Streptococcal protein SIC activates monocytes and induces inflammation

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Highlights

M1 SIC, a secreted S. pyogenes protein interacts with CD14 and TLR2 on monocytes

SIC triggers activation of NF-κB and p38 MAPK pathways and release of TNFα and INFγ

Presence of human plasma enhanced SIC-mediated monocyte activation

M55-SIC homolog has no effect on monocytes

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Article

Streptococcal protein SIC activates monocytes and induces inflammation

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SUMMARY

Streptococcus pyogenes is a major bacterial pathogen in the human population and isolates of the clinically important M1 serotype secrete protein Streptococcal inhibitor of complement (SIC) known to interfere with human innate immunity. Here we find that SIC from M1 bacteria interacts with TLR2 and CD14 on monocytes leading to the activation of the NF-κB and p38 MAPK pathways and the release of several pro-inflammatory cytokines (e.g. TNFα and INFγ). In human plasma, SIC binds clusterin and histidine-rich glycoprotein, and whole plasma, and these two purified plasma proteins enhanced the activation of monocytes by SIC. Isolates of the M55 serotype secrete an SIC homolog, but this protein did not activate monocytes. M1 isolates are common in cases of invasive S. pyogenes infections characterized by massive inflammation, and the results of this study indicate that the pro-inflammatory property of SIC contributes to the pathology of these severe clinical conditions.

INTRODUCTION

Streptococcus pyogenes is a significant pathogen causing a wide range of infections and post-infection sequelae in the human population (Carapetis et al., 2005). M protein is a virulence determinant and a fibrous surface protein in S. pyogenes (for more references, see (Cunningham, 2000)). Based on sequence variation in the NH2-terminal surface-exposed tip of M protein, isolates of S. pyogenes are divided into more than 200 M serotypes, and since the 1980s strains of the M1 serotype have dominated worldwide and are frequently isolated from patients with severe invasive infections causing at least 150,000 deaths annually (Carapetis et al., 2005). The reason for the evolutionary success of the M1 serotype is not clear, but the production of Streptococcal inhibitor of complement (SIC) by M1 strains probably represents a contributing factor.

SIC is a secreted protein which was discovered and isolated from M1 bacteria and found to bind the plasma proteins clusterin and histidine-rich glycoprotein (HRG) and to interfere with the membrane attack complex of complement (Åkesson et al., 1996). The gene encoding SIC is highly polymorphic among M1 isolates and shows variation even during ongoing experimental infection in mice (Hoe et al., 1999), demonstrating a unique degree of adaptation to selective pressure. SIC enhances the ability of M1 bacteria to colonize the mouse mucosal surface after intranasal infection (Lukomski et al., 2000) and blocks the activity of antibacterial proteins and peptides (Fernie-King et al., 2002; Frick et al., 2003; Egesten et al., 2007). In addition, SIC promotes bacterial growth in human blood and virulence in a murine model of systemic infection (Pence et al., 2010). The protein modulates fibrinolysis resulting in enhanced bacterial survival in fibrin clots (Frick et al., 2018). SIC also inhibits the antibacterial activity of histones, and complexes between histones and SIC boost histone-triggered release of cytokines and chemokines in human blood (Westman et al., 2018), further underlining the multiple effects of SIC on innate immunity and the host-bacteria relationship.

Circulating monocytes play an important role in host immune defense against bacterial pathogens. They are rapidly recruited to sites of inflammation and infection where they can differentiate into macrophages and dendritic cells (DCs) or become effector cells with distinct antimicrobial activity (Serbina et al., 2008). Undifferentiated monocytes secrete cytokines when activated by microbial components and following phagocytosis of microbes (Ghebrehiwet et al., 2014); and for this purpose, monocytes carry a broad range of receptors on their surface, which downstream activate various signaling pathways. These microbial pattern-recognition receptors (PRRs) play an important role detecting pathogen-associated molecular patterns, leading to the activation of monocytes and other immune cells (Dale et al., 2008). Toll-like receptors...
Figure 1. Growth medium from *S. pyogenes* secreting SIC activates THP1 cells

(A) Lysates of THP1 cells after incubation with AP1 or ΔSIC growth medium (GM) were subjected to SDS-PAGE and Western blot. The membranes were probed with antibodies against p38 MAP kinase (p38) and phospho-p38 MAP kinase (p-p38). Ctr: medium. Med: concentrated medium.

(B) Band intensity analysis of the Western blot depicted in A. The band intensity of phosphorylated p38 was normalized to the band intensity of p38 MAPK.

(C) TNFa release from THP1 cells incubated with GM from AP1 or ΔSIC was measured by ELISA.

(D) The SIC concentration in AP1 GM was determined by direct ELISA and compared to purified protein.

