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Neuraminidase Reprograms Lung Tissue and Potentiates Lipopolysaccharide-Induced Acute Lung Injury in Mice

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We previously reported that removal of sialyl residues primed PBMCs to respond to bacterial LPS stimulation in vitro. Therefore, we speculated that prior desialylation can sensitize the host to generate an enhanced inflammatory response upon exposure to a TLR ligand, such as LPS, in a murine model of acute lung injury. Intratracheal instillation of neuraminidase (NA) 30 min prior to intratracheal administration of LPS increased polymorphonuclear leukocytes (PMNs) in the bronchoalveolar lavage fluid and the wet-to-dry lung weight ratio, a measure of pulmonary edema, compared with mice that received LPS alone. Administration of NA alone resulted in desialylation of bronchiolar and alveolar surfaces and induction of TNF-α, IL-1β, and chemokines in lung homogenates and bronchoalveolar lavage fluid; however, PMN recruitment in mice treated with NA alone did not differ from that of PBS-administered controls. NA pretreatment alone induced apoptosis and markedly enhanced LPS-induced endothelial apoptosis. Administration of recombinant Bcl-2, an antiapoptotic molecule, abolished the effect of NA treatment on LPS-induced PMN recruitment and pulmonary edema formation. We conclude that NA pretreatment potentiates LPS-induced lung injury through enhanced PMN recruitment, pulmonary edema formation, and endothelial and myeloid cell apoptosis. A similar “reprogramming” of immune responses with desialylation may occur during respiratory infection with NA-expressing microbes and contribute to severe lung injury. The Journal of Immunology, 2013, 191: 4828–4837.

Mammalian cell surfaces are covered with a broad range of carbohydrate structures, glycoproteins and glycolipids, that undergo considerable modification as the cells respond to physiologic and pathologic stimuli. These surface glycans are involved in cell-to-cell interactions and, upon engagement with specific ligands (1–4), can initiate intracellular signaling cascades (5, 6). These events are critical to a wide range of biological processes, including inflammation (7, 8). Further, glycolipid transferases and glycosidases, working in concert, regulate the fine structure and activity of these glycans (9, 10).

Sialic acids are widely distributed in animal tissues as gangliosides or glycoproteins on the cell surface (11). These highly negatively charged molecules are known to be important in T lymphocyte–B lymphocyte interactions, and act by masking Ags and in vivo enhanced PMN recruitment to inflamed sites via modulation of cell surface sialylation, presumably by promoting PMN adherence to and migration across the endothelium (22, 23). Inhibition of PMN sialidase activity either by pharmacologic inhibition or by immune blockade diminished their recruitment to inflamed sites in vivo (23).

Microbial neuraminidases (NAs), enzymes that cleave sialic acid from glycoconjugates in a glycosidic linkage—specific manner, are important virulence factors for pathogens, particularly those that target mucosal surfaces (24–26). For example, influenza virus NA is critical to its infective cycle and is therefore a target of antiviral therapy (24, 25). Pseudomonas aeruginosa and Strepitococcus pneumoniae rely on NAs to colonize the mammalian host (26).

In human acute lung injury (ALI), neutrophilic alveolitis, deposition of hyaline membranes, and formation of microthrombi constitute three key pathological features (27). Although murine models have been established for studying ALI, each displays only one or two characteristics of human ALI, but not all three. Intratracheal (i.t.) deposition of LPS induced intra-alveolar PMN infiltration (28). We hypothesized that during respiratory infection with microbes that express NA, lung tissues may become desialylated. This
desialylation may prime the host inflammatory response to a TLR ligand that may then exacerbate lung injury. To address this question, we adapted an LPS-induced ALI model with NA pretreatment to study the effect of prior desialylation of mouse lung tissue in response to bacterial LPS stimulation in vivo. We now report that NA pretreatment sensitizes the host to LPS-induced PMN recruitment, proinflammatory cytokine production, apoptosis, and severe lung injury. Administration of exogenous recombinant human Bcl-2 (rhBcl-2), an antiapoptotic molecule, partially reverses the lung injury. Our findings indicate that NA is a virulence factor that may potentiate the acute proinflammatory response to NA-expressing respiratory pathogens and exacerbate ALI.

Materials and Methods

Animals

CD1 outbred mice were purchased from Charles River Laboratories. KC knockout mice (KC–/–) on a C57BL/6 background were bred at the University of Maryland, Baltimore, in the colony of Dr. Stefanie Vogel (29). C57BL/6 mice to serve as controls for KC–/– mice were purchased from Charles River Laboratories. All animals were maintained in the University of Maryland, Baltimore, animal facility under an approved Institutional Animal Care and Use Committee protocol.

