Role of human neutrophil peptides in the initial interaction between lung epithelial cells and CD4+ lymphocytes

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Abstract: Human neutrophil peptides (HNP) exert immune-modulating effects. We hypothesized that HNP link innate and adaptive immunity through activation of costimulatory molecules. Human lung epithelial cells and CD4+ lymphocytes were treated with HNP separately or in coculture. Stimulation with HNP induced an increase in cell surface expression of CD54 (ICAM-1), CD80, and CD86 on lung epithelial cells and the corresponding major ligands, CD11a (LFA-1), CD152 (CTLA-4), and CD28 on CD4+ lymphocytes. There was an increased nuclear expression of the transcription factor p53 in human alveolar A549 cells and an elevated NF-κB (p50) and a degradation of I-κB protein in CD4+ lymphocytes following HNP stimulation. HNP enhanced the interaction between A549 cells and CD4+ lymphocytes by increasing cell adhesion and release of IFN-γ, IL-2, and IL-8. This was attenuated by using an α1-proteinase inhibitor to neutralize HNP. We conclude that HNP play an important role in linking innate to acquired immunity by activation of costimulatory molecules in lung epithelial cells and CD4+ lymphocytes. J. Leukoc. Biol. 81: 1022–1031; 2007.

Key Words: infection · immunity · cytokine

INTRODUCTION

The immunological determinants of most inflammatory lung diseases involve lymphocytes including Th cells and associated Th1 and Th2 cytokines [1–4]. Lung diseases, such as community-acquired pneumonia, acute respiratory distress syndrome (ARDS), which is frequently associated with sepsis, and acute lung injury secondary to ischemia/reperfusion and transfusion, are examples of diseases associated with innate immune responses [5]. Although the innate immune system can respond within hours, the course of ARDS/sepsis frequently exceeds 2–3 weeks. Thus, there is sufficient time for T lymphocytes and other parts of the adaptive immune system to take part in the ongoing immune responses [1, 3, 6, 7].

In patients with sepsis, the numbers of circulating CD4+ T lymphocytes are more rapidly increased than was thought previously [7]. In some patients, this leads to a prolonged and severe immune imbalance that is associated with poor prognoses [1–4]. Four hours after i.v. injection of Escherichia coli endotoxin into sheep, ~50% of squashed leukocytes in lung biopsies were T lymphocytes [8]. In an acute lung injury model of lypmphopenic sheep, endotoxin challenge produced a much smaller increase in airway and vascular resistance than in control animals [9]. Manipulation of the T lymphocyte population by pharmacological intervention could reduce mortality in septic mice [10]. These clinical and animal studies suggest that T lymphocytes, acting alone or in conjunction with mediators, are involved in the early pulmonary response to inflammation.

The immune response is initiated when T lymphocytes recognize antigen peptides bound to MHC molecules on the surface of APC. An adaptive immune response requires T lymphocytes to develop a complex cluster of molecules at the T lymphocytes-APC interface. Adhesion, costimulatory, cytoskeletal, and signaling molecules are organized in activation clusters [11–15].

T lymphocytes require two signals for activation: Signal 1 provides activation through the TCR and Signal 2, via costimulatory molecules. The CD28/B7-1 and CD28/B7-2 receptor ligand [i.e., CD28/CD80 (CD86)] system is one of the dominant, costimulatory pathways [16]. In addition, the CD28 family includes CTLA-4 or CD152, which binds to B7-1 (CD80) on APC [17]. Recent studies have suggested that lung epithelial cells exert immune-modulating functions as nonprofessional APC by expressing MHC Classes I and II in response to infection [18–24]. This interaction initiates a cascade of biochemical events in the T lymphocytes, which occur primarily through the increase in IL-2 secretion by the T lymphocytes [16, 25]. Other studies identified ICAM-1 as a costimulatory ligand that binds to LFA-1, thereby promoting the activation of T lymphocytes. As ICAM-1 expression is up-regulated on lung epithelial cells upon a variety of stimulation such as LPS and cytokines, it becomes a crucial molecule for the activation of T lymphocytes, independent of costimulation provided by CD80 and CD86 molecules [26].

