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Macrophage migration inhibitory factor (MIF) controls cytokine release during respiratory syncytial virus infection in macrophages

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Abstract

Objective and design Respiratory syncytial virus (RSV) is the major cause of infection in children up to 2 years old and reinfection is very common among patients. Tissue damage in the lung caused by RSV leads to an immune response and infected cells activate multiple signaling pathways and massive production of inflammatory mediators like macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine. Therefore, we sought to investigate the role of MIF during RSV infection in macrophages.

Methods We evaluated MIF expression in BALB/c mice-derived macrophages stimulated with different concentrations of RSV by Western blot and real-time PCR. Additionally, different inhibitors of signaling pathways and ROS were used to evaluate their importance for MIF expression. Furthermore, we used a specific MIF inhibitor, ISO-1, to evaluate the role of MIF in viral clearance and in RSV-induced TNF-α, MCP-1 and IL-10 release from macrophages.

Results We showed that RSV induces MIF expression dependently of ROS, 5-LOX, COX and PI3K activation. Moreover, viral replication is necessary for RSV-triggered MIF expression. Differently, p38 MAPK in only partially needed for RSV-induced MIF expression. In addition, MIF is important for the release of TNF-α, MCP-1 and IL-10 triggered by RSV in macrophages.

Conclusions In conclusion, we demonstrate that MIF is expressed during RSV infection and controls the release of pro-inflammatory cytokines from macrophages in an in vitro model.

Keywords Respiratory syncytial virus · MIF · Macrophage migration inhibitory factor · Macrophages · Cytokines · Signaling pathways

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Introduction

Respiratory syncytial virus (RSV) is the leading cause of acute bronchiolitis in children under age of 2 years [1]. Approximately, 2–3% of infected children develop severe lower respiratory disease [1, 2] and preterm infants frequently present recurrent wheezing and asthma-like symptoms later in life [3]. Therefore, RSV has been considered the primary cause of hospitalizations due to respiratory disease worldwide, with a huge burden to the public health systems [4–7].

A growing body of evidence suggests that the activation of the innate immune response is crucial for lung homeostasis maintenance [8]. After RSV recognition by pattern recognition receptors (PRR), an innate immune response is triggered and different signaling routes culminate with the transcription of genes mediated by

pro-inflammatory transcription factors, which promote an antiviral response [9, 10]. The chemokines secreted by RSV-infected epithelial cells stimulate the activation and recruitment of immune cells, such as macrophages [11]. Moreover, these recruited cells release pro-inflammatory cytokines, including TNF-α, MCP-1, IL-6 and IL-8, and anti-inflammatory cytokines, such as IL-10 [12–15]. The excessive secretion of these cytokines may contribute to the airway damage caused by RSV infection. Indeed, TNF-α and MCP-1, together with macrophages, have been shown to be critical for RSV-induced asthma exacerbations in mice [16].

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine involved in both the innate and adaptive immune responses [17]. MIF is expressed by different cell types, including monocytes, macrophages and neutrophils, among others [17, 18] and rapidly released in response to a variety of stimuli [17, 18]. MIF has been shown to activate macrophages and to promote the release of TNF-α, IL-1, IL-8, NO, and PGE_2 through the induction of COX-2 [19–21]. More recently, it has been reported that MIF is involved in reactive oxygen species (ROS) generation [22]. Furthermore, MIF plays a critical role in the pathogenesis of several diseases with different etiologies, including autoimmune and allergic disorders [23–25], parasitic, helminthic, bacterial and viral infections [26–32]. Growing evidence has demonstrated that MIF may play a role during respiratory virus infections. It has been previously reported that MIF enhances the pathogenesis of H5N1 virus infection by inducing the downstream release of inflammatory cytokines [33], which may be detrimental to the host. Moreover, MIF is released by lung epithelial cells rendered necrotic by infection with influenza A virus [34]. However, the participation of MIF during RSV infection is completely unknown. Since MIF appears to be involved in the pathogenesis of respiratory viral infections, we aimed to uncover a potential role for MIF during RSV infection. We found that RSV infection triggers the expression of MIF mRNA and protein in both peritoneal and bone marrow-derived macrophages. Interestingly, this effect is dependent on virus replication, since stimulation with UV-inactivated RSV did not induce MIF expression. The induction of MIF expression by RSV requires NADPH oxidase-derived ROS generation and 5-LOX and COX metabolites, as well as PI3K activity. Importantly, RSV promotes TNF-α secretion by macrophages and this secretion is totally dependent on MIF. Additionally, the release of MCP-1 and IL-10 induced by RSV is abolished by the pretreatment of macrophages with the MIF inhibitor ISO-1. These data suggest that MIF may play a harmful role during RSV infection, inducing the overproduction of inflammatory cytokines.