Reagents

Clostridium perfringens NA (type X) and LPS (E. coli O111:B4) were purchased from Sigma-Aldrich. Biotinylated peanut agglutinin (PNA), Sambucus nigra lectin (SNA), and Maackia amurensis lectin II (MAAII) were purchased from Vector Laboratories. The rhBcl-2 was purchased from R&D Systems.

LPS-induced ALI

To induce ALI, 5 μg LPS (25 μl at 0.2 mg/ml) in sterile PBS was administered into the tracheas of anesthetized animals, as described (30). For NA treatment, 100 μM NA (25 μl at 4 U/ml) in PBS was similarly deposited, 30 min before LPS challenge. This was the minimal dose required to desialylate lung tissue to an extent similar to that observed with experimental influenza infection (M. Nita-Lazar, M. Pasek, W. Chen, C. Feng, A. Cross, G. Rabinovich, and G. Vasta). Expression and secretion of galectins in the murine lung is modulated during influenza and pneumococcal infection. Presented at the Society for Glycobiology Annual Meeting, November 2011, Seattle, WA). PBS and heat-inactivated NA (△NA) (100°C, 15 min) were used as controls. The loss of catalytic activity in △NA was confirmed in an NA assay using 2′,4′-((4-methylumbelliferyl)-β-D-N-acetyllactosaminic acid sodium salt hydrate as a substrate (23). Animals were sacrificed the next day or at the indicated time points. Bronchoalveolar lavage fluid (BALF) was collected for WBC count and cytokine determination, and the remaining lung tissues were either stored in TRIzol Reagent (Invitrogen) for RNA extraction or processed to a cell suspension for flow cytometry analysis (see below). TNF-α, IL-1β, CXCL1/ keratinocyte-derived chemokine (KC), CXCL5/LPS-induced CXC chemokine (LIX), and MIP2 concentrations in BALF were determined with ELISA from Charles River Laboratories. All animals were maintained in the suspension for flow cytometry analysis (see below). TNF-α and IL-1β were measured in supernatants from 25 μl wells of 24-well plates. Each upper compartment was seeded with human lung microvascular endothelial cells (HMVEC)-Ls at 2.5 × 10⁵ cells in 0.5 ml media per chamber and cultured for 72 h. The baseline barrier function of each monolayer was determined by applying 14C-BSA to each upper compartment (0.5 ml) for 1 h at 37°C, after which the lower compartment (1.5 ml) was counted for 14C activity. Only monolayers retaining ≥ 97% of the 14C-BSA tracer were used. The monolayers were then exposed for 6 h to NA, △NA, LPS (300 ng/ml), or medium alone. In selected experiments, the HMVEC-L monolayers were pre-incubated for 1 h with either NA or medium alone, after which they were treated for 6 h with LPS (30 ng/ml) or medium alone. In all experiments, transfer of 14C-BSA across HMVEC-L monolayers was again assayed and expressed as picomoles per hour.

Flow cytometry

Cell suspensions from digested lung tissues were prepared as follows: after BALF wash, the lung was excised, minced into fine pieces, and digested in RPMI 1640 (Invitrogen) containing 1 mg/ml collagenase D (Roche), 10 mM HEPES, and 1% FBS for 30 min at 37°C. The cell suspension was separated from tissue debris by passing it through a 40-μm mesh strainer and filtered with PBS for staining. The cells were incubated for 1 h with 1 anti-human surfactant protein A (SurfA) Ab (Millipore) and 3 μl of PE-conjugated anti-mouse IgG (BD Pharmingen); or 3) human CD31 (BD biosciences) for endothelial cells (ECs), followed by 1 h incubation with allophycocyanin-conjugated anti-rabbit IgG (BD Pharmingen); 2) anti-human CD31 (BD biosciences) for endothelial cells (ECs), followed by allophycocyanin-conjugated anti-mouse IgG (BD Pharmingen); or 3) allophycocyanin-conjugated anti-human CD11b (BD Biosciences) for phagocytes. The cells undergoing apoptosis were detected with the ApoFix kit (Sigma-Aldrich) and 2 μl (from 0.02 μg total RNA) was used for each PCR reaction. The cDNA was mixed with SYBR mix (Applied Biosystems) and 1 μM of each primer from the set of Mouse Cytokine Primer Library II (Real Time Primers) to a volume of 20 μl, and amplified in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Each sample was amplified in triplicate, and average values were calculated. The relative gene expression level for each sample was normalized to hypoxanthine phosphoribosyltransferase.