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As an important part of lung innate immune responses, large, circulating, and marginated pools of immune cells such as polymorphonuclear leukocytes (PMN), monocytes, NK cells, and T lymphocytes respond rapidly to chemotactic stimuli in the early phases of lung injury [3, 4, 27, 28]. Once sequestered and activated, immune cells including PMN attack microorganisms with their microbicidal products and remove them by phagocytosis. This host defense activity of PMN should not be viewed simply, however, as an innocent process for waste disposal. Instead, the clearance of microorganisms by PMN clearly adds immunological value. PMN release large amounts of proteins, such as human neutrophil peptides (HNP), into the extracellular milieu as a consequence of degranulation, leakage during phagosome formation, and the death and lysis of the immune cells.

HNP contain six highly conserved cysteine residues forming three intramolecular disulfide bonds [29]. HNP constitute up to 5% of the total protein content in PMN and >50% of the total protein within the azurophilic granules of PMN [30]. In pleural fluid, the concentration of HNP is 13 ± 2 μg/mL in patients with emphysema [31]. In lung lavage fluid, HNP concentrations are 50-fold higher in patients with ARDS than in healthy controls [32, 33]. Sputum HNP levels ranged from 300 to >1600 μg/mL in patients with cystic fibrosis compared with undetectable levels in healthy controls [34]. In septic conditions, the levels of HNP might be elevated up to concentrations reaching mg/mL [29]. There is an excellent correlation between blood HNP concentration and PMN count [29, 31-37], but the exact role of HNP in inflammatory lung diseases remains unknown.

We do know that HNP modulate the cellular immune responses by stimulating the production of IL-8 in lung epithelial cells [38-40] and by exerting chemotaxis to T lymphocytes, immature dendritic cells [41, 42], and monocytes [43]. In addition, intratracheal administration of HNP, at clinically relevant concentrations seen in patients, induced acute lung injury in mice [44]. We thus speculated that HNP can have potent immunological effects, linking an innate response to a more complex, adaptive immune response. To test the hypothesis, we investigated the effect of HNP on expression of co-stimulatory molecules, adhesion molecules on lung structural cells and CD4+ T lymphocytes, on IFN-γ, IL-2, and IL-8 release in cocultures, and on modulation of the transcription factors NF-κB, p53, and IκB, which are involved in modulation of co-stimulatory molecules as well as cytokines.

MATERIALS AND METHODS

Reagents

Mouse antihuman CD89-FITC, CD86-PE-PE, CD28-PE-T, CD154-TCRL-4R-PE, and CD54/ICAM-1-FTC-conjugated mAbs were purchased from BD Systems (Minneapolis, MN, USA). Mouse antihuman CD11a/LEA-1-PE-conjugated mAbs were from BD Biosciences (Franklin Lakes, NJ, USA) and mouse isotype control IgG2a was from Becton Dickinson (San Diego, CA, USA). Polyclonal rabbit antihuman α-HLA primary antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), goat antihuman IgG-HRP secondary antibody was from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), and peroxidase substrate 3,3'-5,5'-tetramethylbenzidine (TMB) was from Sigma Chemical Co. (St. Louis, MO, USA). Antihuman β-actin mAb was obtained from Alpha Diagnostic International Inc. (San Antonio, TX, USA). Purified HNP were a mixture of HNP-1, -2, and -3, as described previously [40]. Briefly, the sputum was pooled from at least 20 patients with cystic fibrosis before purification. With respect to possible variability from one batch to another, at least three different batches of the purified HNP were used to stimulate cells in all experiments reported below. Purified HNP were reconstituted in 0.01% acetic acid and treated by bacterial killing. The myeloblast detection assay was performed by using Luminol amine buffer lyse (Pyrrochrome® Associates of Cape Cod Inc. Falcon, MA, USA), and the LPS content was <100 pg/mL in the purified HNP solution before use. The human α1-proteinase inhibitor (α1-PI; Prostain), purified from the blood-derived α1-PI preparation, was from Bayer HealthCare Canada (Toronto, ON, Canada).

A549 epithelial cell cultures

A human cell line derived from alveolar cell carcinoma of the lung that retains features of Type II epithelial cells (American Type Culture Collections, Manassas, VA, USA) was grown as monolayers in 5% CO₂ at 37°C in DMEM with L-glutamine (Gibco, Grand Island, NY, USA), supplemented with 50 μg/mL gentamicin (Gibco) and 10% heat-inactivated FBS (Gibco).

Human small airway epithelial cell (SAEC) cultures

Human SAEC (Cambrex Bio Sciences Inc., Walkersville, MD, USA), from the distal airspace, were grown in SAEC basal medium (SABM1™ plus SingleQuots® of growth supplements (SAGM BulletKit, Cambrex Bio Sciences Inc.) until confluence. Cells were then washed, and the SACM BulletKit was replaced with epithelial cell basal medium free of serum before HNP stimulation.