Materials and methods

Reagents

DMEM, AIM-V and fetal bovine serum (FBS) were purchased from Gibco. RPMI 1640 and HBSS were from Cultilab. ISO-1 ((*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid) was from Calbiochem. Zileuton was from Cayman Chemical. Mouse anti-RSV monoclonal antibody and goat anti-rabbit IgG secondary antibody HRP were from Millipore. Rabbit anti-mouse IgG secondary antibody HRP, Qubit dsDNA HS assay kit and Qubit Protein assay kit were from Invitrogen. The TNF-α ELISA kit, diphenyleneiodonium (DPI), LY294002, carboxymethylcellulose sodium salt, lipopolysaccharide O111:B4 from *Escherichia coli* (LPS), indomethacin and mouse anti-actin monoclonal antibody were purchased from Sigma-Aldrich. Fluid thioglycollate medium was from Prodimol Biotechnology. Brazol was from LGC Biotecnologia. The RNA isolation: RNeasy kit was from Qiagen. The GoScript reverse transcription system kit was from Promega. Rabbit anti-MIF polyclonal antibody was from Thermo Fisher. The Cytometric Bead Array Mouse Inflammation Kit was from BD Bioscience. TaqMan primers were specific for MIF (Mm01611157_gH), beta actin (ACTB; Mm00607939_s1) and GAPDH (Mm99999915_g1) from Applied Biosystems.

Animals

Balb/c mice that were 8–10 weeks of age were supplied by the breeding facilities of CEMBE, PUCRS. Animals were housed in transparent acrylic boxes with a sterilized shaving bed; the boxes were temperature controlled (24 ± 2 °C) and the animals were subjected to a light/dark cycle of 12 h. All animals followed a standard diet and had access to water ad libitum.

Virus preparation

The virus production of RSV A2 strain (kindly donated by Dr. Fernando Polack, Vanderbilt University School of Medicine, USA) was obtained in VERO cells cultured in Opti-MEM medium with 2% FBS at 37 °C under 5% CO_2. To assess viral titre, VERO cells were infected with RSV in medium without serum followed by a carboxymethylcellulose plaque assay. Lysis plate titration was performed using an anti-RSV antibody and viral titre was expressed as plaque-forming units (PFU). The virus aliquots were stored at − 80 °C.

Macrophage preparation and stimulation

Peritoneal macrophages

Peritoneal macrophages were obtained 3 days after intraperitoneal instillation of 3 mL of thioglycollate 3% by peritoneal washing with chilled RPMI 1640. Cells were seeded at 2×10^5/well in AIM-V medium for 2 h. Afterwards, the non-adherent cells were removed by washing, and adherent cells were stimulated as described below.

Bone marrow-derived macrophages (BMDM)

BMDM were obtained after isolation of cells from murine femurs and tibias. Cells were plated in 24-well plates (10^6 cells/mL) in AIM-V medium and incubated for 7 days at 37 °C under 5% CO_2. For differentiation, bone marrow cells were stimulated with GM-CSF (40 ng/mL) every 3 days. On the 7th day of culture, non-adherent cells were removed by washing and macrophages were stimulated.

Alveolar macrophages

Alveolar macrophages were collected by bronchoalveolar lavage through flushing the lungs three times with 1 mL of chilled PBS supplemented with EDTA (5 mM). The lavage was repeated twice and alveolar macrophages from several mice were pooled. Cells were seeded at 5×10^5/well in AIM-V medium for 2 h. Afterwards, the non-adherent cells were removed by washing, and the adherent cells were stimulated as described below.

BMDM, peritoneal or alveolar macrophages were stimulated with RSV (5×10^4–5×10^5 PFU/mL), LPS (100 ng/mL) or left unstimulated for 24 h at 37 °C with 5% CO_2. To evaluate the role of ROS, PI3K, ERK, p38 MAPK, 5-LOX and COX on RSV-induced MIF expression, macrophages were pretreated with selective inhibitors for 1 h at 37 °C under 5% CO_2, as indicated in figure legends. To evaluate the participation of MIF on RSV clearance and on RSV-triggered cytokine release, cells were pretreated with MIF inhibitor, ISO-1 (100 μM), for 1 h at 37 °C with 5% CO_2. The trypan blue exclusion assay was used to analyze the viability of cells treated with these inhibitors, and at the end of incubation, cell viability was always higher than 97%.

MIF detection by real-time PCR

BMDM, peritoneal or alveolar macrophages (2×10^5 cells/mL) were stimulated with RSV (5×10^4–5×10^5 PFU/mL) or LPS (100 ng/mL) for 24 h at 37 °C with 5% CO_2. After this period, cells were harvested, RNA was extracted using RNeasy kit and cDNA was synthesized using GoScript kit, according to the manufacturer's instructions. MIF gene expression was detected by quantitative real-time PCR (Step One, Applied Biosystems) using specific primers for MIF, ACTB and GAPDH. Data were analyzed using the comparative CT ($\Delta\Delta C_T$) method. The data output is expressed as a fold change over control and normalized using ACTB or GAPDH genes.

