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Distinct intracellular signaling pathways control the synthesis of IL-8 and RANTES in TLR1/TLR2, TLR3 or NOD1 activated human airway epithelial cells

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ABSTRACT

Inflammation is a central feature of many respiratory diseases. Airway epithelial cells are exposed to many agents present in the air that can alter their function and have important structural consequences for the airways. In this study, 19 Toll-Like Receptors (TLRs) and Nucleotide-binding Oligomerization Domain (NOD)1/NOD2 ligands were screened for their capacity to up-regulate Interleukin-8 (IL-8) and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES) in airway epithelial cells. Three ligands (Pam3CSK4, Poly I:C and C12-ie-DAP) were selected for their capacity to activate different receptor complexes (TLR1/TLR2, TLR3 and NOD1 respectively) while leading to the increase of both IL-8 and RANTES albeit with distinct kinetics. Using protein kinase inhibitors we found that the Nuclear Factor κB (NFκB) pathway is essential for the transcriptional regulation of both IL-8 and RANTES following the activation of TLR1/TLR2, TLR3 and NOD1. In contrast, the Mitogen-Activated Protein Kinases (MAPKs) Extracellular signal-Regulated Kinase (ERK)1/ERK2 and p38 MAPK were necessary for the transcription of IL-8 but not RANTES. Moreover, we found that the p38 MAPK was implicated in the post-transcriptional regulation of IL-8 following TLR3 activation. The distinction made between pathways involved in the regulation of IL-8 and RANTES gives rise to the possibility of designing more targeted clinical approaches based on the biological functions to be ablated.

1. Introduction

The World Health Organization estimates that 210 million people suffer from chronic obstructive pulmonary disorder (COPD) and 300 million suffer from asthma worldwide. Inflammation is a central feature of these respiratory diseases. In the lung, inflammation must be resolved efficiently while preventing tissue damage that could impair gas exchange. The airways must also discriminate between the vast amount of non-threatening antigens and the much rarer pathogenic signals. Thus, the pulmonary epithelium not only plays a role as a physical barrier but also forms the first line of defense against infections by secreting cytokines and chemokines that will activate the innate immune defense.

However, in chronic inflammatory disorders like COPD, inflammation has been proposed to be the predominant mechanism of airflow limitation [1] and many inflammatory mediators have been found present in the sputum of COPD patients [2,3]. Monocytes from patients with COPD showed enhanced chemotaxis towards GRO-α [4] and elevated IL-8 levels and increased neutrophil numbers are observed and negatively correlates with pulmonary function [5]. Asthma and COPD patients also suffer from episodes of exacerbations of their condition induced by further amplification of the inflammatory response triggered by various environmental factors, including bacterial and viral infections, which have a profound effect on patients' quality of life [6,7]. During exacerbations, levels of the CC chemokine RANTES are markedly increased in airway epithelial cells and is accompanied by increased eosinophilia [8]. Thus understanding the molecular mechanisms involved in chemokines-initiated inflammation may provide valuable information for new clinical developments in chronic pulmonary diseases.

Human cells have evolved to recognize pathogens through receptors that bind different molecular patterns like lipids, carbohydrates, peptides and nucleic acids expressed by various microorganisms. Once activated, these pattern-recognition receptors (PRRs) trigger a network of intracellular signaling events leading to the production of inflammatory mediators. The two most studied PRR families are the TLR and Nucleotide-binding Oligomerization Domain (NOD)-like receptor (NLR) families. There are currently 12 known mammalian TLRs and more than 20 NLRs [9,10].

Abbreviations: CARD, Caspase-Recruitment Domain; COPD, Chronic Obstructive Pulmonary Disorder; ERK, Extracellular signal-Regulated Kinase; IKK, IκB protein Kinase; IL, Interleukin; IRAK, IL-1-Receptor Associated protein Kinase; IRF, Interferon Response Factor; MAPK, Mitogen-Activated Protein Kinase; MAPKAP-K2, MAPK-Activated Protein Kinase 2; MKK, MAPK Kinase; MyD88, Myeloid Differentiating factor 88; NFκB, Nuclear Factor κB; NLR, NOD-like receptors; NOD, Nucleotide-binding Oligomerization Domain; PRR, Pattern Recognition Receptor; qPCR, real-time quantitative PCR; RANTES, Regulated upon Activation Normal T cell Expressed and Secreted; RIP, Receptor-Interacting Protein; TAK, TGF-β Activated protein Kinase; TBK1, TANK-Binding protein Kinase 1;TIR, Toll/IL-1 receptor domain; TRAF, TNF-Receptor Associated Factor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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Our understanding of TLR-mediated signaling has progressed rapidly in the last few years but many aspects remain unclear. Following dimerization, TLRs bind different adaptor molecules through their Toll/IL-1 receptor domain (TIR) [10]. The best characterized adaptor is MyD88 which was shown to, through the sequential recruitment of IL-1R-associated protein kinases (IRAKs) [11,12], TNF-receptor-associated factor 6 (TRAF6) [13] and TGF-β Activated Kinase (TAK1) [14,15], serve as the template for the activation of four major signaling pathways: the NFκB pathway and the three MAPK pathways, ERK1/ERK2, JNK and p38 MAPK. The TRIF adaptor, which can be recruited directly to TLR3, or indirectly via TRAM to TLR4, leads to the production of type I interferons via the activation of the IKK family members IKKε and TBK1 and phosphorylation of interferon response factors (IRFs) [16]. TRIF can also recruit TRAF6 [17] and in conjunction with Receptor-Interacting Protein 1 (RIP1) activate TAK1 and the downstream signaling pathways associated as outlined above [18]. The NOD receptors bind through their CARD domains to a protein kinase termed RIP2, (also called RICK or CARDIAK) [19], which will initiate downstream signaling via the recruitment of TAK1 and activation of the IKK complex [20,21].