Lectin blot on tissue sections

The tissue sections were deparaffinized in xylene twice for 5 min and rehydrated in the serial ethanol solutions. The sections were washed in PBS; blocked in 3% BSA; and incubated for 1 h with biotinylated PNA, SNA, or MAAII, followed by streptavidin-conjugated Cy2 (a kind gift from Dr. Anna C. Puche, Department of Anatomy and Neurobiology, Program in Neuroscience, University of Maryland School of Medicine). The sections were counterstained with DAPI and mounted with mounting medium for fluorescence microscopy. The images were captured on an Olympus BX61 Fluoview Laser Scanning Microscope with a 60× objective and 2.5× digital amplification with Flouview V5.0. The images from different channels were merged with Adobe Photoshop 4.0.

TUNEL staining

The tissue sections were rehydrated as described above. Rehydrated tissue sections were treated with proteinase K (Roche Applied Science) for 30 min and washed twice with PBS. The sections were stained with Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science) per the manufacturer’s recommendation, counterstained with DAPI, and mounted for microscopic observation.

RNA extraction, reverse transcription, and quantitative RT-PCR array

Total RNA from lung tissue was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s recommendation. Total RNA (1 μg) was treated with 1 U DNase I (Invitrogen) in 10 μl reaction buffer for 15 min. DNase I was inactivated by addition of EDTA solution and heated at 65°C for 10 min. The treated RNA was reverse transcribed into cDNA using the Reverse Transcriptase System (Promega) per the manufacturer’s recommendation. The final products were diluted up to 100 μl with distilled water, and 2 μl (from 0.02 μg total RNA) was used for each PCR reaction.

Expression and secretion of CA2+ channel subunits were measured with a Flow Cytometry Analysis System (see below). TNF-α, IL-1β, CXCL1/keratinocyte-derived chemokine (KC), CXCL5/LPS-induced CXC chemokine (LIX), and MIP2 concentrations in BALF were determined with ELISA kits (R&D Systems).

Lung wet-to-dry weight ratio

To quantify the lung edema in ALI, whole-lung tissue was collected, rinsed to remove surface blood, and patted dry, and the immediate weights of the samples were recorded as the wet weight. The tissues were air dried for 3 d, and their weights were recorded daily until they became stable and recorded as the dry weight. A wet/dry weight ratio for each individual mouse lung was calculated.

Lung histology and H&E staining

After euthanasia, mouse thoracic cavities were opened to expose the trachea. Two sutures were placed around the top and bottom of the trachea. An 18-gauge blunt needle was inserted at the top of the trachea with an incision, and tied with the top suture. About 2 ml Pleurotus odoratus (gloxyl oxide fixative; ANATECH) was slowly perfused to dilate the lung tissue. After perfusion, the bottom trachea was closed by tying the suture. The lungs were dissected from the thoracic cavity and fixed in 10% buffered formaldehyde overnight. Lungs tissues were dehydrated, paraffin embedded, and sectioned (8 μm). Some sections were stained with H&E; the remaining unstained sections were used for tissue lectin blots or TUNEL staining for apoptosis.
twice with cold PBS and and resuspended in Binding Buffer (BD Phar-
mningen) at $1 \times 10^7$ cells per milliliter. Then 100 $\mu$l cell suspension was
transferred to a new tube with 5 $\mu$l Annexin VFITC solution and 5 $\mu$l
propidium iodide (PI) solution (BD Pharmingen) and incubated for 15 min
before analysis. The cells that were stained PI negative, but Annexin V
positive, were considered to have undergone apoptosis; and the cells
stained PI positive were considered to be dead cells.

Statistical analysis

Multiple regression modeling and ANOVA were used in analyzing the
effects of NA, LPS, ANA, and Bcl-2 pretreatment on outcomes of in-
terest. In regression modeling, the effects of NA and LPS, as well as their
interaction, were included initially; the absence of interaction suggests
the effects of NA and LPS are additive, whereas the presence of an in-
teraction suggests the effect of LPS depends on whether NA treatment
was present. In ANOVA, we used the Tukey (or Tukey–Kramer) multiple
comparisons procedure for pairwise comparisons of two or more treatments
(32). A $p$ value $\leq 0.05$ was considered statistically significant. In a few
instances, we considered results suggestive of an effect even though the $p$
value was $>0.05$.

Results

NA desialylated the bronchioles and alveolar surface within 30
min

Airway epithelial cells are heavily sialylated (17, 18). However, the
sialyl linkages from each anatomical section of the airway are
species specific. Using lectin blot techniques established in our
laboratory (33), we determined the sialylation status of murine lung
tissues and their response to sialidase treatment. Lectins are a
group of proteins that bind specifically to different glycoconjugates:
SNA and MAAII recognize terminal sialic acids in $\alpha$-2,6– or
$\alpha$-2,3–linkages, respectively, whereas PNA recognizes a subter-
minal $\beta$-galactose after terminal sialyl residues have been re-
moved (33, 34). PBS (as untreated control) or NA (100 $\mu$U in 25
$\mu$l of PBS) was administered i.t. to mice, and 30 min later lung
tissues were harvested and processed for lectin staining (green).

