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ORIGINAL RESEARCH

Modulation of Human Neutrophil Peptides on *P. aeruginosa* Killing, Epithelial Cell Inflammation and Mesenchymal Stromal Cell Secretome Profiles

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Objective: Neutrophil infiltration and release of the abundant human neutrophil peptides (HNP) are a common clinical feature in critically ill patients. We tested a hypothesis that different cell types respond to HNP differently in lung microenvironment that may influence the host responses.

Methods: Plasma concentrations of HNP were measured in healthy volunteers and patients with sepsis. Cells including the bacteria *P. aeruginosa*, human lung epithelial cells and mesenchymal stromal cells (MSCs) were exposed to various concentrations of HNP. Bacterial killing, epithelial cell inflammation, MSC adhesion and behaviours were examined after HNP stimulation.

Results: Incubation of *P. aeruginosa* or stimulation of human lung epithelial cells with HNP resulted in bacterial killing or IL-8 production at a dose of 50 µg/mL, while MSC adhesion and alternations of secretome profiles took place after HNP stimulation at a dose of 10 µg/mL. The secretome profile changes were characterized by increased release of the IL-6 family members such as C-reactive protein (CRP), leukemia inhibitory factor (LIF) and interleukin (IL-11), and first apoptosis signal (FAS) and platelet-derived growth factor-AA as compared to a vehicle control group.

Conclusion: Stimulation of MSCs with HNP resulted in changes of secretome profiles at 5-fold lower concentration than that required for bacterial killing and lung epithelial inflammation. This undisclosed risk factor of HNP in lung environment should be taken into consideration when MSCs are applied as cell therapy in inflammatory lung diseases.

Keywords: defensins, sepsis, lung injury, cytokines

Introduction

Pulmonary neutrophil infiltration is a hallmark of inflammatory lung diseases such as pneumonia and acute respiratory distress syndrome (ARDS).[1] Neutrophils play an important role in antimicrobial activity during the host defense by oxidative and non-oxidative means. In non-oxidative mechanisms, human neutrophil peptides (HNP) released from neutrophils play a major role to kill bacteria, viruses and fungi through cationic interaction with microorganisms.[2,3] HNP, also known as α-defensin, constitutes about 5% of the total protein content in neutrophils and about 50% of proteins in the azurophilic granules.[4,5] HNP is released into alveoli and circulation after neutrophil activation and phagocytosis in patients with ARDS, pneumonia, cystic fibrosis, bronchiolitis obliterans syndrome (BOS) and COPD.[6–9] Levels of HNP are significantly elevated in the blood and body fluid including bronchoalveolar lavage

fluid (BALF) and pleural fluid from patients with infections[10] and ARDS.[6,11] HNP has also been recently shown to act as an immunoregulatory agent due to its ability to induce cytokine production and to activate immune cells through mechanisms of ligand-receptor (i.e., the purinergic receptor $P2Y_6$) interaction.[12–14] Thus HNP is not only an important biomarker for infection but also an effector contributing to inflammation in lung microenvironment.

Bone marrow-derived mesenchymal stromal cells (MSCs) have been increasingly considered as a therapeutic option in the treatment of ARDS due to their immunomodulatory properties of secretome.[15] Preclinical studies have demonstrated that MSC therapy can be protective or detrimental depending on lung microenvironment at the time of administration.[16] It is noted that MSC retention is prolonged in the injured lung than in normal lung,[17–19] which may contribute to MSC phenotypes changes associated with functional alterations.[16] It has been reported that increased extracellular matrix (ECM) and adhesion molecules, especially fibronectin and integrins may contribute to MSC adhesion,[20] but the exact mechanisms by which lung microenvironment enhance adhesion molecules and MSC retention remain to be elucidated. The study of how HNP affects MSC function will provide a potential target to improve the therapeutic efficacy of MSCs. We tested the hypothesis that different cell types respond to HNP differently in lung microenvironment that may influence the host responses.

