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Involvement of Pattern Recognition Receptors in the Direct Influence of Bacterial Components and Standard Antiacne Compounds on Human Sebaceous Gland Cells

Zouboulis, Christos C ; Oeff, Marina K ; Hiroi, Naoki ; Makrantonaki, Evgenia ; Bornstein, Stefan R

Abstract: INTRODUCTION Pattern recognition receptors are involved in innate and adaptive immunity by detecting microbial components. Bacteria have been accused to play a role in inflammatory acne. We investigated the potential involvement of Toll-like receptor (TLR)2, TLR4, TLR6, and CD14 in the direct influence of bacterial components and standard antiacne compounds on human sebocytes. METHODS mRNA and protein expression of TLR2, TLR4, TLR6, and CD14 in SZ95 sebocytes was evaluated by real-time qRT-PCR and immunocytochemistry. The effects of lipopolysaccharides (LPS) and lipoteichoic acid on TLR2, TLR4, and CD14 expression and of cytokine/chemokine secretion by 13-cis-retinoic acid, all-trans-retinoic acid, retinol, and hydrocortisone at the mRNA and protein levels were assessed by real-time qRT-PCR and ELISA and verified by cocultivation with neutralizing antibodies. RESULTS The constitutive expression of TLR2, TLR4, and CD14 in SZ95 sebocytes was augmented by exposure to LPS. Hydrocortisone induced TLR2, but markedly reduced TLR4 expression. 13-cis-retinoic acid and all-trans-retinoic acid regulated IL-6 release. LPS enhanced and hydrocortisone reduced cytokine and chemokine release. Anti-TLR4 and anti-CD14 mAb blocked LPS-induced IL-8 and IL-6 release. CONCLUSIONS Microbial components use pattern recognition receptors to directly activate sebocytes to express a wide range of proinflammatory molecules and especially IL-8 and IL-6 in a TLR4- and CD14-specific manner. Retinoids, but mostly corticosteroids, also use this pathway to exhibit anti-inflammatory effects.

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Involvement of Pattern Recognition Receptors in the Direct Influence of Bacterial Components and Standard Antiacne Compounds on Human Sebaceous Gland Cells

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Keywords

Human sebaceous gland cells · Toll-like receptors · Retinoids · Hydrocortisone · Innate immunity

Abstract

Introduction: Pattern recognition receptors are involved in innate and adaptive immunity by detecting microbial components. Bacteria have been accused to play a role in inflammatory acne. We investigated the potential involvement of Toll-like receptor (TLR)2, TLR4, TLR6, and CD14 in the direct influence of bacterial components and standard antiacne compounds on human sebocytes. **Methods:** mRNA and protein expression of TLR2, TLR4, TLR6, and CD14 in SZ95 sebocytes was evaluated by real-time qRT-PCR and immunocytochemistry. The effects of lipopolysaccharides (LPS) and lipoteichoic acid on TLR2, TLR4, and CD14 expression and of cytokine/chemokine secretion by 13-*cis*-retinoic acid, all-*trans*-retinoic acid, retinol, and hydrocortisone at the mRNA and protein levels were assessed by real-time qRT-PCR and ELISA and verified by cocultivation with neutralizing antibodies. **Results:** The constitutive expression of TLR2, TLR4, and CD14 in SZ95 sebocytes was augmented by exposure to

LPS. Hydrocortisone induced TLR2, but markedly reduced TLR4 expression. 13-*cis*-retinoic acid and all-*trans*-retinoic acid regulated IL-6 release. LPS enhanced and hydrocortisone reduced cytokine and chemokine release. Anti-TLR4 and anti-CD14 mAb blocked LPS-induced IL-8 and IL-6 release. **Conclusions:** Microbial components use pattern recognition receptors to directly activate sebocytes to express a wide range of proinflammatory molecules and especially IL-8 and IL-6 in a TLR4- and CD14-specific manner. Retinoids, but mostly corticosteroids, also use this pathway to exhibit anti-inflammatory effects.

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Introduction

Human sebocytes are capable of bacterial recognition and may play an important role in initiating and perpetuating the activation of both innate and adaptive immune responses [1, 2]. They participate in skin inflammation by recruiting and communicating with professional immune cells, an interaction, which leads to the generation and differentiation of Th17 cells [3]. Innate immune recognition of pathogens is mediated by germline-encoded receptors specific for conserved structural components of pathogenic microorganisms. These are referred to as pattern recognition receptors (PRR) [4]. Genome-wide analysis of PRR-activated human sebocytes presented a complex immune competence of activated sebaceous glands [5]. Human acne-involved sebaceous glands were shown to express common molecules with other inflammatory diseases, such as psoriasis and lichen ruber [6].

Toll-like receptors (TLR) act as PRR. TLR are transmembrane proteins with an extracellular domain consisting of leucine-rich repeats and cytoplasmic domain similar to the interleukin (IL)-1 receptor and have emerged as key components in the innate immune system [7]. TLR recognize conserved molecular structures, known as pathogen-associated molecular patterns, which are relatively invariant within a given class of microorganisms such as mannans in the yeast cell wall, mycobacterial cell-wall component lipoarabinomannan, lipopolysaccharides (LPS) in Gram-negative bacteria, and lipoteichoic acid (LTA) in Gram-positive bacteria. TLR play a crucial role in the induction of antimicrobial responses in various cells activating multiple steps in those inflammatory reactions that help to eliminate the invading bacteria and coordinate systemic defense [8].

