Aryl Hydrocarbon Receptor Modulates the Expression of TNF-α and IL-8 in Human Sebocytes via the MyD88-p65NF-κB/p38MAPK Signaling Pathways

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**Keywords**
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**Abstract**
Activation of Toll-like receptor (TLR)-2 and subsequent inflammatory response contribute to lesion development in acne vulgaris. A cross-talk between aryl hydrocarbon receptor (AhR), a cytosolic receptor protein that responds to environmental and physiological stress, and TLRs has recently been reported. In this study, we explored the possible role of AhR in the effects induced on cultured human SZ95 sebocytes by peptidoglycan (PGN), a classic TLR2 agonist. PGN-induced secretion of inflammatory factors TNF-α and IL-8 in human SZ95 sebocytes was suppressed after knockdown of AhR and pretreatment with the AhR antagonist CH223191. In addition, the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) enhanced TNF-α and IL-8 secretion in PGN-pretreated sebocytes. Furthermore, PGN-induced expression of myeloid differentiation factor 88 (MyD88), phospho-p38MAPK (p-p38MAPK), and p-p65NF-κB was strengthened by TCDD and repressed by CH223191. AhR inhibition by transfecting shRNA blocked the ability of PGN to stimulate phosphorylation of p38MAPK and p65NF-κB in SZ95 sebocytes. Overall, these data demonstrate that AhR is able to modulate PGN-induced expression of TNF-α and IL-8 in human SZ95 sebocytes involving the MyD88-p65NF-κB/p38MAPK signaling pathway, which probably indicates a new mechanism in TLR2-mediated acne.

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Introduction

Skin represents the first-line defense of the human body against external stimuli. The innate immune system is the major contributor to acute inflammation induced by microbial infection and enables the skin to protect the host from infection. In the skin, nonprofessional immune cells, such as keratinocytes and sebocytes, significantly contribute to its innate immunity. Toll-like receptors (TLRs) are the most important pattern recognition receptors, which are responsible for sensing the presence of microorganisms. TLRs, with the exception of TLR3, accomplish their signals through the myeloid differentiation factor 88 (MyD88)-dependent pathway by activating nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) and subsequently regulating cytokine gene expression [1].

TLR2 is strongly expressed in human basal and infundibular keratinocytes and sebocytes and is involved in the Cutibacterium acnes-mediated enhancement of acne severity. Exposure of microdissected human sebaceous glands to the pathogen-associated molecular pattern of TLR2 in vitro resulted in an interleukin (IL)-1α-like cornification after 7 days of exposure [2]. Moreover, human sebocytes are immunologically activated by bacterial proteins via TLRs and CD14, which are both expressed on their cell surface [3]. Cell-free extracts of C. acnes stimulate cytokine production through activation of TLR in SZ95 sebocytes [4]. Activation of pattern recognition receptors increases the secretion of both pro- and anti-inflammatory cytokines (chemokines [IL-8], TNF-α, antimicrobial lipids, peptides) in sebaceous glands [5–7].

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of TCDD and related compounds [8]. Recent research has shown that AhR not only plays an important role in toxic action, but also in cell physiology including the human immune system [9]. 2,3,7,8-Tetachlorodibenzo-p-dioxin (TCDD) is a high-affinity AhR ligand that is frequently used to investigate biological processes impacted by AhR activation [10]. In addition, close interactions between TLRs, NF-κB, and the AhR signaling pathway have been studied in human dendritic cells [11] and macrophages [12, 13], which contribute to immune dysfunction, metabolism of xenobiotics, and carcinogenesis [14].

In this study, we investigated whether peptidoglycan (PGN) could induce activation of the innate immune response, as well as secretion of the proinflammatory factors TNF-α and IL-8 in human SZ95 sebocytes in vitro. Furthermore, we studied the effect of AhR in the innate immune signaling pathway, by knockdown of the AhR gene and applying the AhR agonist TCDD to the cells, to further explore the possible mechanism of the pathogenesis of TLR-mediated acne.