Materials and Methods

Plasma of Healthy Volunteers and Septic Patients with and Without Sepsis

The plasma of patients was obtained from the DYNAMICS study, a multicenter, prospective, observational study of critically ill septic patients (DYNAMICS Study, ClinicalTrials.gov Identifier: NCT01355042),[21] after approval by the Research Ethics Board at St. Michael's Hospital. The plasma collected from healthy volunteers served as controls after approval by the Research Ethics Board and written informed consent was obtained. Blood samples were collected from the ICU patients within 24 h after admission.

Bacterial Assay

Pseudomonas aeruginosa (ATCC 27853 Manassas, VA) was prepared in tryptic soy broth (TSB) at a concentration of $1x10^8$ CFU/mL measured by spectrophotometer (Spectronic 1001 plus, Milton Roy), and washed with PBS then diluted with SABM before use. After incubation with HNP[22] at different concentrations, the number of bacterial colonies was counted in TSB agar plates after overnight culture.

Human Lung Epithelial Cells

Human small airway epithelial cells (SAECs; Lonza, NJ) were cultured on 24-well cell culture dish at their passage 5 at a density of $5x10^4$ cells/well in the designated medium-containing growth factor and several hormones (SAGM BulletKit; Lonza, NJ). Cells were then subjected to exposure to HNP at different concentrations after 48 h incubation. Basal medium without growth factor or hormone (SABM) was used during the experiment.

HNP and the $P2Y_6$ Antagonist MRS2578

HNP and $P2Y_6$ blocker (MRS2578; Sigma-Aldrich, Oakville, ON) were diluted in SABM. For HNP, the same amount of phosphate-buffered solution (PBS) was used as vehicle control. For MRS2578, because of its solubility, the same amount of DMSO was used as vehicle control. Once the cells had reached 70–80% confluence, cell medium was changed to medium containing MRS2578 or containing DMSO, followed 30 min later by administration HNP at different concentrations and incubated for different time points.

IL-8 ELISA

IL-8 concentrations in cell culture supernatant were measured using Human IL-8 ELISA Kit (Life Technologies Inc. Burlington, ON).

Human MSCs

Human bone marrow-derived mesenchymal stromal cells (MSCs) were provided by Dr. DJ Prockop, Institute for Regenerative Medicine, Texas A&M Health Science Center. The use of the gifted cells was approved by the Research Ethics Board at St. Michael's Hospital (REB-0245). The MSCs were grown as monolayer in a humidified atmosphere of 95% normal air and 5% CO_2 at 37°C in αMEM (Alpha Modification of Eagle's Medium Formulation) (Cat#: 310-010-CL, Wisent Inc. St-Bruno, Quebec) with 1% Antibiotic antimycotic (Cat#: A5955, Sigma-Aldrich, St. Louis, MO) and 16.5% heat-inactivated fetal bovine serum (FBS) (Cat#: SH30070.03, Hyclone, Mississauga, ON). MSCs were used at 3rd and 4th passages for the study.

Cell Adhesion Assay

MSCs were seeded onto 12-well plate and cultured in complete αMEM containing 16.5% FBS, until 75% confluence was reached the cells were washed, starved in serum-free αMEM for 2 h prior to stimulation with HNPs. The MSCs were exposed to either PBS as vehicle control or HNP at 5 or 10 μg/mL in serum-free αMEM for 24 h at 37°C. The medium was removed 24 h later and the MSCs were washed with pre-warmed PBS. Each well was added with 200 μL of 0.25% trypsin-EDTA, incubated at 37°C for approximately 3 min until all the cells were detached in the control group. The trypsin-EDTA was neutralized with complete medium. The remaining adhered MSCs were fixed with 300 μL 4% paraformaldehyde (PFA) for 10 min and permeabilized with 0.025% Triton-X 100 for 3 min. The nuclei were stained with DAPI (Invitrogen, Carlsbad, CA, US) and counted by ImageXpress Micro XLS System (Molecular Devices, San Diego, CA).

Cytokine, Chemokine and Growth Factor Arrays

MSC secretome profiles were analyzed after HNP stimulation in cell supernatants by a protein array (Cat #: AAH-ADI-1-8, RayBiotech, Norcross, GA) consisting of 62 target cytokines, chemokines and growth factors. In brief, 1 mL of medium from PBS- or HNP-treated cells was added in antibody-coated array membranes and incubated overnight at 4°C. Signal was detected by chemiluminescence after biotinylated antibody and horseradish peroxidase-conjugated streptavidin interactions as suggested by manufacturer. Chemiluminescent signal was quantified using Image Lab software (Biorad Laboratories, Hercules, CA).