(E) THP1 cells were incubated for 18 hr with GM from AP1, ΔSIC or ΔSIC + 3 μg/mL purified SIC. Absorbance of QuantiBlue color change as indicator for NF-kB activation was measured at 655 nm.
Figure 1. Continued

(F) Quantitative M5 analysis of purified SIC. Three fractions of purified SIC were digested with trypsin and analyzed with mass spectrometry. The resulting data was searched against the S. pyogenes AP1 proteome and the identified peptides were further analyzed with MS1 precursor intensity-based quantification. The figure shows the top 4 most abundant streptococcal proteins identified in the fractions. The y axis is relative percentage of top4 protein intensity.

All data represent mean ± SEM of 3 independent experiments, one-way ANOVA, Dunnett’s multiple comparison test, with single pooled variance. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(TLRs) are type I membrane proteins with an extracellular leucine-rich domain (Kawasaki and Kawai, 2014), constituting an essential class of PRRs. Within the TLR superfamily, TLR2 has been associated with the recognition of Gram-positive bacteria and their derived products, like M1 protein (Ozinsky et al., 2000; Henneke et al., 2001; Joosten et al., 2003; Pålman et al., 2006; Wu et al., 2016). The activity of TLR2 in response to bacterial products can be elevated by the interaction between TLR2 and glycosphatidylinositol (GPI)-anchored CD14 (Schwandner et al., 1999; Yoshimura et al., 1999), which for instance acts as a co-receptor for LPS and peptidoglycan (Henneke et al., 2001). Signaling via TLR2 might lead to downstream pathways resulting in the release of pro-inflammatory cytokines. The observation that this activity of SIC was enhanced in the presence of plasma or purified plasma proteins known to form complexes with SIC, further support the notion that SIC is an important virulence factor in severe S. pyogenes infection.

RESULTS

SIC in bacterial growth medium triggers activation of NF-κB and p38 MAP kinase

Supernatants from streptococcal cultures were tested on the monocyte THP1 cell line. S. pyogenes strains AP1 and ΔSIC were grown in RPMI +10% FBS and growth media (GM) were collected and added to THP1 cells. The GM from both bacterial strains clearly mediated phosphorylation of the p38 MAP kinase as compared to medium alone (Figures 1A and 1B). However, when band intensities were normalized to the loading control, the GM from the ΔSIC strain was significantly less efficient in activating the MAP kinase (Figure 1B). The release of TNFα was significantly lower when THP1 cells were incubated with the ΔSIC GM as compared to AP1 GM (Figure 1C). Next, the impact of S. pyogenes GM on nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells, NF-κB activation, was analyzed; GM from AP1 triggered the activation, which was only slightly but still significantly higher compared to when THP1 cells were incubated with GM from ΔSIC bacteria (Figure 1E). A direct ELISA was performed to determine the concentration of SIC in the GM from AP1 bacteria. Purified SIC protein at a concentration of 5 μg/mL served as a control (Figure 1D), and the GM from the S. pyogenes AP1 strain was found to contain around 3 μg SIC/ml (Figure 1D). When SIC was added to the GM from ΔSIC bacteria to obtain a concentration of 3 μg/mL, NF-κB activation increased to levels similarly to that of AP1 GM (Figure 1E), indicating that SIC produced and secreted during bacterial growth is responsible for the activation of THP1 cells. Purified SIC was analyzed by quantitative mass spectrometry (MS), revealing an SIC protein content of over 90% in 3 different fractions (Figure 1F).

SIC influences the release of various pro-inflammatory cytokines

When AP1 bacteria is grown to early stationary phase the concentration of SIC in the GM reaches 5 μg/mL (Frick et al., 2003), and this concentration was used in the following experiments. Apart from monocytes, we wanted to analyze the effect of SIC also on the nasopharyngeal epithelial cell line Detroit 562. To exclude cytotoxic effects of SIC on the cells, LDH release was measured (Figure S1A). No detrimental effect was observed in either of the cell types when incubated for 18 hr with 5 μg/mL of SIC in the presence or absence of plasma (Figure S1A). Subsequently, THP1 monocytes and Detroit cells were incubated with SIC alone or in the presence of plasma for 2 hr, and the supernatants were collected and analyzed for cytokine content (Figures 2A–2I). Detroit 562 cells showed no upregulation or downregulation in response to SIC alone or in combination with plasma (data not shown). In contrast, incubation of THP1 cells with SIC caused a significant release of several pro-inflammatory cytokines like IL-1β, IL-2, IL-8, and TNFα (Figures 2A, 2B, 2D, and 2I). SIC-induced cytokine release was significantly enhanced by plasma in case of IL-1β (Figure 2A) and...
TNFα (Figure 2I), while plasma presence slightly reduced the SIC-mediated cytokine release for IL-2, MIP-1α/CCL3, IFNγ, and CCL4 (Figures 2B, 2E, 2F, and 2H).