MIF detection by Western blotting

BMDM or peritoneal macrophages (2×10^5 cells/mL) were stimulated with RSV (5×10^4–5×10^5 PFU/mL) or LPS (100 ng/mL) for 24 h at 37 °C with 5% CO_2. Afterwards, cells were harvested and lysed in protein extraction buffer (10 mM Tris HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA pH 8.0, 5 mM B-mercaptoethanol, 0.1 mM PMSF, 0.5% CHAPS, 10% glycerol). Cell lysates were centrifuged and supernatants were boiled and subjected to electrophoresis in SDS–polyacrylamide gel (15%) in reducing conditions. The quantified proteins were transferred to a nitrocellulose membrane at 4 °C for 2 h. Then, the membranes were blocked with non-fat milk diluted in PBS for 30 min at room temperature and incubated overnight with anti-MIF antibody (1:500) followed by HRP anti-rabbit secondary antibody (1:1000). As an endogenous control, anti-actin antibody (1:1000), followed by HRP anti-mouse secondary antibody (1:500) was used. The detection of MIF expression was performed using the ECL system. The densitometry analysis was performed using ImageJ 1.43 software (NIH). MIF bands were normalized to β-actin bands.

Viral load quantitation by real-time PCR

To evaluate the role of MIF on RSV clearance, peritoneal macrophages (2×10^5 cells/mL) were pretreated with ISO-1 (100 μM) for 1 h at 37 °C with 5% CO_2. Afterwards, cells were stimulated with RSV (5×10^5 PFU/mL) for 24 h at 37 °C with 5% CO_2. After this period, cells were harvested, RNA was extracted using Brazol and cDNA was synthesized using GoScript kit, according to the manufacturer's instructions. For viral load quantitation, real-time PCR was performed to RSV F protein gene amplification using specific primers and probes. For the standard curve, tenfold serial dilution of 6×10^7 copies of a plasmid containing RSV F protein sequence were added to the same plate of qPCR in duplicate.

Cytokine measurements

The concentrations of TNF-α, MCP-1 and IL-10 were determined in cell supernatants using Cytometric Bead Array (BD Biosciences) or ELISA (Sigma-Aldrich) following the manufacturer's instructions. The cytometric bead array data were acquired using FACSCanto II flow cytometer

(Becton–Dickinson) and analyzed with FCAP Array software (Soft Flow Hungary Ltd; v.3).

Statistical analyses

Data are presented as mean ± SEM. Results were analyzed using GraphPad Prism 5 statistical software package. Comparisons between multiple groups were analyzed with one-way ANOVA and a posteriori Bonferroni test. When appropriate, unpaired Student's *t* test or Mann–Whitney test were employed. The level of significance was set at $p \leq 0.05$.

Ethics statement

This study was reviewed and approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA/PUCRS) under protocol number 13/00328.

Results

RSV infection induces MIF mRNA and protein expression in BMDM, peritoneal and alveolar macrophages

Macrophages are central cells for the innate immune response, playing a crucial role in host defense against different types of pathogenic microorganisms [35, 36] and constitute an important source of MIF [37]. Moreover, the levels of MIF have been found to be elevated in the lungs and serum of mice infected with influenza H5N1 virus [33]. However, the expression of MIF during RSV infection has not been studied. Therefore, we were interested in determining whether RSV would induce MIF expression in macrophages by infecting bone marrow-derived, peritoneal or alveolar macrophages with increasing concentrations of RSV and analyzing the expression of MIF mRNA and protein levels. Indeed, RSV was able to stimulate both MIF mRNA and protein expression in BMDM in a concentration-dependent manner (Fig. 1a, b). Likewise, the increasing concentrations of RSV induced MIF mRNA and protein expression in peritoneal macrophages (Fig. 1c, d). Typically, RSV is recognized by alveolar macrophages in the lungs. Indeed, RSV triggered MIF mRNA expression from alveolar macrophages in vitro (Fig. 1e). As expected, LPS also induced MIF expression at mRNA and protein levels in BMDM, peritoneal and alveolar macrophages (Fig. 1a–e). Importantly, the pretreatment of cells with the MIF inhibitor ISO-1 abolished the expression of MIF mRNA and protein (Fig. 1c, d). Furthermore, the effect of RSV on MIF mRNA expression was totally dependent on virus replication, since ultraviolet radiation-inactivated RSV (UV-RSV) was not capable of inducing such an expression (Fig. 1f).

RSV-induced MIF expression is dependent on NADPH oxidase-derived ROS generation

It has been recently shown that *T. gondii*-triggered MIF expression requires ROS generation through NADPH oxidase activation [38]. Thus, we sought to verify whether RSV would induce MIF expression through a similar mechanism. Pretreating peritoneal macrophages with DPI (an irreversible inhibitor of flavoenzymes including NADPH oxidase) profoundly inhibited MIF mRNA expression stimulated by RSV (Fig. 2). These data indicate that RSV triggers MIF expression through ROS generation by a functional NADPH oxidase.