Platelet Derived-Growth Factor-AA (PDGF-AA) Measurement

PDGF-AA levels in the concentrated samples were detected by ELISA (Cat #: ab100622, Abcam, Toronto, ON).

Western Blot

The expression level of integrin β1 was detected in MSCs by means of Western blot after stimulation with HNP. Total proteins were obtained by cell lysis and separated by 10% SDS-PAGE gel under reducing conditions. The proteins were then transferred onto nitrocellulose membranes, which were blocked with 5% bovine serum albumin in TBS (20 mM Tris, pH 7.5, and 150 mM NaCl) containing 0.1% Tween-20 for 1 h. The membranes were probed with antibodies against human integrin β1 (Cat #: ab30394, Abcam, Toronto, ON) and GAPDH (Abcam, Toronto, Ontario, Canada) respectively, overnight at 4°C. After washing, the membranes were incubated with appropriate secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, Texas) conjugated with horse-radish peroxidase, and the signals were detected with an enhanced chemiluminescence kit (Pierce, Rockford, IL). GAPDH was used as a gel loading control. The images were developed and visualized with ChemiDo Touch imaging system (Biorad Laboratories, Hercules, CA).

Statistical Analysis

Data were analyzed by GraphPad Prism (version 5, GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM. Comparison of means between two groups of data was made using the unpaired, two-tailed Student *t* test. p values < 0.05 were considered as statistically significant difference.

Results

HNP Levels in Healthy Controls and Patients

A total of 62 septic patients and 9 healthy volunteers were included in the study. The main demographic and clinical characteristics of the patients are provided in Table 1. Plasma levels of HNP in septic patients were higher than those of healthy volunteers (Table 1).

Microbicidal Effect of HNP on *P. aeruginosa* Was Independent Upon P2Y$_6$ Signaling

Incubation of *P. aeruginosa* with HNP at doses of 0, 10, 20 and 50 μg/mL resulted in a time-dependent effect of bacterial killing at the dose of 50 μg/mL in 6 h (Figure 1A). In subsequent experiments, when *P. aeruginosa* was treated with a solution of HNP (50 μg/mL) that was mixed with various doses of MRS2578 at 1, 3 and 10 μM, the microbicidal property of HNP was not affected by blocking the P2Y$_6$ signaling using MRS2578 (Figure 1B).

Pro-Chemoattractant Effect of HNP on Human Lung Epithelial Cells via P2Y$_6$ Signaling

When human small airway epithelial cells were incubated with *P. aeruginosa* at a concentration of 3x10^5 CFU/mL, an increase in IL-8 production was observed although it did not

Table 1 Demographic and HNP Levels in Healthy Subjects and Patients with Sepsis

Variables	Healthy	Sepsis
Number (n)	9	62
Age (year)	46.3 ± 2.2	67.4 ± 4.5
Gender (M/F)	5/4	34/28
Lactate (mmol/L)		4.5 ± 0.5
HNP (ng/mL)	1.4 ± 0.3	4.5 ± 0.5[a]
WBC (10^9/L)	5.5 ± 0.3	17.6 ± 1.1[a]
Neutrophils (10^9/L)	1.5 ± 0.6	14.8 ± 7.2[a]
APACHE II		26.0 ± 0.4
MODS Score		8.1 ± 3.3
Site of infection[a] (n)		
Bacteremia		26 (41.9%)
Lung		28 (45.2%)
Abdomen		5 (8.1%)
Urinary tract		13 (21.0%)
Soft tissue and skin		3 (4.8%)
Nervous system		2 (3.2%)
Other		1 (1.6%)

Notes: [a]$p < 0.05$ vs Healthy; [a]Some patients had more than one site of infection.
Abbreviations: HNP, human neutrophil peptide; WBC, white blood cell; APACHE II, acute physiology and chronic health evaluation II; MODS, multiple organ dysfunction syndrome.

reach statistical significance (Figure 1C). However, when the epithelial cells were treated with HNP at 50 μg/mL the level of IL-8 increased significantly (Figure 1C). The elevated IL-8 production by HNP stimulation was attenuated by the use of MRS2578 in a dose-dependent manner (Figure 1C), suggesting that the pro-inflammatory effect by HNP was mediated through $P2Y_6$ signaling.