Materials and Methods

Cell Culture and Reagents

The immortalized human sebaceous gland cell line SZ95 [15] was routinely maintained in Sebomed© basal medium-Biochrom, Berlin, Germany) supplemented with 5 ng/mL human epidermal growth factor, 10% fetal calf serum, 1% penicillin/ streptomycin (all from Gibco, Invitrogen, Carlsbad, CA, USA), and 1 mM CaCl2 (Sigma, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO2 at 37 °C. The medium was replaced daily, and cells were used at passage 30–40. Small hairpin RNA (shRNA) targeted against AhR (shAhR) and shCon SZ95 sebocytes were generated according to our previous study [16]. shAhR and shRNA consisting of a scrambled sequence that would not lead to specific degradation of any cellular message (shCon) were generated (GeneChem, Shanghai, PR China). These plasmids were further co-transfected into 293T cells with lentiviral packaging plasmids to generate an AhR shRNA-expressing lentivirus (LV-shAhR) or a control shRNA-expressing one (LV-shCon). SZ95 sebocytes cultured in 6-well plates were infected with titrated viral supernatant at an MOI of 20 in the presence of 5 g/mL polybrene (Sigma) to generate AhR-silenced SZ95 cells and controls.

Activation of Cell Cultures

Cell cultures were grown to approximately 70–80% confluence and were treated with medium only, PGN (20 μg/mL) (from Staphylococcus aureus; Sigma), TCDD (10 nM/L) (Sigma), and CH223191 (10 μM) (Tocris Bioscience, Minneapolis, MN, USA). CH223191 was added in cell medium 1 h prior to PGN and TCDD. PGN was prepared in culture medium, and TCDD and CH223191 were prepared in DMSO. In PGN-treated cells, medium without the additives to be tested served as control. In TCDD and CH223191 treatment experiments, DMSO (0.1% v/v) was added as control. Cell counting was conducted using a cell counting chamber slide and cell viability was determined using the MTT assay.

Real-Time RT-PCR

SZ95 sebocytes were seeded into 24-well plates (Corning) at a density of 1 × 105 cells/well. Total RNA was isolated by Trizol reagent (Invitrogen). RNA concentration was determined at 260 nm (NanoDrop; Thermo Scientific). Reverse transcript was performed using an mRNA reverse transcription kit (Takara, Tokyo, Japan). The mRNA level of selected genes was measured with SYBR Green (Applied Biosystems®). The primers (Sangon, Shanghai, PR China) used were as follows:

- **TNF-α** forward: 5′-ATGGTTTAGCAACCCCTCAAGC-3′ and reverse: 5′-GAGGAGCACATGGGTGGAG-3′, IL-8 forward: 5′-CCTGATTTCCTCAGCTCTCTG-3′ and reverse: 5′-CCAGA-
AhR-Mediated PGN Induction of Cytokine Secretion in Sebocytes

CAGAGCTCTCTCTCCAT-3’, MyD88 forward: 5’-CTGCTCGAGGTGCTTACCAA-3’ and reverse: 5’-GATGGCACCTGGAGAGGG-3’, IL-1α forward: 5’-GTTTGATGCAGAAAAGATTCA-3’ and reverse: 5’-GGAGTGCCCATACTTACAT-3’, CYP1A1 forward: 5’-TGACCTCAACAGGACACCCA-3’ and reverse: 5’-CACCCTGTGCTGTAGCCAAA-3’. Fold differences in expression were calculated using the comparative CT method by standardizing against GAPDH expression.

Enzyme-Linked Immunosorbent Assay
Quantikine enzyme-linked immunosorbent assay (ELISA) kits (MultiSciences, Biotech, Hangzhou, PR China) were used to detect IL-8 and TNF-α. SZ95 sebocytes were seeded into 24-well plates (10^5 cells/well) and maintained in culture medium alone or treated with stimuli as indicated above. After incubation for 48 h, culture supernatants were collected and centrifuged for removing cell debris. Aliquots were stored at -80°C until use. TNF-α and IL-8 protein levels were then determined according to the manufacturer’s recommendation.

