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Vigilant Keratinocytes Trigger PAMP Signaling in Response to Streptococcal M1 Protein

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Author contribution: S T Persson performed the research, analyzed the data and wrote the paper. L Wilk performed the research and analyzed the data, M Mörgelin contributed by performing experiments and H Herwald supervised the study.

ABSTRACT

The human skin exerts many functions in order to maintain its barrier integrity and protect the host from invading microorganisms. One such pathogen is *Streptococcus pyogenes*, which can cause a variety of superficial skin wounds that may eventually progress into invasive deep soft tissue infections. Here we show that keratinocytes recognize soluble M1 protein, a streptococcal virulence factor, as a PAMP to release alarming inflammatory responses. We found that this interaction initiates an inflammatory intracellular signaling cascade involving the activation of mitogen-activated kinases, ERK, p38 and JNK, and the subsequent induction and mobilization of the transcription factors NF- κ B and AP-1. We also determined the imprint of inflammatory mediators released, such as IL-8, GRO α , MIF, EMMPRIN, IL-1 α , IL-1Ra, and ST2 in response to streptococcal M1 protein. The expression of IL-8 is dependent on TLR2 activity and subsequent activation of the MAP kinases ERK and p38. Notably this signaling seems distinct for IL-8 release and it is not shared with the other inflammatory mediators. We conclude that keratinocytes participate in a proinflammatory manner in streptococcal pattern recognition and that expression of the chemoattractant IL-8 by keratinocytes constitutes an important protective mechanism against streptococcal M1 protein.

Keywords: *Streptococcus pyogenes*, skin infection, innate immunity, keratinocytes, M1 protein, interleukin-8, mitogen-activated protein kinase (MAPK), pathogen-associated molecular pattern (PAMP)

INTRODUCTION

In order to cause infection, invasive microorganisms have to surmount protective barriers such as skin, respiratory mucosa, or the gastrointestinal tract. Among these potential ports of entry, the skin is the largest integumentary organ. Notably, the skin is constantly colonized, not only with commensals but sometimes also with pathogenic bacteria (1), and thus special precaution is needed to prevent their invasion. This is indeed a difficult task and because of their large size, phagocytosing cells cannot continuously patrol the entire skin surface and scan for intruders. Therefore the skin has to rely on sophisticated alert systems sending inflammatory signals that in turn evoke the recruitment of neutrophils, macrophages, and other immune cells. Keratinocytes are the most prevalent cell type of the skin and they are equipped with such alert systems (2, 3). Among these, Toll-like receptors (TLRs) play an important role in the early host response to an invading pathogen. Once activated, TLRs can trigger a number of immune reactions such as the secretion of chemotactic cytokines (4) and mobilization of antimicrobial peptides (5, 6). The TLR arsenal in keratinocytes is extensive and it has been found that these cells express functional TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9 (7, 8).

The Gram-positive bacterium *Streptococcus pyogenes* (Group A Streptococcus) is an important human pathogen causing a variety of superficial infections of the skin (impetigo and erysipelas) and throat (pharyngitis) (9). Under rare and unfortunate circumstances, these complications can lead to severe life threatening systemic infections such as necrotizing fasciitis and streptococcal toxic shock syndrome, often associated with high morbidity and mortality (10-12). To cause these conditions, *S. pyogenes* has evolved a panel of secreted and surface bound virulence factors that enable the bacteria to effectively infect the human host. Among these factors, M and M-like proteins are probably the best-characterized streptococcal virulence determinants. Due to their high abundance, these proteins are used to classify different serotypes and currently over 200 have been identified (13). M and M-like proteins are composed of two polypeptide chains that form an alpha-helical coiled-coil structure, which can interact with a number of plasma proteins such as factor H, fibrinogen, and serum albumin. It is worth mentioning that M1 protein is normally anchored to the bacterial cell wall, but it can also be released from the bacterial

surface as seen for instance in patients with necrotizing fasciitis (14). The release can occur endogenously or by the action of host-derived proteinases (14, 15). Once released, M1 protein can cause systemic inflammatory reactions by targeting neutrophils, monocytes, and T cells (14, 16-18). Interestingly, the interaction of M1 protein with these cells involves different receptors. While the interaction with neutrophils is mediated by crosslinking β_2 integrins (14), M1 proteins binds to TLR2 on monocytes (16) and to T-cell receptors on T-cells (18). These interactions can cause a number of inflammatory reactions such as the mobilization of heparin binding protein, a potent inducer of vascular leakage and sepsis biomarker (19), and various pro-inflammatory and Th1-type cytokines (14, 16-18). Though it has been shown that the release of these proteins contributes to the pathology of an infection, it has not been reported whether the release of M1 protein in proximity to the site of infection can act as a pathogen associated pattern molecule (PAMP) that helps to alert the innate immune system.

