Molecular Immunology 91 (2017) 105–113

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Calcitonin gene-related peptide exerts anti-inflammatory property through regulating murine macrophages polarization *in vitro*

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ARTICLE INFO

Keywords:
Calcitonin gene-related peptide
Macrophages
Alternatively activated macrophage
NLRP3 inflammsome
Inflammation

ABSTRACT

Acute lung injury (ALI) is a condition resulting from direct or indirect lung injury associated with high mortality and morbidity. The phenotype of macrophages in lung contributes to the pathological progress of ALI. Calcitonin gene-related peptide (CGRP) is one of the most abundant neuropeptides in lung, and attenuates lipopolysaccharide (LPS)-induced ALI in rats. However, the exact effect of CGRP on the activation of macrophages remains unknown. Here we investigate the effect of CGRP on the macrophages activation and inflammation in murine macrophages *in vitro*. We found that LPS increased the expression of CGRP in a LPS-induced ALI murine model and LPS-stimulated murine macrophages. Although CGRP didn't alter the expression of tumor necrosis factor-α (a marker of pro-inflammatory phenotype of macrophages, M1 macrophages) or Arginase 1 (Arg1, a marker of M2 macrophages) in non-differentiated macrophages, CGRP significantly reduced the *NLRP3* and *pro-IL-1β* mRNA expression induced by LPS, as well as NLRP3 protein and IL-1β secretion induced by LPS + ATP in macrophages *in vitro*. On the other hand, CGRP dramatically enhanced the Arg1 expression and activity induced by IL-4 in the time- and dose-dependent manners. CGRP also promoted the expression of markers of M2 macrophages (IL-10, Fizz1 and Mrc1) induced by IL-4 in murine macrophages. These effects of CGRP were also observed in primary murine peritoneal macrophages. In addition, we found that CGRP regulated macrophages polarization partially through calmodulin, PKC and PKA pathways. Specifically, CGRP could inhibit the degradation of I-κB induced by LPS, and enhance the phosphorylation of STAT6 induced by IL-4 in macrophages. In conclusion, our results indicate that CGRP regulates macrophage polarization and inhibits inflammation in murine macrophages.

1. Introduction

Acute lung injury (ALI) and its severe form acute respiratory distress syndrome (ARDS) are diseases with high mortality (Zhao et al., 2016). ALI is characterized by inflammatory injury, lung edema and refractory hypoxemia, among which inflammatory injury mainly contributes to the tissue and cell injury (Mishra et al., 2016). Abnormal activation of macrophages, infiltration of neutrophils, and excessive pro-inflammatory cytokines are responsible for inflammatory responses (Kobayashi et al., 2016). As the first defensive line in lung, macrophages are the major factors in the initiation, propagation and resolution of inflammation (Ying et al., 2015). Thus, thorough illumination of macrophages activation is vital to reveal the pathophysiology process of ALI.

The phenotypes of macrophages depend largely on local conditions, including pathogen, chemokine, and cytokine (Pope et al., 2016). Upon exposure to TLR ligands (such as lipopolysaccharide, LPS), macrophages undergo a phenotype referred to classically activated macrophages or M1 macrophages. M1 macrophages produce a high level of pro-inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6. Recently, it was reported that the expression of NLRP3 inflammasome is up-regulated in M1 macrophages (Awad et al., 2017). The release of IL-1β is mainly mediated by NLRP3 inflammasome in mammal (Zhang et al., 2016). Under the stimulation of IL-4 or IL-13, macrophages program alternatively activation (as the M2 macrophages) *via* phosphorylation of STAT6 (Brunn et al., 2014; Kapoor et al., 2015). M2-associated genes include IL-10, arginase 1 (Arg1), Fizz1 and Mrc1 (Sun et al., 2015; Ying et al., 2015). A line of researches

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http://dx.doi.org/10.1016/j.molimm.2017.08.020
Received 16 June 2017; Received in revised form 4 August 2017; Accepted 22 August 2017

find that the phenotypes of macrophages play a vital role in the development and outcome of ALI. IL-4-mediated therapeutic macrophage reprogramming, M2 macrophages, can accelerate the resolution and lung repair in the ARDS rats (D'Alessio et al., 2016). And depletion of $CD206^+$ M2 macrophages exaggerates lung injury in endotoxemic mice *in vivo* (Kambara et al., 2015). These findings indicate that the increase of M2 macrophages promotes the inflammation resolution of ALI. Ginsenoside Rg1 attenuates LPS-induced ALI in mice, partly by regulating the infiltration of M2 macrophages and inhibiting pulmonary cell apoptosis (Bao et al., 2015). On the other hand, the inhibition of M1 macrophage attenuates the ALI by dampening the NLRP3 inflammasome activation (Zhang et al., 2016, 2017a). We have reported that blockade of triggering receptor expressed on myeloid cell-1 attenuates LPS-induced ALI *via* inhibiting NLRP3 inflammasome activation (Liu et al., 2016). Interestingly, matrix metalloproteinase (MMP)-$10^{-/-}$ results in the decrease of M2 markers but increase of M1 markers *in vivo* and in cultured alveolar macrophages, contributing to the enhanced inflammatory response (McMahan et al., 2016). So, it is important for the therapy of ALI to find an efficient strategy regulating the profiling of macrophages activation.