MSC Adhesion by HNP

Human MSCs stimulated with HNP at 0, 5 and 10 μg/mL for 24 h showed cell adhesion in a dose-dependent manner (Figure 2A). This was associated with increased expression of integrin β1 (Figure 2B). There were two bands on the integrin β1 blots (Figure 2B) in which the upper band represented the activated functional form of integrin β1 together with the heavy chain fragment of α5. The lower band represented the non-activated form of integrin β1.[23]

MSC Secretome Profile Changes by HNP

Human MSCs stimulated with HNP at 10 μg/mL for 24 h resulted in increased production of IL-11, leukocyte inhibitory factor (LIF), C-reactive protein (CRP), first apoptosis signal (FAS) and platelet-derived growth factor AA (PDGF-AA) while the levels of IL-6 and monocyte chemotactic protein-1 (MCP-1) decreased in cell supernatant as compared to the vehicle control group (Figure 3).

Discussion

Our study has several notable findings. First, plasma concentrations of HNP were higher in patients with sepsis than in healthy controls. Second, the mechanisms of HNP were distinct for bacterial killing and for the induction of inflammatory response in lung epithelial cells. Third, MSC appeared to be more vulnerable than *P. aeruginosa* and human lung epithelial cells in response to a given concentration of HNP. These findings may have important implications in cell therapy using MSCs in the microenvironment where neutrophil infiltration and thus released HNP is present that may alternate MSC secretome profiles.

Circulating concentrations of HNP were higher in our patients with sepsis than in the healthy controls. The increased HNP levels could be due to the greater neutrophil numbers.[24] These observations are consistent with those previously reported in which high concentrations of HNP were detected in blood of patients with inflammatory lung diseases.[6,9,10,24–31] The increased HNP levels seen in septic patients brought our attention to examine whether and how bacterial killing activity and behaviours of lung epithelial cells and MSCs are modulated by HNP.

We observed that HNP exerted microbicidal properties in clearing *P. aeruginosa* per se, while also increasing the *P. aeruginosa*-induced inflammatory response by enhancing IL-8 production in human lung epithelial cells. Since the use of the $P2Y_6$ antagonist MRS2578 attenuated the HNP-induced IL-8 production and did not affect the microbicidal activity of HNP, our results indicated that 1) HNPs acted on eukaryotic cells through ligand-receptor mechanisms[12–14] and kill prokaryotic cells via different approaches such as charge–charge interaction;[32] and 2) the ligand-receptor mechanism seemed to override that of charge interaction when eukaryotic and prokaryotic cells were co-existent.

We believe that through the same ligand-receptor mechanism, HNP acts on MSCs to modulate the cell secretome profiles. It is noteworthy to mention the fact that MSCs showed significant cell adhesion and change of secretome profiles in responses to a much lower concentration of HNP (i.e., 5 x) than that required for human lung epithelial cells to be activated. This observation is in agreement with previous findings reporting that HNP stimulation increased expression of adhesion molecule on human primary small airway epithelial cells and alveolar type II-like cells[33] at a dose 10 times higher than that used