5-LOX, COX and PI3K are required to MIF expression induced by RSV infection

RSV has been shown to induce the expression of 5-LOX and COX-2 in lung epithelial cells [39, 40]. Moreover, it has been previously reported that there is a crosstalk between MIF and the arachidonic acid pathways that can enhance inflammatory responses [20, 41]. Considering that, we tested the effect of selective inhibitors of 5-LOX and COX in murine macrophages infected with RSV. Pretreating macrophages with zileuton and indomethacin abrogated MIF mRNA expression triggered by RSV (Fig. 3a), suggesting that 5-LOX and COX products, such as prostaglandins and leukotrienes, are likely necessary for MIF expression during RSV infection in macrophages.

Mitogen-activated protein kinases (MAPK) have been reported to be important for MIF release [20, 21]. To investigate the role of PI3K/AKT, and MAPK pathways in RSV-infected macrophages, we tested whether MIF mRNA expression could be decreased in the presence of specific inhibitors. The pretreatment of macrophages with LY294002, a selective PI3K inhibitor, was able to abolish RSV-induced MIF expression (Fig. 3b). However, inhibition of ERK and p38 MAPK with PD98059 and SB203580, respectively, partially diminished MIF mRNA expression stimulated by RSV infection (Fig. 3b). These data suggest that PI3K is essential for RSV-induced MIF expression, but ERK and p38 MAPK are only partially necessary.

The essential role of MIF in RSV-triggered cytokine secretion

As part of the immune response, several cytokines and chemokines are released during RSV infection and likely directly dictate the severity of the pathophysiology [10, 42], by facilitating or not the viral clearance. Therefore, we

