The release of pro-inflammatory cytokines is mediated via mitogen-activated protein kinases rather than by the inflammasome signalling pathway in keratinocytes

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The release of pro-inflammatory cytokines is mediated via MAP-kinases rather than inflammasome signaling pathway in keratinocytes

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Short title: Cytokines and signaling pathway in keratinocytes

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Abstract

Toll like receptors (TLRs) are expressed in the skin and airway epithelial tissues, which are the most important sites of host-pathogen interactions. TLRs recognize the three-dimensional structure of pathogen associated molecules and are thus useful markers of the innate immune response. Here, we investigated the role of lipopolysaccharides (LPS) and monosodium urate (MSU) crystals in the activation of the TLR and Nod Like Receptors (NLR) pathways in human keratinocytes. Analysis of the inflammasome compounds revealed that NLRP3 and TLR4, both of which are components of

inflammasome complexes involved in the activation of IL-1β, were not expressed in keratinocytes. Transcriptomic analysis showed that combination of both MSU and LPS priming do not elicit significant result compare to MSU treatment that induced the expression of TLR2, IL-6 and IL-8/CXCL8 in the keratinocyte cell line HaCaT. Furthermore, MSU promoted the phosphorylation of Erk1/2 and MAPK14/p38α MAP kinases. We concluded that MSU stimulates a pro-inflammatory response in keratinocytes via MAP Kinase pathway to induce production of IL-8/CXCL8 and TLR2.

Key words

Keratinocytes, Toll like receptors, Inflammation, MAP kinase

Introduction

The skin is the first line of defense against invading pathogens [1]. Its epithelial tissue structure is mainly composed of keratinocytes, which form the largest surface of the body in contact with the outside. Keratinocytes play a prominent role in primary immunity. Moreover, the cross-talk between keratinocytes and fibroblasts is important for the inflammatory process of wound healing and control extracellular matrix homeostasis [2].

The inflammatory process is associated with the activation of cell signaling pathways leading to the release of various mediators including cytokines and chemokines, which in turn leads to tissue remodeling, wound healing and skin repair. One intracellular signaling pathway associated with the inflammatory process is the NLRP3 inflammasome, which is characterized by the activation and the secretion of IL-1β [3,4].

Toll-like receptors (TLR) are pattern recognition receptors (PRR) directed against highly conserved molecular patterns called pathogen-associated molecular patterns (PAMPs). Ten functional TLRs targeting specific structures (lipids, proteins, nucleic acids or crystals) have been identified in humans [1,5]. In monocytes, TLRs activate several signaling pathways such as MAP kinases, leading to the production of several cytokines, namely TNF, IL-1β, IL-6 and IL-8/CXCL-8. Keratinocytes express these cytokines constitutively in very low amounts in resting conditions and their production is increased upon stimulation [5]. TLR4 constitutes the main receptor of LPS [6]. Several studies have found that TLR4 is expressed on keratinocytes [7] whereas others have not [8]. Others still have found that TLR4 expression depends on the stage of keratinocyte differentiation [9,10], which is correlated with the presence or absence of the inflammasome in keratinocytes.

Several inflammasome pathways have been described, including the canonical pathway which consists of three main effectors, namely NLRP3, the pro-caspase-1 and the apoptosis speck-like protein containing a CARD (ASC) adapter, the later bridging the interactions between NLRP3 and the pro-caspase-1. Assembly of the NLRP3 inflammasome leads to the cleavage of cytosolic pro-IL-1β into the mature pro-inflammatory cytokine IL-1β by activated caspase-1 [4]. Monosodium uric (MSU) crystals activate the inflammasome via the ASC adapter protein, leading to the activation of caspase-1 by the complex, which cleaves the immature form of pro-IL-1β into an active form[3].

We previously reported that the combination of MSU crystals with LPS activates the NLRP3 inflammasome in human macrophages and results in the production of IL-1β [11]. In this study, we investigated the mechanisms of production of pro-inflammatory cytokines from keratinocytes activated with MSU, notably the involvement of NLR-inflammasome, TLR and MAP kinase pathways.

Results

Synthesis of pro-inflammatory cytokines from keratinocytes stimulated with MSU and LPS

MSU treatment significantly increased the abundance of IL-1β, IL-6 and IL-8/CXCL8 mRNA in both HEK and HaCaT cells (Figure 1). Up-regulation of the pro-IL-1β gene suggests that keratinocytes may produce activated IL-1β through the inflammasome signaling pathway. To investigate the involvement of the inflammasome in keratinocytes, cells were stimulated by LPS, as TLR4 agonist, in association with MSU. Nevertheless, LPS priming before MSU stimulation or LPS alone did not resulted in IL-1β expression (data not shown), suggesting that keratinocytes are unable to produce IL-1β despite of the increase of mRNA transcript.

In contrast, MSU treatment significantly induced the production of IL-6 and IL-8/CXCL8 proteins in keratinocytes whereas LPS alone did not (Figure 2). Moreover, LPS priming before MSU treatment did not further boost IL-6 and IL-8/CXCL8 expression. The glucocorticoid dexamethasone significantly impaired the production of both IL-6 and IL-8/CXCL8 in MSU and LPS plus MSU-stimulated cells.

Investigation of TLR and NLR inflammasome pathways in keratinocytes

In order to identify signaling pathways involved, we further examined the mRNA expression of various Nod-like (NLRP1, NLRP2, NLRP3, NLRP6, NLRC4, AIM2) and toll-like receptors (TLR 1 to 10) in keratinocytes (Figure 3). We used THP-1 macrophages as a comparative positive control because these cells are described to express high levels of various TLR and NLR inflammasomes [4,12].