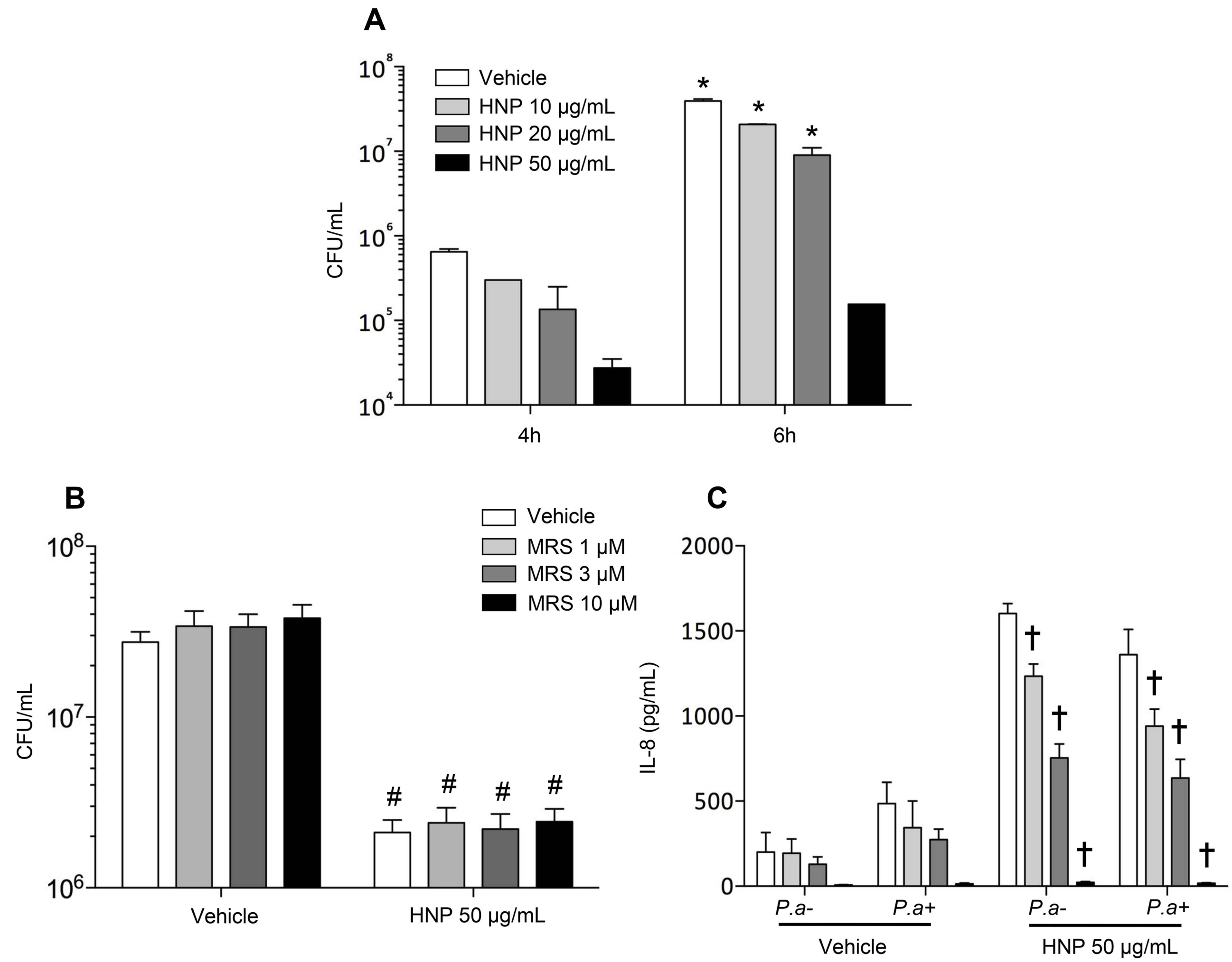

Figure 1 Bacterial count and IL-8 production. (**A**) *P. aeruginosa* was incubated with HNP at indicated concentrations for indicated time points, and bacterial CFU was counted in TSB agar plates after overnight culture (n=4) (**B**) *P. aeruginosa* was treated with a solution containing HNP (50 µg/mL) and indicated doses of MRS2578 for 6 h, CFU was counted in diluted cell supernatants after overnight incubation (n=6). (**C**) Human small airway epithelial cells were co-cultured with and without *P. aeruginosa* in the medium containing indicated concentration of MRS2578 or DMSO (Vehicle), followed by 30 min later by administration of PBS or HNP at 50 µg/mL for 6 h. IL-8 levels were measured in cell supernatants by specific ELISA (n=6). *$p < 0.05$ vs vehicle at identical conditions at 4 h, respectively; #$p < 0.05$ vs vehicle at identical MRS concentration, respectively; †$p < 0.05$ vs identical conditions at HNP 50 µg/mL, respectively.