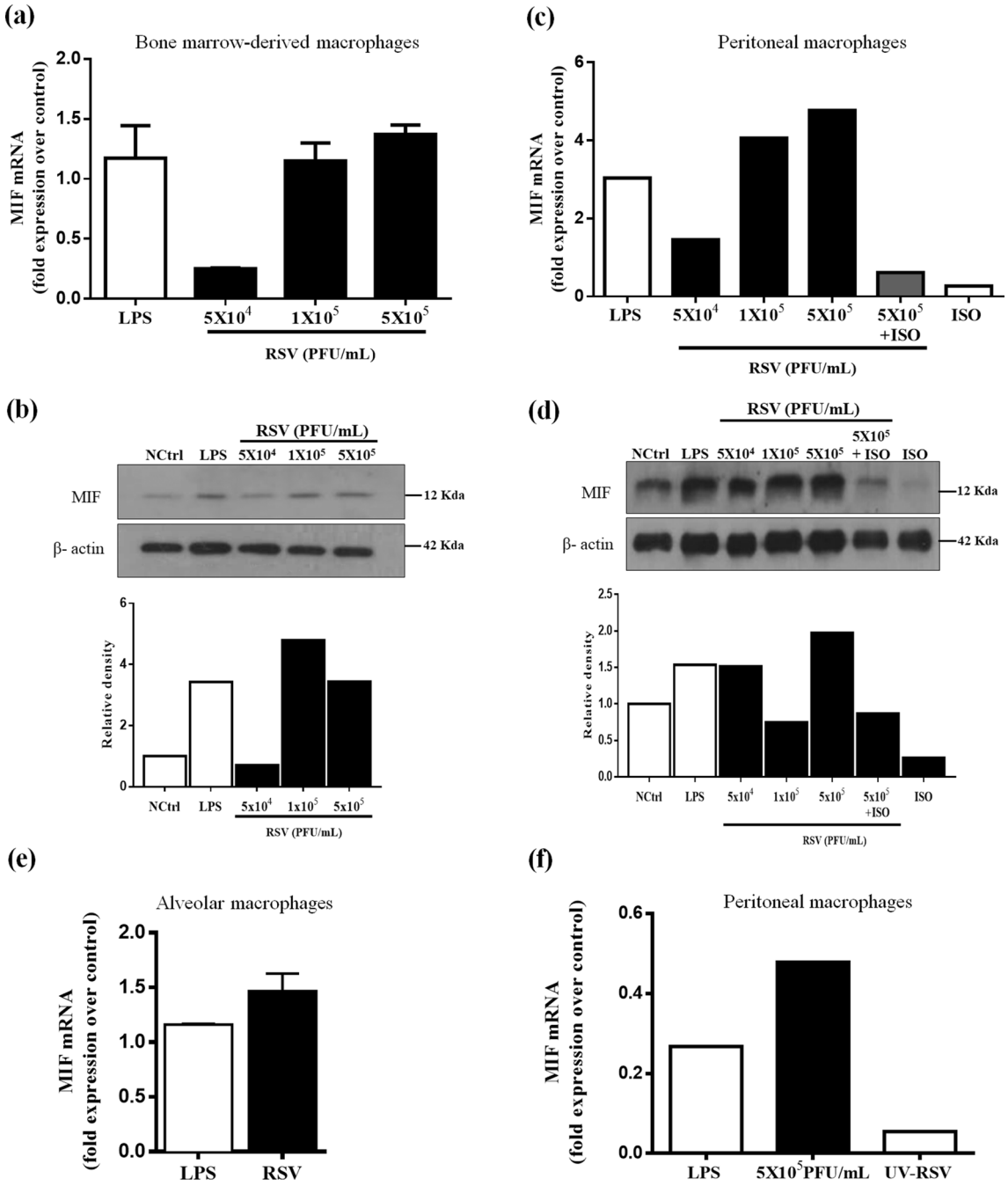

Fig. 1 RSV induces MIF expression by bone marrow-derived and peritoneal macrophages. Bone marrow-derived (**a**, **b**) or peritoneal macrophages (**c**–**e**) from BALB/c mice were infected with RSV (5×10^4–5×10^5 PFU/mL), UV-inactivated RSV or LPS (100 ng/mL) for 24 h. Cells were pretreated with ISO-1 (100 μM) when necessary for 1 h before stimulation. MIF expression was quantified by RT-PCR (**a**–**e**) using $\Delta\Delta C_T$ method and expressed as fold expression over control. MIF protein detection (**b**, **d**) was performed by the ECL technique and staining with anti-MIF antibody. Mouse GAPDH and ACTB were used as endogenous control. Data are representative of at least two independent experiments performed in triplicate and represent mean ± SEM

sought to determine the role of MIF in RSV clearance in macrophages. Surprisingly, our results show that MIF contributes to RSV clearance in peritoneal macrophages, since the inhibition of MIF with the use of ISO-1 increased the viral load in macrophages (Fig. 4a). We next determined whether RSV would induce TNF-α release by macrophages. Indeed, TNF-α was released in response to RSV infection in macrophages (Fig. 4b). Interestingly, pretreating cells with MIF inhibitor, ISO-1, abolished TNF-α secretion triggered by RSV (Fig. 4c). Similarly, RSV was able to stimulate

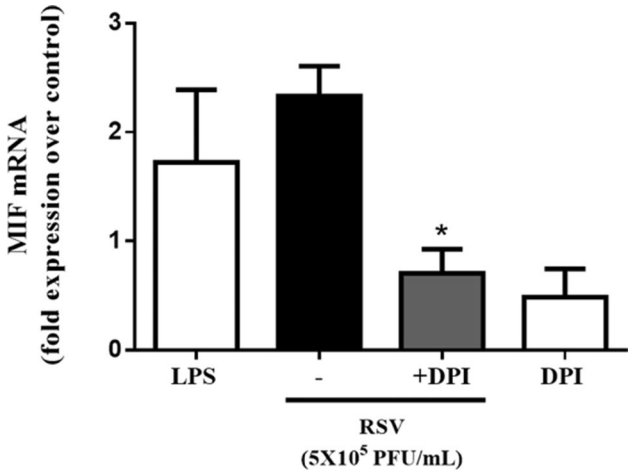

Fig. 2 ROS inhibitors reduce the expression of MIF in RSV-infected peritoneal macrophages. Peritoneal macrophages were collected by peritoneal lavage from BALB/c mice. Cells were pretreated for 1 h with the selective inhibitor of NADPH oxidase, diphenyleneiodonium chloride (DPI; 10 μM) before stimulation with RSV (5×10^5 PFU/mL) for 24 h. MIF expression was quantified by RT-PCR using $\Delta\Delta C_T$ method and expressed as fold expression over control. Data are representative of two independent experiments performed in triplicate and represent mean ± SEM using one-way ANOVA with post hoc Tukey test. *$p < 0.05$

MCP-1 secretion from macrophages, but surprisingly, MCP-1 levels could not be detected after treatment of macrophages with ISO-1 (Fig. 4d), suggesting a key role of MIF for cytokine release during RSV infection.

The release of the anti-inflammatory cytokine IL-10 could contribute to worsening inflammation because of its involvement with Th2 type response triggered by RSV infection [4, 42, 43]. Thus, we aimed to elucidate whether RSV could lead to IL-10 release from macrophages and whether MIF would play a role in this effect. RSV was able to induce IL-10 secretion from macrophages and MIF inhibition by ISO-1 completely abrogated IL-10 release triggered by the virus (Fig. 4e). Altogether, these data indicate that MIF is necessary for RSV clearance and controls the production of TNF-α, MCP-1 and IL-10 induced by RSV in macrophages.

Discussion

MIF is a multifunctional cytokine that plays key roles in both the antimicrobial host defense and in the pathogenesis of several infectious and autoinflammatory diseases [27, 44, 45]. Although several studies have reported a role for MIF during virus infection [46–50], the literature regarding the participation of MIF in respiratory virus infections is scarce. In this study, we show for the first time that increasing concentrations of respiratory syncytial virus upregulate MIF mRNA and protein expression levels in peritoneal, alveolar and bone marrow-derived macrophages. MIF has been shown to be released by lung epithelial cells rendered necrotic by infection with influenza A H1N1 virus [34]. Furthermore, H5N1 virus-infected mice presented high levels of MIF in the lungs and serum [33]. Interestingly, MIF mRNA expression in the lungs of infected animals could be detected at later time points, suggesting that a massive virus replication results in long-lasting induction of MIF in this organ [33]. Likewise, UV-inactivated RSV was not able to induce MIF mRNA expression in macrophages, indicating that virus replication is essential to trigger MIF expression.