The possible role of TLR in acne pathogenesis has been investigated by targeting *Propionibacterium acnes* (*P. acnes*) as the most obvious source of the pathogen-associated molecular pattern recognized by TLR2 [2, 9, 10]. TLR2 and TLR4 as well as CD14 are expressed in human monocytes. TLR2 is upregulated on perilesional macrophages of acne lesions, and *P. acnes* can activate TLR2 and trigger IL-8 release from peripheral blood mononuclear cells (PBMC) [11].

IL-8 is chemotactic for neutrophils, and early acne lesions are characterized by a paucity of neutrophils [12, 13]. They are most likely to be involved later in the generation of pustules. Several putative physiological ligands of TLR, such as – most intriguingly – fatty acids, are likely to be involved in acne pathogenesis [14, 15].

TLR, such as TLR2 and TLR4, have also been identified activated on human epidermal keratinocytes [16, 17] and sebocytes in vitro [5, 18, 19]. Moreover, TLR2 is expressed in basal and infundibular keratinocytes and in sebaceous glands in vivo and its activation provoked the release of IL-1 α from primary human keratinocytes in vitro. The exposure of microdissected human sebaceous glands to the TLR ligands LPS, peptidoglycan, and LTA resulted in a pattern of IL-1 α -like cornification after 7 days of exposure [20]. Clinical isolates of *P. acnes* significantly induced IL-8 mRNA expression in human keratinocytes in vitro [21]. *P. acnes*-induced increase in IL-8 gene expression could be inhibited by anti-TLR2 and anti-TLR4 neutralizing antibodies.

A major target of acne therapy is the reduction of inflammation. Retinoids and hydrocortisone (HC) have shown anti-inflammatory activity in the treatment of severe acne [22–24], whereas dexamethasone increased TLR2 mRNA expression in sebocytes by inhibiting 11 β -hydroxysteroid dehydrogenase type 1 and activating mitogen-activated protein kinase phosphatase-1 [18]. Treatment of primary human monocytes with all-*trans* retinoic acid (atRA) led to downregulation of TLR2 and the TLR4 coreceptor CD14 [25]. The ability of a TLR2/1 ligand to trigger monocyte cytokine release was inhibited by pretreatment and co-treatment with atRA, which also downregulated monocyte cytokine induction by *P. acnes*. At last, systemic isotretinoin (13-*cis*-retinoic acid [13cRA]) therapy normalized the exaggerated TLR2-mediated innate immune response in acne patients [26]. The goal of the present study was to investigate or confirm the potential involvement of Toll-like receptor (TLR)2, TLR4, TLR6, and CD14 in the direct influence of bacterial components and standard antiacne compounds on human sebocytes in vitro.

Materials and Methods

Cell Culture

Immortalized human facial SZ95 sebocytes [27], which have been shown to conserve the major characteristics of normal sebocytes [28], were maintained in Sebomed® Basal Medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (Biochrom), 5 ng/mL human epidermal growth factor (Sigma, Deisenhofen, Germany), 50 μ g/mL gentamicin (Gibco/BRL, Berlin, Germany), and 1 mM Ca²⁺ in a humidified atmosphere of 5% CO₂ at 37°C. Cell culture medium was changed every 2 days. SZ95 sebocytes were allowed to grow to 70% confluence. The human acute myeloid leukemia cell line HL-60 [29] was cultured under the same conditions with SZ95 sebocytes. PBMC were prepared from peripheral blood from healthy individuals as

previously described [30]. HL-60 cells and PBMC served as positive controls. Keratinocytes from neonatal foreskin cultured in serum-free keratinocyte medium (Gibco/BRL) supplemented with 5 ng/mL epidermal growth factor, 50 µg/mL bovine pituitary extract, and 50 µg/mL gentamicin (all from Gibco/BRL) in a humidified atmosphere of 5% CO₂ at 37°C served as additional control. Since the cells used in this study either originate from immortalized human cell lines or from previously established normal human cell lines and did not derive from tissue specimens of human subjects obtained for the presented studies no written consent or ethics approval was required.

Reagents

For in vitro stimulation of SZ95 sebocytes, the following substances were used: 13cRA, atRA, retinol (ROL), HC, LPS from *Escherichia coli* 026:B6, and LTA from *Bacillus subtilis*. Phorbol 12-myristate 13-acetate (PMA) served as positive control (all from Sigma). Mouse monoclonal antibodies (mAb) were used for immunocytochemistry and neutralization of microbial component-induced chemokine and cytokine production: anti-human CD14 (OriGene, Rockville, MD, USA), anti-human TLR2 TL2.1 (Thermo Fisher, Waltham, MA, USA), anti-human TLR4 HTA125 (Novus, Centennial, CO, USA), and IgG2a isotype control (Thermo Fischer). All secondary antibodies used and the alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were from Dako (Hamburg, Germany). ELISA kits were from R&D Systems (Wiesbaden, Germany).