Immunohistochemistry
SZ95 sebocytes were grown on coverslips in 6-well plates (5 × 10^5 cells/well) in culture medium alone or treated with stimuli as indicated above. After incubation for 48 h, culture supernatants were collected and centrifuged for removing cell debris. Aliquots were stored at -80°C until use. TNF-α and IL-8 protein levels were then determined according to the manufacturer’s recommendation.

Cell Transfection
Human SZ95 sebocytes were seeded into 6-well plates at a density of 5 × 10^5/well for 24 h. AhR or control shRNA obtained from GeneChem was transfected into the cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 48 h of transfection, the cells were treated as indicated above for further studies.

Western Blot Analysis
SZ95 sebocytes were seeded into 6-well plates (5 × 10^5 cells/well) in culture medium alone or treated with stimuli as indicated above, washed with PBS, and then lysed in cell lysis buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, and 2% mercaptoethanol) for 30 min. Protein concentration was determined using a BCA protein assay kit (Thermo Fisher, Shanghai, PR China). Equal protein amounts were separated on SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked in TBST (20 mM Tris-HCl at pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% nonfat powder milk. The membranes were then incubated with antibodies against AhR (#83200, 1:1000), phospho-p38MAPK (Thr 180/Tyr 182) (#4511, 1:1000) and phospho-p65NF-κB (Ser536) (#3033, 1:1000) (all Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After three washes with PBS, the membranes were incubated with a secondary antibody (#7074, 1:2000) (Cell Signaling Technology) for 1 h at room temperature and washed again. The blots were developed by the chemiluminescence system ECL (Amersham, UK). Densitometry was performed by ImageJ.

Statistical Analysis
Values are presented as the mean of three represented independent experiments in each condition ± SEM. A comparison of two groups was made with an unpaired, two-tailed Student’s t test. A comparison of multiple groups was made with analysis of variance (ANOVA). A two-way ANOVA was used when data with more than one factor were assessed. Differences were considered significant when: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. All statistics were performed using GraphPad Prism version 6.0 software (San Diego, CA, USA).
Fig. 2. PGN-induced secretion of TNF-α and IL-8 is inhibited in AhR knockdown SZ95 sebocytes and CH223191-pretreated cells. Production of TNF-α (a) and IL-8 (b) in Lv-shAhR-transfected SZ95 sebocytes and Lv-shCon-transfected cells treated with PGN (20 μg/mL). Sebocytes were pretreated with the AhR antagonist CH223191 for 1 h in comparison with control cells (maintained in 0.1% DMSO), then the cells were treated with PGN (20 μg/ml) (c, d). mRNA was extracted from cells treated with PGN for 3 h. The culture supernatants were harvested from the cells treated with PGN for 48 h. mRNA was detected by RT-PCR and relative results were normalized to GAPDH. Cytokines in culture supernatants were assayed by ELISA. Data are presented as the mean ± SEM of at least 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
**Results**

**PGN-Induced TNF-α and IL-8 Production in Human SZ95 Sebocytes**

First, we studied the effect of PGN (20 μg/mL), TCDD (10 nM), and CH223191 (10 μM) on the viability of SZ95 sebocytes by the MTT assay after 48 h of treatment. As shown in Figure 1a, the investigated compounds had barely any effect on SZ95 sebocyte viability.

Second, human SZ95 sebocytes exposed to PGN (20 μg/mL) were investigated for cytokine synthesis. PGN stimulated cytokine mRNA levels at different time points. TNF-α mRNA expression increased 20-fold and IL-8 100-fold at 3 h of treatment (Fig. 1b, c). Both mRNA levels were reduced over time up to 24 h. These findings indicate an early stimulation of TNF-α and IL-8 synthesis in SZ95 sebocytes by PGN.