The mechanisms underlying MIF expression and secretion induced by different microorganisms have been extensively studied. However, the precise mechanisms involved in RSV-promoted MIF expression are yet to be elucidated.

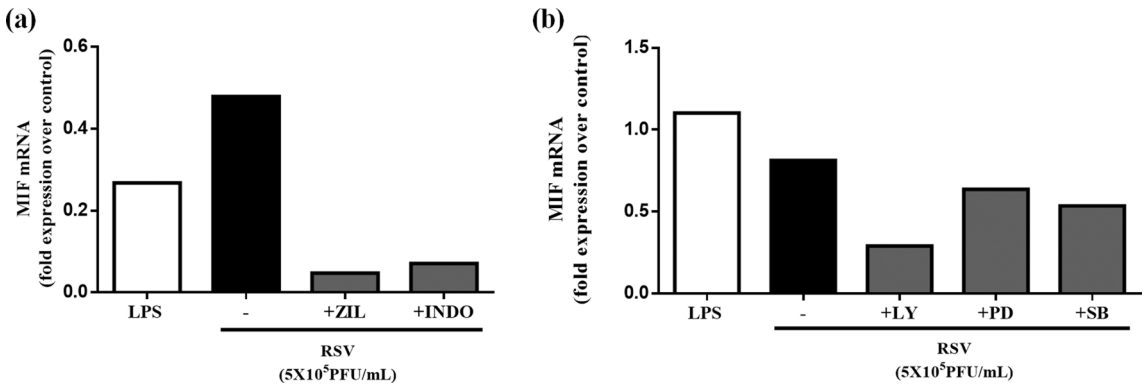

Fig. 3 5-LOX, COX and PI3K are required for MIF expression induced by RSV infection. Peritoneal macrophages were collected by peritoneal lavage from BALB/c mice, pretreated for 1 h with the following inhibitors zileuton (ZIL; 1 μM), indomethacin (INDO; 100 nM); LY294002 (LY; 50 μM), PD98059 (PD; 30 μM) or SB203580 (SB; 10 μM) and stimulated with RSV (5×10^5 PFU/mL) for 24 h. MIF expression was quantified by RT-PCR using $\Delta\Delta C_T$ method and expressed as fold expression over control. **a** Inhibition of LOX-5 and COX pathways. **b** Inhibition of PI3K, ERK, p38/MAPK pathways. Mouse GAPDH was used as endogenous control. Data are representative of at least two independent experiments performed in triplicate and represent mean ± SEM