In this study we investigated if keratinocytes react immunologically to M1 protein, released from the bacterial surface. We further wished to gain insight into the molecular mechanisms by which these cells respond to streptococcal infection. Our data show that when keratinocytes encounter M1 protein, it induces an inflammatory intracellular pathway resulting in the release of pro-inflammatory mediators. Together our findings suggest that the recognition and pro-inflammatory response of keratinocytes to this streptococcal surface protein is an important step in alarming the host defense about the intruder and to prevent further development into a severe infection.

MATERIALS AND METHODS

Cell culture and reagents

HaCaT cells, a spontaneously immortalized human keratinocyte cell line, were cultured in serum-free Keratinocyte-SFM without calcium chloride (Invitrogen, Carlsbad, CA) and were supplemented with 25 µg/ml bovine pituitary extract, 0.2 ng/ml epidermal growth factor and 1x Gibco's 100X Antibiotic-Antimycotic (Life technologies, Carlsbad, CA) (20). The M1 protein was purified according as previously described (21). Briefly, an isogenic AP1 mutant strain expressing an M1 protein lacking the cell-wall-anchoring region was used. Consequently, the streptococcal protein accumulates in the growth medium, from which it was purified based on its high affinity to human fibrinogen. M1 protein is the only fibrinogen-binding protein of the AP1 strain (15, 22) and after purification no other contaminants have been detected (16). All other streptococcal proteins (protein M4, protein M5, protein M22, and protein SIC) were purified as described earlier (23-25). For Western blot analysis, antibodies to phospho-ERK1/2, -RSK1, -JNK, -p38, and IκBα were purchased from Cell Signaling Technologies (Beverly, MA). TLR blocking antibodies, PAb Control, PAb hTLR2 and PAb hTLR4, were purchased from Invivogen (San Diego, CA). The ERK-, p38- and JNK inhibitors, FR180204, SB202190, and SP600125, respectively, was purchased from Sigma Aldrich (St. Louis, MO, USA)(26). A pre-incubation with blocking antibodies and inhibitors were used before M1 protein was added to the cell-antibody/inhibitor supernatants.

RNA isolation and microarray analysis

Total cellular RNA was isolated from cultured HaCaT cells using GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich, St Louis, MO) according to the manufacturer's instructions. Cells were seeded into 12-well plates and treated with 5 µg/ml M1 protein for 6 h or left untreated before isolation. Gene expression analyses were performed at the SCIBLU Genomics Centre, Lund University, Sweden. Briefly, purity and concentration was determined with an ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE) and integrity was determined using a 2100 bioanalyzer (Agilent

Technologies Inc., Santa Clara, CA). Microarray experiments were performed in triplets; three control samples and three M1 protein stimulated samples. Gene expression was analyzed using the Human Gene 2.0 ST array (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Affymetrix chip and experimental quality analyses were performed using Expression Console software v1.1.2 (Affymetrix) applying the Robust Multichip Average normalization method. Genes were considered differently expressed when fold change was either ≥ 1.6 (up-regulated) or ≤ 0.6 (down-regulated) and a p-value below 0.0001. The data received for this analysis have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE61993 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61993>).

Western blot analysis

Cells were lysed at 4 °C, 30 min, in Lysis Buffer 6 (R&D Systems, Minneapolis, MN). Total protein concentrations in lysates were measured with ND-1000 spectrophotometer (Nanodrop Technologies Inc.). Equal amounts of total protein were loaded onto 10 % SDS-polyacrylamide gels in sample buffer. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA) and blocked with Tris-buffered saline containing 0.1 % Tween-20 and 5 % nonfat dry milk. Membranes were incubated with the appropriate primary antibody overnight at 4 °C followed by incubation with horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. Blots were developed by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA) and visualized with ChemiDocTM MP Imaging System (Bio-Rad Laboratories, Hercules, CA).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from 8×10^6 cells and lysed using Pierce Nuclear and Cytoplasmic Extraction Reagent kit (Thermo Fisher Scientific). EMSA were performed using Pierce LightShift Chemiluminescent EMSA Kit according to the manufacturer's instructions. Briefly, AP-1 or NF- κ B

consensus oligonucleotide probes (AP-1; 5'-CGC TTG ATG AGT CAG CCG GAA-3', NF- κ B; 5'-AGT TGA GGG GAC TTT CCC AGC C-3') were end-labeled with biotin (Invivogen, San Diego, CA). Reaction mixtures consisted of 10 μ g nuclear extract in binding buffer and the probe-protein complexes were separated by a 6 % polyacrylamide gel before electrotransferred onto a 0.45 μ m Biodyne B nylon membrane (Thermo Fisher Scientific). Membranes were cross-linked using ChemiDoc MP Imaging System (Bio-Rad Laboratories) with UV-light for 10 min. Probes were detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific) and visualized with ChemiDoc MP Imaging System (Bio-Rad Laboratories).

