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Neutrophils augment LPS-mediated pro-inflammatory signaling in human lung epithelial cells

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ABSTRACT

Background: The role of polymorphonuclear neutrophils in pulmonary host defense is well recognized. The influence of a pre-existing inflammation driven by neutrophils (neutrophilic inflammation) on the airway epithelial response toward pro-inflammatory exogenous triggers, however, is still poorly addressed. Therefore, the aim of the present study is to investigate the effect of neutrophils on lipopolysaccharide (LPS)-induced pro-inflammatory signaling in lung epithelial cells. Additionally, underlying signaling pathways are examined.
Methods: Human bronchial epithelial cells (BEAS-2B) were co-incubated with human peripheral blood neutrophils or bone-marrow derived neutrophils from either C57BL/6J wild type or nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase deficient ($p47^{phox-/-}$) mice. Upon stimulation with LPS, interleukin (IL)-8 production and reactive oxygen species (ROS) generation were measured. Additionally, activation of the extracellular signal-regulated kinases (ERK) 1/2 and nuclear factor (NF)-κB signaling pathways was analyzed.
Results: Our studies show that the presence of neutrophils synergistically increases LPS-induced IL-8 and ROS production by BEAS-2B cells without inducing cytotoxicity. The observed IL-8 response to endotoxin increases in proportion to time, LPS-concentration and the number of neutrophils present. Moreover, this synergistic IL-8 production strongly correlated with the chemotactic properties of the co-incubations and significantly depended on a functional neutrophilic NADPH oxidase. The presence of neutrophils also augments LPS-induced phosphorylation of ERK1/2 and IκBα as well as NF-κB RelA DNA binding activity in BEAS-2B cells.
Conclusions: Our results indicate that the pro-inflammatory effects of LPS toward lung epithelial cells are amplified during a pre-existing neutrophilic inflammation. These findings support the concept that patients suffering from pulmonary neutrophilic inflammation are more susceptible toward exogenous pro-inflammatory triggers.

1. Introduction

Although breathing is mandatory for all aerobic life forms, it also implicates constant exposure of the respiratory tract to noxious environmental stimuli and airborne pathogens. As the front line of pulmonary defense against these potential threats, the airway epithelial cells are not only providing a physical barrier but will also enhance the clearance of these invading micro-organisms by stimulating the expression of various inflammatory mediators including mucus proteins and cytokines [1,2]. In general, NOX enzymes can produce large amounts of ROS, mainly superoxide anion radical and H_2O_2, upon stimulation with various bacterial and environmental triggers including endotoxins and ambient particulate matter [2]. Classically, the phagocytic NOX has been regarded as the main player of systemic innate immune responses [3]. Upon intrusion of microbial pathogens, neutrophils will be recruited from the circulation to attack and neutralize these pathogens by producing ROS via their NOX-driven respiratory burst. Alternatively, it has become appreciated that the lung also comprises other NOXs capable of producing ROS. Indeed, it is shown that the NADPH oxidase homologs DUOX1/2 are the primary sources of pulmonary epithelial production of H_2O_2 [4,5]. Being a substrate for lactoperoxidase and

Abbreviations: BEAS-2B, human bronchial epithelial cultured cell line; CPH, 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine; DUOX1/2, dual oxidases 1 and 2; EPR, electron paramagnetic resonance; ERK1/2, extracellular signal-regulated kinases 1/2; H_2O_2, hydrogen peroxide; IκBα, inhibitory part of NF-κB; IL, interleukin; KO, $p47^{phox-/-}$ knockout; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NOX, NADPH oxidase; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; TLR, toll-like receptor; WT, wild type

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myeloperoxidase, H_2O_2 contributes to the formation of respectively hypothiocyanous acid and hypochlorous acid, which both possess strong bactericidal capacities [6–9]. Other NOX enzymes displaying pulmonary expression include NOX2 and, to a lesser extent, NOX4, although the expression of these enzymes largely resides in non-epithelial cells present in the lung including respectively phagocytic inflammatory cells (neutrophils and macrophages) and fibroblasts [2,4,10,11]. Interestingly, it has recently been suggested that ROS produced by NOX4 play a role in tissue repair functions and pulmonary fibrogenesis as this homolog is upregulated in lungs of mice subjected to noninfectious injury as well as in lungs of pulmonary fibrosis patients [12].

An important player in the respiratory epithelial defense, which is also highly ROS-inducible, is the chemokine IL-8/CXCL8. Although IL-8 displays various inflammatory effects, including induction of ROS production and modulation of histamine release, its key activity is the chemotaxis of PMN toward infectious sites [13,14]. Expression of the IL-8 gene is regulated by several pathways, as can be deducted from the presence of binding sequences for various transcription factors, including NF-κB and AP-1, on its promoter region [15,16]. The transcription factor AP-1 is composed of members of the jun and fos DNA-binding protein families [15,17]. The abundance and activity of AP-1 relies on the phosphorylation of both the jun and fos proteins [18] by respectively c-Jun NH_2-terminal kinases and ERKs. Upon activation, ERKs translocate into the nucleus and phosphorylate fos proteins. For c-fos, fosB and fra1 it has already been shown that this phosphorylation occurs on serine and/or threonine residues in the COOH-terminal domain [19]. The transcription factor NF-κB is present in the cytoplasm as an inactive complex, typically existing as a dimer of the RelA and p50 subunits that is bound to the inhibitory protein IκB [20,21]. The classical NF-κB activation involves phosphorylation of the cytosolic inhibitor protein IκBα at the serine residues 32 and 36 by the IKK enzyme complex, leading to the protein's rapid ubiquitination at lysines 21 and 22 and subsequent annihilation by the 26S proteosome [20]. Consequently, the dissociated NF-κB dimer is released and translocates to the nucleus.