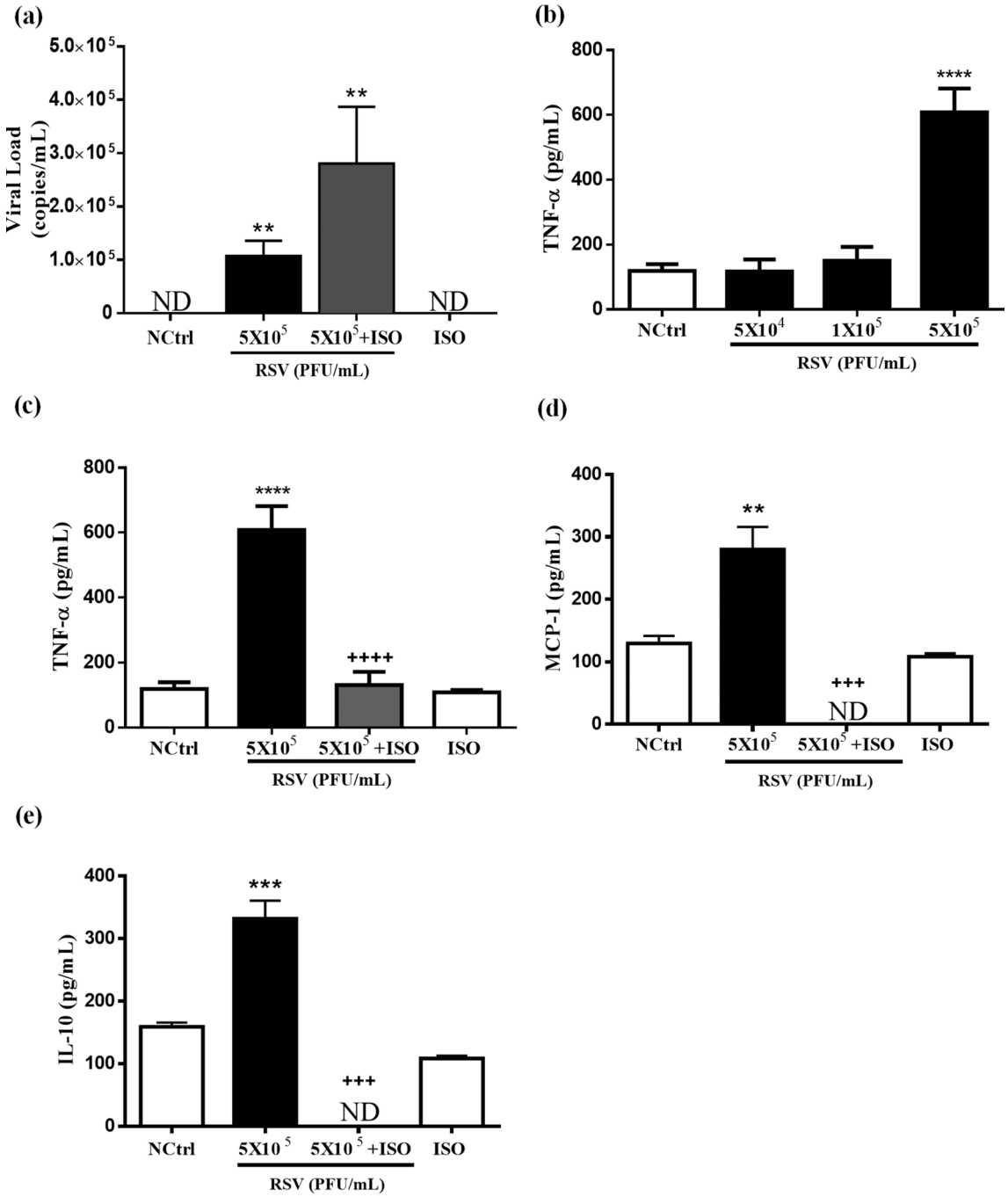

Fig. 4 MIF contributes to the inflammatory response of murine macrophages during RSV infection. Peritoneal macrophages of BALB/c mice elicited with thioglycollate 3% were infected with RSV (1×10^5 and 5×10^5 PFU/mL) for 24 h, cells were pretreated with ISO-1 (100 μM) when necessary for 1 h before stimulation. The culture supernatant was collected and cytokines (TNF-α, MCP-1 and IL-10) were measured by ELISA (**a**) or CBA method using flow cytometry (**b**–**d**). Data were acquired in FACSCanto II cytometer and analyzed in FCAP Array software. Data are representative of at least two independent experiments performed in and represent mean ± SEM using one-way ANOVA with post hoc Tukey test. $^{**}p < 0.01$, $^{****}p < 0.0001$ when compared to negative control (NCtrl); $^{+++}p < 0.001$ when compared to RSV-infected cells. *ND* not detected

To shed light on the signaling pathways activated by RSV that culminate in MIF expression by macrophages, we took advantage of selective inhibitors of specific signaling routes. We found that RSV-triggered MIF mRNA expression levels were profoundly attenuated by the pretreatment of cells with DPI, an irreversible inhibitor of NADPH oxidase. This result indicates that RSV stimulates MIF expression dependently of a functional NADPH oxidase-derived ROS generation.

In phagocytes, ROS are usually generated through the activation of the NADPH oxidase enzyme to play key roles as messengers and to kill pathogens [51, 52]. Although NOX2 is an important ROS-producing isoform of NADPH oxidase in phagocytic cells, other isoforms, such as NOX1 and NOX4 are involved in ROS generation and cell activation in response to inflammatory stimuli [53]. It has been recently reported that NOX4 is necessary for the generation of MIF by macrophages and host defense against *T. gondii* infection [38]. Our findings clearly show that RSV activates NADPH oxidase to upregulate MIF expression; however, the required isoform of NADPH oxidase remains elusive.

Previous studies demonstrated that MIF induces the phosphorylation and activation of cytosolic PLA2 [20, 54]. The release of arachidonic acid by cPLA2 is the first step in the downstream synthesis of prostaglandins and leukotrienes, which have potent pro-inflammatory activities. MIF has been shown to directly stimulate the secretion of PGE_2 and LTB_4 from RAW 264.7 macrophages and overrides the inhibitory effect of dexamethasone on PGE_2 and LTB_4 release [54]. Moreover, MIF upregulates the expression of COX-2 and the production of PGE_2 in primary rat microglial cells [55]. In addition, RSV infection of lung epithelial cells induces the expression of 5-LOX and COX-2 [39, 40]. Importantly, COX-2 has been reported to mediate lung pathology in both influenza and RSV infections [40, 56]. However, the effect of COX and 5-LOX products on MIF expression triggered by RSV has not been investigated. To elucidate the role of COX and 5-LOX on RSV-induced MIF expression in macrophages, we pretreated the cells with selective inhibitors of these enzymes. Our results demonstrate that 5-LOX inhibition by zileuton and COX suppression by indomethacin abrogated MIF mRNA expression induced by RSV in macrophages. These data likely imply leukotrienes and prostaglandins as mediators of MIF expression during RSV infection.