Calcitonin gene-related peptide (CGRP), identified in the early 1980s (Rosenfeld et al., 1983), is a 37 amino acid neuropeptide as a member of the calcitonin family of peptides (Kuzawinska et al., 2016). CGRP receptor belongs to the G protein-coupled receptor superfamily. Binding of CGRP results in activation of cyclic adenosine monophosphate (cAMP)-signaling pathway and increases cAMP levels, involved in many physiological and pathophysiological event, such as chronotropic and inotropic actions in the heart, dilatation of arterial vessels and relaxation of urinary smooth muscle (Kuzawinska et al., 2016). PKA inhibitor H89 (Permpoonputtana et al., 2016) could abolish the effect induced by CGRP. As early as in 1996, researchers found that Ca^{2+} also is the second messengers probably involved in the action of CGRP (Castronuovo et al., 1996). Calmodulin inhibitor W7 could abolish the effect induced by CGRP (Castronuovo et al., 1996). CGRP is also found in other organs, such as lung (Yang et al., 2015). In addition, our previous research points out that CGRP promotes the wound healing of human bronchial epithelial cells *via* PKC (Zhou et al., 2013). Targeting CGRP is a new strategy for migraine treatment (Kuzawinska et al., 2016). CGRP down-regulates bleomycin-induced pulmonary fibrosis, and sensory CGRP depletion by capsaicin exacerbates pulmonary fibrosis in rats (Li et al., 2016). Yang and his colleague have proved that exogenous α-CGRP attenuates LPS-induced ALI in rats (Yang et al., 2015). But the effect of CGRP on the alternative activation of macrophages is still unclear. Since adrenomedullin 2 (ADM2), an endogenous bioactive peptide belonging to the CGRP family, can activate M2 macrophages (Lv et al., 2016), we hypothesize that CGRP regulates the activation of macrophages, dampening the inflammatory response of murine macrophages.

In this study, intraperitoneal instillation of LPS was used to induce ALI in mice. The expression of CGRP in lung of ALI mice was evaluated. And the effect of CGRP on the LPS-induced classically activation and IL-4-induced alternative activation of murine macrophages was investigated *in vitro*.

2. Materials and methods

2.1. Animal

Eight- to twelve-week-old male C57BL/6 mice were provided by the laboratory animal unit of Central South University. Mice were housed in a specific pathogen-free, temperature-controlled (22–24 °C) room with 12 h dark/light cycle. Mice were fed a standard laboratory diet. Experimental use of mice in the present study was performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the animal protocol was approved by the institutional animal care committee of Xiangya Medical College.

2.2. Animal treatment procedures

Mice were randomly divided into two groups: the control group and the ALI group. The mouse model of ALI was established by an intraperitoneal injection of LPS from O111:B4 *Escherichia coli* (10 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). Mice in the control group received sterile saline only. The mice were sacrificed 8 h, 16 h or 24 h after the LPS injection (n = 8 at each time point) under anesthesia with pentobarbital sodium (50 mg/kg). The lung histology and expression of *CGRP* mRNA were tested. Other batch of mice were sacrificed at 8 h, 16 h or 24 h (n = 8 at each time point) for bronchoalveolar lavage to harvest the bronchoalveolar lavage fluid (BALF).

2.3. Lung histology and injury score analysis

Right upper lung lobes of mice sacrificed 8 h after the LPS injection were used for histological evaluation. Lungs were fixed in 10% formalin for 24 h, embedded in paraffin, cut into 5 μm section, and then stained with hematoxylin-eosin (HE). HE staining was done by deparafinizing and hydrating the slides to water. Slides were stained with Harris hematoxylin for 15 min and eosin for 30 s. Slides were dehydrated, cleared, and mounted with Cytoseal. A designated scoring system to quantify the extent of histological lung injury in animals was used to assess lung injury (Gonzales et al., 2014). Lung injury score was measured by two blinded pathologists. Five independent variables were evaluated with a 0 to 4 point scale. The five variables were neutrophils in the alveolar space, hemorrhage, hyaline membranes, pertinacious debris filling the airspaces, and septal thickening. A score of 0 represented no damage; l represented mild damage; 2 represented moderate damage; 3 represented severe damage and 4 represented very severe histological changes.

2.4. Mouse macrophage cell culture

Mouse macrophage cells (RAW264.7, ATCC, Manassas, VA, USA) were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Hyclone, Logan, UT, USA) with 10% FBS (Gibco Invitrogen, Grand Island, NY, USA) at 37 °C in a humidified 5% CO_2 incubator.

2.5. Primary murine peritoneal macrophage isolation

The primary peritoneal macrophages were isolated as our previous report (Dong et al., 2017). Briefly, 8-week-old male C57BL/6 mice were injected (*i.p.*) with 3 mL of 3% thioglycolate (Sigma-Aldrich, St. Louis, MO, USA). Three days later, peritoneal macrophages were harvested by peritoneal lavage with pre-cooled RPMI 1640 (Hyclone, Logan, UT, USA). Macrophages were collected by centrifugation at 1500 rpm for 10 min at 4 °C. Cells were plated in cell culture plates 1×10^6 cells/well for 12-well plate or 2×10^6 cells/well for 6-well plate. After 2 h, culture medium was changed completely to remove the non-adherent cells. Macrophages were cultured with 10% FBS in a humified CO_2 incubator at 37 °C, and rested for 24 h before subsequent experiments.

2.6. Cell treatment

Macrophages cells line (RAW264.7) or primary peritoneal macrophages were seed into 12-well plate (1×10^6 cells/well) for gene expression detection, or 6-well plate (2×10^6 cells/well) for protein expression or Arg-1 activity detection. To investigate the effect of CGRP on the inflammatory reaction of macrophages, cells were treated with LPS (100 ng/mL) for 3 h to prime the NLRP3 inflammasome or treated with LPS (100 ng/mL) for 135 min plus ATP (5 mM) for 45 min to activate the NLRP3 inflammasome as our previous report (Liu et al., 2016), following the pretreatment of CGRP for 30 min. In order to test whether the CGRP promotes IL-4-induced alternative activation in murine macrophage, series of concentration of CGRP (1, 10 and