SNA stained most of the alveolar areas in PBS-treated lung tissue
(Fig. 1a), and the SNA signal slightly decreased after NA treat-
ment (Fig. 1b); MAAII staining of the alveolar area was less in-
tense (Fig. 1c), but also diminished after NA treatment (Fig. 1d).

In PBS-treated tissues, PNA binding was found only on a few
bronchi/bronchiole surfaces, likely indicative of sialyl residues
masking underlying galactose molecules (Fig. 1e). After NA

FIGURE 1. The i.t. instillation of NA desialylates mouse lung tissue. PBS (a, c, e) or NA (b, d, f) was administered i.t. into the lungs of CD1
outbred mice. After 30 min, the lungs were fixed, paraffin embedded, and
processed for probing with biotinylated SNA (a, b), MAAII (c, d), or PNA
(e, f) lectins, followed by Cy2-conjugated streptavidin (green) and
counterstained with DAPI (blue). Original magnification $\times 60$; arrows in-
dicate positive lectin staining in alveolar area. Each photomicrograph is
representative of three independent experiments. Scale bar in (e), lower
left, 50 $\mu$m.

BALF PMNs above the level of the PBS/PBS treatment group, but
NA greatly increased BALF PMNs after LPS challenge [NA i.t.
followed by LPS i.t. administration (NA/LPS)], compared with
that observed with PBS/LPS ($p < 0.0001$, Fig. 2A). This NA-
induced enhancement in BALF PMNs was not found using
NA/PBS (Fig. 2A), which confirmed the involvement of the heat-
labile NA catalytic activity. These data indicate that prior desia-
ylation enhances the LPS-induced PMN recruitment into the
bronchoalveolar compartment.

NA (NA/PBS) or LPS challenge (PBS/LPS) each alone
increased the mean lung wet-to-dry weight ratio, a measure of lung
edema, compared with PBS-treated mice (PBS/PBS) (Fig. 2B).
However, NA/LPS challenge further increased the mean wet-to-
dry weight ratio compared with PBS/LPS (Fig. 2B). In multiple
linear regression modeling, both the LPS ($p = 0.009$) and NA
effects ($p = 0.002$) were independently significant, and the effects
of NA and LPS appeared to be additive. These data indicate that
prior desialylation further enhanced pulmonary edema formation
in response to LPS challenge.

Next, we studied the histological changes in the lung tissue
following LPS challenge in the presence or absence of NA pre-
treatment (Fig. 2C). At 18 h following LPS i.t. challenge, PBS/
LPS increased PMN recruitment to alveolar tissues (Fig. 2Cii)
compared with the PBS/PBS controls (Fig. 2Ci), but no damage to
either endothelium or epithelium was evident. NA/PBS treatment
did not induce histological changes compared with control PBS/
PBS mice (Fig. 2Ciii). However, PMN infiltration was dramati-
cally increased in lung tissue after NA/LPS challenge (Fig. 2Civ).
These findings are consistent with our results showing increased
PMN infiltration from the BALF (Fig. 2A).
Desialylation increased PMN recruitment in the absence of KC

KC, a murine functional homolog to human IL-8, is a potent PMN attractant and has been reported to be induced in lung tissue in an ALI model (35). We speculated that if the LPS-induced KC expression in lung tissues was further increased with NA pretreatment, it would potentiate PMN infiltration. Accordingly, we determined the KC concentration in the BALF 18 h after LPS challenge. Unexpectedly, although KC was increased after LPS challenge (p < 0.0001), there was no apparent effect of NA, either with or without LPS challenge (Fig. 3A). A similar result was obtained for two other chemotactic chemokines, LIX and MIP2 (Fig. 3B). ∆NA also had no apparent effect on KC (Fig. 3A).

Because PMN recruitment into the lung was observed as early as 3 h after LPS challenge (data not shown), we analyzed chemokine expression in BALF at early time points following LPS challenge. Surprisingly, NA treatment alone appeared to increase KC biosynthesis and release within 30 min, the time point that immediately preceded LPS administration (t = 0). This finding was based, however, on only two mice with and two mice without NA treatment observed before challenge (p = 0.097). At 1 h after LPS challenge, the chemokine production from mice receiving NA pretreatment only slightly surpassed chemokine levels in mice receiving PBS/LPS challenge. Although both NA pretreatment and LPS challenge appeared to increase chemokine levels, at neither 1 h nor 3 h were treatments significantly different from each other by the Tukey multiple comparisons procedure, except for a significant increase (p < 0.05) with NA/LPS treatment compared with the PBS/PBS control at 3 h (Fig. 3C). LIX production at 3 h with NA/PBS treatment was similar to that observed after PBS/LPS challenge and with NA/LPS treatment. All showed significant increases (p < 0.01) over the level with the PBS/PBS control (Fig. 3D). Other chemokines, such as MIP2, also did not increase with NA pretreatment (data not shown). Thus, increased BALF PMNs associated with NA pretreatment could not be attributed to potentiation of chemokine protein expression.