Human XL Cytokine Array

Keratinocyte culture supernatants were analyzed with Proteome Profiler Human XL cytokine array (R&D Systems) according to the manufacturer's protocol. Cells were seeded in 12-well plates to 70-80 percent confluency, and then either treated with 5 μ g/ml M1 protein or left untreated for 24 h. Supernatants were collected and centrifuged at 500x g for 5 min. Membranes were incubated with 400 μ l of the supernatant overnight at 4 °C. Chemiluminescence was detected by using ChemiDocTM MP Imaging System and the mean pixel density was analyzed by Image Lab 4.1 (Bio-Rad Laboratories).

ELISA

Cytokine levels in the keratinocyte culture supernatants were measured by sandwich ELISA (DuoSet ELISA Development kit, R&D Systems) according to the manufacturer's protocol. Briefly, wells were incubated overnight at 4 °C with 100 μ l capture antibody diluted in coating buffer. Wells were blocked with coating buffer containing 0.5 % bovine serum albumin. Standards and samples were added to wells diluted in assay buffer and then incubated with detection antibody diluted in assay buffer. Wells were incubated with streptavidin conjugated to horseradish-peroxidase before visualization of the antibody-binding by adding 100 μ l 3,3',5,5'-tetramethylbenzidine (TMB) solution and the reaction was stopped by the addition of 1.8 M H₂SO₄. The absorbance was measured with a microplate reader, Viktor3 Model

1420 (Perkin Elmer, Waltham, MA) at 450 nm. All measurements were performed in doublets. Data are presented as percent cytokine level compared to M1-induced cytokine expression.

Immunoelectron microscopy

HaCaT cells were incubated with M1 protein for 30 min at 37 °C. For transmission electron microscopy, samples were embedded in Epon resin, sectioned and subjected to antigen retrieval with metaperiodate as recently described in detail (27). Sections were labeled with rabbit anti-M1 protein and rat anti-TLR2 followed by gold-conjugated goat-anti rabbit (5 nm) and goat-anti rat (10 nm) antibodies. Samples were observed in a Philips/FEI CM 100 transmission electron microscope at the Core Facility for Integrated Microscopy, Panum Institute, University of Copenhagen.

Statistical analysis

All data are expressed as mean \pm SD from at least three independent experiments. Differences were assessed by Student t-test. A probability of $p < 0.05$ was considered significantly different. Analyses were performed using statistical software GraphPad Prism version 6.0a for Mac OS X, (GraphPad Software Inc., La Jolla, CA).

GEO Series accession number:

GSE61993 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61993>).

RESULTS

M1 protein triggers an inflammatory response in human keratinocytes

In order to study the role of M1 protein in evoking an inflammatory response in human keratinocytes, RNA extraction and microarray analysis were performed following 6 h of incubation with M1 protein. The data received from this analysis have been deposited in NCBI's Gene Expression Omnibus (28) and are accessible through GEO Series accession number GSE61993 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61993>). The analysis revealed 498 differently expressed genes, of which 304 genes were up- and 194 genes down-regulated. Having a closer look at up-regulated genes with a function in inflammation, we found 18 genes coding for proteins that are involved in intracellular signaling. Out of those, *TLR4*, *TRAF4*, *TICAM1*, and *MYD88* are parts of the TLR and IL-1R pathways (Table 1). In addition, we identified a panel of genes that act downstream of these signaling networks and function as transcription factors in the AP-1 (*FOSB*, *FOS*, *JUN*, *JUND*, *JUNB*) or NF- κ B (*REL*, *NFKB1*, *NFKB2*) complexes. Other genes involved in inflammation and intracellular signaling code for transcription factors (*CEBPB*, *CEBPD*, *CITED4*), bradykinin receptor (*BDKRB1*), as well as the plasminogen activator and its receptor (Table 1). Together, these findings suggest that M1 protein is capable to trigger inflammatory signaling cascades in human keratinocytes.