Abbreviations: HNP, human neutrophil peptide; CFU, colony-forming unit; TSB, tryptic soybroth. IL, interleukin; PBS, phosphate-buffered saline; HNP, human neutrophil peptide; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethylsulfoxide.

in the MSC stimulation of the present study. Our results demonstrated that MSCs appeared to be very susceptible to HNP stimulation than lung epithelial cells. The increased MSC adherence by HNP may provide a viable explanation for the prolonged MSC retention in animal models of lung injury[16] and in human inflammatory disease conditions. The prolonged retention would increase the exposure of MSCs to the inflammatory environment and respond accordingly producing mediators that normally are quiescent.

The increased MSC adhesion was associated with the upregulation of the adhesion molecule integrin β1 upon HNP stimulation. In a previous study,[34] investigators have shown that human MSCs express highly integrin β1, contributing to cell adhesion. Adhesion of MSC to fibronectin, through α5β1-integrin, specifically induced MSC migration from circulation by activating platelet-derived growth factor receptor-β (PDGFR-β) signaling during vascular remodelling.[35] The upregulation of PDGF-AA, the most common PDGF secreted from MSCs[36] observed in our study may also play a major role as a niche to support MSC adherence.

The known protective effects of MSCs in cell therapy are due to their paracrine effects by releasing secretome in the milieu.[37] However, our results may suggest that MSCs could modulate the courses of lung injury when neutrophil