Cell Challenge

SZ95 sebocytes grown to about 70% confluence were maintained further under serum-free conditions and were either left untreated or challenged for 24 h with 10⁻⁶ M 13cRA, 10⁻⁶ M atRA, 10⁻⁶ M ROL, 10⁻⁶ M HC, 100 ng/mL LPS, 100 ng/mL LTA, and 100 ng/mL PMA. Following incubation, the supernatants were collected and the cells were harvested for detection of mRNA and protein levels.

RT-PCR Analysis

Total RNA was extracted from SZ95 sebocytes, keratinocytes, PBMC, and HL-60 by standard methods (RNeasy Midi Kit; Qiagen, Hilden, Germany). The total RNA was then treated with RNase-Free DNase Set (Qiagen). An aliquot of total RNA (1 µg) was reverse-transcribed into single-stranded cDNA with oligo(dT)15 (1 µg) and Moloney murine leukemia virus reverse transcriptase in a final volume of 33 µL as indicated in the manufacturer's instructions (First-strand cDNA Synthesis Kit; GE Healthcare, Freiburg, Germany). Reverse transcription mixture (2 µL) was used for the PCR reaction. PCR was carried out in a total volume of 50 µL with a primer pair at 0.5 µM in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 200 µM each of dATP, dCTP, dTTP, and dGTP, and 1.25 units Ampli Tag DNA polymerase (Applied Biosystems, Weiterstadt, Germany) in an Eppendorf Mastercycler gradient-automated thermal cycler. The amplification protocol contained 1 cycle of initial denaturation at 94°C for 5 min followed by varying number of cycles of denaturation at 94°C for 1 min, annealing at the individual annealing temperature (*T_a*) for 1 min, extension at 72°C for 2 min, and 1 cycle of terminal extension at 72°C for 10 min. PCR products were separated electrophoretically on 1.5–2% Tris-acetate EDTA-agarose gels, stained with ethidium bromide, and photographed under UV light. Table 1

Table 1. RT-PCR primers

Gene	Forward primer	Reverse primer	Expected size, bp	Annealing temperature (<i>T_a</i>), °C	Cycles, <i>n</i>
TLR2	5'-GCC AAA GTC TTG ATT GAT TGG-3'	5'-TTG AAG TTC TCC AGC TCC TG-3'	346	54	35
TLR4	5'-TGG ATA CGT TTC CTT ATA AG-3'	5'-GAA ATG GAG GCA CCC CTT C-3'	506	56	35
TLR6	5'-CTA TTG TTA AAA GCT TCC ATT TTG T-3'	5'-ACC TGA AGC TCA GCG ATG TAG TTC-3'	186	56	35
CD14	5'-ACT TAT CGA CCA TGG AGC-3'	5'-AGG CAT GGT GCC GGT TA-3'	407	52	33
IL-1α	5'-ATG GCC AAA GTT CCA GAC ATG TTT GAA-3'	5'-CTA CGC CTG GTT TTC CAG TAT CTG AAA-3'	816	60	34
IL-1β	5'-ATG GCA GAA GTA CCT GAG CTC GCC AGT-3'	5'-CTC CAG CTG TAG AGT GGG CTT ATC ATC-3'	578	60	33
IL-6	5'-GCT ATG AAC TCC TTC TCC ACA-3'	5'-ATG CTA CAT TTG CCG AAG AGC-3'	644	62	33
IL-8	5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'	5'-TCT CAG CCC TCT TCA AAA ACT TCT-3'	290	60	33
IL-10	5'-AAG CCA TGA GTG AGT TTG ACA-3'	5'-GGG AAT GAG GTT AGG GGA ATC-3'	613	60	33
TNFα	5'-ATG AGC ACT GAA AGC ATG ATC-3'	5'-TCA CAG GGC AAT GAT CCC AAA GTA-3'	695	60	35
GAPDH	5'-GAA GGT GAA GGT CCG AGT-3'	5'-GAA GAT GGT GAT GGG ATT TC-3'	225		

TLR, Toll-like receptor; IL, interleukin; TNFα, tumor necrosis factor alpha.