Isolation of CD4+ lymphocytes

CD4+ lymphocytes were isolated from human PBMC by using a Dynal™ CD4+ lymphocyte negative isolation kit containing Dynabeads® and antibody mix (Invitrogen Canada Inc., Burlington, Ontario, Canada). The Dynabeads® are uniform, superparamagnetic beads coated with a Fe-specific, human IgG4 antibody against mouse IgG. The antibody mix contains a mixture of mouse mAb for CD4, CD16, CD56, CD123, CD36, CD8, HLA Class II DR/DP, and Glyphorin A. Thus, the kit depletes B cells, NK cells, CD8+ T lymphocytes, activated T cells, monocytes, erythrocytes, and granulocytes. The coated cells were separated with a magnet particle concentrator (Dynal magnetic particle concentrator) and discarded. The isolated CD4+ lymphocytes were viable (>98%). The CD4+ lymphocytes were subsequently in RPMI-1640 with L-glutamine (Gibco), supplemented with 50 μg/mL gentamicin (Gibco) and 10% heat-inactivated FBS (complete medium, Gibco).

Cell adhesion assays

A549 cells (2.5x10⁵ cells/well) were grown in 500 μL DMEM containing 10% serum using 24-well plates. When confluent, the cells were washed twice and treated with or without HNP at 0-20 μg/mL in serum-free DMEM for 4 h. After washing with PBS, the cells were incubated with 0.2% trypsin-EDTA (Gibco) at 37°C for 5 min. The wells were washed thoroughly and incubated for 5 min with the fixative solution of methanol (Hemacore® stain set, Solution 1, EM Diagnostic Systems, Gibbstown, NJ, USA). After rinsing over the plates to remove the fixative solution, the cells (Hemacore® stain set, Solution 3, EM Diagnostic Systems) were added, and the plates were incubated for 5 min to stain cell nuclei. After washing, the plates were left in room air until the wells were dry; then, the slides were washed with PBS, rinsed with distilled water, and air-dried. The slides were mounted with Permount (Fisher Scientific) and analyzed with a Leica DMRB microscope equipped with a Leica DFC 420 digital camera (Leica Microsystems, Wetzlar, Germany). In separate experiments, A549 cells and SAEC were grown on glass coverslips. When confluent, the cells were washed twice in PBS and incubated in serum-free medium. In parallel, freshly isolated, human CD4+ lymphocytes were labeled for 30 min with 5 μM calcein-AM (Molecular Probes, Eugene, OR, USA). After the incubation, 250 μL CD4+ lymphocyte suspension (2x10⁶ cells/ml) was added to each well of a 24-well plate containing confluent A549 cells or SAEC for 4 h in the presence and absence of HNP at 0, 10, 50, or 100 μg/mL. The coverslips were then washed extensively, fixed

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in cold PBS containing 4% paraformaldehyde, and mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA) on a glass slide. The number of adherent CD4+ lymphocytes was calculated by counting the number of fluorescent cells from six randomly selected, high-power fields (X400) using a Nikon Eclipse E800 fluorescence microscope.

Coculture of A549 cells, SAEC, and CD4+ lymphocytes

A549 cells were grown on 24-well plates. When confluent, the cells were washed twice in serum-free medium and treated with or without HNP at 0, 10, 50, or 100 μg/mL, followed by addition for 4 h of 250 μL of a CD4+ lymphocyte suspension (2×10^5 cells/mL) to each well of the 24-well plates. Additionally, freshly isolated, human CD4+ lymphocytes were treated with or without HNP at 0, 10, 50, or 100 μg/mL for 4 h.

To confirm the results seen with A549 cells, the experiments were repeated with SAEC with 10 μg/mL HNP for 4 h, and after the incubation, the culture supernatants were collected for cytokine measurements.

Immunofluorescence staining

A549 cells were seeded in a four-well chamber slide system (Lab-Tek, Nalgene Nunc International, Naperville, IL, USA). Cells formed a subconfluent monolayer after overnight culture and were treated with HNP at different concentrations in serum-free medium. In separate experiments, CD4+ lymphocytes were cocultured with A549 cells for 2 h in the presence or absence of HNP. After washing, the cells were fixed in cold PBS containing 4% paraformaldehyde, incubated for 2 h at 4°C with mouse isotype control IgG2a or conjugated mAb to human CD45, CD80, or CD86, mounted with fluorescent mounting medium, and examined with a confocal scanning laser microscope (Zeiss510, Zeiss, Oberkochen, Germany). The fluorescence densities were measured using a molecular imaging system (Kodak Image Station 2000MM digital imaging system, Mandel Scientific Company Inc., Guelph, ON, Canada).