To determine if KC played a role in PMN infiltration and/or increased PMNs in BALF, we challenged KC−/− mice with LPS, with or without NA pretreatment. After LPS challenge of PBS-pretreated KC−/− mice, a small amount of PMN infiltration was observed (Fig. 3E), which was much lower than that observed in similarly treated wild-type C57BL/6 mice (p = 0.014). This finding emphasizes the critical role of KC in attracting PMNs into lung tissues during LPS-induced ALI. Nevertheless, even with minimal KC production, NA pretreatment combined with LPS challenge increased the PMN infiltration (p < 0.05 by the Tukey procedure for NA/LPS compared with PBS/PBS and NA/PBS; p = 0.067 for NA/LPS compared with PBS/LPS) (Fig. 3E). These results suggest that prior desialylation can partially increase PMN recruitment to the BALF through a KC-independent mechanism.

NA treatment alters expression of multiple genes

Although treatment with NA alone induced KC and LIX production (Fig. 3B–D), it only slightly increased PMNs in BALF (Fig. 2A). These findings suggest that elevation of these chemokines alone was insufficient to explain the increased PMN recruitment in response to LPS challenge after NA pretreatment. To determine the alteration of inflammatory cytokine/chemokine gene expression in lung tissue after desialylation, we carried out a Real Time PCR array and compared inflammatory gene expression in the lung homogenates after 30 min of NA treatment. ∆NA was used as the untreated control to offset any stimulation effect by potential contaminants in the NA preparation. In comparison with ∆NA-treated tissues, 9 of 88 genes (www.realtimeprimers.com/mocyprliii.
were upregulated, mostly chemokines, whereas expression of one chemokine gene, CCL27, was downregulated with desialylation (Fig. 4A). In addition to the chemokines, TNF-α was upregulated, as we previously reported (7).

TNF-α, a major inflammatory cytokine produced in response to LPS stimulation, can activate ECs (22). We therefore measured the effect of NA pretreatment on LPS-induced TNF-α protein production in BALF. NA treatment alone appeared to increase TNF-α production within 30 min (p = 0.094 for two mice with and two mice without NA pretreatment; Fig. 4B). At 1 h after LPS challenge, TNF-α production was higher with LPS challenge compared with treatment with PBS, but no differences among treatments were statistically significant (Fig. 4B). NA pretreatment with LPS challenge increased IL-1β production in the lung (Supplemental Fig. 1A); however, LPS-induced PMN infiltration was increased with NA pretreatment in caspase-1-deficient mice and IL-1R1 knockout mice (Supplemental Fig. 1B, 1C), which lack functional IL-1β and its receptor, respectively. This suggests that despite increased IL-1β expression, IL-1β and its signaling pathway may not be critical for LPS-induced PMN infiltration.

**NA treatment disrupted endothelial barrier integrity**

As TNF-α, which was upregulated by NA treatment (Fig. 4A), has the capability to increase endothelial paracellular permeability (22, 31), we asked whether prior desialylation increases PMN recruitment to BALF through opening of the endothelial paracellular pathway. To address this question, we treated postconfluent HMVEC-L monolayers with NA and measured transendothelial flux of 14C labeled-BSA (31). Although LPS treatment increased BSA flux (p < 0.05 compared with each of the other treatments by the Tukey multiple comparisons procedure), there was no evidence of barrier disruption after treatment with a high concentration of NA alone (100 mU/ml), compared with treatment with medium alone or ΔNA (Fig. 5A).

We then asked whether NA pretreatment was able to enhance endothelial barrier responsiveness to LPS stimulation. For this purpose, a concentration of LPS (30 ng/ml), which alone did not induce EC permeability, was used. Indeed, NA pretreatment combined with a subthreshold LPS exposure stimulation increased...
transendothelial 14C-albumin flux ($p < 0.001$ by the Tukey procedure for NA + LPS compared with all other treatments; Fig. 5B).

Because EC apoptosis could underlie the observed loss of barrier function, we next studied whether NA pretreatment, alone or in combination with LPS, increased HMVEC-L apoptosis. The ECs were suspended and stained with Annexin V and PI for flow cytometry analysis. Whereas LPS alone did not induce more apoptotic cells, NA treatment alone induced cellular apoptosis. Moreover, NA treatment plus LPS induced even more apoptotic cells (Fig. 5C). In contrast, although LPS stimulation induced TNF-α production in EC culture supernatant ($p < 0.01$ compared with medium), NA treatment alone did not (Fig. 5D). However, NA/LPS treatment induced more TNF-α than LPS alone in this in vitro system ($p < 0.01$, Fig. 5D), as it did in vivo (Fig. 4B).