Upon secretion of IL-8 by the airway epithelium, large amounts of PMN will migrate into the lung lumen where they become activated and undergo a respiratory burst [22]. This burst, initiated by NOX2, is a self-destructing process characterized by an extensive ROS production. In resting cells, NOX2 is a fully inactive enzyme that exists as a cytosolic complex, containing $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ [3,23]. On activation, its regulatory subunit $p47^{phox}$ becomes phosphorylated and translocates to the membrane where it will bind to its transmembrane cytochrome unit comprising both NOX2 and the closely associated $p22^{phox}$ [2,3,23]. The distinct movement of GTP-binding protein Rac to the membrane enables the combination of all required regulatory units to form an active enzyme, capable of transferring electrons from NADPH via a bound flavin adenine nucleotide to molecular oxygen to form superoxide anion radical [3,23,24].

Due to their biological activity, stimulated neutrophils may represent a double-edged sword for pulmonary homeostasis. On the one hand, the appropriate recruitment of neutrophils toward the infected part of the airway results in killing the invading microorganisms and thus preserving the homeostasis. On the other hand, those same activated neutrophils also possess the ability to seriously damage host tissue. For example, activated neutrophils have been shown to induce oxidative DNA-lesions [25,26] and to inhibit DNA nucleotide excision repair [27] in pulmonary epithelial cells. Moreover, excessive neutrophil influx and/or incomplete apoptosis of activated neutrophils may result in disproportionate ROS production and prolonged inflammation. Indeed, neutrophil dysregulation has been associated with various pulmonary inflammatory diseases including asthma, adult respiratory distress syndrome and chronic obstructive pulmonary disease [28,29]. However, the influence of a pre-existing pool of neutrophils, resulting from either a yet-to-be resolved inflammation or a chronic lung condition, on the airway epithelial response toward noxious stimuli is not known. Therefore, the aim of the current study is to investigate the effect of neutrophils on LPS-induced pro-inflammatory signaling in lung epithelial cells.

2. Materials and methods

2.1. Chemicals

LPS from *Escherichia coli* (0.26:B6), PMA, transferrin, insulin, dexamethasone, bovine pituitary extract, trypsin, 4-aminobenzoic acid hydrazide (4-ABAH) and luminol (3-aminophthalhydrazide) were purchased from Sigma (St. Louis, MO). Hoechst 3342, penicillin, streptomycin, HBSS, DMEM/Ham's F12 (1:1), trizol and BSA were obtained from Gibco Invitrogen (Paisley, UK). Epidermal growth factor was acquired from Biomol (Hamburg, Germany) while CPH was purchased from L-Optik (Berlin, Germany). The RNeasy mini kit was provided by Qiagen (Germany). The iScript cDNA Synthesis kit and SYBR© Green Supermix were supplied by Biorad (Germany). TLR-4, IL-8 and β-actin primers were ordered at Eurofins MWG Operon (Germany) with the following sequences: β-actin forward: 5′-CCC CAG GCA CCA GGG CGT GAT-3′; β-actin reverse: 5′-GGT CAT CTT CTC GCG GTT GGC CTT GGG GT-3′; TLR-4 forward 5′-TGC ATG GAG CTG AAT TTC TAC-3′; TLR-4 reverse: 5′-TGA GCC ACA TTA AGT TCT TTC-3′. Cholera toxin was generously provided by Dr J. de Jonge (Maastricht University, the Netherlands). All other chemicals were of analytical grade.

2.2. Cytotoxicity

Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay as a marker of plasma membrane damage using a colorimetric kit (Roche, Switzerland) according to the manufacturer's instructions.

2.3. Isolation of human and murine PMN

The fresh isolation of human PMN, using blood of 3 healthy male and 3 healthy female volunteers (ages 30–45), was performed as described previously with minor modifications [26]. In short, blood was diluted with cold HBSS (1:1) after which gradient centrifugation was used to remove the lymphocytes. The remaining lowest layer containing PMN and erythrocytes was then suspended in cold lysis buffer to lyse the erythrocytes. The remaining pellet containing PMN only was suspended in HBSS and counted using a Bürker chamber. Viability was tested using Trypan Blue dye exclusion (0.4%). Keeping all solutions and PMN on ice all the time to prevent premature activation, this isolation method consistently yielded PMN with a viability >95%.

For the fresh isolation of murine PMN, the femurs and tibias of specific pathogen-free C57BL/6J (WT) and $p47^{phox-/-}$ knockout (KO) mice were used [30]. Animals were obtained from Taconic (Lille Skensved, Denmark) and had ad libitum access to food and water containing antibiotics (800 mg sulfamethoxazol and 160 mg trimethoprim per liter). Upon sacrificing the animals using a phentobarbital overdose, bone-marrow derived PMN were obtained according to the method originally described by Boxio et al. [31]. Upon harvesting the PMN fraction using a three-layer Percoll gradient, the similar procedure as for the human PMN was used with the exception that HBSS was replaced by HBSS containing 0.5% BSA. Consequently, this isolation method, combined with cooling all the solutions and PMN again, also resulted in a constant and viable (>95%) yield. No remarkable differences regarding reactivity were observed between PMN isolated from male and female volunteers.