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Anti-inflammatory roles of mesenchymal stromal cells during acute *Streptococcus pneumoniae* pulmonary infection in mice

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Abstract

Background. Pneumonia is the fourth leading cause of death worldwide, and *Streptococcus pneumoniae* is the most commonly associated pathogen. Increasing evidence suggests that mesenchymal stromal cells (MSCs) have anti-inflammatory roles during innate immune responses such as sepsis. However, little is known about the effect of MSCs on pneumococcal pneumonia. *Methods.* Bone marrow–derived macrophages (BMDMs) were stimulated with various ligands in the presence or absence of MSC-conditioned medium. For *in vivo* studies, mice intranasally-inoculated with *S. pneumoniae* were intravenously treated with MSCs or vehicle, and various parameters were assessed. *Results.* After stimulation with toll-like receptor (TLR) 2, TLR9 or TLR4 ligands, or live *S. pneumoniae,* TNF-α and interleukin (IL)–6 levels were significantly decreased, whereas IL-10 was significantly increased in BMDMs cultured in MSC-conditioned medium. In mice, MSC treatment decreased the number of neutrophils in bronchoalveolar lavage fluid (BALF) after pneumococcal infection, and this was associated with a decrease in myeloperoxidase activity in the lungs. Levels of proinflammatory cytokines, including TNF-α, IL-6, GM-CSF and IFN-γ, were significantly lower in MSC-treated mice, and the bacterial load in the lung after pneumococcal infection was significantly reduced. In addition, histopathologic analysis confirmed a decrease in the number of cells recruited to the lungs; however, lung edema, protein leakage into the BALF and levels of the antibacterial protein lipocalin 2 in the BALF were comparable between the groups. *Conclusions.* These results indicate that MSCs could represent a potential therapeutic application for the treatment of pneumonia caused by *S. pneumoniae*.

Key Words: *cell therapy, mesenchymal stromal cells, pneumonia,* Streptococcus pneumoniae

Introduction

Pneumonia is the fourth leading cause of death worldwide, and *Streptococcus pneumoniae* is a major cause of community-acquired pneumonia, a disease that can result in significant morbidity in addition to mortality [1]. To overcome pneumococcal infection, a vaccine has been developed and is recommended, especially for patients with underlying disease. However, some cases are still associated with poor outcomes, and, thus, the development of new treatment strategies for pneumococcal pneumonia, in addition to antibiotics, is required.

Macrophages and neutrophils, part of the innate immune system, are the first line of defense against invading pathogens such as *S. pneumoniae* [2]. Toll-like receptors (TLRs), which are major pattern recognition receptors (PRRs), play pivotal roles in the innate immune response to pathogens such as those causing pneumococcal infections. Immune cells such as macrophages use these receptors to recognize specific pathogen-associated molecular patterns (PAMPs); TLR2 recognizes gram-positive bacterial cell wall components such as peptidoglycan, lipoteichoic acid (LTA) and lipoproteins [3,4]. TLR9 recognizes viral and bacterial CpG-DNA [5]. TLR4 recognizes lipopolysaccharides (LPS) of gram-negative bacteria and pneumolysin, a putative virulence factor of *S. pneumoniae* [2,6]. During *S. pneumoniae* infection, TLR2, TLR4 and TLR9 are especially important because pneumococcal cell wall components, peptidoglycan and pneumococcal LTA, are recognized by

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TLR2 [7], pneumococcal pneumolysin is recognized by TLR4 [7] and pneumococcal CpG-DNA is recognized by TLR9 [8].