SIC mediates p38 MAP kinase phosphorylation and triggers NF-κB activation in THP1 monocytes

Next, possible downstream effects of TNFα release and other cytokines was investigated. Secretion of TNFα and its subsequent binding to a cell surface receptor is involved in p38 MAP kinase activation and phosphorylation (Liu et al., 2000). This activation in turn leads to a secondary release of cytokines including TNFα. Thus, THP1 and Detroit cells were incubated with SIC in the presence and absence of plasma and cell lysates were analyzed by Western blotting (Figures 3A–3D). Phosphorylation of p38 MAP kinase in THP1 cells was mediated by SIC alone or by SIC in plasma (Figures 3A and 3B), whereas no increased phosphorylation was observed in the Detroit cells (Figures 3C and 3D). The phosphorylation of p38 MAP kinase can
response to fragment II alone and in the presence of plasma (Figure 4E). To investigate whether the plasma amino acids 33–101, fragment II aa 102–198, and fragment III aa 199–305 (Figure 4A). The combination of the mature secreted SIC protein (amino acids 1–33 correspond to the signal sequence): fragment I covers HRG, but not lysozyme, significantly triggered NF-κB described above, and SEAP expression was analyzed. In combination with fragment II, clusterin and proteins known to interact with SIC were responsible for the enhanced effect of fragment II in combination with plasma, THP1 cells were incubated with fragment II together with the purified plasma proteins, as mentioned, phosphorylation of p38 MAP kinase may result in secretion of pro-inflammatory cytokines like TNFα, but not lysozyme, significantly triggered NF-κB.

**The central region of SIC is responsible for the activation of NF-κB and p38 MAP kinase**

To map the region of SIC responsible for the observed activation of THP1 cells, three recombinant fragments of SIC were expressed and utilized (Fernie-King et al., 2004; Frick et al., 2018). The fragments span the mature secreted SIC protein (amino acids 1–33 correspond to the signal sequence): fragment I covers amino acids 33–101, fragment II aa 102–198, and fragment III aa 199–305 (Figure 4A). The combination of fragment II and plasma was found to mediate phosphorylation of p38 MAP kinase (Figures 4B and 4C). As mentioned, phosphorylation of p38 MAP kinase may result in secretion of pro-inflammatory cytokines like TNFα, and similar to the effect observed for the p38 MAP kinase, fragment II in combination with plasma triggered a significant release of TNFα (Figure 4D). A strong activation of NF-κB was also detected in response to fragment II alone and in the presence of plasma (Figure 4E). To investigate whether the plasma proteins known to interact with SIC were responsible for the enhanced effect of fragment II in combination with plasma, THP1 cells were incubated with fragment II together with the purified plasma proteins, as described above, and SEAP expression was analyzed. In combination with fragment II, clusterin and HRG, but not lysozyme, significantly triggered NF-κB activation (Figure 4F).

**SIC interacts with TLR2 and CD14 on THP1 cells**

The demonstrated interaction between SIC and monocytes raised the question whether SIC, similarly to M1 protein (Valderrama et al., 2017), could be taken up by monocytes. Indeed, live imaging of THP1 cells...
incubated with AlexaFluor 633-labeled SIC protein, showed a pattern indicating surface binding and/or cellular uptake of the protein (Figure 5A). To quantify the uptake/binding, flow cytometry analyses were performed (Figures 5B–5F). As compared to the control, the number of cells positive for SIC suggested binding or uptake of the protein (Figures 5B, 5C, and 5F). Incubation of the cells on ice significantly reduced uptake/binding of SIC (Figures 5D and 5F), whereas pre-incubation with cytochalasin D almost completely abolished uptake/binding (Figures 5E and 5F). The results indicated an interaction between SIC and monocyte surface receptors.

Figure 4. Fragment II of SIC (aa 102–198) is responsible for the activation of THP1 cells
(A) Schematic structure of SIC from strain AP1 and localization of the three fragments used. The signal sequence (Ss) is cleaved off during maturation. The mature secreted protein contains a short repeat region (SRR), as well as three tandem repeats (R1-R3). The numbers indicate amino acid positions, and also refer to the length of each of the SIC fragments (I-III), which are recombinantly expressed in E. coli. Based on previous publications (Fernie-King et al., 2004; Binks et al., 2005), the sizes of the fragments were chosen.
(B) Western blot analyses of THP1 cell lysates after stimulation with 5 μg/mL SIC fragments (I, II, III) +/- 2.5% plasma (P). Membranes were incubated with antibodies against p38 MAPK (p38) and phosphorylated p38 MAPK (p-p38).
(C) Quantification of phosphorylation of Western blots depicted in B. The band intensities of p-p38 samples were normalized to loading control (p38 band) and values analyzed.
(D) THP1 cells were incubated with 5 μg/mL SIC fragments +/- 2.5% plasma (P) and the release of TNFα was measured by ELISA.
(E) Activation of NF-κB by SIC fragments +/- plasma was analyzed by detecting QuantiBlue color change at 655 nm.
(F) Absorbance of QuantiBlue color change at 655 nm, as indicator of NF-κB activation after THP1 cell incubation with 5 μg/mL SIC fragment II alone and in combination with 2.5% whole plasma (P), 5 μg/mL clusterin (Clu), 5 μg/mL lysozyme (Lys) or 200 ng/mL HRG. All data represent mean ± SEM of 4-6 independent experiments, one-way ANOVA, Dunnett’s multiple comparison test, with single pooled variance. *p < 0.05, **p < 0.01, ****p < 0.0001.