In this study we have investigated the PRR responsible for the production of IL-8 and RANTES by airway epithelial cells, and identify key protein kinases involved in their synthesis.

2. Materials and methods

2.1. Materials

Human TLR1-9 Agonist kit, Human and Mouse NOD1/2 Agonist kit and SB203580 were obtained from Invivogen (San Diego, CA, USA). PD184352 was bought from USBiological (Swampscott, MA, USA). BIRB07896 and PS1145 were kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). All chemicals were bought from Fisher Scientific (Fair Lawn, NJ, USA). All enzymes were bought from Invitrogen (Carlsbad, CA, USA). Complete protease inhibitor cocktail tablets were from Roche (Mannheim, Germany).

2.2. Antibodies

Anti-phospho-ERK1/ERK2 (Thr202/Tyr204) (AB3826; dilution 1:3000) and anti-ERK1/ERK2 (AB3053; 0.3 μg/mL) were purchased from Chemicon-Millipore (Temecula, CA, USA). Anti-phospho p38 MAPK (Thr180/Tyr182) (09-272; dilution 1:1000), anti-p38 MAPK (05-454; 0.4 mg/mL) and anti-IκB (22631; 1.5 mg/mL) were bought from Upstate-Millipore (Lake Placid, NY, USA). Anti-Rabbit-HRP (HAF008; dilution 1:5000) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-Mouse-HRP (31450; 1:10,000) was purchased from Thermo Scientific (Rockford, IL, USA).

2.3. Cell culture

Primary and immortalized human bronchial epithelial cells Beas-2B cells were maintained at 37 °C, 5% CO_2, 100% humidity, in DMEM (4.5 g/L D-glucose) supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. Prior to stimulation with agonists and inhibitors, cells were serum starved overnight in 0.5% (v/v) FBS. After stimulation of Beas-2B cells, the medium was collected and snap-frozen in liquid-nitrogen for quantification of IL-8 and RANTES protein levels by ELISA (R&D Systems, Minneapolis, USA).

2.4. Cytokine array

1 mL of culture media was centrifuged to remove insoluble material and the supernatants analyzed by following the protocol of the Human Cytokine Array panel A kit (purchased from R&D systems).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The RNA was quantified and 1 μg was treated with DNAse I Amp Grade (Invitrogen, Carlsbad, USA) and reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA), according to the manufacturer's protocols.

2.6. Real-time PCR

Real-time PCR (qPCR) was performed in 96 well plate format using SYBR Green based detection on a Step-One-Plus machine (ABI) with each 20 μL reaction containing approximately 50 ng cDNA, 0.3 μM of sense and antisense primers, and 1× Quantitect SYBR Green supermix (Qiagen). The plate was sealed and cycled under the following conditions: 95 °C/10 min, 50 cycles of 95 °C/10 s and 60 °C/45 s. Each reaction was performed in duplicate, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization and fold induction was determined from Ct values using Pfaffl method [22]. PCR efficiency was determined from the slope of a standard curve generated using five-fold dilution series of the DNA template. The sequences of primers were as follows: IL-8 (5′-GTGCAGTTTTGCCAAGGAGT-3′; 5′-CTCTGCACCCAGTTTTCCTT-3′), RANTES (5′-TACACCAGTGGCAAGTGCTC-3′; 5′-GAAGCCTCCCAAGCTAGGAC-3′) and GAPDH (5′-AGCAATGCCTCCTGCACCACC-3′; 5′-CCGGAGGGGCCATCCACAGTC-3′).

2.7. ELISA

Human CXCL8/IL-8 (DY208) and Human CCL5/RANTES (DY278) DuoSet ELISA kits were purchased from R&D Systems (MN, USA). 100 μL of supernatant collected after cell stimulation was directly used for RANTES quantification or diluted ten times for IL-8 quantification according to the manufacturer's protocol.

2.8. Cell lysis and immunoblotting

Following stimulation, cells were lysed in ice-cold buffer A (50 mM Tris–Cl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, Complete Mini protease inhibitor cocktail and 2 mM DTT). Proteins were quantified using the Bradford method, 30 μg of lysates was submitted to SDS-PAGE, transfer to nitrocellulose and immunoblotted with specified antibodies. Chemiluminescence was performed using Immun-Star Western Chemiluminescent kit (Bio-Rad).

3. Results

3.1. RANTES and IL-8 are up regulated by similar TLR and NOD ligands in airway epithelial cells

When epithelial cells from a human bronchoalveolar carcinoma (A549) are stimulated with Flagelline to activate TLR5, three chemokines (namely RANTES, GRO-α and IL-8) were greatly up-regulated out of 36 common inflammatory mediators screened (Fig. 1A and B). GRO-α and IL-8 are two members of the same family of CXC chemokines that bind the CXCR2 receptor, whereas RANTES is a member of the CC chemokine family. In order to better understand which PRRs lead to an increase in synthesis of two distinct family of chemokines, 9 TLR ligands and 10 NOD ligands were screened for their ability to up-regulate IL-8 and RANTES in a human cell line derived from a normal bronchial epithelium (Beas-2B), a more relevant cellular model than the A549 cells. The results show that Pam3CSK4 (TLR1/TLR2), Poly I:C (TLR3), Flagelline (TLR5), FSL-1 (TLR2/TLR6) and C12-ie-DAP (NOD1) lead to the greatest increase in IL-8 synthesis as judged by ELISA following a 24 h stimulation of Beas-2B cells (Fig. 1C). Similar results were obtained by real-time PCR (qPCR; data not shown). When