Table 2. Quantitative RT-PCR primers

Gene	Forward primer	Reverse primer	TaqMan probes	Expected size, bp
TLR2	5'-TGT GAA GAG TGA GTG GTG CAA GT-3'	5'-ATG GCA GCA TCA TTG TTC TCA T-3'	5'-FAM-TGA ACT GGA CTT CTC CCA TTT CCG TCT TT-TAMRA-3'	78
TLR4	5'-AGA GTT TCC TGC AAT GGA TCA AG-3'	5'-TTA TCT GAA GGT GTT GCA CAT TCC-3'	5'-FAM-TTC GTT CAA CTT CCA CCA AGA GCT GCC T-TAMRA-3'	82
CD14	5'-CGC TCC GAG ATG CAT GTG-3'	5'-AGC CCA GCG AAC GAC AGA-3'	5'-FAM-TCC AGC GCC CTG AAC TCC CTC-TAMRA-3'	60
IL-1α	5'-CAA TTG TAT GTG ACT GCC CAA GA-3'	5'-ACT ACC TGT GAT GGT TTT GGG TAT C-3'	5'-FAM-CAG GCA TCT OCT TCA GCA GCA CTG GTT-TAMRA-3'	84
IL-1β	5'-CTG ATG GCC CTA AAC AGA TGA AG-3'	5'-GGT CGG AGA TTC GTA GCA GCT GGA T-3'	5'-TTC CAG GAC CTG GAC CTC TGC CCT C-3'	87
IL-6	5'-AGC CGC CCC ACA CAG A-3'	5'-CCG TCG AGG ATG TAC CGA AT-3'	5'-FAM-CCA CTC ACC TCT TCA GAA CGA ATT GAC AAA CA-TAMRA-3'	72
IL-8	5'-CTG GCC GTG GCT CTC TTG-3'	5'-TTA GCA CTC CTT GGC AAA ACT G-3'	5'-FAM-CCT TCC TGA TTT CTG CAG CTC TGT GTG AAG-TAMRA-3'	77
IL-10	5'-ACG GCG CTG TCA TCG ATT-3'	5'-TGG AGC TTA TTA AAG GCA TTC TTC A-3'	5'-FAM-TGT GAA AAC AAG AGC AAG GCC GTG GA-TAMRA-3'	81
TNFα	5'-CTC GAA CCC CGA GTG ACA A-3'	5'-GGA GCT GCC CCT CAG CTT-3'	5'-FAM-CCT GTA GCC CAT GTT GTA GGC AAA CCC-TAMRA-3'	67

TLR, Toll-like receptor; IL, interleukin; TNFα, tumor necrosis factor alpha.

Fig. 1. SZ95 sebocytes express TLR2, TLR4, TLR6, and CD14 mRNA. TLR2, TLR4, TLR6, and CD14 mRNA expression was examined by RT-PCR with specific primer sets in SZ95 sebocytes (1), human keratinocytes (2), PBMC (3), and HL-60 cells (4) cultured for 24 h. While TLR2, TLR4, TLR6, and CD14 mRNA expression was detectable in untreated SZ95 sebocytes and PBMC, keratinocytes and HL-60 cells only expressed detectable TLR2, TLR4, TLR6, and CD14 mRNA levels after stimulation with 100 ng/mL LPS. mRNA expression was normalized to GAPDH. TLR, Toll-like receptor; PBMC, peripheral blood mononuclear cells.

shows primer sequences and experimental conditions for RT-PCR as well as size of the cDNA fragments. The primers were purchased from Metabion (Berlin, Germany).

Real-Time Quantitative RT-PCR Analysis

One microgram of total RNA from SZ95 sebocytes or HL-60 cells treated with RNase-Free DNase Set (Qiagen) was reverse-transcribed to cDNA using a First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) with random hexamers as primers in a 33-μL reaction solution. To quantify expression of IL-1α, IL-1β, IL-6, IL-8, IL-10, tumor necrosis factor (TNFα), CD14, TLR2, and TLR4 mRNA, real-time quantitative RT-PCR (TaqMan PCR; Applied Biosystems) was performed using a Perkin-Elmer 7700 sequence detector [31]. The reaction contained 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the forward and reverse primers, and 200 nM of TaqMan probes. Table 2 shows primer sequences for quantitative RT-PCR. All primers and probes were purchased from Eurogentec (Cologne, Germany). 18S rRNA primers and probe (Applied Biosystems) were added at 50 nM to serve as the internal control. Thermal cycling proceeded at 50 cycles of 95°C for 15 s and 60°C for 1 min. Input RNA amounts were calculated with a multiplex comparative method for the mRNAs of interest and 18S rRNA. Experiments were performed in triplicate for each condition.

TLR2, TLR4, and CD14 Protein Detection

SZ95 sebocytes cultured in two-chamber culture slides for 1, 6, 12, and 24 h or cytocentrifuged on microscopic slides at a density of 25,000 cells per 200 μL in a Shandon Cytospin 2 cytocentrifuge

Fig. 2. Human SZ95 sebocytes express TLR2, TLR4, and CD14 protein. Protein expression was examined by immunocytochemistry with mouse anti-human TLR2 (a), TLR4 (b), and CD14 (c) antibodies. d Unlabeled control. TLR2 could be mainly attributed to the cell membrane, while TLR4 and CD14 were mainly distributed diffusely in the cytoplasm of SZ95 sebocytes. TLR, Toll-like receptor.

were subjected to immunocytochemistry. The preparations were fixed in methanol at -20°C for 5 min and incubated with anti-CD14 (diluted 1:50), anti-TLR2 (diluted 1:20), or anti-TLR4 (diluted 1:20) antibodies for 30 min. The secondary antibody used was APAAP complex-conjugated rabbit anti-mouse (diluted 1:20), and the reaction was performed according to the instructions for the APAAP labeling method (all incubation steps at room temperature for 30 min). The preparations were developed with new fuchsin counterstained with hematoxylin and mounted. Triplicate slides were stained for each data point.

Determination of IL-1 α , IL-1 β , IL-6, IL-8, IL-10, and TNF α Release

Chemokines and cytokines were measured in the supernatants of challenged SZ95 sebocytes using Quantikine ELISA kits (R&D System, Wiesbaden, Germany) according to the manufacturer's protocols. IL and TNF α release was measured in triplicate wells for all individual treatments. The results are presented as the relative fold increase compared to against the results from the untreated controls at the same time, whereas the ratio 1 represents the IL release of the controls.