Flow cytometric analysis

In the experiments assessing the effect of CD28, CD152/CTLA-4, and CD11a/LFA-1, CD4+ lymphocytes were incubated with HNP for 2 h and washed. For each analysis, 1×10^6 cells were incubated with mouse isotype control IgG2a or CD28-FITC, CD152/CTLA-4-R-PE, or CD54/ICAM-1-FITC-conjugated mAb at 4°C for 30 min. After washing, the cells were analyzed with a FACSCanto (Becton Dickinson, Mountain View, CA, USA) using BD FACSDiva software. For each analysis, 5,000 events were collected, and the percent of positive cells was determined.

NF-κB p50/p65 assays

The p50/p65 (NF-κB/RelA) heterodimers and the p50 homodimers are the most common dimers found in the NF-κB signaling pathway. In testing cells, NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins. Treatment of cells with various inducers results in the phosphorylation, ubiquitination, and subsequent degradation of IκB proteins. This results in the release of NF-κB dimers, which subsequently translocate to the nucleus, where they activate appropriate target genes. To detect NF-κB activation, NF-κB p50 ChemiChip65 Chemiluminescence transcription factor assay kits (TransAM™, Active Motif North America, Carlsbad, CA, USA) were used. Briefly, the kits contain a nuclear extract kit and a 96-well plate, on which oligonucleotide containing the NF-κB consensus site (5′-GGCAATTCCTCC-3′), has been immobilized. The primary antibodies used to detect NF-κB recognize an epitope on p50 or p65, which is accessible only when NF-κB is activated and bound to its target DNA. A HRP-conjugated secondary antibody provides a chemiluminescent readout, which was quantified by a molecular imaging system (Kodak Image Station 2000MM digital imaging system, Mandel Scientific Company Inc.).

p53 Transcription factor assays

To quantify p53 activation, commercial kits (TransAM p53 transcription factor assay kits, Active Motif North America) were used. Briefly, the nuclear extract binds specifically to an immobilized oligonucleotide that contains a p53 consensus binding site (5′-GGCATGACCCGTGACGTCGAG-3′) sequence on a 96-well plate. A primary antibody used to detect p53 recognizes an epitope on p53 protein upon DNA binding. A HRP-conjugated, secondary antibody provides a chemiluminescent readout and quantification using the Kodak Image Station 2000MM digital imaging system.

SDS-PAGE and I-κB Western blotting

A549 cells were detached with cell scrapers in cold cell lysis buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.05% Triton X-100, 100 μg/mL PMSF, and 1:1000 protease inhibitor cocktail (Sigma Chemical Co.)]. CD4+ lymphocytes were lysed using the cell lysis buffer, and the lysed cells were passed through a 21-G needle 10 times, resuspended in PBS, and spun down at 4°C at 10,000 g for 10 min. The protein concentrations of the total cell lysates were measured (Bio-Rad protein assay, Bio-Rad, Mississsauga, ON, Canada). Each cell lysate (5 μg) was resolved on a 12% SDS-PAGE (Mini-PROTEAN 3 electrophoresis cell, Bio-Rad) and transferred to nitrocellulose membrane (Bio-Rad Trans-Blot semi-dry system). After washing, the membrane was blocked with PBS containing 0.1% Tween-20 and 0.5% nonfat dry milk powder overnight incubation with a polyclonal rabbit antihuman IκBα primary antibody (1 μg/mL) at 4°C, followed by 1:5000 goat antirabbit IgG-HRP secondary antibody for 1 h at room temperature, followed by the addition of peroxidase substrate TMB. Antihuman β-actin mAb was used as a loading control for Western blots. The intensity of the bands of IκBα and β-actin was measured using a molecular imaging system (Kodak Image Station 2000MM digital imaging system, Mandel Scientific Company Inc.).

Lactate dehydrogenase (LDH) cytotoxicity detection

LDH is a stable cytoplasmic enzyme present in most cells. LDH is released into cell culture supernatant upon damage of the cell membrane. To confirm constant cell viability before and after treatment with HNP, cytotoxicity was evaluated in all the reported experiments conducted in A549 cells, SAEC, and CD4+ lymphocytes, as described previously [40]. Briefly, the supernatants of cells were collected, and the cells were washed with FBS, serum-free DMEM at 200 μL was added, and the cells were lysed by freeze-thaw cycles (frozen at −80°C for 30 min and thawed at 37°C). The supernatants were pooled and centrifuged at 250 g for 5 min. LDH concentrations in cell culture supernatants and cell lysates were measured using a cytotoxicity detection (LDH) kit (Roche Applied Science, Penzberg, Germany).