These data suggest that desialylation of ECs potentiates LPS-induced apoptosis, which may contribute to ALI, perhaps in part through a TNF-α–mediated mechanism. However, although administration of anti–TNF-α Ab reduced TNF-α levels in the BALF, it did not reduce the increased PMN infiltration of NA/LPS-challenged mice (Supplemental Fig. 2), which suggests a TNF-α–independent mechanism is involved.

**Desialylation increased the apoptosis in lung tissue after LPS challenge**

Because TNF-α is associated with cellular apoptosis (36–38) and was induced by LPS challenge (Figure 4), we asked whether apoptosis was induced in lung tissues after LPS challenge, and, if so, whether such LPS-induced apoptosis could be enhanced by prior desialylation. Lung tissues from mice after overnight LPS challenge were sectioned and TUNEL stained. As expected, although...
As Bcl-2 pretreatment reversed the enhanced PMN infiltration and wet-to-dry ratio in lungs with NA/LPS challenge, we wondered whether this was associated with suppression of NA-induced apoptosis in lung tissue. Therefore, we pretreated mice with saline or rhBcl-2 before PBS/LPS or NA/LPS challenge, harvested the single-cell suspension from the lung, and quantified the number of apoptotic cells (Fig. 7C). With either prior saline or prior rhBcl-2 infusion, the NA/LPS-challenged mice had more apoptotic cells than did those challenged with PBS/LPS ($p < 0.001$ for NA/LPS compared with PBS/LPS with saline, $p < 0.05$ for NA/LPS compared with PBS/LPS with rhBcl-2). As with PMN infiltration and wet-to-dry ratio, the level for NA/LPS was lower with prior rhBcl-2 than with prior saline, but the difference was not statistically significant. These results are consistent with the hypothesis that Bcl-2 inhibits the NA-mediated apoptosis in lung tissue.

We further analyzed the effect of Bcl-2 on apoptosis in endothelial (CD31$^+$), epithelial (SurfA$^+$), and myeloid (CD11b$^+$) populations (Fig. 7D). With prior saline treatment, the number of apoptotic cells increased in all populations with NA treatment ($p < 0.0001$ for CD11b$^+$, $p = 0.0002$ for CD31$^+$, $p = 0.077$ for SurfA$^+$). In contrast, Bcl-2 pretreatment appeared to decrease the number of apoptotic cells in the CD11b$^+$ ($p = 0.032$) population, but not the CD31$^+$ ($p = 0.063$) and SurfA$^+$ ($p > 0.50$) populations.

Taken together, these data suggest that the desialylation-mediated increase of LPS-induced PMN infiltration and pulmonary edema are dependent in part on apoptosis and can be alleviated by the administration of antiapoptotic agents such as Bcl-2, whereas the LPS-induced PMN infiltration may involve additional mechanisms, such as the opening of the endothelial paracellular pathway.

**Discussion**

The airway epithelial cell surface is armed with numerous receptors that recognize host mediators and exogenous danger signals. Many of these receptors contain oligosaccharide chains whose outermost positions terminate with sialic acid. These highly electronegative, hydrophilic sialyl residues influence protein tertiary conformation, and in their terminal location are strategically positioned to influence intermolecular and cell–cell interactions through steric hindrance and/or electrostatic repulsion. Further, these sialyl residues may mask “activation epitopes,” as we have reported for β2 integrin and the TLR4 receptor complex (21, 32). Current studies support the hypothesis that removal of sialyl residues from lung tissue potentiates LPS-induced ALI through enhanced PMN recruitment and transendothelial migration, proinflammatory cytokine and chemokine production, pulmonary edema formation, and apoptosis.

Mammalian lung tissues are sialylated with both α-2,6–linked and, less consistently, α-2,3–linked sialic acids (17, 18, 41, 42). Our data confirmed the presence of α-2,6–linked sialic acid and, to a lesser extent, α-2,3–linked sialic acid in CD1 outbred mouse lungs (Figure 1). PNA lectin binding to lung tissue was increased following i.t. instillation of exogenous NA, thereby confirming the loss of sialyl residues (Fig. 1).