The expression of TLR2 was moderately expressed in both HEK and HaCaT but was significantly up-regulated after MSU treatment. Others TLRs were moderately or not at all expressed in both HEK and HaCaT. For example, TLR4 is not expressed in primary keratinocytes and barely detectable in the HaCaT cell line.

We did not detect NLRP3 mRNA in keratinocytes suggesting that the NLRP3-inflammasome pathway is not involved in the activation of keratinocytes. NRLP6, NLRC4 and AIM2 mRNA were barely detectable whereas NLRP1 and NLRP2 mRNA were present at higher levels (Figure 3).

Involvement of the MAP kinase pathway in MSU stimulation

To investigate the signaling pathways associated with MSU activation, we used a proteome profiler array to analyze the status of 43 kinase phosphorylation sites in keratinocytes stimulated with MSU and in unstimulated control cells. MSU significantly induced the phosphorylation of ERK1/2 and MAPK14/p38α, both of which are involved in the MAP kinase pathway. In contrast, the phosphorylation of WNK1 kinase was significantly down-regulated by MSU) (Figure 4a).

Then, we also analyzed the time course of MAPK14/p38α phosphorylation in MSU-stimulated keratinocytes by western blotting, using unstimulated keratinocytes as a control. MAPK14/p38α phosphorylation appears to be maximum after 45 minutes of MSU treatment, whereas total MAPK14/p38α showed no variation (Figure 4b).

Involvement of the MAP kinase pathway in the production of IL-8

To confirm the involvement MAPK14/p38α and ERK1/2 in HaCaT, we used selective inhibitors of these kinases (Figure 5). U0126 is a potent and selective inhibitor of MEK1 and MEK2 which blocks intracellularly the phosphorylation and activation of ERK [13] and SB239063 is a MAPK14/p38α inhibitor selective for the p38 α and β isoforms [14]. Both inhibitors (U0126 and SB239063) significantly reduced the production of IL-8/CXCL8 by HaCaT Cells stimulated with MSU (Figure 5).

Gene expression profiling of keratinocytes stimulated with MSU

To investigate the mecanisms involved in the inflammatory process in keratinocytes by MSU, we analyzed the gene expression profile of HaCaT cells incubated for 12 h with MSU at 1mg/ml (Figure 6). 12h was determined as an optimal time point for gene expression analysis by qPCR analysis (data not shown). We selected 12h rather than 24h to obtain a moderate inflammation response. 24h of MSU stimulation corresponds to the mRNA maximun expression of cytokines expression (IL-6 & CXL8/IL8) but could decrease other proteins of interests.

Gene expression analysis resulted in the modulation of 128 genes ($P < 0.005$, fold change > 2), including 91 up-regulated and 37 down-regulated genes.

Gene ontology analysis revealed that various family of genes up-regulated by MSU treatment were involved in inflammation, including cytokine of the CCL family (5 genes) and chemokines of the CXCL family (7 genes). Consequently of pro inflammatory conditions, keratinocytes response leads to keratinocytes differentiation by producing S100 proteins family (S100A8, S100A9, S100A12) and small proline-rich protein involved in cross-bridging proteins for the keratinocytes differentiation (SPRR1A, SPRR2A, SPRR2C, SPRR2D, SPRR2D, SPRR2E, SPRR2F) (Error! Reference source not found.).

The second family of genes of interest is involved in response to MSU includes innate response genes as defensin (defensin, beta 4A) and gene associated to Fas- or tumor necrosis factor type alpha-induced apoptosis immediate early response genes (IER3). Of note, IERS and some other proteins including dual specificity phosphatase (DUSP6) are known to be involved in phosphorylation for ERK1/2 proteins [15,16] (Error! Reference source not found.).

Discussion

Keratinocytes are the main cellular constituents of the skin epidermis and are an important line of defense against pathogens. When activated, they participate of the inflammatory process through the increased production of several cytokines and/or chemokines [17]. In this study, we investigated how different signaling pathways related to innate immunity are involved in this production. We used the activation of HEK and HaCaT keratinocyte cells by MSU as an experimental model. Indeed, various studies showed that MSU is important for the induction of pro-inflammatory responses and monocyte recruitment, leading to the secretion of large amounts of IL-6 and IL-8/CXCL8 proteins [24-26].

LPS has been largely reported to be a potent activator for the production of IL-1β from macrophages [21]. In the present study, we found that LPS neither stimulates nor potentiates the production of IL-1β from keratinocytes. However, it is still unclear whether keratinocytes synthesize IL-1β. Some studies have detected IL-1β protein in these cells [22,23] whereas others found that although keratinocytes express IL-1β, they do not produce its active form [24]. Given that the production of IL-1β depends on the NLRP3-inflammasome pathway, we further explored the involvement of this signaling pathway in keratinocytes stimulated with MSU. Interestingly, by investigating the expression of various NLR-inflammasomes, we observed that NLRP3 was not expressed in keratinocytes, whereas NLRP1, NLRP2, NLRP6, NLRC4 and AIM2, were moderately expressed.