LiquiChip multiple cytokine assay

Cell culture supernatants from indicated experiments were collected for simultaneous measurement of multiple cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, and GM-CSF) using the LiquiChip Human 10 cytokine kit (Quigen, Valencia, CA, USA) [40].

Statistical analysis

Data are expressed as means ± SD or indicated specifically otherwise. A one-way ANOVA, followed by the Tukey/Kramer test, was used for statistical analysis. Differences were considered statistically significant at P < 0.05.

RESULTS

HNP enhanced cell adhesion and interaction

We noted that the HNP-treated A549 cells were resistant to Trypsin-EDTA: the cells remained largely attached on the culture plate after treatment with Trypsin-EDTA. We thus assessed quantitatively the effect of HNP (5–20 μg/mL) on epithelial cell adhesion and found that HNP enhanced A549 cell adhesion in a dose-dependent manner (Fig. 1A). In contrast, no adhesion of A549 cells to culture plates was observed after vehicle control solution or after HNP, at a concentration of 1 μg/mL, was administered (Fig. 1A).

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Adhesion of lymphocytes to epithelial cells is an important part of the inflammatory process. We determined if treatment of A549 cells with HNP could influence the adherence of CD4+ lymphocytes. As shown in Figure 1B, the adhesion of human CD4+ lymphocytes to A549 cells was increased by approximately fourfold after stimulation with HNP. The maximal response was observed with HNP at a concentration of 50 μg/mL. Portions of fields were observed at a magnification of ×400 to better appreciate the effect of HNP (Fig. 1E).

The concentrations of HNP were chosen based on the previous clinical observations, where lung HNP levels increased from nondetectable in healthy controls to high μg/mL levels in patients with inflammatory lung diseases [31–35]. We demonstrated that stimulation of the cells with HNP at 10, 50, or 100 μg/mL for 4 h did not induce cytotoxicity, as reflected by a constant LDH level before and after HNP administration (Fig. 1C). As a positive control, LPS at 100 ng/mL induced a significant increase in LDH levels in SAEC at 4 h (Fig. 1D).

We thus subsequently stimulated the cells by using HNP at 10 μg/mL as a positive control for 4 h in the study. In contrast, prolonged incubation of lung epithelial cells with HNP at 100 μg/mL over 24 h has been reported to cause cytotoxicity associated with a decrease in IL-8 production [45].

HNP induced cytokine responses

Given that HNP induced a rapid, significant cell adhesion and enhanced interaction between A549 cells and CD4+ lymphocytes, we examined whether the cell adhesion and interaction stimulated CD4+ lymphocytes to produce IFN-γ and IL-2 at a dose of 10 μg/mL HNP in the subsequent experiments. Figure 2, A and B, illustrates that the basal levels of IFN-γ and IL-2 were slightly higher in CD4+ lymphocytes than in A549 cells, and HNP, at the dose used, was unable to increase the release of IFN-γ and IL-2 by the two cell types at 4 h. The coculture of A549 cells and CD4+ lymphocytes showed much higher levels of IFN-γ and IL-2 than observed with either of the single cell cultures, and the levels of the cytokines were increased further by HNP stimulation. Clearly, HNP modulate interactions between A549 cells and CD4+ lymphocytes.

Figure 2, C and D, illustrates that 2 h of incubation with HNP did not induce IL-8 release significantly by A549 cells, SAEC, or CD4+ lymphocytes alone. It is interesting that the basal IL-8 level increased by threefold in the coculture conditions compared with the single cultures, and the level of IL-8 was increased further following stimulation with HNP. The HNP-induced IL-8 production was blunted by treatment of the
cells with an equimolar amount of Prolastin, a human α1-PI, which forms complexes with and inactivates HNP [46].

Of the 10 cytokines assayed, there was no significant change in the production of cytokines other than IFN-γ, IL-2, and IL-8.