Mesenchymal stromal cells (MSCs) are heterogeneous multipotent fibroblast-like cells that can be isolated from various sources including bone marrow, adipose, skeletal muscle, heart, umbilical cord, lung, liver and placental tissue, in addition to amniotic fluid [9]. These cells are capable of differentiating into mesodermal lineage cells such as bone, cartilage and fat cells [10]. The immunomodulatory effect of MSCs during inflammation, mediated by the innate immune system, has been reported, for example, MSCs represent a promising therapeutic option for various chronic inflammatory lung diseases such as interstitial pulmonary fibrosis, chronic obstructive pulmonary disease and bronchiolitis obliterans syndrome [11]. MSCs have been shown to attenuate experimental sepsis through the production of prostaglandin E_2 (PGE_2), which acts on macrophages that produce the anti-inflammatory molecule, interleukin (IL)-10 [12]. MSCs also play protective roles in LPS-induced acute lung injury [13]. In addition, treatment with MSCs has been shown to improve lung function in a human *ex vivo*–perfused TLR4-mediated acute lung injury model [14]. MSCs were also shown to improve survival during experimental pneumonia caused by *Escherichia coli* by secreting antimicrobial molecules, such as lipocalin 2 [15] and LL-37 [16], and were also shown to improve resistance to *Klebsiella pneumoniae* [17]. Therefore, MSCs represent a promising option for cell-mediated therapies to treat inflammatory diseases [18]. Indeed, there are currently several clinical trials underway to evaluate the safety and efficacy of such MSCs in different diseases [11].

The mechanism of MSC-mediated immunomodulation has been studied; MSCs secrete various soluble factors such as transforming growth factor β-1 (TGF-β1) [19], PGE_2 [12], hepatocyte growth factor (HGF) [20], inducible nitric-oxide synthase (iNOS) [21], and heme oxygenase-1 (HO-1) [22], which modulate the proliferation of T and B lymphocytes, natural killer (NK) cells and dendritic cells, in addition to stimulating the induction of regulatory T cells (Tregs) [10,23]. MSCs also possess immunomodulatory properties that are mediated by direct cell-cell contact [24]. However, little is known about the protective roles of MSCs during pneumococcal pneumonia.

In the present study, we hypothesized that MSCs and their soluble factors have anti-inflammatory roles that contribute to protection against acute pulmonary infection by *S. pneumoniae*. Thus, we investigated the effect of MSCs on pulmonary pneumococcal infection. MSC-conditioned medium modulated the production of various cytokines in bone marrow–derived macrophages (BMDMs) after stimulation with pneumococcal infection-associated TLR ligands and heat-killed *S. pneumoniae* (HKSP). In addition, MSCs exhibited anti-inflammatory protective effects during acute pulmonary infection by *S. pneumoniae*. The results of this study suggest a novel treatment option for pneumococcal pneumonia.

Methods

Reagents

Pam3CSK4 (a TLR2 ligand) and HKSP were purchased from Invivogen. Mouse CpG-DNA (a TLR9 ligand) was purchased from Cell Science. LPS from *E. coli* 055:B55 was purchased from Sigma-Aldrich.

Mice

Eight to ten-week-old male C57BL/6 mice were purchased from CLEA. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were approved by the Animal Use Committee at the Keio University School of Medicine (protocol numbers 12109 and 12110).

Culture of MSCs

Mouse MSCs (also called mouse bone marrow–derived stromal cells) were kindly provided by Dr. Akihiro Umezawa through the RIKEN BioResource Center. The cells were cultured in alpha-minimum essential medium (MEM) supplemented with 20% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin, and sub-cultured every 3–4 days with fresh media. The culture medium was collected and centrifuged at 600*g* for 10 min. The supernatant was used as MSC-conditioned medium in further experiments, whereas cell-free medium incubated using the same conditions served as the control. All media were kept at −80°C until use.

Culture of BMDMs

Bone marrow cells were harvested from 8- to 10-week-old male C57BL/6J mice by flushing the femur and tibia with Roswell Park Memorial Institute medium (RPMI) 1640. Recovered cells were then cultured in bone marrow cell medium (20% FBS, 30% L-cell supernatant, 2 mmol/L L-glutamine, 1% penicillin/streptomycin and 0.25 μg/mL amphotericin B in RPMI 1640). Fresh bone marrow cell medium was added on day 3. On day 6, adherent cells were re-plated in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine and 1% penicillin/streptomycin for use as BMDMs.

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