To investigate why IL-1 β protein is not produced in keratinocytes and to gain insight into the role of the innate immune response and LPS, we analyzed the mRNA levels of the 10 functionally active TLRs in humans in the HaCaT cell line and HEK human keratinocyte progenitors stimulated with MSU. TLR1, TLR2, TLR3 and TLR5 were expressed in both HaCaT and HEK cells. TLR4 and TLR10 were specifically expressed in HaCaT cells whereas TLR6 was specifically expressed in HEK cells. The remaining TLR transcripts were not detected in either cell types. These results suggest that TLR2 can form heterodimers with TLR1 to recognize triacyl lipopeptide, and to a lesser extent with TLR6 to recognize diacyl lipopteptide [25], in keratinocytes. These heterodimers can recognize many lipopeptides from the cell wall of bacteria and homodimers of TLR2 can recognize bacterial peptidoglycan and some heat shock proteins [26]. We found that TLR7, TLR8, and TLR9 are not expressed in keratinocytes, suggesting that these cells are not able to recognize single stranded RNA or CpG from bacteria or viruses [10]. The function of TLR10 is still unclear. This receptor was only expressed in HaCaT cells and was significantly down-regulated by MSU stimulation. Nevertheless, our results confirm new observations linking the up-regulation of TLR2 to the anti-inflammatory properties of TLR10 [27]. Our finding that keratinocytes express TLR3 confirms previous results showing that these cells are able to sense genetic material from necrotic cells, including UV-damaged keratinocytes [28]. These results are consistent with previous *in vivo* observations showing that TLR2 is up-regulated in response to pro-inflammatory stimuli in psoriatic skin [7,29]. Recent studies imply a role for TLRs in wound healing and the maintenance of skin barrier homeostasis, and demonstrate that TLR2 stimulates the formation of tight junctions [30]. TLR3 and TLR4 may also promote wound healing [31–33]. These mechanisms remain poorly understood but may involve the hyperproliferation of keratinocytes associated with the up-regulation TLR2 in psoriatic skin [29].

Thus, we observed that the main components of the NLRP3-inflammasome and TLR4, are not expressed in primary keratinocytes and are moderately expressed in HaCaT cells. These data are consistent with the absence of the production of IL-1β from keratinocytes in our study and are not consistent with the proposal of the involvement of NLRP3-caspase 1 pathway in keratinocytes [22,34].

We also clearly showed that following MSU stimulation, HEK and HaCaT cells release IL-6 and IL-8/CXCL8. Both proteins are strongly up-regulated in skin during pro-inflammatory disorders such as psoriasis, and lead to leucocyte migration and keratinocyte proliferation and differentiation in the upper level of the epidermis [35,36]. Interestingly, the gene expression profiling showed that several cytokines were also up-regulated by MSU. A similar cytokine expression profile is observed in skin lesions, such as psoriatic skin, to which both Th2 and Th1 cells are frequently recruited [37,38].

It remains unclear how MSU is recognized by the cell and leads to cytokine release [39]. One study [40] suggests that TLRs are able to recognize the crystal structure of MSU. Based on the Proteome Profiler™ Array results, we found that MSU selectively leads to the phosphorylation of two MAPkinase proteins: MAPK14/p38α and ERK1/2. This observation suggests that these two kinases are involved in the recognition of the MSU and their activation leads to the up-regulation of TLR2 as occurs in several skin disorders for the recognition of microbial pathogens [5,41]. This mechanism is similar to that reported for Poly(I:C), a TLR3 agonist that stimulates IL-8/CXCL8 production in keratinocytes [10,42]. We then used selective inhibitors to confirm the involvement of MAPK14/p38α and ERK1/2 in IL-8/CXCL8 expression. These inhibitors impaired IL-8/CXCL8 expression in response to MSU, suggesting the involvement of ERK1/2 and MAPK14/p38α in this process as described previously in monocytes [43]. The inflammasome-independent pro-inflammatory NF-kB signaling pathways in keratinocyte proliferation and differentiation has been largely demonstrated [29,44,45].

Here we have demonstrated that alternative pathway involving the MAPkinase ERK1/2 or MAPK14/p38α are also involved in keratinocyte inflammatory response. Inhibition of MAPkinase ERK1/2 or MAPK14/p38α did not completely abolish IL-8 synthesis, which confirms the involvement of additional signaling pathway including NF-KB signaling. This data are in correlation with genes profile expression results, several genes coding for proteins involved in the ERK1/2 phosphorylation pathway including DUSP6 and IERS3 [15,16] are upregulated upon MSU stimulation.

In conclusion, we showed that MSU may induce the production of IL-6 and IL-8/CXCL8 proteins and up-regulates TLR2 in keratinocytes. However, we failed to detect IL-1β protein in keratinocytes, although MSU stimulated the production of IL-1β mRNA. MSU also up-regulated the phosphorylation of ERK1/2 and MAPK14/p38α proteins in the MAP Kinase pathway, which in turn stimulated the production of IL-6 and IL-8/CXCL8. Our results are consistent with *in vivo* observations in psoriasis [29], this disease is associated to keratinocytes hyper proliferative activity, keratinocytes differentiation, cytokines expression, and TLR2 up-regulation.