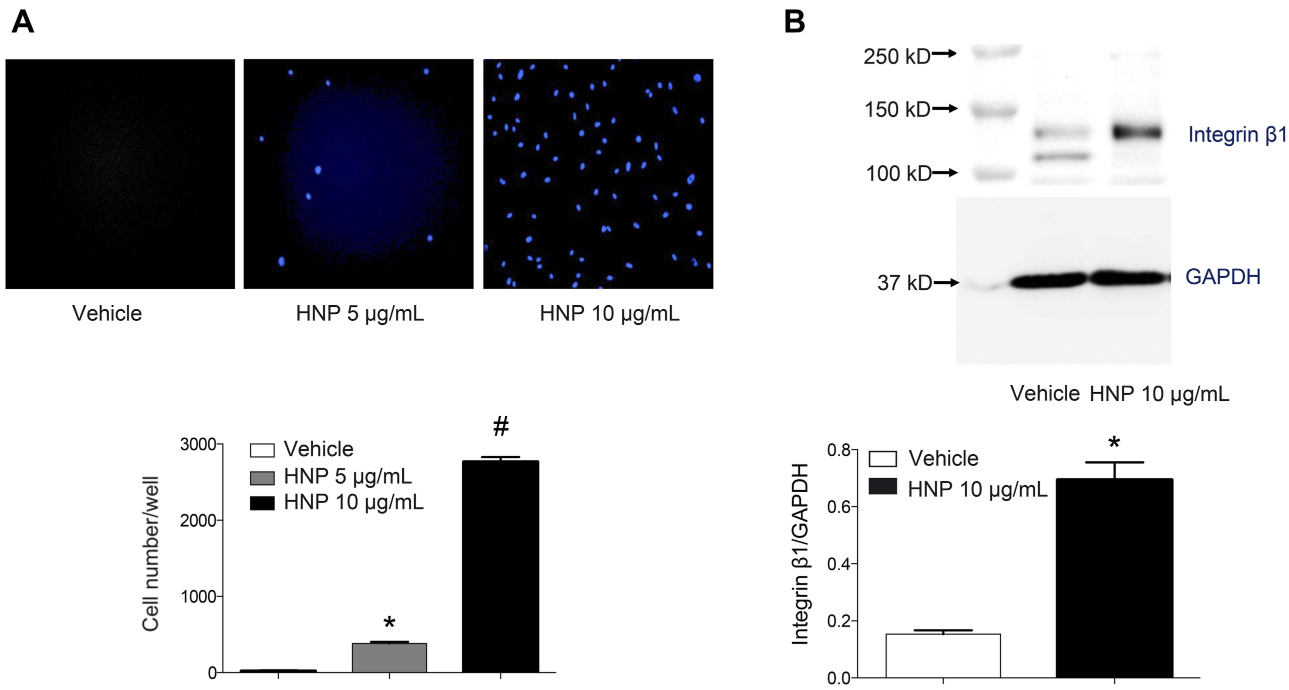

Figure 2 MSC adhesion and integrin β1 expression increased upon stimulation with HNP in a dose-dependent manner. (**A**) MSCs were stimulated with PBS (Vehicle), 5 or 10 μg/mL HNP for 24 h. After cell detachment with trypsin-EDTA, the remaining cells were fixed and nuclei were stained with DAPI (in blue), and detached cells were counted (n=3); (**B**) Expression of integrin β1 in MSCs after stimulation with PBS or 10 μg/mL HNP for 24 h, and the mean value of integrin β1 expression (n=31). *$p < 0.05$ vs Vehicle, $^{\#}p < 0.05$ vs other groups.

Abbreviations: MSC, mesenchymal stem cell; HNP, human neutrophil peptide; EDTA, ethylene diamine tetraacetic acid; DAPI, diaminophenyl indole.

infiltration and HNPs are present. The altered MSC secretome profiles included upregulation and downregulation of pro- or anti-inflammatory mediators as discussed briefly below.

The increased pro-inflammatory mediators in MSC secretome after HNP stimulation included PDGF-AA, IL-11, CRP and FAS. Since the role of PDGF-AA has been discussed above, we will discuss other mediators. IL-11 is an IL-6 like cytokine, and has been reported to play an important role in Th2- and IL-13-induced lung inflammation and mucus production,[38] and in cardiovascular[39] and pulmonary fibrotic responses in infectious human airway disorders.[40] C-reactive protein (CRP), is an acute-phase protein that can rapidly and dramatically increase in response to inflammation, cell damage or tissue injury.[41] High concentrations of CRP have been reported in both alveolar fluid and plasma suggesting its important role in lung injury.[42] Patients with pneumonia showed increased levels of CRP that is correlated with high mortality.[43] Fas belongs to the TNF receptor superfamily, which is also known as CD95/Apo-1. Fas-Fas ligand has been reported to induce apoptosis, promotes lung inflammation, neutrophil extravasation and loss of epithelial cells.[44,45] Taken together, our results and those from others[42–45] suggests that the increase of these factors secreted by MSCs after HNP stimulation may aggravate lung injury and even induce adverse prognosis.

The decreased pro-inflammatory mediators in MSC secretome after HNP stimulation included IL-6 and MCP-1. Although IL-11 is a member of IL-6-type cytokine it interacts with IL-11Rα subunit expressed highly in fibroblasts and stromal cells and not in immune cells, while IL-6 receptors are expressed lowly in stromal cells but highly in immune cells.[39,46] Investigators have demonstrated that IL-11 and IL-6 negatively regulate between each at posttranscriptional level.[47] This mechanism may explain the increased expression of IL-11 and low expression of IL-6 in MSC secretome after HNP stimulation observed in our study. One expects to observe an upregulation of chemokines by MSCs upon stimulation with HNPs. However, the production of CCL2, CCL3 and CXCL8 chemokine members all decreased although only the reduction of MCP-1 reached statistically significant. This observation maybe speculated by senescence of the cultured MSC in response to oxidative stress after HNP challenge.[33,48] The senescence of UCB-MSCs is orchestrated by MCP-1, which is secreted as a major component of the SASP and is epigenetically

