during infection. The dynamics of sCD14 release by other leukocytes and non-immune cells needs to be further investigated.

Taken together, our findings highlight the detrimental role of CD14 in *A. xylosoxidans*-induced pneumonia, mediated by pulmonary and systemic release of sCD14, which likely maintains leukocyte recruitment to the lungs and impairs pulmonary homeostasis. Furthermore, these results reinforce that CD14 inhibition may be a possible alternative therapy for patients with chronic lung inflammation caused by *A. xylosoxidans*.

Declarations

Conflict of interest The authors declare no competing interests.

Compliance with ethical standards All experiments carried out in this work were approved by the Ethics Committee on the Use of Animals (CEUA) of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, protocol 17.1.820.60.0.

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