RSV has been shown to activate PI3K to inhibit granulocyte spontaneous apoptosis [57]. Moreover, we have previously reported that RSV fusion protein is able to phosphorylate ERK and p38 MAPK to induce the release of neutrophil extracellular traps (NETs) from human neutrophils [58]. Thus, we hypothesized that RSV could activate these signaling pathways in macrophages to trigger MIF expression. Indeed, the treatment of cells with the PI3K inhibitor LY294002 profoundly decreased MIF mRNA expression, while inhibiting ERK and p38 MAPK only partially suppressed MIF expression. These results suggest that RSV signaling requirements are selective, depending on the response activated by the virus in the cell.

During RSV infection, there is a greater secretion of cytokines due to immune cell migration and subsequent release of more inflammatory cytokines at the site of infection, that end up contributing to the immunopathology of the airways [11]. Additionally, it is believed that a "cytokine storm" underlies the harmful inflammatory consequences associated with severe RSV infection [59]. In this context, TNF-α and MCP-1 have been shown to play critical roles during RSV infection [60, 61] and RSV-induced asthma exacerbations [16]. Based on previous data from the literature and on our findings showing that RSV induces TNF-α, MCP-1 and IL-10 release from macrophages, we sought to elucidate whether these inductions would be mediated by MIF. Strikingly, MIF blockade during RSV infection abolished the release of these cytokines by macrophages. The sharp reduction in the production of TNF-α, MCP-1 and IL-10 upon MIF blockade indicates that MIF acts in an autocrine manner, modulating the secretion of these cytokines. These results suggest that MIF expression precedes the amplification of the inflammatory response and point out to a detrimental role for MIF during RSV bronchiolitis in young children. Although MIF seems to be necessary to host defense [17, 62–64], high levels of MIF are detrimental during acute infections, since high doses of recombinant MIF exacerbate lethal endotoxemia and *E. coli* sepsis in mice [17, 27]. Furthermore, patients with severe sepsis presented high levels of MIF and IL-10 in the serum during the early phase of sepsis and had a rapidly fatal outcome within 48 h [65]. In addition, dengue patients and MIF-deficient mice presented a lower production of inflammatory mediators and low score of tissue damage when MIF was inhibited [47].

In conclusion, our study demonstrates that RSV is able to induce MIF expression at mRNA and protein levels, and this effect is dependent on virus replication. RSV-triggered MIF expression was dependent on a functional NADPH oxidase-derived ROS generation and required PI3K activity. Furthermore, 5-LOX and COX metabolites are likely to mediate MIF expression induced by RSV. Importantly, we show that MIF expression controls the secretion of TNF-α, MCP-1 and IL-10 during RSV infection, which may be harmful to the host (Fig. 5). We propose that targeting MIF could represent an additional therapeutic approach to help prevent RSV-induced inflammatory consequences and pathogenesis of viral bronchiolitis in young children and babies.

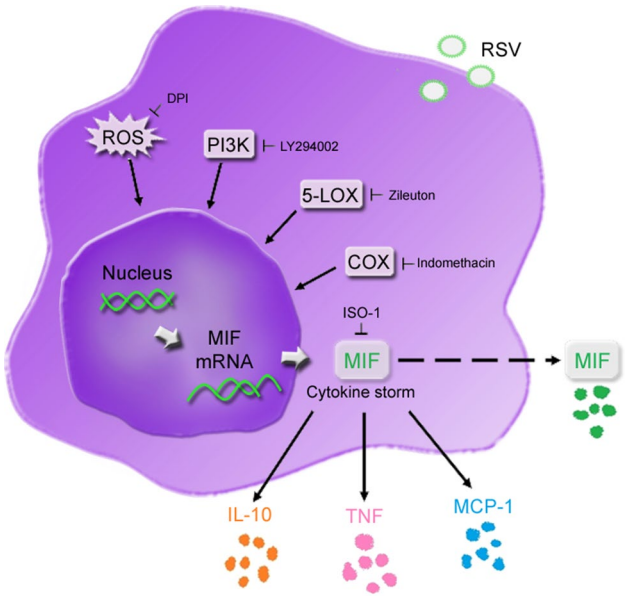

Fig. 5 Mechanisms underlying RSV-induced MIF expression in mouse macrophages. MIF is a pro-inflammatory cytokine regulated by different pathways that act together to promote its expression and release during inflammation and infection. RSV triggers MIF mRNA and protein expression in both bone marrow-derived and peritoneal macrophages. RSV-induced MIF expression is dependent on NADPH oxidase-derived ROS generation, since the oxidase inhibitor, DPI, blocked MIF expression in mouse macrophages. Similarly, PI3K, 5-LOX and COX activities are necessary for MIF mRNA expression triggered by RSV. Interestingly, MIF expression controls the secretion of TNF-α, MCP-1 and IL-10 from macrophages during RSV infection

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Author contributions BNP and GFS conceived and designed the study. GFS, SPM, APTM, LDS and AGS performed the experiments. BNP, GFS and SPM performed the statistical analysis and interpreted the data. BNP, GFS and SPM wrote the manuscript. BNP, APDS, RTS and PTB critically revised the draft. All authors contributed to the manuscript revision and approved the submitted version.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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