Once activated, keratinocytes are known to release a number of soluble factors involved in cell proliferation, host defense, or inflammation (29-31). Table 2 provides a summary of 20 up-regulated genes of effector molecules that are produced upon stimulation with M1 protein. The expression of genes coding for interleukin-8 (IL-8) and chemokine c-c motif ligand 20 (CCL20) were increased as much as 32.9 and 20.2 fold, respectively, but also a considerable rise for *CXCL1* and *CXCL3* was noted. The four genes code for chemokines that are known for their ability to attract immune cells including neutrophils, lymphocytes and monocytes (32-34). Other genes that were found up-regulated are *TNFAIP*, *IL1RL1*, *CSF2*, *IL1A*, *IL1B*, and *IL1F9*, which are important in either inducing or modulating an inflammatory environment. Finally, we identified a third group of genes, namely *HAS2*, *CTGF*, *HBEGF*, *IL24*, *IL20*, *RNASE7*, *VEGFA*, *F2RL1* and *TGFA*, that have a more direct effect on wound healing and the location of

an infection, rather than supporting immune responses (Table 2). The remaining genes that were up-regulated are mainly coding for transcription factors with unknown or undefined targets, while most of the down-regulated genes identified play a role in cell cycle regulation, cell replication, metabolic processes, and transcription of regulatory genes. Altogether, the microarray analysis revealed that stimulation of keratinocytes with M1 protein leads to multiple intracellular signaling events and induction of inflammatory reactions.

M1 protein triggers MAPK activation and subsequent AP-1 and NF- κ B transcription factor activity

TLR signaling has been described to activate MAPKs and NF- κ B transcription factor activity in order to induce inflammatory responses (35). As our microarray analysis also revealed increased expression of AP-1 and NF- κ B subunits, downstream targets of MAPKs, we wished to study if stimulation of keratinocytes by M1 protein leads to MAPK-activation. Thus, whole cell lysates from M1 protein activated keratinocytes were subjected to Western blot analysis using antibodies against the phosphorylated state of extracellular signal-regulated kinases (ERK), downstream ribosomal r6 kinase (RSK), p38 MAP kinase (p38), and Jun N-terminal kinase (JNK) (Fig.1a). GAPDH was used as a loading control and non-stimulated keratinocytes to determine background phosphorylation. The results show a fast activation of ERK, especially ERK2, already 30 min after M1 protein exposure. RSK activation was also noted after 30 min, however, in contrast to ERK1/2, the signal continued to rise for up to 1 h. This was followed by p38 activation after 1 h and an activation of JNK where phosphorylation was detected after 2 h. Together, these findings suggest that activation of MAPKs is highly regulated and has a specific order to orchestrate their executive functions.

Activation and translocation of the transcription factor NF- κ B from the cytoplasm into the nucleus is an important step in the TLR signaling pathway. This is initiated by phosphorylation, ubiquitination and subsequent degradation of the NF- κ B inhibitor, I κ B α . Indeed, we observed that approximately 2 h after stimulation with M1 protein I κ B α starts to disappear, pointing to activation of NF- κ B (Fig. 1b).

AP-1 is also a transcription factor and, like NF- κ B, induced upon TLR signaling. To follow the mobilization of the two transcription factors into their target destination, electrophoretic mobility shift assays (EMSA) were carried out. The EMSA results show binding of both AP-1 and NF- κ B consensus-binding sequence probes, demonstrating the presence and DNA binding activity of both AP-1 and NF- κ B inside the nucleus in keratinocytes 2-4 h of stimulation with M1 protein (Fig.2). In summary, the data show that stimulation with M1 protein triggers a chain of signaling events in keratinocytes that eventually leads to the translocation and activation of transcription factors inside the nucleus.

Determination of the cytokine imprint from keratinocytes exposed to M1 protein

In a next series of experiments we characterized the cytokine response to M1 protein-activated keratinocytes. We used the human XL cytokine array kit, which allows the simultaneous measurement of 102 different cytokines, chemokines, and growth factors. Keratinocytes were incubated with M1 protein or left untreated. After 24 h incubation, supernatants were collected and subjected to semiquantitative cytokine analysis. Figure 3a-b shows that 17 secreted proteins were identified of which 10 were up-regulated upon stimulation. For further characterization we focused only on those proteins with a major function in inflammation. This group of proteins includes IL-8, growth related oncogene-alpha (GRO α), macrophage migration inhibitory factor (MIF), extracellular matrix metalloproteinase inducer (EMMPRIN), IL-1 α , IL-1Ra, and ST2. The secretion of these seven proteins was further confirmed by ELISA. We also included TLR2 and 4 blocking antibodies to investigate the role of TLR signaling for the induction of these cytokines (Fig.4a). The expression of IL-8 was significantly decreased when the inhibitors were used (TLR4-inhibitor $p=0.0080$, TLR2-inhibitor $p<0.0001$), especially by blocking TLR2. The experiments also show that treatment with the TLR blocking antibodies seems to slightly up-regulate the mobilization of some cytokines such as IL-1 α , GRO α , and MIF. Although some of the observed increases are significant, this does not necessarily need to have a biological impact.

Based on these findings we next investigated whether M1 protein is able to interact with TLR2 on the surface of keratinocytes. Figure 4b depicts immune electron micrographs of HaCaT cells that were

stimulated with M1 protein. Indeed, immuno-staining revealed that M1 protein and TLR2 co-localize, corroborating TLR2-dependence of IL-8 release.