Material and methods

Cell culture

Normal human keratinocytes (HEK) were obtained from Cellntec (Bern, Switzerland) and grown in CnT-57 PCT Epidermal Keratinocyte Medium supplemented with bovine pituitary extract (Cellntec, Bern, Switzerland). HaCaT cells were obtained from Cell Lines Service (Eppelheim, Germany) [46], and were grown in DMEM (PAN biotech, Aidenbach, Germany) supplemented with 10% FBS (Life Technology, Grand Island, NY, USA), L-glutamine and non-essential amino acids. Cells and supernatant were harvested in 24-well plates after stimulation for 24 h with either lipopolysaccharide (LPS) tlrl-3pelps LPS from E. coli O111:B4 (Invivogen, Toulouse, France) at 1µg/ml

or MSU (MonoSodium Urate) at 1mg/ml in endotoxin free conditions. MSU was obtained as described previously [3] and optimal concentration was determined at 1000µg/ml without cytotoxicity. Cells were also stimulated with dexamethasone at 10µM in serum-free conditions (Sigma, Saint-Quentin Fallavier, France). Signaling pathway inhibitors were tested in the HaCaT cell line. HaCaT were incubated for 2 h with either the MEK inhibitor U0126 monoethanolate (3µM, 5µM) or the MAPK14/p38α inhibitor SB239063 (20µM, 10µM), and were then incubated for 24 h with MSU at 1mg/ml [47]. THP-1 cells were purchased from American Type Culture Collection (Manasass, VA, USA.) and maintained in RPMI 1640 medium supplemented with 10% FCS, 50 UI/mL penicillin, 50 µg/mL streptomycin, 2mM L-glutamine and 1mM sodium pyruvate at 37°C and 5% CO_2 in a humidified incubator. THP-1 cells were differentiated by 10ng/ml of Phorbol-myristate-acetate (PMA) for 3 days, followed by 24 h without PMA in RPMI 1640 medium supplemented with 2% FCS.

RT-qPCR

Total RNA was extracted with the RNeasy Plus Mini Kit Qiagen and quantified with the nanodrop sytem (Wilmington, DE, USA). RT-PCR was performed with 1µg of RNA that had been reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Life Technology, Grand Island, NY, USA). The SYBR Green PCR Master Mix from Life Technology (Grand Island, NY, USA) was used for all reactions. The expression of the gene of interest was normalized to that of the housekeeping gene GAPDH.

Gene expression profiling

Transcriptomic analysis was performed as described previously [48]. The cells were incubated with MSU (1mg/ml) for 12 h. RNA was extracted from cells at 80% confluence (n=3) with the QIAzol Lysis Reagent RNeasy kit (Qiagen) and 200ng of total RNA was transcribed and labeled *in vitro* using the Agilent Low-Input QuickAmp Labeling kit (Agilent Technologies) in the presence of Cy3-CTP

according to the manufacturer's protocol. After purification using the RNeasy mini kit (Qiagen), the yield and specific activity of cRNA were determined using a NanoDrop Spectrophotometer (yield, 6.2±0.85µg cRNA; specific activity, 13.46±1.59 pmol Cy3/µg cRNA).

An equal amount (600ng) of Cy3-labeled cRNA was fragmented and subsequently hybridized for 18 h onto human SurePrint G3 8x60K pangenomic microarrays (Agilent Technologies), which were then washed and scanned according to the manufacturer's instructions (Agilent Technologies). Gene expression data were further processed using Feature Extraction (version 10.7) and GeneSpring (version 11.0) software (Agilent Technologies). Filtration of array data resulted in the selection of 24,198 non-flag positive and significant gene features. Inter-array normalization was performed using the 75th percentile signal value. The results of MSU-treated and control samples were compared with a t-test (p>0.05 and fold change FC>2). Modular and Singular Enrichment Analysis was performed from GeneCodis analysis [49–51]

Microarray data are publicly available from the gene expression omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo; GSE65931).

ELISA

Cytokines were quantified by ELISA with the Human DuoSet from R&D systems (Minneapolis, MN), for, IL-6 (DY206) and IL-8 (DY208) according to the manufacturer's protocol.

Western blotting

Cells were collected and washed with cold PBS. Phospho-p38α (#9211) antibody and total-p38α (#9212) antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Cells were harvested in lysis buffer (ref: 895561, R&D Systems, Minneapolis, USA). The Micro BCA Protein Assay

Kit was purchased from Pierce (Rockford, IL, USA). Acrylamide gel NuPAGE® Novex® 4-12% Bis-Tris Midi Protein Gels (Life technology, Grand Island, NY, USA) and Nitrocellulose Trans-Blot® Turbo™ Mini membranes (Biorad, Hercules, CA, USA) were used.

Human Phosphoprotein Array

Cells were harvested at 10^7cells/ml after 45 min incubation with MSU. Protein was quantified with the BCA Protein Assay Kit from Pierce (Rockford, IL, USA). A total of 700µg of whole cell lysate was applied to the phosphoprotein array according to the manufacturer's instructions (Proteome Profiler Human Phosphokinase Array kit, R&D Systems, Minneapolis, USA).

Statistical analysis

Experiments were performed in triplicate. Results are presented as the mean ± STDV. Comparisons between the experimental and control conditions were performed with a Mann Whitney test.

For transcriptomic analysis, comparisons were made with an unpaired t-test. The cut off fold change was 2 with a *p* value of 0.05.

Conflict of Interest

The authors state no conflict of interest.

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Figures legends

Figure 1 : Expression of pro inflammatory cytokines in keratinocytes (HEK and HaCaT). Cells were incubated in serum-free medium for 24 h with MSU (1mg/ml) or medium alone (DMEM control). The abundance of IL-1β, IL-6 and IL-8/CXCL8 mRNA was analyzed by RT-qPCR. The mean ± STDV of three experiments is shown. * p<0.05 compared to control.

Figure 2: Effects of dexamethasone on the production of IL-6 and IL-8/CXCL8 from HaCat cells treated with LPS and MSU and both LPS+MSU. HaCaT cells were treated for 24 h with LPS (1µg/ml), MSU (1mg/ml) or with LPS for 24 h followed by MSU and dexamethasone (10µM) for another 24 h. Cells were cultured in serum-free conditions. The mean ± STDV of three experiments is shown. IL-6 and IL-8/CXCL8 were measured by ELISA, * *p*<0.05 compared to control, α *p*<0.05 compared to MSU.