HNP induced cell surface expression of costimulatory molecules

As adhesion of CD4+ lymphocytes to epithelial cells occurs via costimulatory molecules, we investigated the expression of CD80, CD86, and CD54/ICAM-1 on the A549 cells and SAEC and their corresponding ligands CD28, CD152/CTLA-4, and CD11c/LFA-1, respectively, on the CD4+ lymphocytes. As illustrated in Figure 3A, A549 cells showed no basal expression of CD80 and weak expression of CD86 and ICAM-1 but had increased surface expression of the three molecules in response to stimulation with HNP. SAEC showed higher expression of CD80, CD86, and ICAM-1 at basal conditions, and the expression was increased further by the stimulation with HNP (Fig. 3B). Figure 3C shows that the expression of CD28, CD152, and LFA-1 was increased on CD4+ lymphocytes in a dose-dependent manner by stimulation with HNP.

The expression of the costimulatory molecules is summarized in Table 1. Taken together, these results suggest that the enhanced cell adhesion and the interaction between A549 cells and CD4+ lymphocytes induced by HNP are associated with an up-regulation of cell surface expression of the costimulatory molecules.

HNP induced NF-κB (p50) activation and degradation of I-κB in CD4+ lymphocytes

As production of cytokines in response to costimulatory molecules involves the translocation of NF-κB/Rel transcription factors from cytoplasm to nuclei, we next examined the nuclear expression of the NF-κB subunits p50 (NF-κB1) and p65 (RelA) in A549 cells and CD4+ lymphocytes, which were stimulated with various concentrations of HNP. There was no difference in nuclear expression of p65 before and after HNP stimulation, but stimulation of the cells with TNF-α (20 ng/mL) induced p65 activation (data not shown); thus, only p50 translocation is reported in the present study. As shown in Figure 4, stimulation of the cells with HNP at concentrations as low as 1–2 μg/mL for 30 min led to a significant p50 translocation in CD4+ lymphocytes. This p50 translocation was not seen by stimulation with HNP at concentrations of up to 5 μg/mL in A549 cells. The increased expression of nuclear p50 was associated with a reduced expression of I-κB proteins. These results indicate that CD4+ lymphocytes but not A549 cells activate the p50 pathway, which is associated with up-regulated immune responses upon HNP stimulation.

HNP induced p53 activation in A549 cells

In some situations, the transcription factors p53 and p50 are competitor transcriptional activators inversely regulating each other's activation [47]. We thus investigated if p55 could be an alternative activator of the immune responses in the cells tested. Exposure of A549 cells to HNP resulted in a 2.5- and threefold increase in p53 expression at 2 and 5 μg/mL, respectively, but no significant p50 response at the same doses of HNP (Fig. 4). On the contrary, the CD4+ lymphocytes show no change in p53 expression in response to the same concentrations of HNP, which result in activation of p50. Taken together, the study suggests that HNP stimulation is associated with activation of the transcription factor p50 in CD4+ lymphocytes and p53 in lung epithelial cells.

DISCUSSION

The clinical course of inflammatory lung diseases frequently exceeds a few days and lasts several weeks. In an animal model
Fig. 3. HNP increased surface expression of adhesion and costimulatory molecules in single cell-type cultures. A549 cells or SAECs were seeded in a four-well chamber slide system, formed a subconfluent monolayer after overnight culture, and were then treated with HNP at indicated concentrations in serum-free medium for 2 h. CD4+ lymphocytes were incubated in Eppendorf tubes for 2 h with HNP at indicated concentrations. After washing, the cells were fixed in cold PBS containing 4% paraformaldehyde, incubated for 2 h at 4°C with mouse isotype control IgG2a, or conjugated mAb to human CD80, CD86, CD54/ICAM-1, CD28, CD152, or CD11a/LFA-1 (n=7 experiments each). Surface expression of the CD markers was examined using a confocal microscope and a flow cytometry FACSCanto, respectively.

TABLE 1. Quantification of CD80, CD86, and ICAM-1 Densities on A549 Cells and SAEC, and Percentage of Positive CD4+ Lymphocytes Expressing CD28, CD152, and LFA-1