In order to study if the MAPK signaling pathway is involved in IL-8 release, we also employed a panel of MAPK inhibitors. Figure 5a shows that an ERK-inhibitor (FR180204) and a p38-inhibitor (SB202190) significantly block the release of the chemokine ($p < 0.0001$). A combination of both inhibitors did not show further down-regulation of IL-8 secretion. When testing a JNK-inhibitor, SP600125, no effect on IL-8 release was seen (data not shown). Interestingly, no significant effect on the secretion pattern was observed for the other 6 proteins analyzed (GRO α , MIF, EMMPRIN, IL-1 α , IL-1Ra, and ST2) upon ERK- and p38-inhibition, except for GRO α levels which were lowered in the presence of the p38-inhibitor (p38-inhibition $p = 0.0271$, ERK- and p38-inhibition $p = 0.0372$). Finally, we tested whether the release of IL-8 is restricted to stimulation with M1 protein or if other M proteins are also capable of inducing its release from keratinocytes. Therefore, we stimulated cells with M1, M4, M5 and M22 protein, while protein SIC, a non M- or M-like soluble streptococcal protein, served as control. Figure 5b depicts that in addition to M1 protein, M5 protein was also able to stimulate the cells, while M4 and M22 protein as well as protein SIC had no effect. Taken together, these data show that the secretion of IL-8 is dependent on TLR signaling and MAPK pathways, summarized in Figure 6, while other inflammatory mediators may utilize other pathways.

DISCUSSION

Streptococcal skin infections are often initiated by adherence of the bacteria to epidermal cells. This can eventually provoke local inflammatory reactions, which then can lead to suppurative lesions. The clinical spectrum of the resulting cutaneous and soft tissue infections is broad and ranges from localized impetigo to deeply invasive necrotizing fasciitis. As to which extend these conditions will develop into more systemic complications, depends on the depth the bacteria reach within the tissue (11, 36). Though much effort has been undertaken to unravel the bacterial strategies employed to spread and become more invasive, the molecular mechanisms involved are still far from being completely understood.

Streptococcal M- and M-like proteins are important virulence determinants that interact not only with cell types such as neutrophils, monocytes, and endothelial cells, but also with keratinocytes (16, 37-39), which were the focus of this study. Already in 1995, it was described that M and M-like proteins are responsible for bacterial adherence to keratinocytes by binding to membrane cofactor protein (CD46) (39). Later in 2003, it was reported that this interaction is also important for bacterial entry into epithelial cells (40). However, whether this leads to an induction of host responses was not addressed in these studies.

In the present paper we examined the effect of soluble M1 protein on human keratinocytes to show that the protein is able to trigger multiple immunologic responses in these cells (summarized in Figure 6). It should be noted that the M1 serotype is considered one of the most invasive streptococcal serotypes (41) and soluble M1 protein is a potent inducer of inflammation. Once the protein has been released from the bacterial surface, it can cause systemic reactions such as severe pulmonary damage, before the bacteria have entered the circulation (14). These previous findings suggest that soluble M1 protein contributes significantly to pathological complications distal to the primary site of infection. To our knowledge, the present study is the first to show that M1 protein can also act as a PAMP, thereby evoking innate immune responses. Interestingly, we observed that M5 protein can cause a similar response than M1 in keratinocytes when the effect of other M proteins was tested. Since the M5 serotype is also regarded as an invasive strain (42), this points to a stronger response by the host defense to such strains.

The immune responses following M1 exposure involve the activation of the TLR signaling pathway, through MAPKs and the transcription factors NF- κ B and AP-1. The effects of this activation include the keratinocytic production and release of pro-inflammatory mediators such as IL-8, GRO α , MIF, IL-1 α , IL-1Ra, and ST2. The M1 protein has been described to interact with TLR2 on human peripheral monocytes in order to trigger cytokine release (16), but the cell type specific cytokine imprint in response to M1 protein seems very different.

Particularly, IL-8 and GRO α signaling have been described to play an important function in recruiting neutrophils to the site of infection or inflammation (43, 44). Interestingly, our data point to a special role for IL-8, because the chemokine is not only the most up-regulated gene upon stimulation with M1 protein, but it also utilizes a distinct signal pathway via the TLRs and MAPKs, ERK and p38, that was not shared with the other inflammatory mediators tested. M proteins have been described to bind the membrane cofactor protein CD46, although the receptor has not been described to be involved in cytokine production. It is therefore not likely that CD46 is responsible for the other inflammatory mediators that are released upon stimulation with M1 protein. One could speculate that the production and release of these mediators is a consequence of autocrine signaling from pre-stored vesicles containing IL-1 α or TNF α to their receptors, IL-1R and TNFR respectively (30), however, additional experimental support is needed to confirm this hypothesis.