Figure 3: Comparison of TLR and NLR gene expression in HEK and HaCat keratinocytes and THP-1 macrophages. a) The amount of mRNA detected by qPCR is indicated as follows: (–) no detectable expression or Ct value above 32, (+) normal expression between 28 and 32 Ct, and (++) under 28 Ct. RT-PCR for TLRs and NLRs in HaCaT and HEK, THP-1 was used as a positive control. b) HEK and HaCaT were stimulated for 24 h with MSU at 1mg/ml. The abundance of each TLR mRNA was quantified by qPCR and compared with that in control cells. Only detectable mRNA is shown on graphs. The mean ± STDV of three experiments is shown. * *p*<0.05 compared to control.

Figure 4: Effect of MSU on the phosphorylation status of various kinases involved in key signaling pathways in HaCaT and kinetics of MAPK14/p38α phosphorylation. a) Cells were incubated for 45 min in serum-free medium with MSU (which corresponds to the time at which MAPK14/p38α phosphorylation is maximal). Human phosphoprotein arrays were then probed with cell extracts. The relative signal intensity is expressed as a percentage of the unstimulated control. b) Cells were incubated at different time points and activation was stopped in lysis buffer. MAPK14/p38α phosphorylation after MSU treatment was compared to that in control conditions and normalized to total MAPK14/p38α.The mean ± STDV of three experiments is shown. * *p*<0.05 compared to control.

Figure 5: Inhibitors of MEK1/2 (U0126 monoethanolate) and MAPK14/p38α (SB 239063) significantly impair IL-8/CXCL8 production in response to MSU in HaCaT cells. Cells were incubated in serum-free conditions for 2 h in the presence of the MEK1/2 inhibitor U0126 or the MAPK14/p38α inhibitor SB 239063 and then incubated without MSU (Vehicle 1) or 24 h with MSU at 1mg/ml (vehicle 2). IL-8/CXCL8 was quantified by ELISA. The mean ± STDV of three experiments is shown. * $p<0.05$ compared to control. α $p<0.05$ compared to treatment.

Figure 6 : Gene expression profiling of keratinocytes (HaCaT) incubated for 12 h with MSU (1mg/ml). a) Hierarchical clustering analysis of genes differentially expressed between MSU and control (CTRL) samples. Each column represents a gene. b) Gene ontology analysis classifies the differentially expressed genes according to their function. The categories containing the largest numbers of up-regulated genes are protein binding, receptor, chemokine and cytokine activity. Up-regulated genes included TLR2 (included in protein binding and receptor activity categories), IL-8/CXCL8 (chemokine activity) and IL-1β (cytokine and growth factor activity). The pie chart shows the main families of up-regulated genes.

Table 1: Deposition of Microarray Data

Genes up-regulated by MSU in keratinocytes					
GeneSymbol	**FCAbsolute**	***p*-value**	**EntrezGeneID**	**RefSeqAccession**	**GeneName**
CCL2	13.43511	0.008603666	6347	NM_002982	chemokine (C-C motif) ligand 2
STEAP1	2.6475887	0.0032068007	26872	NM_012449	six transmembrane epithelial antigen of the prostate 1
ABCA12	2.4267173	0.0023810326	26154	NM_173076	ATP-binding cassette, sub-family A (ABC1), member 12
ANKK1	3.1858988	0.0027284503	255239	NM_178510	ankyrin repeat and kinase domain containing 1
BCL3	2.0136309	0.013269338	602	NM_005178	B-cell CLL/lymphoma 3
BIRC3	5.319158	0.017337652	330	NM_001165	baculoviral IAP repeat containing 3
CCL17	4.020964	0.011919154	6361	NM_002987	chemokine (C-C motif) ligand 17
CD47	2.023733	5.98002E-4	961	NM_198793	CD47 molecule
CD83	2.6760464	0.04108729	9308	NM_004233	CD83 molecule
CH25H	2.6119664	0.004160655	9023	NM_003956	cholesterol 25-hydroxylase
CXCL1	3.6461887	0.0015183772	2919	NM_001511	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CXCL1	4.473043	2.130726E-4	2919	NM_001511	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CXCL2	4.432413	0.002278508	2920	NM_002089	chemokine (C-X-C motif) ligand 2
CXCL5	8.463041	0.010513073	6374	NM_002994	chemokine (C-X-C motif) ligand 5
CXCL6	6.2538757	0.0051556756	6372	NM_002993	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
CYB5R2	2.0837421	0.023170486	51700	NM_016229	cytochrome b5 reductase 2
DEFB4A	2.678952	0.039558947	1673	NM_004942	defensin, beta 4A
DUSP5	2.2878127	0.012084142	1847	NM_004419	dual specificity phosphatase 5
DUSP6	2.4933317	0.0012751031	1848	NM_001946	dual specificity phosphatase 6
FOSL1	2.4588754	0.0061446475	8061	NM_005438	FOS-like antigen 1
GFPT2	8.238503	0.0051870644	9945	NM_005110	glutamine-fructose-6-phosphate transaminase 2
GOLGA7B	2.2784798	0.00750335	401647	NM_001010917	golgin A7 family, member B
HAS3	2.03934	0.01052052	3038	NM_005329	hyaluronan synthase 3

