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# Glucocorticoids and Toll-like receptor 2 cooperatively induce acute-phase serum amyloid A

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ABSTRACT

Serum amyloid A (SAA) is a highly conserved acute-phase protein and extrahepatic produced SAA1/2 contributes to cutaneous inflammation. Prolonged systemic or topical treatment with glucocorticoids can provoke skin diseases such as steroid-induced acne. Glucocorticoids increase Toll-like receptor 2 (TLR2) expression, however, an inflammatory mediator linked to this side effect remains elusive. We report that TLR2 agonists in combination with dexamethasone substantially increase SAA expression and production in human keratinocytes and epithelial cells. Dexamethasone-mediated SAA1 induction depends on the glucocorticoid receptor (GR). In response to *Propionibacterium acnes*, TLR2-activated signal transducer and activator of transcription 3 (STAT3) and nuclear factor κB (NF-κB) signaling pathways are critically involved in dexamethasone-induced SAA1 production. The formation of transcription factor complexes between GR or p300 and phospho-STAT3 was confirmed by co-immunoprecipitation in dexamethasone- and *P. acnes*-stimulated keratinocytes. Furthermore, dexamethasone and *P. acnes*-increased TLR2 and mitogen-activated protein kinase phosphatase-1 (MKP-1) contribute to induction of SAA1 and 2. Likewise, tumor necrosis factor (TNF) induces SAA1 in combination with dexamethasone. GR, transcription factors STAT3 and NF-κB, but not MKP-1, mediate TNF- and dexamethasone-induced SAA1. Conclusively, we provide evidence that glucocorticoids promote SAA1 production under infectious and sterile inflammatory conditions which may provide significant insights to the pathogenesis of steroid-induced acne.



## 1. Introduction

Serum amyloid A (SAA) is a family of apolipoproteins mainly synthesized in the liver. Extrahepatic production of SAA has been demonstrated in other cells including macrophages, neutrophils and smooth muscle cells [1]. Humans express two groups of different isoforms: acute-phase SAA (A-SAA: SAA1 and SAA2) and constitutive SAA (c-SAA: SAA4). During the acute phase of inflammation SAA1/2 are secreted rapidly, and the levels positively correlate with the severity of inflammatory diseases such as rheumatoid arthritis and osteoarthritis [2]. Thus, SAA is suggested as a clinical marker for disease progression.

SAAs elicit their functions through binding to multiple proteins and receptors [1]. More recently the role of SAA1 in cutaneous inflammation has been described. Excessive ultraviolet irradiation-induced SAA1 is recognized by Toll-like receptors (TLRs) and promotes expression of proinflammtory cytokines and matrix metalloproteinase-1 in fibroblasts [3,4]. Moreover, increased SAA1 levels in psoriatic epidermis contribute to IL-1β secretion via inflammasome activation [5]. In contrast, the molecular mechanisms modulating SAA expression are poorly understood. Previous studies suggest cell-type dependent involvement of CCAAT/enhancer binding proteins (C/EBP), signal transducers and activators of transcription (STAT) 3 and NF-κB [6,7].

As a side effect, prolonged systemic or topical treatment with glucocorticoids may cause acne or exacerbate existing acne lesions [8]. This clinical observation is paradoxical to the well-known immune suppression effect of glucocorticoids. Glucocorticoids induce TLR2 expression [9] and signaling in keratinocytes [10]. *Propionibacterium acnes* contributes to inflammation in acne vulgaris by activation of TLR2 heterodimers in human cells [11], however, an inflammatory mediator linked to TLR2 signaling in the presence of *P. acnes* and glucocorticoids is still missing. Interestingly, glucocorticoids alone or in combination with proinflammatory cytokines induce SAA1/2 production in non-hepatic cell lines [12–14] and SAA2 expression is upregulated in acne lesions [15].

In the present study, we aimed to examine the expression of SAA1/2 in primary human keratinocytes stimulated with glucocorticoids under infectious and sterile inflammatory conditions and characterized the underlying molecular mechanisms modulating SAA1 production.

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## 2. Materials and methods

### 2.1. Cell culture

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. For primary cultures, normal human epidermal keratinocytes and dermal fibroblasts were isolated from human juvenile foreskin and cultured as described [16,17]. 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. Fibroblasts, the immortalized keratinocyte cell line HaCaT (CLS Cell Lines Service, Eppelheim, Germany) passage 45–52 and the oral epithelial cell line TR146 were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (Sigma-Aldrich, Steinheim, Germany), 10% fetal calf serum (FCS, Biochrom AG, Berlin, Germany), and 5 mM L-glutamine (PAA Laboratories, Pasching, Austria). Cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air. HEK-Blue hTLR2 cells were obtained from InvivoGen (Toulouse, France). HEK cells passage 10–15 were cultured as described [11]. The cell lines were regularly tested negative for mycoplasma contamination (Venor GeM Classic Mycoplasma PCR detection kit, Minerva Biolabs, Berlin, Germany).