Previous work by Hoffmann and colleagues has shown that the three MAPKs (ERK, p38, and JNK, respectively) are important for generating maximum IL-8 expression (45). In our study, inhibition of ERK and p38 significantly reduced the IL-8 expression in M1 protein stimulated keratinocytes and similar findings have been made when gastric epithelial cells were treated with *Helicobacter pylori* (46). However, JNK, a c-JUN activating MAPK (47), had no effect on the generation of IL-8 in our experiments and also others have reported similar findings such as Bennett and colleagues who, in 2001, described that JNK is not involved in IL-8 expression in lipopolysaccharide stimulated human monocytes (48).

Our results further show that the two MAPKs ERK and p38 are activated in a time-space manner, but since a combination of both inhibitors did not further decrease the IL-8 expression, they might share the same downstream targets for IL-8 induction. We noted that p38 inhibition had the strongest effect on IL-8 expression. This could be due to p38-related phosphorylation of both AP-1 and NF- κ B, which leads to transcriptional activation (49, 50), whereas ERK-phosphorylation of AP-1 only has a stabilizing effect (51) and no direct activity on NF- κ B has been described. ERK can initiate NF- κ B signaling by activation of RSK- or MSK-related phosphorylation of the NF- κ B inhibitor, I κ B α (52, 53). In addition, p38 has been found to have a stabilizing effect on mRNA for IL-8 (54), which could provide another explanation for our results. Our findings further implicate that streptococcal M1 protein induces highly regulated and complex signaling networks through different MAPKs, triggering pro-inflammatory actions in keratinocytes such as IL-8 release.

In this study we demonstrate that keratinocytes recognize M1 protein as a PAMP to alert the innate immune system by releasing alarming inflammatory mediators. To this end, we examined the interaction between keratinocytes and streptococcal surface protein M1 and determined the immunologic response of these cells when encountering the bacterial protein. The data presented thus far suggest a keratinocytic release of inflammatory mediators most likely designated for the recruitment of neutrophils. Although it remains to be clarified how critical IL-8 signaling, together with the other inflammatory mediators released, is for preventing severe streptococcal skin infections, our data suggest that neutrophil attraction through IL-8 signaling could be an important protection keratinocytes exert in response to streptococcal M1 protein.

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Conflict of interest

The authors state no conflict of interest.

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Table 1. Increased gene expression of inflammatory signal transducer receptors and intracellular signaling in keratinocytes in response to streptococcal M1 protein¹

Gene	Gene name	FC ²	Function
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	7.3	AP-1 transcription factor complex
FOS	FBJ murine osteosarcoma viral oncogene homolog	5.8	AP-1 transcription factor complex
JUN	jun proto-oncogene	4.2	AP-1 transcription factor complex
TLR4	toll-like receptor 4	2.7	Recognition and activation of innate immunity
JUND	jun D proto-oncogene	2.5	AP-1 transcription factor complex
REL	c-REL, v-rel reticuloendotheliosis viral oncogene homolog (avian)	2.5	NF-κB transcription factor complex
TRAF4	TNF receptor-associated factor 4	2.4	Mediate signal transduction of IL-1R/Toll-like and TNF receptors
JUNB	jun B proto-oncogene	2.3	AP-1 transcription factor complex
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	2.3	Transcription factor with inflammatory responses
PLAUR	plasminogen activator, urokinase receptor	2.3	The receptor of urokinase, plasminogen activator
TICAM1	toll-like receptor adaptor molecule 1	2.0	Mediate signal transduction of Toll-like receptors
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1.9	NF-κB transcription factor complex (p105, p50)
MYD88	myeloid differentiation primary response gene (88)	1.9	Essential signal transducer of the interleukin-1 and Toll-like receptors
BDKRB1	bradykinin receptor B1	1.8	The receptor of bradykinin
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	1.7	Transcription factor with inflammatory responses
PLAU	plasminogen activator, urokinase	1.7	A serine protease converting plasminogen to plasmin and degrading extracellular matrix
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	1.7	NF-κB transcription factor complex (p100, p52)
CITED4	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	1.7	Transcriptional co-activator

¹Microarray Analysis

²FC: Fold change between M1 induced and control mRNA expression

Table 2. Increased gene expression of inflammatory mediators in keratinocytes in response to streptococcal M1 protein¹