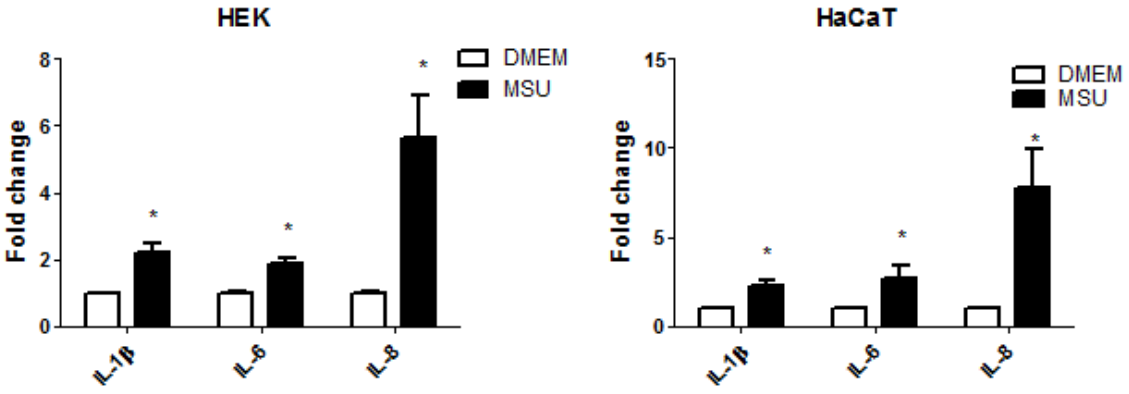
HBEGF	2.5357227	9.93069E-4	1839	NM_001945	heparin-binding EGF-like growth factor
HDAC9	2.717065	3.5141406E-4	9734	NM_014707	histone deacetylase 9
HIVEP2	2.0187995	8.480973E-4	3097	NM_006734	human immunodeficiency virus type I enhancer binding protein 2
HSD17B2	3.5992804	0.0015635884	3294	NM_002153	hydroxysteroid (17-beta) dehydrogenase 2
ICOSLG	2.4058344	0.0059850607	23308	unknown	inducible T-cell co-stimulator ligand
IER3	2.3516538	8.420515E-5	8870	NM_003897	immediate early response 3
IER3	2.3281276	1.4141662E-5	8870	NM_003897	immediate early response 3
IFNGR1	2.2897298	0.0029388058	3459	NM_000416	interferon gamma receptor 1
IL1B	2.6368616	0.005424856	3553	NM_000576	interleukin 1, beta
IL23A	2.043089	5.9619884E-4	51561	NM_016584	interleukin 23, alpha subunit p19
IL32	4.6174574	0.020101381	9235	NM_001012631	interleukin 32
IL32	3.2542193	0.036551133	9235	NM_001012633	interleukin 32
IL4I1	17.166391	0.0027565965	259307	NM_172374	interleukin 4 induced 1
IL7R	2.157746	0.031783726	3575	NM_002185	interleukin 7 receptor
IL8	14.576107	3.232352E-4	3576	NM_000584	interleukin 8
INHBA	2.505979	0.0014575184	3624	NM_002192	inhibin, beta A
IRS1	2.120465	0.031377267	3667	NM_005544	insulin receptor substrate 1
ITGAM	2.1192982	0.015472453	3684	NM_000632	integrin, alpha M (complement component 3 receptor 3 subunit)
KIAA1462	2.6853669	5.274683E-4	57608	NM_020848	KIAA1462
KIAA1462	2.047554	0.020300413	57608	NM_020848	KIAA1462
KLHDC7B	2.7646654	0.017606499	113730	NM_138433	kelch domain containing 7B
KLHDC7B	2.7886536	0.007835362	113730	NM_138433	kelch domain containing 7B
LIF	2.3029518	0.006888382	3976	NM_002309	leukemia inhibitory factor (cholinergic differentiation factor)
LOC645638	2.9905028	0.04061634	unknown	NR_030732	WDNM1-like pseudogene
LOC645638	3.2154202	0.026736347	unknown	NR_030732	WDNM1-like pseudogene
MMP9	3.5994515	0.0070882635	4318	NM_004994	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
NFE2L3	2.3559108	0.0401264	9603	NM_004289	nuclear factor (erythroid-derived 2)-like 3
NFKBIA	2.7625787	0.0016015236	4792	NM_020529	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NMB	2.5604427	0.019079503	4828	NM_021077	neuromedin B
NTRK2	2.6577046	0.003528536	4915	NM_001018064	neurotrophic tyrosine kinase, receptor, type 2
OLR1	2.9886293	1.4390222E-4	4973	NM_002543	oxidized low density lipoprotein (lectin-like) receptor 1
PAPPA	2.906035	0.004017578	5069	NM_002581	pregnancy-associated plasma protein A, pappalysin 1
PCOLCE2	2.1458278	0.014328724	26577	NM_013363	procollagen C-endopeptidase enhancer 2
PI3	4.9683604	0.0043469253	5266	NM_002638	peptidase inhibitor 3, skin-derived
PRDM1	2.207393	0.015570803	639	NM_001198	PR domain containing 1, with ZNF domain
PRRX2	2.2362597	0.003084618	51450	NM_016307	paired related homeobox 2
RELB	5.127107	0.001143309	5971	NM_006509	v-rel reticuloendotheliosis viral oncogene homolog B
S100A12	3.8538802	1.8653568E-4	6283	NM_005621	S100 calcium binding protein A12
S100A8	5.116005	0.005851642	6279	NM_002964	S100 calcium binding protein A8
S100A9	4.026237	1.7029754E-4	6280	NM_002965	S100 calcium binding protein A9
SAA3P	2.1914887	0.0069663706	6290	NR_026576	serum amyloid A3 pseudogene
SERPINB4	2.2102022	0.04723509	6318	NM_002974	serpin peptidase inhibitor, clade B (ovalbumin), member 4
SERPINE2	2.0049095	0.03960766	5270	NM_006216	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
SLC5A1	2.242327	0.025657842	6523	NM_000343	solute carrier family 5 (sodium/glucose cotransporter), member 1
SLC6A14	5.537535	0.043936387	11254	NM_007231	solute carrier family 6 (amino acid transporter), member 14
SOCS3	2.0151772	0.0036186043	9021	NM_003955	suppressor of cytokine signaling 3