### 2.2. Cell stimulation

Primary cells from the third passage were used and pooled from at least three donors to reduce donor-specific properties. $1.8 \times 10^5$ (6-well) or $7 \times 10^4$ (12-well) cells were seeded into culture plates (TPP, Trasadingen, Switzerland) and incubated in growth medium for 24 h. Before stimulation, keratinocytes, fibroblasts, HaCaT and TR146 cells were washed with phosphate-buffered saline (PBS; Sigma-Aldrich) and KBM (without hydrocortisone) or basal medium without FCS and antibiotics was added for 24 h. Cells were washed and stimulated with 10 ng/ml TNF (eBioscience, San Diego, USA), or different inhibitors (Ro 31-8220, mifepristone (RU486), pimozide, S3I-201, BAY 11-7082 and CU-CPT22; all from Sigma-Aldrich) in the presence or absence of dexamethasone (Sigma-Aldrich). The TLR2 ligands $Pam_3CSK_4$ (TLR2/1; 1 μg/ml), $Pam_2CSK_4$ (TLR2/6; 1 μg/ml), and HKLM (TLR2; $1 \times 10^8$ cells/ml), as well as anti-hTLR2-IgA (clone B4H2), anti-hTLR6-IgG (clone C5C8) and isotype-matched control antibodies were purchased from Invivogen (San Diego, USA). The vehicles used were ethanol (dexamethasone, RU486), DMSO (S3I-201, pimozide, CU-CPT22, BAY 11-7082), and cell culture grade water (TLR ligands, TLR antibodies). Final vehicle concentrations in cell culture were below 1% (v/v). Vehicle controls showed no significant difference to non-stimulated controls (data not shown) and cell viability of primary keratinocytes was at least 75% in the presence of the inhibitors (Fig. S1). HEK-Blue cells were stimulated in Opti-MEM (ThermoFisher Scientific, Darmstadt, Germany). At the end of the experiments, cell culture supernatants were collected and SEAP production was determined [11].

### 2.3. ELISA

The cell culture supernatant was assayed for IL-8 (ELISA-Ready Set Go; eBioscience) and SAA1 (DuoSet, R&D Systems, Wiesbaden, Germany) by using commercially available ELISA kits.

### 2.4. RNA isolation and quantitative RT-PCR

Total RNA isolation, cDNA synthesis and quantitative RT-PCR (qPCR) were performed as described [16]. Primers (synthesized by TIB Molbiol, Berlin, Germany) with the following sequences were used: *YWHAZ*, *MKP1* and *TLR2* as published previously [18], *SAA1* 5′-CCTGGGCTGCAGAAGTGATCAGCGA-3′ and 5′-AGTCCTCCGCACCATGGCCAAAGAA-3′, and *SAA2* 5′-AGCCAATTACATCGGCTCAG-3′ and 5′-ATTTATTGGCAGCCTGATCG-3′. Fold difference in gene expression was normalized to the housekeeping gene *YWHAZ*.

### 2.5. Bacteria

*Propionibacterium acnes* (ATCC 11827; DSMZ, Braunschweig, Germany) was cultured as described previously [10,11]. Bacteria were heat-inactivated at 95 °C for 10 min.

### 2.6. Co-immunoprecipitation

Nuclear extracts were prepared from keratinocytes following the protocol (Nuclear Extract Kit, Active Motif, La Hulpe, Belgium). Total protein content was determined by using the BCA Protein Assay kit (Pierce, Thermo Scientific) and an equivalent amount was used for co-immunoprecipitation. Lysates were immunoprecipitated with rabbit anti-phospho-Stat3 (Tyr705) (D3A7) antibody (Sepharose Bead conjugate) or rabbit anti IgG isotype control (Sepharose Bead conjugate) (NEB, Germany) and incubated overnight at 4 °C. Samples were washed 5 times with IP buffer (Thermo Scientific). Immunoprecipitated proteins were eluted with standard SDS-PAGE sample buffer in the presence of DTT, boiled and analysed by Western blot. The non-immunoprecipitated nuclear extracts served as loading control (10% input).

### 2.7. Western blotting

Cells were lysed and prepared as described previously [19]. After gel electrophoresis and blotting, membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at 37 °C, membranes were incubated with rabbit anti-phospho-Stat3 (Tyr705) (D3A7), rabbit anti-stat3 (79D7), rabbit anti-phospho-NF-κB p65 (Ser536) (93H1), rabbit anti-Acetyl-CBP (K1535)/p300 (K1499), rabbit anti-glucocorticoid receptor (D6H2L), rabbit anti-TLR2 (D7G9Z) and rabbit anti-β-actin (13E5) (all 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 or Elite ECL reagent (NEB) and visualized by PXi Touch gel imaging system (Syngene, Cambridge, UK). All western blots were performed at least three times independently and the results shown are from one representative experiment. Quantifications were obtained by densitometric analysis using ImageJ version 1.46r and normalized to the respective β-actin loading controls.

### 2.8. RNA interference

siRNA duplexes for TLR2 and a nonsilencing control siRNA duplex (synthesized by Eurofins Genomics, Ebersberg, Germany) were tested for knockdown efficiency. The siRNA target sequences were as follows: TLR2: 5′-GGCUUCUCUGUCUUGUGAC-3′ and control: 5′-UUCUCCGAACGUGUCACGU-3′. TLR2 and control siRNA stock solutions were prepared according to the manufacturer's instructions. Keratinocytes were incubated with 100 nM siRNA and TransIT-X2 transfection reagent (Mirus, Darmstadt, Germany) for 24 h prior to stimulation. Knockdown was confirmed by qRT-PCR and western blot.

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