Neutralization of Microbial Component-Induced Chemokine and Cytokine Production by Anti-TLR2, Anti-TLR4, and Anti-CD14 Antibodies

Cultured cells were preincubated for 1 h at 37°C with $10\text{ }\mu\text{g}$ per mL anti-TLR2, anti-TLR4, or anti-CD14 antibodies followed by the addition of 100 ng/mL LPS, LTA, or PMA. To determine the specificity of these antibody studies, control cultures were preincubated with a mouse IgG2a isotype control antibody followed by exposure to 100 ng/mL LPS, LTA, or PMA. After 24 h, the culture supernatants were harvested and assessed by Quantikine ELISA kits according to the manufacturer's protocols. Details of proliferation, differentiation, or viability of SZ95 sebocytes are given in online suppl. data; for all online suppl. material, see www.karger.com/doi/10.1159/000513259.

Statistical Analysis

All data are presented as the mean $\pm$ SEM for at least 3 experiments. Statistical significance was calculated by the Yates method-corrected two-sample independent-group Student's *t* test. Mean differences were considered significant at values of $p < 0.05$.

Results

SZ95 Sebocytes Express TLR2, TLR4, TLR6, and CD14 at the mRNA Level

TLR2, TLR4, TLR6, and CD14 mRNA were detected in SZ95 sebocytes by RT-PCR and their levels, especially those of TLR2 and CD14, increased with the duration of SZ95 sebocyte culture (Fig. 1). PBMC, LPS-stimulated HL-60 cells, and epidermal keratinocytes served as positive controls and sterilized water as negative control. We detected TLR2, TLR4, TLR6, and CD14 mRNA expression in SZ95 sebocytes and all positive controls.

Expression of TLR2, TLR4, and CD14 at the Protein Level in SZ95 Sebocytes and Their Cellular Localization

Labeling of SZ95 sebocytes with anti-TLR2, anti-TLR4, and anti-CD14 mAb revealed a positive staining for all molecules tested (Fig. 2). TLR2 could be mainly attributed to the cell membrane, while TLR4 and CD14 were mainly distributed diffusely in the cytoplasm of SZ95 sebocytes. These results have been corroborated by Western blot detection of abundant TLR2 and TLR4 protein levels in SZ95 sebocytes, shown in another own work submitted for publication.

Effect of LPS and LTA on TLR2, TLR4, and CD14 mRNA Levels in SZ95 Sebocytes

As expected, the proinflammatory/cytotoxic agent PMA used as positive control markedly upregulated TLR2 ($2.55 \pm 0.77x$) and TLR4 ($2.63 \pm 0.58x$) mRNA levels in SZ95 sebocytes, being the only compound tested to induce an increase in mRNA levels of over 2.5-fold in all experiments performed (Fig. 3). Challenging SZ95 sebocytes with LPS slightly increased mRNA levels of TLR2

Fig. 3. TLR2 and TLR4 mRNA levels in SZ95 sebocytes are regulated by microbial components and anti-inflammatory agents. TLR2 and TLR4 mRNA levels in SZ95 sebocytes were assessed by real-time quantitative RT-PCR 24 h after treatment with 10^{-6} M 13cRA, 10^{-6} M atRA, 10^{-6} M ROL, 10^{-6} M HC, 100 ng/mL LPS, 100 ng/mL LTA, and 100 ng/mL PMA. TLR2 and TLR4 mRNA levels were normalized with 18S rRNA levels and are presented as the x-fold increase over the untreated controls at 24 h. LPS up-regulated TLR2 mRNA levels. HC slightly upregulated TLR2 mRNA levels but markedly reduced TLR4 mRNA levels. PMA markedly upregulated TLR2 and TLR4 mRNA levels in SZ95 sebocytes. The chart shows the mean $\pm$ SEM of 10 independent experiments. Statistical analysis was performed by Student's *t* test using a two-tailed hypothesis. * $p < 0.05$; *** $p < 0.01$. TLR, Toll-like receptor; 13cRA, 13-*cis*-retinoic acid; atRA, all-*trans* retinoic acid; ROL, retinol; HC, hydrocortisone; LPS, lipopolysaccharides; LTA, lipoteichoic acid; PMA, phorbol 12-myristate 13-acetate.

($1.77 \pm 0.24x$), while LTA did not affect TLR2 and TLR4 mRNA levels. CD14 mRNA levels were affected by neither LPS nor LTA, indicating CD14 is present in human sebocytes as a co-player for the induction of inflammatory processes by TLR.

Effect of Retinoids and HC on TLR2, TLR4, and CD14 mRNA Levels in SZ95 Sebocytes

TLR2, TLR4, and CD14 mRNA levels in SZ95 sebocytes were assessed after treatment with the retinoids 13cRA, atRA, and ROL as well as with HC, compounds known to modify the inflammatory response in vivo and

in vitro (Fig. 3). All retinoids tested did not affect TLR2 and TLR4 mRNA levels. HC slightly enhanced TLR2 mRNA levels ($1.44 \pm 0.17x$) and reduced TLR4 mRNA levels ($0.46 \pm 0.20x$). We could reproduce this HC effect with additional experiments using dexamethasone (data not shown).