Of note, whereas desialylation upregulated gene expression in lung tissue, including genes encoding for certain chemokines and TNF-α (Fig. 4), in the absence of LPS challenge, NA treatment did not cause ALI, as measured by PMNs in the BALF (Fig. 2); however, this desialylation-induced “reprogramming” of the lung tissues dramatically altered the responses to LPS challenge (Figs. 2, 6). It is possible that mucosal pathogens that express a sialidase may induce a similar desialylation-induced reprogramming of lung tissue. If that were the case, then the pulmonary innate im-

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**FIGURE 6.** Desialylation prior to LPS challenge enhances lung and immune cell apoptosis. CD1 outbred mice were pretreated with PBS or NA before i.t. challenge with LPS or PBS. (A) After 18 h, lung tissues were fixed, then processed for TUNEL staining (green) and counterstaining with DAPI (blue). Each photomicrograph is representative of three independent experiments. Original magnification ×60. Arrows indicate apoptotic cells. (B) After 18 h, single-cell suspensions were prepared from the lungs of challenged mice using collagenase and DNase I digestion, and stained with SurfA$^-$, CD11b$^-$, or CD31-specific Abs, followed by Annexin V and PI staining for flow cytometric analysis. Each vertical bar represents the percentage of PI$^+$ Annexin V$^+$ cells (±SEM). Data are a composite of four separate experiments in which groups of five mice for each treatment were pooled, stained, and analyzed in each experiment. PBS/LPS versus PBS/ PBS, $p < 0.05$. NA/LPS versus PBS/LPS, $p < 0.01$. Among the three cell types, the difference between NA/LPS and PBS/LPS was statistically significant only for CD11b$^+$.
Polarization of murine responses to subsequent pathogens or endogenous alarmins could be similarly altered.

PMN recruitment into inflamed tissues is a critical step in the host innate immune response. This multistep process is tightly orchestrated to diminish collateral tissue damage. To initiate PMN transmigration through the endothelium, EC activation is required, either directly, as with exposure to LPS, or indirectly, through the induction of cytokines. This leads to de novo synthesis and surface expression of adhesion molecules (43, 44). The interactions between the selectins on “inflamed” endothelium and their counterligands on PMNs slow the circulating cells, enabling firm adhesion through the interactions between activated ICAMs and integrins. The induced firm adhesion enables the PMNs to transmigrate through the EC–EC junction down a chemokine gradient toward the inflamed site (43, 44).

In the current study, the increased protein content of KC, MIP-2, LIX, and TNF-α in BALF was evident after LPS challenge (Figs. 3, 4, 5). KC is a critical chemokine that locally attracts PMNs into tissues (29, 45, 46). Its importance in LPS-provoked PMN recruitment is clear, based on the observation that the LPS-induced PMN infiltration is nearly absent in KC−/− mice (Fig. 3C). In the absence of KC, other redundant chemokines, such as MIP-2 and LIX, might contribute to PMN recruitment into lung tissues. However these two chemokines are less potent chemotactically than KC (IL-8) (46). Because NA treatment alone upregulated expression of KC and other chemokines, but failed to increase PMN infiltration in lung tissues without a subsequent LPS challenge, these chemokines alone may be insufficient to attract PMNs into lung tissue in our murine model of ALI (Fig. 4). Further, the enhanced LPS-induced PMN infiltration with NA pretreatment was not simply due to elevated KC or other chemokines (MIP-2, LIX) production because the concentration of these chemokines from LPS-challenged mice with NA pretreatment did not differ substantially from those without NA treatment (Fig. 3). Therefore, elevation of these PMN-attracting chemokines alone cannot explain the increased cell infiltration with NA pretreatment followed by LPS challenge. In contrast, in the absence of KC, the minimal PMN infiltration was still enhanced with NA pretreatment (Fig. 4). Because KC induces inflammatory cytokine expression such as TNF-α, IL-6, and IL-8 (19, 36, 47) in ECs that can upregulate its surface expression of E-selectin and ICAM-1 (48–50), adhesion molecules important in leukocyte infiltration. In addition, monocytes, macrophages, dendritic cells, and epithelial cells in the lung enhance proinflammatory cytokines in response to LPS stimulation (19, 20). In LPS-induced ALI, intrapulmonary cytokine expression is upregulated at the protein (Fig. 4B, Supplemental Fig. 1) level. As TNF-α is induced in lung tissue and is capable of activating ECs, it likely contributes to the LPS-induced...
PMN infiltration, as we previously reported (22); however, neutralizing anti-TNF-α Abs did not reduce PMN recruitment (Supplemental Fig. 2). In contrast, another proinflammatory cytokine, IL-1β, which is capable of activating ECs and inducing neutrophilia, was also expressed after LPS administration, but, based on experiments in caspase-1− and IL-1R−/− deficient mice, did not appear to contribute to PMN infiltration in our model of LPS-induced lung injury (Supplemental Fig. 1).