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<th>Isotype control</th>
<th>0</th>
<th>10</th>
<th>50</th>
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<tr>
<td>A549 cells</td>
<td>1.3 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>50.6 ± 16.1&quot;</td>
<td>65.4 ± 15.4&quot;</td>
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<tr>
<td>CD80</td>
<td>16.1 ± 1.6</td>
<td>10.6 ± 2.2</td>
<td>61.9 ± 10.3&quot;</td>
<td>66.2 ± 11.0&quot;</td>
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<tr>
<td>CD86</td>
<td>12.0 ± 3.1</td>
<td>13.1 ± 2.7</td>
<td>46.3 ± 12.7&quot;</td>
<td>44.9 ± 13.6&quot;</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>18.9 ± 2.3</td>
<td>21.9 ± 3.9</td>
<td>48.3 ± 12.1&quot;</td>
<td>46.6 ± 10.1&quot;</td>
</tr>
<tr>
<td>CD80</td>
<td>15.6 ± 1.3</td>
<td>16.2 ± 4.1</td>
<td>41.8 ± 10.0&quot;</td>
<td>45.2 ± 7.6&quot;</td>
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<tr>
<td>CD86</td>
<td>12.2 ± 0.9</td>
<td>12.2 ± 1.8</td>
<td>25.3 ± 2.2&quot;</td>
<td>31.8 ± 9.1&quot;</td>
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<tr>
<td>SAEC</td>
<td>41.8 ± 12.1</td>
<td>46.6 ± 20.1</td>
<td>66.3 ± 13.4</td>
<td>96.8 ± 20.9&quot;</td>
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<tr>
<td>CD28</td>
<td>4.5 ± 1.6</td>
<td>5.7 ± 2.3</td>
<td>88.5 ± 23.1&quot;</td>
<td>99.5 ± 22.3&quot;</td>
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<tr>
<td>CD152</td>
<td>1.8 ± 0.4</td>
<td>2.1 ± 0.4</td>
<td>48.9 ± 3.7&quot;</td>
<td>25.3 ± 8.6&quot;</td>
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<tr>
<td>LFA-1</td>
<td>11.0 ± 0.5</td>
<td>11.2 ± 1.0</td>
<td>52.3 ± 12.7&quot;</td>
<td>55.3 ± 13.6&quot;</td>
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<td>Mean ± str; n = 3 experiments. *P &lt; 0.05 versus 0 μg/mL.</td>
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and exert chemotaxis for T lymphocytes [41], we demonstrated that CD4+ lymphocyte adhesion to human A549 cells was increased in the presence of HNP. The ability of HNP to increase CD4+ lymphocyte adhesiveness corresponds to the expression of cell surface, costimulatory molecules.

We first demonstrated that HNP stimulated A549 cells to produce the costimulatory molecules CD80 and CD86 on the cell surface. A direct effect of HNP on the expression of costimulatory molecules has not been reported previously, but our results are supported by other studies showing that lung epithelial cells are not quiescent and may actually act as nonprofessional APC, as primary epithelial cells and the A549 alveolar epithelial cells express MHC Classes I and II and costimulatory molecules [22, 23, 49] whose levels are increased by infection [22–24]. A recent study has demonstrated that the A549 cells abundantly expressed the CD80 and B7 homologue, whose levels were augmented further by stimulation with the proinflammatory cytokine TNF-α [24].

Physiologic activation of naive CD4+ lymphocytes requires two signals. The first signal is initiated by the binding of a peptide-MHC complex to the antigen TCR; the second is initiated by the binding of a costimulatory ligand to a costimulatory receptor [50]. The peptide-MHC complex and the costimulatory ligand are presented to the T cell by an APC.

Stimulation of naive CD4+ lymphocytes through the TCR in the presence of a costimulatory signal leads to cell proliferation and differentiation. In the absence of a costimulatory signal, activation of naive CD4+ lymphocytes is nonproductive. Thus, the costimulatory signal is essential for CD4+ lymphocyte function. We next clarified the role of HNP on the expression of costimulatory molecules on CD4+ lymphocytes, an area of research that is poorly documented. We demonstrated that stimulation of CD4+ lymphocytes with HNP increases the expression of CD80 and CD152, the corresponding costimulatory receptors for the costimulatory ligands CD80 and CD86 expressed on lung epithelial cells. It is noteworthy to mention that in a previous study using Peyer's patch-derived, naive CD4+ lymphocytes from mice immunized with OVA, HNP enhanced cell proliferation and Th cell cytokine secretion [51], suggesting an activation role of HNP.

The expression of surface costimulatory molecules seen in single types of cells was confirmed in the cultures of A549 cells and CD4+ lymphocytes following HNP stimulation. The CD80, CD86, and CD28/CD152 are the best-characterized costimulatory pathways, which not only provide critical positive second signals promoting CD4+ lymphocyte responses but also contribute to critical negative second signals that down-regulate CD4+ lymphocytes [52–55]. CD28 delivers signals important for lymphocyte activation and survival, whereas CD152 inhibits lymphocyte responses and regulates lymphocyte tolerance [52–55]. Although HNP appeared to induce a greater expression of CD152 than CD28, in our experimental conditions, the overall biological consequence was an increased CD4+ lymphocyte immune response, as reflected by an up-regulation of IFN-γ and IL-2, the markers of CD4+ lymphocyte activation. These observations may be explained by the differences in the expression of transcription factors that play an important role.
in balancing positive and negative signals after HNP stimulation.