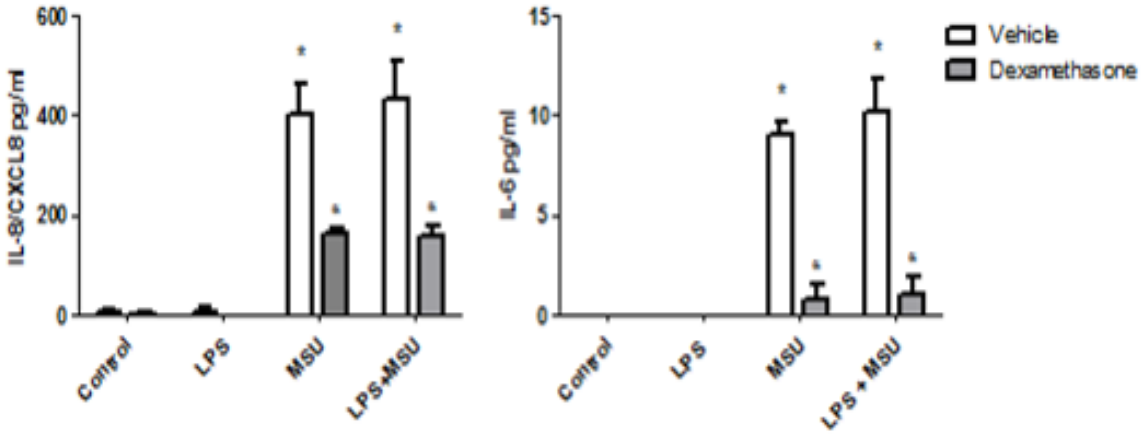
SPRR1A	4.8986077	0.008468201	6698	NM_005987	small proline-rich protein 1A
SPRR2A	11.498696	0.0037288724	6700	NM_005988	small proline-rich protein 2A
SPRR2A	11.735891	0.0023093529	6700	NM_005988	small proline-rich protein 2A
SPRR2C	11.968722	0.0027338644	6702	NR_003062	small proline-rich protein 2C (pseudogene)
SPRR2D	12.43513	0.0022396913	6703	NM_006945	small proline-rich protein 2D
SPRR2D	2.6497638	0.036530297	6703	NM_006945	small proline-rich protein 2D
SPRR2E	12.462624	0.002826207	6704	NM_001024209	small proline-rich protein 2E
SPRR2F	9.693043	0.0040087244	6705	NM_001014450	small proline-rich protein 2F
STEAP1	2.771142	0.0062769824	26872	NM_012449	six transmembrane epithelial antigen of the prostate 1
STEAP1B	2.5698094	0.007341518	unknown	NM_001164460	STEAP family member 1B
TLR2	2.434586	9.984365E-4	7097	NM_003264	toll-like receptor 2
TMEM171	2.066541	0.023592334	134285	NM_173490	transmembrane protein 171
TNFAIP3	5.844732	2.09863E-5	7128	NM_006290	tumor necrosis factor, alpha-induced protein 3
TNFRSF6B	2.1609578	0.025099022	8771	NM_003823	tumor necrosis factor receptor superfamily, member 6b, decoy
TRIB2	2.4692175	0.026945762	28951	NM_021643	tribbles homolog 2 (Drosophila)
TRIB2	2.3218746	0.006010035	28951	unknown	tribbles homolog 2 (Drosophila)
XDH	2.2054226	0.009534106	7498	NM_000379	xanthine dehydrogenase
XLOC_005478	3.5730193	0.027137967	unknown	unknown	unknown
XLOC_014418	2.0016391	0.009228317	unknown	unknown	unknown
XLOC_l2_015752	2.202458	0.0329782	unknown	XR_132685	unknown
ZC3H12A	2.9859571	0.0014432394	80149	NM_025079	zinc finger CCCH-type containing 12A
ZC3H12C	2.8214753	0.002250323	85463	NM_033390	zinc finger CCCH-type containing 12C