**PGN-Induced Release of TNF-α and IL-8 is AhR Dependent**

To investigate if PGN induction of TNF-α and IL-8 synthesis by SZ95 sebocytes was followed by enhanced cytokine secretion, we investigated the PGN-induced secretion of TNF-α and IL-8 in both Lv-shAhR-transfected SZ95 sebocytes and SZ95 sebocytes treated with the AhR antagonist CH223191.

SZ95 sebocytes transfected with Lv-shCon and Lv-shAhR were exposed to PGN (20 μg/mL). mRNA expression of TNF-α and IL-8 was detected by RT-PCR after 3 h of treatment and protein levels in supernatants were measured by ELISA after 48 h of treatment. Lv-shCon- and Lv-shAhR-transfected SZ95 sebocytes have been established and described in previous work [16]. In the present study, TNF-α and IL-8 protein levels in the supernatants of Lv-shAhR-transfected cells were markedly reduced compared those of Lv-shCon-transfected SZ95 sebocytes (Fig. 2a, b).

To corroborate if PGN-induced secretion of proinflammatory cytokines was, indeed, AhR dependent, we repeated the experiments after pretreatment with the AhR antagonist CH223191 for 1 h. SZ95 sebocytes were then treated with PGN alone or in the presence of CH223191 for 3 and 48 h. TNF-α and IL-8 mRNA levels were both increased 100-fold under treatment with PGN in comparison with controls, while a 60% reduction in TNF-α mRNA levels and a 50% reduction in IL-8 mRNA levels compared to PGN-treated cells were detected when cells were pretreated with CH223191 (Fig. 2c, d). Supernatant protein amounts confirmed the PGN-induced TNF-α and IL-8 production. Pretreatment of SZ95 seocytes with CH223191 reduced TNF-α and IL-8 protein levels (Fig. 2c, d).

Taken together, we provided evidence that the PGN-induced secretion of TNF-α and IL-8 was, at least partially, AhR dependent in human SZ95 sebocytes.

**AhR Ligand TCDD Synergistically Enhanced the Production of TNF-α and IL-8 in PGN-Stimulated SZ95 Sebocytes**

SZ95 sebocytes were treated with PGN and the AhR agonist TCDD for 48 h and cytokine secretion was assessed by supernatant ELISA. The detected data suggest
that the combination treatment with TCDD and PGN resulted in an increase in secreted TNF-α and IL-8 protein levels compared to cells treated with PGN alone (Fig. 3).

**TCDD Synergistically Enhanced the Expression of MyD88 in PGN-Treated SZ95 Sebocytes**

PGN has been reported to be sensed by cell surface TLR2, resulting in secretion of inflammatory proteins [17]. Since we have shown that PGN-induced TNF-α and IL-8 secretion can be enhanced by TCDD in SZ95 sebocytes and MyD88 is a downstream gene of TLRs, we further studied the effect of TCDD on PGN-induced MyD88 expression. The presented data showed that PGN treatment of SZ95 sebocytes induced elevated levels of MyD88 mRNA and protein expression. Compared with cells treated with PGN, PGN + TCDD co-treatment of SZ95 sebocytes resulted in increased levels of MyD88 mRNA and protein (Fig. 4).

**Fig. 4.** MyD88 mRNA and protein expression in SZ95 sebocytes. (a) Cells were treated with PGN and TCDD for 3 h. MyD88 mRNA expression was assessed by RT-PCR. Data are presented as the mean ± SEM of at least 3 independent experiments. *p < 0.05, **p < 0.01. (b) SZ95 sebocytes were treated with PGN and TCDD for 24 h. Anti-MyD88 immunocytology was used to evaluate localization and protein levels in SZ95 sebocytes. Representative expression patterns are shown of at least 3 independent experiments. Original magnification, ×100. Scale bars, 200 μm.