Given the prevalence of sialylated glycoconjugates on the surface of mammalian cells, including lung tissue, there is a large array of potential molecular targets for NA. We reported previously that desialylation of monocytes in vitro activated phosphorylation of ERKs and induced cytokine production in response to LPS (19). In a subsequent publication, we demonstrated that removal of sialyl residues from the TLR4 receptor complex accelerated and enhanced LPS-initiated signaling (21). Further, removal of sialyl residues led to activation of PMN β2 integrins and enhanced adhesion, whereas desialylation of ICAM-1 promoted leukocyte arrest in an in vivo model of cell migration (33). Recently, we reported that MUC1 and the EGF receptor on airway epithelial cells were highly sialylated and that desialylation enhanced their responsiveness to their cognate ligands (51). Thus, desialylation of cell surface glycoconjugate molecules relevant to ALI potentiated inflammatory responses to LPS.

The molecular mechanism or mechanisms by which desialylation enhanced LPS-mediated ALI remains to be determined. Edema (wet-to-dry weight) in lung tissues, suggestive of loss of endothelial barrier function, was induced after LPS challenge and was enhanced upon desialylation (Figs. 2B, 7). Consistent with this observation, LPS increased endothelial permeability in vitro, and NA pretreatment lowered the threshold at which LPS induced endothelial permeability (Fig. 5).

An earlier investigation showed that LPS-induced vascular collapse resulted from disseminated endothelial apoptosis mediated sequentially by the generation of TNF-α and ceramide (52). The disseminated endothelial apoptosis preceded nonendothelial parenchymal tissue damage. It is possible that desialylation potentiated the TLR4 receptor complex–mediated signaling, as we recently reported (21). This might result in the enhanced expression of TNF-α and/or ceramide. Alternatively, desialylation of proteins located at the inter-EC junction could contribute to the ALI. EC adherens junction proteins, such as VE-cadherin, are sialylated and could be an additional target of NA (53). It is likely that the NA-associated increase in endothelial “leak” contributes to the leukocyte infiltration.

LPS administration induced ALI, as measured by recruitment of PMNs to the BALF and increased wet-to-dry weight in the lungs, a measure of pulmonary edema. These effects were substantially increased by NA pretreatment and were accompanied by apoptosis, as evidenced by both TUNEL and Annexin V staining (Fig. 6). The exogenous administration of the antiapoptosis reagent rhBcl-2 abolished the NA treatment effect, but, interestingly, did not alleviate the ALI induced by LPS alone (Fig. 7). This observation suggests that there may be two apparently distinct and independent signaling mechanisms by which NA and LPS induce apoptosis.

Because NA treatment can induce apoptosis in the absence of causing endothelial barrier dysfunction (Fig. 5), apoptosis alone cannot explain the development of ALI. In our murine model, a “second hit,” as provided by LPS, either to enlist additional pathogenic mechanisms or to increase the level of apoptosis above that induced by NA alone appears to be required for the development of ALI.

The antiapoptotic protein Bcl-2 is a member of a larger family of Bcl-2 intracellular proteins that may be released extracellularly during cecal ligation and puncture-induced sepsis (54) or during cell necrosis. In previous studies, treatment of mice with rhBcl-2 protected them from sepsis by reducing apoptosis in multiple target tissues (55), including ECs (56). Bcl-2 inhibits ceramide-mediated apoptosis (52). Thus, extracellular Bcl-2 may be considered a damage-associated molecular pattern or alarmin that modulates the response of the innate immune system to tissue injury (40). Administration of rhBCL2A1 to mice also increased expression of endogenous Bcl-2 protein in mice and was associated with increased recruitment of PMNs to the inflammatory site, but not the induction of proinflammatory cytokines (55). Most likely, LPS-induced PMN infiltration is an active mechanism mediated predominantly by IL-8 (KC in mice) and is apoptosis independent, whereas desialylation triggers another pathway that is apoptosis dependent, but KC independent, which additively enhances the PMN infiltration in response to LPS. This is consistent with our observation that NA pretreatment was able to increase LPS-induced PMN recruitment even in the absence of KC (Fig. 3E).

Microbial NAs could be important for the pathogenesis of infection, particularly for those agents that target mucosal surfaces (24, 26, 57). Thus, mucosal surfaces are likely to be desialylated during or post infection, and this may increase the likelihood of further lung injury or susceptibility to secondary infection. Indeed, we have recently shown that nonlethal infection of mice with influenza results in greatly increased severity of S. pneumoniae infection (58). Further, host-derived sialidases, or NEU proteins—the NA homolog present in myeloid cells, epithelial cells, and ECs—may play an important role in cellular function by desialylating MUC1 or EGFR, or other surface glycoconjugate receptors (23, 51). Therefore, it is important to more precisely define the role of sialidases in the host immune response to infections. Such studies may lead to novel therapeutic applications of NA inhibitors, including a more expansive role in treating pulmonary hyperinflammatory conditions.

Disclosures

The authors have no financial conflicts of interest.

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