Effect of Microbial Components on Cytokine and Chemokine Synthesis in SZ95 Sebocytes

To detect the effects of LPS and LTA on cytokine/chemokine synthesis in SZ95 sebocytes, we measured the production of selected proinflammatory chemokines and cytokines by real-time quantitative RT-PCR and ELISA. The positive control substance PMA increased IL-1 α , IL-1 β , IL-6, and IL-8 mRNA levels and protein secretion (IL-1 α [$4.44 \pm 0.76x$], IL-1 β [$4.94 \pm 0.44x$], IL-6 [$4.34 \pm 0.68x$], and especially IL-8 secretion levels [$32.25 \pm 15.90x$]) from SZ95 sebocytes (Fig. 4). In contrast to LTA treatment, which did not affect cytokine and chemokine protein levels, LPS enhanced cytokine and chemokine release (IL-1 α [$1.65 \pm 0.15x$], IL-1 β [$2.08 \pm 0.20x$], IL-6 [$2.97 \pm 0.40x$], and IL-8 [$4.83 \pm 1.11x$]) from SZ95 sebocytes. IL-10 and TNF α mRNA expression and release were only markedly induced by PMA, but not by LPS or LTA.

Effect of Retinoids and HC on Cytokine and Chemokine Synthesis in SZ95 Sebocytes

To detect the effects of retinoids and HC on cytokine/chemokine synthesis in SZ95 sebocytes, we also measured their effect on the production of selected proinflammatory chemokines and cytokines by real-time quantitative RT-PCR and ELISA. As expected from the TLR pattern regulation reported above, retinoids did not affect mRNA levels of IL-1 α , IL-1 β , IL-6, and IL-8 and only induced minor effects on cytokine/chemokine release (Fig. 4). 13cRA slightly reduced IL-1 α ($0.83 \pm 0.03x$) and IL-6 ($0.42 \pm 0.10x$) secretion and atRA IL-6 ($0.50 \pm 0.10x$) and ROL IL-1 α ($0.71 \pm 0.10x$) secretion from SZ95 sebocytes. In contrast, HC reduced cytokine and chemokine release (IL-1 α [$0.45 \pm 0.08x$], IL-1 β [$0.59 \pm 0.05x$], IL-6 [$0.28 \pm 0.04x$], and IL-8 [$0.26 \pm 0.06x$]) from SZ95 sebocytes. IL-10 and TNF α release was affected neither by retinoids nor by HC.

LPS Induces SZ95 Sebocyte IL-8 and IL-6 Release by a TLR4- and CD14-Dependent Mechanism

To determine whether IL-8 and IL-6 synthesis in SZ95 sebocytes is regulated by TLRs and/or CD14, we investigated the effect of anti-TLR2, anti-TLR4, and anti-CD14 antibodies on LPS- and LTA-induced IL-8 and IL-6 re-

Fig. 4. Cytokine and chemokine production in SZ95 sebocytes is regulated by microbial components and anti-inflammatory agents. The effect of 10^{-6} M 13cRA, 10^{-6} M atRA, 10^{-6} M ROL, 10^{-6} M HC, 100 ng/mL LPS, 100 ng/mL LTA, and 100 ng/mL PMA on IL-1α, IL-1β, IL-6, and IL-8 release from SZ95 sebocytes was measured by ELISA in 24-h cell culture supernatants. In contrast to LTA, which was found inactive, LPS enhanced and HC reduced the release of all cytokines and chemokines tested. 13cRA reduced IL-1α and IL-6 secretion and atRA IL-6 and ROL IL-1α secretion from SZ95

sebocytes. PMA markedly upregulated the secretion of all cytokines tested. Data are presented as the x-fold increase over the untreated control at 24 h. The chart shows the mean $\pm$ SEM of 7 independent experiments. Statistical analysis was performed by Student's *t* test using a two-tailed hypothesis. **p* < 0.05; ****p* < 0.01. 13cRA, 13-*cis*-retinoic acid; atRA, all-*trans* retinoic acid; ROL, retinol; HC, hydrocortisone; LPS, lipopolysaccharides; LTA, lipoteichoic acid; PMA, phorbol 12-myristate 13-acetate.

lease. No effect was detected on LPS- and LTA-induced SZ95 sebocyte IL-8 and IL-6 release after incubation with the IgG2a isotype antibody used as negative control. As expected, PMA-induced IL-8 and IL-6 release used as the positive control was not affected by incubation of SZ95 sebocytes with anti-TLR2, anti-TLR4, and anti-CD14 antibodies (Fig. 5, 6). IL-8 release induced by LPS was reduced by anti-TLR4 mAb (4.28 ± 0.89 to $1.80 \pm 0.35x$), as expected and given the lack of IL-8 stimulation by LTA basal IL-8 release was slightly reduced by anti-TLR2 mAb (1.30 ± 0.19 to $0.74 \pm 0.09x$) (Fig. 5). The anti-CD14 mAb caused a reduction in IL-8 release by LPS (4.78 ± 1.05 to

$3.14 \pm 0.40x$), as also expected since CD14 is a co-receptor for TLR4. Moreover, anti-TLR4 and anti-CD14 mAb inhibited IL-6 release induced by LPS (anti-TLR4: 2.74 ± 0.34 to $1.72 \pm 0.26x$; anti-CD14: 3.62 ± 0.52 to $1.42 \pm 0.26x$). Given the lack of IL-6 stimulation by LTA, anti-TLR4 and anti-CD14 mAb seemed to also slightly reduce the basal IL-6 release (anti-TLR4: 1.21 ± 0.11 to $0.51 \pm 0.17x$; anti-CD14: 1.26 ± 0.21 to $0.50 \pm 0.23x$), respectively (Fig. 6). On the other hand, incubation with the anti-TLR2 antibody inhibited neither IL-8 nor IL-6 release induced by LPS in SZ95 sebocytes (Fig. 5, 6).