A striking finding in the present study is the dramatic effect of HNP on epithelial cell adhesion. We believe that the increased cell adhesion caused by HNP is mediated largely by the up-regulation of the ICAM-1 protein. The adhesion molecule ICAM-1 belongs to the Ig superfamily and possesses costimulatory activity in lung epithelial cells [56]. The interaction of LFA-1 with its ligand ICAM-1 is well known to be an important step in the activation of lymphocytes [57–59]. ICAM-1 plays a dynamic role in the initiation of CD4+ lymphocyte and APC interaction compared with other adhesion molecules [15, 26], and a similar mechanism may apply in the present coculture model with lung epithelial cells. It was not our focus to investigate the relationship between ICAM-1 and MHC-I, but it is of interest to point out that ICAM-1 is associated with MHC-I proteins [60], and enhanced engagement of ICAM-1 on target cells leads to recruitment of the MHC-I proteins to the contact area and enhances presentation of cognate peptide MHC-I complexes to lymphocytes [15].

A continuous monolayer typical of the epithelial cells appeared to lose the cohesive phenotype, exhibiting stellate-shape morphology with long, filopodia-like extensions after HNP stimulation in the coculture with CD4+ lymphocytes. The exact mechanisms remain to be elucidated, but it has been shown that an altered modulation of Ig superfamily proteins such as junctional adhesion molecules can produce dramatic changes in epithelial cell morphology [61], which are similar to our observations in the present study. Further investigation is thus required to examine the effects of HNP on the regulation of junctional adhesion molecules.

NF-κB is involved in regulating the expression of many molecules in immune response pathways including MHC molecules, adhesion molecules, and costimulatory molecules. In this study, we showed that stimulation with HNP for 2 h resulted in an up-regulation of nuclear p50 in CD4+ lymphocytes. We speculated that the increase in p50 activation was a negative signal to modulate the overwhelming immune responses induced by HNP, as p50 homodimers bind to histone deacetylase1 (HDAC1), and the p50-HDAC1 complex can then bind to DNA and repress transcription [62]. It is interesting that no significant nuclear p65 activation was found at the time that p53 was up-regulated after the stimulation with HNP. It is possible that the peak signaling of p65 activation was missed at an early time-point, which was associated with a degradation of IκB. However, a previous study showed that degradation of IκB is largely dependent on the CD28 costimulatory signal [63].

The NF-κB signaling pathway is known to be important for activation of ICAM-1 transcription. Here, we demonstrate that HNP stimulation did not result in p50 translocation but induced p53 activation in A549 cells. Other investigators have reported that NF-κB inhibition does not prevent the effect of p53 on ICAM-1 expression after DNA damage, and induction of ICAM-1 is abolished after treatment with the specific p53 inhibitor pifithrin-α and is abrogated in p53-deficient cell lines [64]. Taken together, these results suggest HNP can modulate nuclear transcriptional factors contributing to immune responses.

HNP markedly induced the production of the potent chemokine IL-8 as a biological readout in the interaction between A549 cells and SAEc with CD4+ lymphocytes, which was blunted by blocking HNP activity using the α1-PI. It is known that IL-8 exerts chemotaxis of PMN, which in turn, release HNP in the lung. The blocking effects on IL-8 production by the α1-PI are of particular interest, as patients with inflammatory lung diseases, including pneumonia and ARDS, showed α1-PI deficiency as a result of elevation of plasma elastase, which consumes endogenous PIs [65]. In addition, it has been demonstrated that although concentrations of HNP were undetectable in volunteers, they were increased markedly to high μg/mL levels in patients with α1-antitrypsin deficiency and moderate to severe lung disease [66]. Taken together, the data suggest that there may be a missing link between an increased concentration of HNP and a reduction of α1-Pis in the inflammatory lung diseases.

In conclusion, HNP possesses immune-modulating properties by linking innate to acquired immunity via induction of adhesion and costimulatory molecules, activation of transcription factors, and increasing the production of IL-8. CD4+ lymphocyte adhesion to lung structural cells may further provoke and prolong the immune responses. Modulation of HNP may be a therapeutic target in inflammatory diseases.

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