PGN Induces a Time-Dependent Phosphorylation of p38MAPK and p65NF-κB in Human SZ95 Sebocytes

NF-κB and MAPK are downstream genes in the innate immune signaling pathway and play a pivotal role in inflammatory and immune response. The Gram-positive bacterium *C. acnes* activates MAPKs in human keratinocytes [18]. We investigated, therefore, the effect of PGN (which is produced – among others – by the Gram-positive bacterium *Staphylococcus aureus*) on the expression of p38MAPK and p65NF-κB in SZ95 sebocytes. Incubation of SZ95 sebocytes with PGN (20 μg/mL) significantly induced phosphorylation of p65NF-κB with peak at 15 min and p38MAPK with peak at 120 min, and both exhibited a strong expression at 30 min (Fig. 5).

AhR Inhibition Blocks the Ability of PGN to Stimulate Phosphorylation of p38MAPK and p65NF-κB in SZ95 Sebocytes

To elucidate if the signaling pathways that are activated by PGN are influenced by AhR expression level in
SZ95 sebocytes, we transfected SZ95 sebocytes with shRNAs, which specifically block AhR expression (AhR shRNA), or scrambled shRNA and subsequently treated the cells with PGN for 30 min. First, we evaluated the efficiency of AhR shRNA in SZ95 sebocytes. Figure 6 shows that the transfection of SZ95 sebocytes with AhR shRNA significantly decreased AhR protein expression compared to the scrambled shRNA (negative control). Knockdown of AhR by AhR shRNA resulted in significantly decreased phosphorylation of p38MAPK and p65NF-κB in comparison with cells transfected with a scrambled control (Fig. 6). Therefore, PGN, indeed, downregulated AhR expression in the scrambled control and also in the AhR knockout cells.

**TCDD Enhanced PGN-Induced Phosphorylation of p38MAPK and p65NF-κB in SZ95 Sebocytes**

To further understand the molecular basis of TCDD potentiation of PGN-induced activation of inflammatory signaling in SZ95 sebocytes, we next examined whether the phosphorylation of p38MAPK and p65NF-κB could be enhanced by TCDD. SZ95 sebocytes were pretreated with CH223191 for 1 h and subsequently treated with PGN and TCDD for 30 min. Phosphorylation of p65NF-κB and p38MAPK was assessed by Western blot. As shown in Figure 7, treatment of SZ95 sebocytes with TCDD also caused a significant increase in phospho-p38 (p-p38) and p-p65 like that of PGN, whereas the combination treatment of PGN and TCDD induced a stronger effect than each compound alone. Pretreatment of cells with CH223191 significantly inhibited p-p38 and p-p65 levels, compared with PGN + TCDD-treated cells. These data indicate that the AhR agonist TCDD can enhance the phosphorylation of p65NF-κB and p38MAPK and the effect can be inhibited by an AhR antagonist, facts which suggest AhR to play a pivotal role in PGN-induced inflammatory response in human SZ95 sebocytes.
Discussion

Acne is a chronic inflammatory disease of the sebaceous follicle [19]. Inflammation is a crucial factor at every stage of acne development. In inflammatory acne lesions, TNF-α and IL-8 expression is increased compared with the adjacent normal skin [20]. Cytokines, such as TNF-α and IL-8, propagate the inflammatory response by acting on endothelial cells to elaborate adhesion molecules (e.g. ICAM-1) to facilitate recruitment of professional inflammatory cells towards the skin lesions [21]. The transcription factor NF-κB is activated in acne lesions [20], and cell-free extracts of C. acnes have been shown to promote protein phosphorylation of p38MAPK in SZ95 sebocytes [4].

Increasing interest in AhR biology has currently transferred its focus from the toxic effects of dioxins and other environmental pollutants to its endogenous biological roles [22]. Indeed, AhR plays an important role in the human immune system [9] and its interaction with the TLR/NF-κB signaling pathway has recently been elucidated [11–13]. The presence of bacterial originating TLR2 ligands seems to be crucial for detoxification of luminal carcinogens by CYP1A1 in the intestine and indicates a
complex interplay between the immune system of the host and intestinal bacteria with detoxification mechanisms [23]. TCDD was shown to upregulate the expression and secretion of TNF-α and IL-6 by increasing Akt phosphorylation and nuclear translocation of p65 NF-κB in cultured hepatic stellate cells [24] and TCDD induced MAPK phosphorylation in HepG2 cells [25].