Fig. 5. Microbial components induce SZ95 sebocytes to release IL-8 by a TLR4- and CD14-dependent mechanism. LPS and LTA induction of SZ95 sebocyte IL-8 secretion was examined by ELISA. SZ95 sebocytes were pretreated with 10 µg/mL anti-TLR2 mAb (a), anti-TLR4 mAb (b), anti-CD14 mAb, (c) or mouse IgG2a isotype control antibody for 1 h and then challenged with 100 ng/mL LPS, LTA, or PMA for 24 h. Anti-TLR4 mAb and anti-CD14 mAb blocked LPS-induced but not LTA-induced IL-8 release. Anti-TLR2 mAb slightly reduced basal IL-8 release. Control IL-8 levels (under IgG2a isotype control antibody treatment): 339 ± 28 pg/mL. The chart shows the mean ± SEM of 7 independent experiments. Statistical analysis was performed by Student's *t* test using a two-tailed hypothesis. **p* < 0.05; ****p* < 0.01. TLR, Toll-like receptor; LPS, lipopolysaccharides; LTA, lipoteichoic acid; PMA, phorbol 12-myristate 13-acetate; mAb, mouse monoclonal antibodies.

Fig. 6. Microbial components induce SZ95 sebocytes to release IL-6 by a TLR4- and CD14-dependent mechanism. LPS and LTA induction of SZ95 sebocyte IL-6 secretion was examined by ELISA. SZ95 sebocytes were pretreated with 10 µg/mL anti-TLR2 mAb (a), anti-TLR4 mAb (b), anti-CD14 mAb (c), or mouse IgG2a isotype control antibody for 1 h and then stimulated with 100 ng/mL LPS, LTA, or PMA for 24 h. LPS-induced IL-6 release was blocked by anti-TLR4 and anti-CD14 mAb. Anti-TLR4 and anti-CD14 mAb also slightly reduced the basal IL-6 release. Control IL-6 levels (under IgG2a isotype control antibody treatment): 506 ± 37 pg/mL. The chart shows the mean ± SEM of 7 independent experiments. Statistical analysis was performed by Student's *t* test using a two-tailed hypothesis. **p* < 0.05; ****p* < 0.01. TLR, Toll-like receptor; LPS, lipopolysaccharides; LTA, lipoteichoic acid; PMA, phorbol 12-myristate 13-acetate; mAb, mouse monoclonal antibodies.

Discussion

This study confirms previous evidence that human sebocytes express functional TLR2 and TLR4 [5, 18–20] and detects for the first time the expression of the TLR4 co-receptor CD14 in human sebocytes. We could further confirm TLR6 mRNA expression, which is known to form a heterodimeric pair with TLR2 [32]. Thus, the expression pattern of evolutionarily conserved TLR might enable human sebocytes to respond directly to microorganisms, as already shown for keratinocytes in vitro [21]. The activation of TLR and CD14 with subsequent release of cytokines, which regulate the local immune response, is a mechanism by which bacterial products may enhance inflammation in acne [5, 18–20].

The parallel detection of TLR2, TLR4, TLR6, and CD14 mRNA in LPS-stimulated primary human keratinocytes in our study also confirms the findings by Song et al. [17], Pivarcsi et al. [33], and Nagy et al. [21], who reported that human keratinocytes constitutively express TLR2, TLR4, and CD14 responding to low concentrations of LPS.

TLR2 and TLR4 are the most widely studied PRR. TLR2 is involved in the recognition of a variety of microbial products such as lipopeptides and lipoproteins from Gram-negative and Gram-positive bacteria as well as LTA, peptidoglycans from Gram-positive bacteria, mycobacterial lipoarabinomannan, neisserial porins [34], zymosan from yeasts [35], atypical LPS, and heat-shock proteins [36, 37]. Peptidoglycan recognition requires the TLR2/TLR6 complex, whereas recognition of LTA only needs the presence of TLR2 [16]. In contrast, TLR4 primarily mediates cellular signaling induced by Gram-negative bacteria and was characterized as a receptor specific for LPS [17, 38]. LPS are a major component in the Gram-negative bacterial cell wall, whereas only saturated fatty acids containing LPS act as a ligand for TLR4 [39, 40]. CD14 is a co-receptor of TLR4 but has the ability to also recognize LTA and peptidoglycans from Gram-positive bacteria [41, 42]. In our study, LPS already at low concentrations, which do not affect cell proliferation and differentiation, slightly upregulated mRNA expression of TLR2 in SZ95 sebocytes.

