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Short Communication

Recognition of *Propionibacterium acnes* by human TLR2 heterodimers

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ABSTRACT

Propionibacterium acnes has been considered as a crucial contributor to the pathogenesis of acne vulgaris. The interaction between *P. acnes* and the host is mainly mediated by Toll like receptor (TLR) 2 recognition. TLR2 homodimers recognize *P. acnes* in mice, but here we describe the prerequisite of TLR2/1 and TLR2/6 heterodimers in human cells for *P. acnes* recognition. *P. acnes*-induced NF-κB and AP-1activation observed in HEK hTLR2-transfected but not control cells confirmed the specificity of TLR2 recognition. The activation was blocked by neutralizing antibodies against TLR2, TLR1 and TLR6, as well as the TLR2 antagonist CU-CPT22, which showed no selectivity towards human TLR2 heterodimers. The combination of anti-TLR1 and anti-TLR6 antibodies completely abrogated activation by *P. acnes*. In primary human keratinocytes, *P. acnes*-increased NF-κB phosphorylation was inhibited by anti-TLR6 and anti-TLR2 antibodies. Furthermore, *P. acnes*-induced inflammatory responses were impaired by anti-TLR2 neutralizing antibodies and fully blocked by CU-CPT22. Our study suggests species-specific recognition of *P. acnes* by TLR2 heterodimers which can be exploited therapeutically by small molecules targeting TLR2 for the control of inflammatory responses.

1. Introduction

Toll-like receptor 2 (TLR2) has been identified as the functional receptor for bacterial lipoproteins on gram-positive and gram-negative bacteria (Brightbill et al., 1999). TLR2 heterodimerizes with either TLR1 or TLR6 to recognize triacylated and diacylated lipoproteins, respectively. TLR2/1 and TLR2/6 heterodimers form different lipid-binding pockets (Jin et al., 2007; Kang et al., 2009) and may activate distinct signaling pathways depending on the intracellular domains and adaptor proteins (van Bergenhenegouwen et al., 2013).

TLR2 plays an important role in the pathogenesis of dermatological diseases (McInturff et al., 2005). Acne vulgaris is one of the most prevalent skin diseases and microbial involvement has been considered to contribute to the development of acne (Fitz-Gibbon et al., 2013). In inflammatory acne lesions, increased epidermal TLR2 expression facilitates recognition of *Propionibacterium acnes* and contributes to inflammatory responses (Kim, 2005). In mice and possibly humans, *P. acnes* is recognized by TLR2 homodimers but not heterodimers (Kim et al., 2002), however, small but important species-differences of the TLR2 ligand binding pocket have been noticed (Jin et al., 2007). Therefore, we questioned whether human cells recognize *P. acnes* differently from mouse cells. To address this question, we used human embryonic kidney (HEK)-Blue hTLR2 cells and human primary keratinocytes. HEK-Blue hTLR2 cells are well established systems to monitor the activation of TLR2 signaling and selectivity of TLR2 modulators (Cheng et al., 2015). Keratinocytes are one of the major players in the pathophysiology of acne, in which TLR2 recognition and activation can be the initiating step in comedogenesis (Selway et al., 2013). Here, we describe a distinct recognition of *P. acnes* by human TLR2 heterodimers and provide novel mechanistic insights into *P. acnes*-mediated inflammatory responses.

2. Materials and methods

2.1. Cell culture

HEK-Blue hTLR2 cells and HEK-Blue Null1 cells were obtained from InvivoGen (Toulouse, France). HEK-Blue cells passage 6–15 were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (Sigma-Aldrich, Steinheim, Germany), 10% heat-inactivated fetal calf serum (FCS, Biochrom AG, Berlin, Germany), 5 mM l-glutamine (PAA Laboratories, Pasching, Austria), 100 μg/ml streptomycin (PAA Laboratories), 100 units penicillin (PAA Lab-

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; MOI, multiplicity of infection; SEAP, secreted embryonic alkaline phosphatase; TLR, Toll-like receptor.

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oratories) and 100 μg/ml Normocin (InvivoGen). 1x HEK-Blue Selection or 100 μg/ml Zeocin (InvivoGen) was added to the medium for hTLR2 cells or Null1 cells, respectively. HEK-Blue cells were stimulated in Opti-MEM (ThermoFisher Scientific, Darmstadt, Germany). After stimulation, cell culture supernatants were collected and 20 μL of the supernatant was transferred to a 96-well plate (TPP, Trasadingen, Switzerland) in duplicates and treated with 200 μL QUANTI-Blue (InvivoGen) buffer. After incubation at 37 °C, optical density (OD) was determined at 640 nm using a FluoStar Optima microplate reader (BMG Labtech, Offenburg, Germany). The SEAP activity levels shown were corrected by blank OD (QUANTI-Blue plus Opti-MEM). The cell line was regularly tested negative for mycoplasma contamination (Venor GeM Classic Mycoplasma PCR detection kit, Minerva Biolabs, Berlin, Germany).

For primary cultures, normal human epidermal keratinocytes were isolated from human juvenile foreskin and cultured as described (Bätz et al., 2013; Weindl et al., 2011). Keratinocytes were grown in keratinocyte basal medium (KBM; Lonza, Basel, Switzerland) supplemented with insulin, hydrocortisone, human epidermal growth factor and bovine pituitary extract (keratinocyte growth medium, KGM) as provided by the manufacturer. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Primary cells from the third passage were used and pooled from at least three donors to reduce donor-specific properties. Before stimulation, keratinocytes were washed with phosphate-buffered saline (PBS; Sigma-Aldrich) and KBM was added for 24 h. All donor and patient samples were obtained after written informed consent and only anonymized samples were used for the experiments. All experiments were performed in accordance with relevant guidelines and regulations and were approved by the ethics committee of the Charité – Universitätsmedizin Berlin, Germany.