Gene	Gene name	FC ²	Function
IL8	Interleukin 8	32.9	Induces chemotaxis in target cells, primarily neutrophils
CCL20	Chemokine (C-C motif) ligand 20, MIP3A	20.2	Strongly chemotactic for lymphocytes but can also attracts neutrophils
HAS2	Hyaluronan synthase 2	17.7	Produce hyaluronan molecules
TNFAIP	Tumor necrosis factor, alpha-induced protein 3	10.6	Anti-inflammatory protein
CXCL1	Chemokine (C-X-C motif) ligand 1, (GRO α)	10.0	Neutrophil chemoattractant activity
CTGF	Connective tissue growth factor	9.8	Matricellular protein important for wound healing
HBEGF	Heparin-binding EGF-like growth factor	9.5	Membrane-anchored chemotactic mitogen
IL1RL1	Interleukin 1 receptor-like 1, (ST2)	7.5	The receptor of interleukin 33
IL24	Interleukin 24	5.5	Cytokine of the IL-10 family, important for wound healing
IL20	Interleukin 20	5.4	Regulates proliferation and differentiation of keratinocytes during inflammation
CSF2	GM-CSF, colony stimulating factor 2	4.9	Cytokine that controls the production, differentiation, and function of granulocytes and macrophages
RNASE7	Ribonuclease, RNase A family, 7	4.8	Skin-derived antimicrobial protein 2 (SAP-2) with ribonuclease activity.
IL1B	Interleukin 1, beta	4.7	Proinflammatory cytokine
VEGFA	Vascular endothelial growth factor A	3.7	Mediating increased vascular permeability
IL1A	Interleukin 1, alpha	3.4	Proinflammatory cytokine
IL11	Interleukin 11	3.1	Proinflammatory cytokine
CXCL3	Chemokine (C-X-C motif) ligand 3, (GRO γ)	2.8	Chemotactic for monocytes and neutrophils
F2RL1	Coagulation factor II (thrombin) receptor-like 1, Protease activated receptor 2 (PAR2)	2.7	Modulates inflammatory responses and acts as a sensor for proteolytic enzymes
TGFA	Transforming growth factor, alpha	2.6	Ligand for the epidermal growth factor receptor
IL1F9	Interleukin 1 family, member 9	2.5	Ligand to interleukin 1 receptor-like 2

¹Microarray Analysis

²FC: Fold change between M1 induced and control mRNA expression

FIGURE LEGENDS

Figure 1. MAPK and NF- κ B activation following M1 protein exposure. Lysates from HaCaT cells stimulated with 5 μ g/ml streptococcal M1 protein collected at the indicated time points were analyzed by Western blotting. (a) Phosphorylation of ERK 1/2 at Thr202/Tyr204 and Thr185/Tyr187, RSK at Ser380, p38 at Thr180/Tyr182, and JNK at Thr183/Tyr185. p, phosphorylated. (b) Degradation of I κ B α . Representative blots from three independent experiments are shown. GAPDH was used as a loading control.

Figure 2. M1 protein induces nuclear localization and activation of AP-1 and NF- κ B transcription factors in keratinocytes. The results of EMSAs of nuclear extracts from keratinocytes cultured in the absence or presence of M1 protein (5 μ g/ml) obtained at the indicated time points are shown. Ten-microgram nuclear extracts were analyzed by EMSA using labeled oligonucleotide probes corresponding to the consensus AP-1 DNA binding sequence (a) and to the consensus NF- κ B DNA binding sequence (b). The results shown are representative of those from three independent experiments yielding comparable results.

Figure 3. Cytokine imprint from keratinocytes exposed to M1 protein. Keratinocytes were incubated in the presence or absence of M1 protein (5 μ g/ml) for 24 h. a) Cell supernatants were analyzed using a human cytokine XL array. The numbers correspond to the numbers in the bar graph in panel b. (b) Mean pixel densities were quantified by Image Lab software (v4.1; Bio-Rad Laboratories), and the identities of the respective cytokines are indicated. Dkk-1, Dickkopf/WNT signaling pathway inhibitor 1; FGF-19, fibroblast growth factor 19; PDGF-AA, platelet-derived growth factor, AA chain; PLAUR, plasminogen activator, urokinase receptor; VEGF, vascular endothelial growth factor.

Figure 4. M1 protein-induced release of IL-8 is dependent on TLR2.

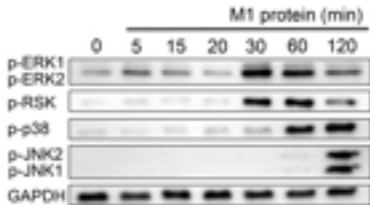
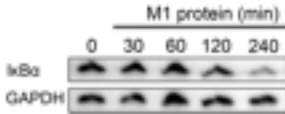
(a) Keratinocytes were pre-incubated with TLR blocking antibodies (5 $\mu\text{g/ml}$) for 30 min and then incubated in the presence or absence of M1 protein (5 $\mu\text{g/ml}$) for 24 h. Cell supernatants were analyzed by ELISA. CTRL, control; AB, antibody. The data represent the average percent \pm SD from at least three independent experiments analyzed by the Student *t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (b) HaCaT cells were incubated with M1 protein for 30 min at 37°C. Cells were then fixed and prepared for transmission electron microscopy. Samples were first labeled with rabbit anti-M1 protein and rat anti-TLR2 followed by gold-conjugated goat anti-rabbit immunoglobulin (5 nm) and goat anti-rat immunoglobulin (15 nm). The colocalization of M1 protein and TLR2 was observed. Bar, 500 nm.