The marked induction of IL-1 α , IL-1 β , IL-6, and IL-8 by LPS in SZ95 sebocytes confirms earlier reports of cytokine expression in and around the pilosebaceous unit at the preclinical and initial stages of acne lesions [15, 43–45]. In addition, our experiments on blocking TLR2, TLR4, and CD14 indicate that microbial components might induce human sebocyte IL-8 and IL-6 release by a mechanism probably dependent on TLR4/CD14 under the conditions tested. These experiments demonstrate that microbial components are capable of directly activating

SZ95 sebocytes to express and release IL-8 and IL-6 in an orchestrated TLR4- and CD14-dependent manner.

In acne, the host response to *P. acnes* may result in the production of proinflammatory cytokines and contribute to the clinical manifestations of the disease. Our data suggest that wall components of Gram-positive bacteria, such as LTA, might be less able to trigger an inflammatory cytokine response in human sebocytes than the Gram-negative bacterial product LPS, indicating the possibility that other bacteria than *P. acnes* might be responsible for the initiation of acne [46].

A major goal of acne therapy is to reduce inflammation. 13cRA is used to reduce sebaceous gland size by decreasing proliferation and inflammation [22, 47]. Corticosteroids, such as prednisolone or dexamethasone, modify the inflammatory response in vivo and in vitro and are used in combination with retinoids in severe inflammatory acne and acne fulminans [24]. Since TLR may mediate inflammation in acne [11, 15], we examined whether the mechanism of anti-inflammatory activity of retinoids and corticosteroids correlates with regulation of TLR expression and function. Retinoids exhibited a rather weak overall effect on cytokine release from SZ95 sebocytes. However, IL-6, which induces lymphocyte chemotaxis and is constitutively expressed in human sebocytes, was affected by retinoids due to a significant release reduction, especially through 13cRA and atRA. This finding indicates a selective anti-inflammatory effect of retinoids on human sebocytes. In contrast to our findings, Liu et al. [25] reported that atRA was able to inhibit cytokine release from monocytes by downregulating TLR2 and CD14 expression and function, while TLR4-induced cytokine release was not affected. On the other hand, atRA reduced TLR2 expression in human keratinocytes while simultaneously increased the levels of IL-8 and TLR4 [48]. Evidently, the mode of retinoid-regulated gene expression depends on cell type with exertion of an anti-inflammatory effect via two pathways, one specifically affecting TLR2 and CD14 expression and another TLR-independent, the latter probably is common with human sebocytes.

As previously reported for dexamethasone [49], HC upregulated TLR2 mRNA levels, but markedly reduced TLR4 and CD14 mRNA levels. This confirms our hypothesis that TLR2 acts as a “guard” for human sebocytes, as it is upregulated after treatment with anti-inflammatory agents. HC induced an overall reduction of cytokine/chemokine release in SZ95 sebocytes, a finding that emphasizes the global anti-inflammatory activity of corticosteroids, also on sebocytes, which is clinically obvious in severe acne [23, 24].

This study provides further support for an active role of human sebocytes in innate immune response at least through the constitutive expression of functional TLR2, TLR4, and CD14. Obviously, the expression of functional receptors for pathogen-associated molecular patterns is required not only in professional epidermal immune cells, such as Langerhans cells, but also in human sebocytes and epidermal keratinocytes to guarantee normal skin homeostasis. Since the pilosebaceous unit is in direct contact with microorganisms, it is not surprising that human sebocytes are endowed with the capacity to initiate innate immune responses. Georgel et al. [1], Nagy et al. [2], Lovászi et al. [50], and Sanford et al. [45] have provided evidence for the potential of human sebocytes to kill pathogens by various mechanisms such as the production of antibacterial lipids and the expression of antimicrobial peptides.

On the other hand, an important role for TLR2 and TLR4 receptors in the hypothalamus-pituitary adrenal axis has previously been suggested [51]. Human sebocytes express their own neuroendocrine stress system including the capacity to modify local steroid metabolism [52]. Thus, sebocytes are responsive to stress, providing a potential link to the pathogenesis of acne [53]. The present data underline the close functional interdependence of both the classical and local endocrine stress system with the key mediators of the cutaneous innate system.

In conclusion, human sebocytes express functional PRR. Microbial components use them to directly activate the cells to express a wide range of proinflammatory molecules and especially IL-8 and IL-6 in a TLR4- and CD14-specific manner, thus initiating and perpetuating the activation of both innate and adaptive immune responses in human skin [3]. Retinoids, but mostly corticosteroids, also use this pathway to exhibit anti-inflammatory effects.

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Statement of Ethics

The authors have no ethical conflicts to disclose. The reported research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors declare no conflicts of interest regarding this manuscript. C.C. Zouboulis is the owner of an international patent on the immortalized human sebaceous gland cell line SZ95 (WO0046353).

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Author Contributions

Study design: C.C. Zouboulis. Performance of the experiments: M.K. Oeff, H. Naoki, and E. Makrantonaki. Statistical analysis: M.K. Oeff. Preparation and revision of the manuscript: C.C. Zouboulis, M.K. Oeff, H. Naoki, E. Makrantonaki, and S.R. Bornstein.

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