2.2. Cell stimuli and bacteria

The TLR ligands Pam_3CSK_4 and Pam_2CSK_4 as well as anti-hTLR2-IgA (clone B4H2), anti-hTLR1-IgG (clone H2G2), anti-hTLR6-IgG (clone C5C8) and isotype-matched control antibodies were purchased from InvivoGen. The TLR2 antagonist CU-CPT22 was obtained from Sigma-Aldrich.

Propionibacterium acnes (ATCC 6919, ATCC 11827; DSMZ, Braunschweig, Germany) was cultured in Hungate anaerobic tubes (Chemglass Life Sciences, Vineland, NJ, United States) in cooked meat medium (Difco, Becton Dickinson, Heidelberg, Germany) supplemented with yeast extract (5 mg/ml, Sigma-Aldrich), $K_2HPO_4{\cdot}3H_2O$ (6.5 mg/ml, Merck Group, Darmstadt, Germany), resazurin (1 μg/ml, Sigma-Aldrich) and cysteine chloride (0.5 μg/ml, Sigma-Aldrich) at 37 °C. Bacteria were harvested by centrifugation at 2600 × *g* for 15 min, washed in PBS and resuspended in cell culture medium and stored at −80 °C. The number of bacteria was counted by Bacteria counting kit (ThermoFisher Scientific) using a Cytoflex flow cytometer (Beckman Coulter, Krefeld, Germany). Human cells were exposed to heat-inactivated (10 min at 95 °C) or live *P. acnes*.

2.3. ELISA

The cell culture supernatant was assayed for IL-8 by using commercially available ELISA kits (ELISA-Ready Set Go; eBioscience).

2.4. Cell viability

Cell viability was determined by the MTT assay in keratinocytes as described (Do et al., 2014). Viability of untreated cells was set at 100%. LDH assay was performed according to the manufacturer's instructions (Thermo Scientific, Darmstadt, Germany). The percentage of LDH release was calculated compared to 100% cell lysis control.

2.5. RNA isolation and quantitative RT-PCR

Total RNA isolation, cDNA synthesis and quantitative RT-PCR (qPCR) were performed as described. Primers (synthesized by TIB Molbiol, Berlin, Germany) with the following sequences were used: YWHAZ and IL8 as published previously (Weindl et al., 2011; Weindl et al., 2007) and 11β-HSD1 5′-AGCAGGAAAGCT CATGGGAG-3′ and 5′-CCACGTAACTGAGGAAGTTGAC-3′. Fold difference in gene expression was normalized to the housekeeping gene YWHAZ which showed the most constant level of expression.

2.6. Western blotting

Cells were lysed and prepared as described previously (Hamidi et al., 2014; Pfalzgraff et al., 2016). After gel electrophoresis and blotting, membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature, membranes were incubated with rabbit anti-phospho-NF-κB p65(Ser536) (93H1) and rabbit anti-β-actin (13E5) (both 1:1000, from NEB) over night at 4 °C and incubated with anti-rabbit horseradish-peroxidase (HRP)-conjugated secondary antibody (NEB; 1:1000) for 1 h. Then blots were developed with SignalFire ECL reagent (NEB) and visualised by PXi Touch gel imaging system (Syngene, Cambridge, UK).

2.7. Statistical analysis

Data are depicted as means + SD. Statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc analysis and considered significant at $P \leq 0.05$. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad software, San Diego, USA).

3. Results and discussion

To investigate recognition of *P. acnes* by human TLR2 homo- and heterodimers, we used HEK-Blue cells overexpressing hTLR2. In parallel, HEK-Blue Null1 cell line serves as a control for TLR2 activation, expressing only the NF-κB-inducible SEAP gene. Thus, HEK-Blue Null1 cells still produce SEAP when stimulated with various pathogen recognition receptor agonists, such as TLR3, TLR5 and NOD1 agonists, but not TLR2 agonists (Salyer et al., 2016). Furthermore, HEK cells are capable of forming TLR2 heterodimers with endogenous expressed TLR1 and TLR6 (Westwell-Roper et al., 2016). In this study, HEK-Blue hTLR2 but not HEK-Blue Null1 cells responded to the synthetic TLR2/1 and TLR2/6 ligands Pam_3CSK_4 and Pam_2CSK_4 (Supplemental Fig. S1A and B), respectively, while TLR1 or TLR6 neutralizing antibodies blocked this effect (Supplemental Fig. S1C and D). In addition, the TLR2/1 specific antagonist in mice (Cheng et al., 2012), CU-CPT22, inhibited both Pam_3CSK_4- and Pam_2CSK_4-mediated responses in HEK-Blue hTLR2 cells (Supplemental Fig. S1E), confirming non-selectivity towards human TLR2 heterodimers (Bock et al., 2016).

Two strains classified as acne associated types of *P. acnes* (Fitz-Gibbon et al., 2013) were used in this study. Heat-inactivated *P. acnes* strain ATCC 11827 activated SEAP signaling in a multiplicity of infection (MOI) dependent manner in HEK-Blue-hTLR2 cells, but not HEK-Blue Null1 cells (Fig. 1A). TLR2 neutralizing antibodies and CU-CPT22 dose-dependently reversed the activation (Fig. 1B and C). These data suggest a TLR2-dependent NF-κB activation by *P. acnes*. TLR2 signaling was also induced by heat-inactivated *P. acnes* strain ATCC 6919 and inhibited by anti-TLR2 antibodies and CU-CPT22 (Fig. 1E).