Figure 5. Effect of signal transduction inhibitors and different M-protein serotypes on IL-8 release from keratinocytes.

(a) HaCaT cells were preincubated with MAPK inhibitors (10 μM the ERK-inhibitor FR180204 or 1 μM the p38-inhibitor SB202190, or both) for 1 h or left untreated before the addition of M1 protein. Cell supernatants were collected after 24 h and analyzed by ELISA. The data represent the average percent \pm SD of at least three independent experiments. (b) HaCaT cells were incubated with M1, M4, M5 and M22 proteins and protein SIC (5 $\mu\text{g/ml}$) for 24 h, and the release of IL-8 was determined by ELISA. The data represent the average protein levels \pm SDs from at least three independent experiments, which were analyzed by the Student *t* test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 6. Schematic overview of M1 protein-induced IL-8 expression in human keratinocytes. The binding of M1 protein to TLR2 on keratinocytes induces activation of intracellular myeloid differentiation primary response gene 88 (MyD88). MyD88 is then responsible for transmitting the inflammatory signal by recruiting interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4 together with TNF receptor-associated factor 6 (TRAF6). This complex activates transforming growth factor β -activated kinase

(TAK) and TAK-binding protein (TAB), both of which can activate the MAPK signaling cascade and the NF- κ B pathway. At the end of this chain of events, the localization and promoter binding of AP-1 and NF- κ B trigger the transcription of IL-8 inside the nucleus and its subsequent secretion. IKK, I κ B kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MAPKK, mitogen-activated protein kinase kinase.

a**b**

a

AP-1

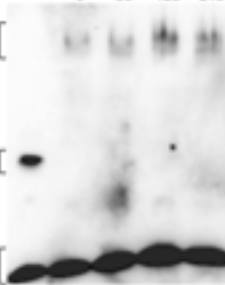
M1 protein (min)

0 30 120 240

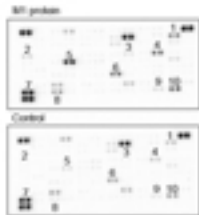
AP-1/probe
complexFree
probe**b**NF- κ B

M1 protein (min)

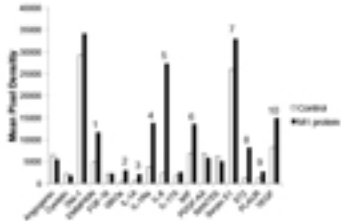
0 30 120 240

NF- κ B/probe
complexProbe
complexFree
probe

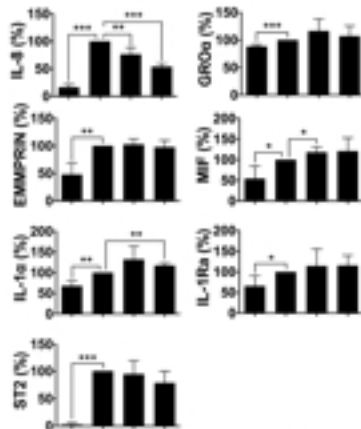
a



b

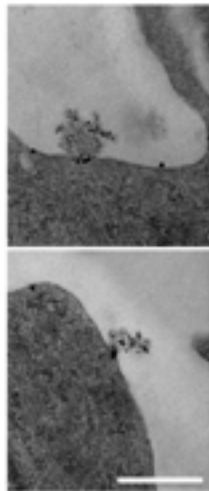


a

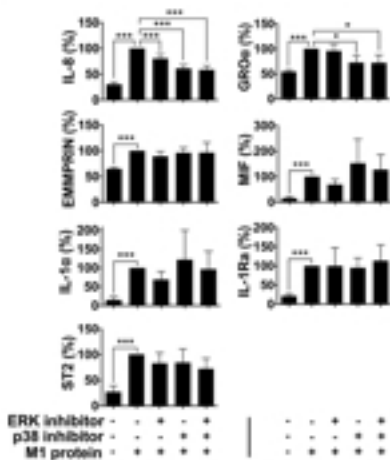


CTRL-AB	-	+	-	-	-	+	-	-
TLR4-AB	-	-	+	-	-	-	+	-
TLR2-AB	-	-	-	+	-	-	-	+
M1 protein	-	+	+	+	-	+	+	+

b



a



b