The relationship between AhR and sebaceous glands is especially interesting, since AhR and CYP1A1 are both strongly expressed in human sebocytes and environmental contaminants TCDD and benzo(a)pyrene inhibit lipogenesis via switching sebocyte into keratinocyte differentiation [16, 26, 27]. Moreover, AhR may be involved in sebaceous gland homeostasis under both steady-state and stress (inflammatory) conditions [28].

In the current work, we used human SZ95 sebocytes to further elucidate the role of AhR in the inflammation process. Interestingly, a PGN-induced release of TNF-α and IL-8 was detected to be – at least partially – AhR dependent, as shown in both AhR knockdown SZ95 sebocytes and by the AhR antagonist CH223191 administration. The AhR agonist TCDD enhanced the PGN-induced TNF-α and IL-8 cellular secretion.

Upon pathogen-associated molecular pattern recognition, TLRs are activated and subsequently transduce signals from the cell membrane to the nucleus [29]. Currently, two main branches of the TLR signal transduction pathway have been identified, namely the MyD88- and the TRIF-dependent pathways [30]. The principal signaling pathways downstream to MyD88 are the NF-κB and MAPK-AP-1 pathways [31]. We demonstrated that PGN-induced MyD88 mRNA levels and protein expression in SZ95 sebocytes as well as TCDD strengthen this effect (Fig. 4). Our results also showed a PGN-induced phosphorylation of p65NF-κB and p38MAPK in SZ95 sebocytes, whereas p65 and p38 phosphorylation could be disrupted by knocking down AhR. Moreover, TCDD exhibited a synergistic effect on the PGN-induced phosphorylation of p65 and p38, which could be inhibited by the AhR antagonist CH223191. Taken together, these ob-

Fig. 7. TCDD treatment enhances PGN-induced phosphorylation of p65 NF-κB and p38MAPK in SZ95 sebocytes. The cells were pretreated with the AhR antagonist CH223191 for 1 h, then the CH223191-treated cells and controls were treated with PGN (20 μg/mL) and TCDD (10 nM) for 30 min. Cell lysates were used for Western blot analyses performed as described in Materials and Methods. The density of phosphorylated p38MAPK and p65 NF-κB band was normalized to its loading control GAPDH. Mean ± SEM of relative arbitrary units of the bands from 3 immunoblots conducted in separate experiments are presented. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 versus the control. ## p < 0.01 versus the PGN + TCDD group. A representative Western blot from 1 of 3 experiments is shown.
servations clearly indicate that AhR is critically involved in PGN-induced TNF-α and IL-8 synthesis in human SZ95 sebocytes via MyD88-p38MAPK/p65NF-kB signal transduction pathways. Since this project focused on the TLR and PGN downstream pathway, it apparently represents a classic TLR2 agonistic mode of action. The investigation of AhR-associated influence of TLR2 expression is the target of future studies.

As shown in Figure 6, PGN, indeed, downregulated AhR expression in the scrambled control and also in the AhR knockout cells. PGN is a classic ligand of TLR2, but in our work it could also directly influence the AhR signal pathway. Downregulation of AhR expression in SZ95 sebocytes at the protein level has been proven in our previous studies under treatment with the typical AhR agonists TCDD [26] and benzo(a)pyrene [16]. On the other hand, this study has shown that PGN activates the AhR signal pathway, whereas the exact molecular mechanism still needs to be studied in the future.

In recent years, increasing evidence indicates a link between acne and exposure to environmental pollutants through unclear mechanisms [32]. Our data elaborate the molecular mechanisms of AhR – a transcription factor that senses environmental stimuli to regulate innate immunity in human SZ95 sebocytes – and provide a new insight into the association between environmental pollution and regulation of innate immunity in acne.

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Disclosure Statement

The authors declare that they have no conflict of interest.