Genes down-regulated by MSU in keratinocytes

GeneSymbol	FCAbsolute	p-value	EntrezGeneID	RefSeqAccession	GeneName
ADRA2C	2.3994887	0.034613658	152	NM_000683	adrenergic, alpha-2C-, receptor
ATP6V0A4	2.4191291	0.027209284	50617	NM_020632	ATPase, H+ transporting, lysosomal V0 subunit a4
ATP8B3	2.3210068	0.014976682	148229	NM_138813	ATPase, aminophospholipid transporter, class I, type 8B, member 3
BNIPL	2.259942	0.035787836	149428	NM_138278	BCL2/adenovirus E1B 19kD interacting protein like
C17orf109	2.564169	0.010708096	643008	NM_001162995	chromosome 17 open reading frame 109
CALML5	2.0325718	6.106207E-4	51806	NM_017422	calmodulin-like 5
CCDC80	2.4860718	0.031756032	151887	NM_199511	coiled-coil domain containing 80
CDKN1C	2.023164	0.0075730374	1028	NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CLIC3	2.0375786	0.015674096	9022	NM_004669	chloride intracellular channel 3
DSG4	2.1074948	0.0029668862	147409	NM_177986	desmoglein 4
FAM171B	2.0474072	0.01053821	165215	NM_177454	family with sequence similarity 171, member B
FLJ13197	2.01631	0.024708144	79667	NR_026804	uncharacterized FLJ13197
INHBB	2.071415	0.008051887	3625	NM_002193	inhibin, beta B
KCNH2	2.4417326	0.007148539	3757	NM_000238	potassium voltage-gated channel, subfamily H (eag-related), member 2
LDHD	2.2502933	0.009157377	197257	NM_153486	lactate dehydrogenase D
LDLRAD1	3.0002525	0.01176602	388633	NM_001010978	low density lipoprotein receptor class A domain containing 1
LDLRAD1	3.090969	0.009562125	388633	NM_001010978	low density lipoprotein receptor class A domain containing 1
LEFTY1	2.435346	0.0109418295	10637	NM_020997	left-right determination factor 1
LMO1	2.1862698	0.042113435	4004	NM_002315	LIM domain only 1 (rhombotin 1)
LOC100129363	2.0230477	0.00538203	100129363	unknown	uncharacterized LOC100129363
LOC100505478	2.6196442	0.031026466	unknown	NM_001199233	uncharacterized protein LOC100505478

LOC100505664	2.4712846	0.02371401	unknown	XR_109653	uncharacterized LOC100505664
LOC283050	2.3637078	0.0034261094	283050	NR_024431	uncharacterized LOC283050
METTL7B	2.1271703	0.044615388	196410	NM_152637	methyltransferase like 7B
MYZAP	2.1682537	0.021380391	100820829	NM_001018100	myocardial zonula adherens protein
PLA2G10	2.1331096	0.04945346	8399	NM_003561	phospholipase A2, group X
PLA2G10	2.086816	0.042921778	unknown	NM_003561	phospholipase A2, group X
PPP1R1B	2.7082868	0.0019258258	84152	NM_032192	protein phosphatase 1, regulatory (inhibitor) subunit 1B
RASL11B	2.1184278	0.0031235833	65997	NM_023940	RAS-like, family 11, member B
SLC12A3	2.9377286	0.033505958	6559	NM_000339	solute carrier family 12 (sodium/chloride transporters), member 3
TMEM229B	2.003447	0.049651455	161145	NM_182526	transmembrane protein 229B
VWF	2.3352153	0.009994977	7450	NM_000552	von Willebrand factor
WFDC11	2.189715	0.008648113	259239	NM_147197	WAP four-disulfide core domain 11
XLOC_007832	2.0569808	0.03749021	unknown	unknown	unknown
XLOC_011305	2.3527958	0.0050483444	unknown	unknown	unknown
XLOC_011305	3.131674	0.0070146034	unknown	unknown	unknown
XLOC_011951	2.1209884	0.037107155	unknown	unknown	unknown





a)

TLR & NLR	HEK	HaCaT	Macrophages (THP-1)	Primers (5'->3')
TLR1	+	++	++	F: TCACAGTGTCTGGTACACGC R: CCAACTCAGTAAGGTGCCCA
TLR2	+	+	++	F: TCTGTAGCAACTGGCTTAGTTCA R: TGGCCACAGAGGAGTCTCTTA
TLR3	+	++	+	F: ACATCCCTGAGCTGTCAAGC R: AGAGTTCAAAGGGGGCACTG
TLR4	-	+	++	F: CAGGATGATGTCTGCCTCGC R: TTAGGAACCACCTCCACGCA
TLR5	+	++	++	F: TCATCATGGTGGTGGTTGGG R: GATCCTCAGGCCACCTCAAA
TLR6	+	-	++	F: GAGTGTTTGCTGGAGTCCGA R: GCCAACCTTTCACCTCCTGA
TLR7	-	-	++	F: CCTTGTGCGCCGTGTAAAAA R: GGGCACATGCTGAAGAGAGT
TLR8	-	-	++	F: CATGGAAAACATGTTCCTTCAGTC R: TCTTCGGCGCATAACTCACA
TLR9	-	-	+	F: ACTTCTTCCAAGGCCTGAGC R: GGCCAGGTAATTGTCACGGA
TLR10	-	+	+	F: TTGCTTTTGCCACCAACCTG R: AGGCCAGACTCAATACCCTCT
NLRP1	++	++	+	F: GGACTGACGATGACTTCTGG R: ATCACAAAGCAGAGACCCG
NLRP2	++	++	++	F: TCGGGTTGGTGTCTTGTTC R: CAACTTAGCACCCTCATCCAG
NLRP3	-	-	++	F: GTGTTTCGAATCCCACTGTG R: TCTGCTTCTCACGTACTTTCTG
NLRP6	+	+	++	F: TCTTCATCCACTCTTTCAGGC R: CTCAGAAAGGTCTCGGCAG
NLRC4	+	+	+	F: CAGTCCCCTCACCATAGAAG R:TCAAGTTACCCAAGCTGTCAG
AIM2	+	+	++	F: TGAAACCCCGAAGATCAACAC R: CCCAGTACTTCCATTTTCCCAG

b)