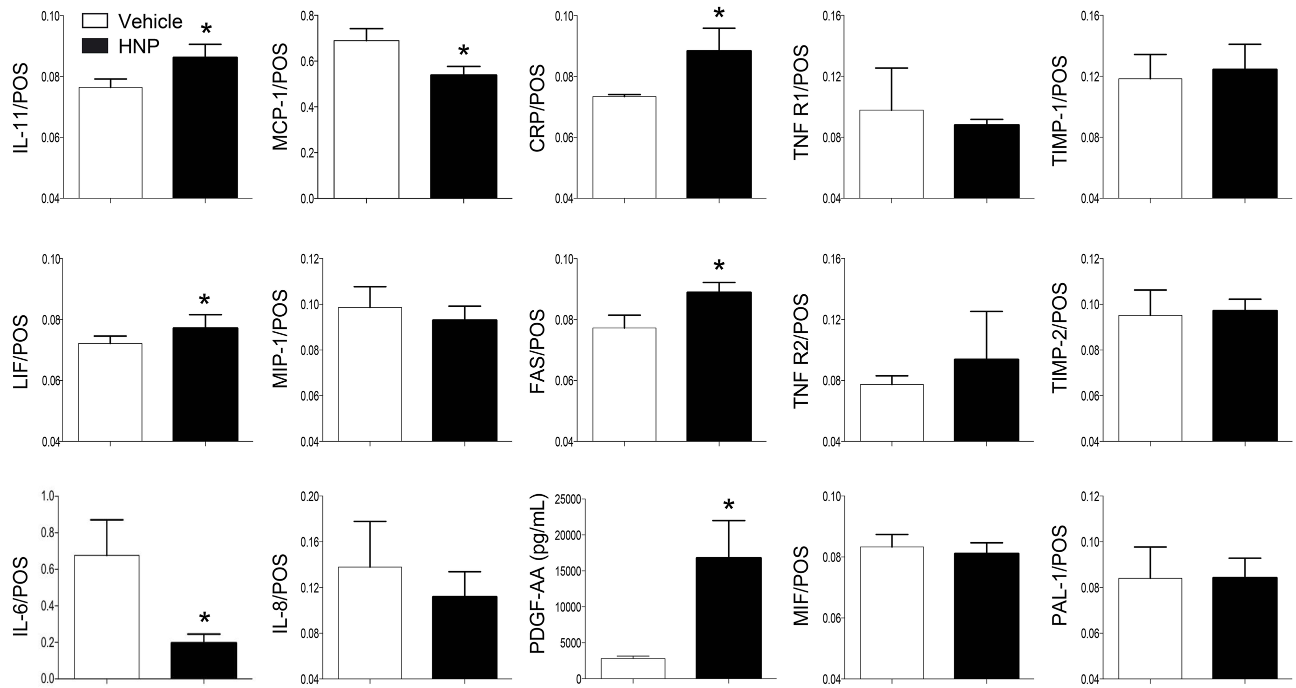

Figure 3 Changes of secretome profiles by MSCs after stimulation with PBS (Vehicle) or HNP at 10 μg/mL for 24 hrs. The cell culture supernatants were added in antibody-coated array membranes and incubated overnight at 4°C. Signal was detected by chemiluminescence after biotinylated antibody and horseradish peroxidase-conjugated streptavidin interactions. Quantification of protein expression was compared between Vehicle and HNP-treated groups (n=3). Expression of 6 out of 14 proteins detected reached statistical difference. Levels of PDGF-AA were measured by human-specific ELISA (n=17). *p < 0.05 vs Vehicle.
Abbreviations: MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; HNP, human neutrophil peptide; PDGF, platelet-derived growth factor.

regulated by BMI1. The BMI1, a polycomb protein, expressed in senescence MSCs could suppress the gene expression of MCP-1 by binding to its regulatory elements resulting in depression of MCP-1.[49]

We observed that LIF, an anti-inflammatory mediator, increased in MSC secretome after HNP stimulation. Studies have previously shown that LIF protects the lung from viral infection, bacterial pneumonia by its anti-inflammatory effects.[50–52]

There are limitations in our study. The sensitivity of the protein array membrane might be limited to the most striking changes of secretome in the MSC supernatants. Furthermore, the effects of altered secretome profiles by MSCs after HNP challenge in lung injury are yet to be examined in future studies.

In conclusion, our results provide novel mechanistic insights with respect to bacterial killing, lung epithelial reaction and MSC behaviours upon exposure to different levels of HNPs. MSCs are highly vulnerable to HNP exposure by expressing different secretome profiles, and thus this undisclosed risk factor of HNP in lung environment should be taken into consideration when MSCs are applied as cell therapy in inflammatory lung diseases.

Data Sharing Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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