incubated with AlexaFluor 633-labeled SIC protein, showed a pattern indicating surface binding and/or cellular uptake of the protein (Figure 5A). To quantify the uptake/binding, flow cytometry analyses were performed (Figures 5B–5F). As compared to the control, the number of cells positive for SIC suggested binding or uptake of the protein (Figures 5B, 5C, and 5F). Incubation of the cells on ice significantly reduced uptake/binding of SIC (Figures 5D and 5F), whereas pre-incubation with cytochalasin D almost completely abolished uptake/binding (Figures 5E and 5F). The results indicated an interaction between SIC and monocyte surface receptors.
Figure 5. Analysis of the interaction between SIC and THP1 cells
(A) THP1 cells were incubated for 45 min with 5 μg/mL AlexaFluor 633-labeled SIC protein (red). Live imaging of the cells was used to observe the interaction of SIC with the THP1 cells. Scale bar represents 20 μm.
(B-E) Flow cytometry analysis of SIC-monocyte interaction. B: THP1 cells incubated with medium (Ctrl) C: THP1 cells incubated with 5 μg/mL SIC (SIC) D: THP1 cells incubated with 5 μg/mL SIC on ice (SIC ice) E: THP1 cells pre-incubated with 20 μM CytD for 30 min, then incubated with 5 μg/mL SIC (SIC CytD).
(F) Median fluorescent intensity analysis of SIC interaction with the cells in B-E. Data shown from 3 independent experiments, *p < 0.05, **p < 0.01.
(G) THP1 cells, untreated or trypsinized, were incubated with 5 μg/mL SIC +/- 2.5% plasma (P). Lysates of the cells were subjected to SDS-PAGE analysis and Western blot. Membranes were probed with antibodies against p38 MAPK (p38) and phosphorylated p38 MAPK (p-p38). Band intensities were analyzed.
(H) TNFα secretion by untreated and trypsinized THP1 cells, incubated with 5 μg/mL by SIC +/- 2.5% plasma (P) was measured by ELISA at 450 nm.
Monocytes, like macrophages and neutrophils, express various receptors on their surface to recognize invading pathogens, e.g., TLR2 or FcγRI (CD64) (Juarez et al., 2010; Bournazos et al., 2016). To remove all surface proteins the THP1 cells were pre-treated with trypsin, and after incubation with SIC in the presence and absence of plasma, cell lysates were analyzed by Western Blotting for p38 MAPK phosphorylation. Trypsin treatment reduced the phosphorylation of p38 MAPK mediated by SIC in the presence of plasma (Figure 5G) and significantly reduced the release of TNFα (Figure 5H), as well as the activation of NF-κB by SIC in the presence and absence of plasma (Figure 5I). The reduction in monocyte responses following trypsin digestion of surface proteins was also recorded for fragment II of SIC in the presence and absence of plasma (Figure 5J). To investigate whether FcγRI (CD64) might be an interaction partner of SIC, the effect of blocking the Fc receptor with IgG and IgG Fc fragments was analyzed. Pre-treatment of the cells with the FcγRI ligands had no impact on SIC-mediated NF-κB activation (Figure 6A). S. pyogenes bacteria as well as M1 protein are known to bind to TLR2 on host cells surfaces (Sigurdardottir et al., 2010). DimORIZATION of TLR2 with a receptor interaction partner induces a signaling cascade involving NF-κB activation and subsequent cytokine release (Shmuel-Galia et al., 2016). To explore the possibility that the binding of SIC to TLR2 leads to activation of NF-κB, this pathway was blocked with a specific antibody against TLR2 (Figure 6B). Pre-treatment with this antibody was found to significantly decrease the activation of THP1 cells when incubated with SIC, both in the presence or absence of plasma (Figure 6B). This was further shown by a reduced phosphorylation of p38 MAP kinase when TLR2 was blocked, in the case of SIC + P (Figure 6C). Mass spectrometry analysis of THP1 cells treated with or without trypsin revealed a decrease of TLR2 peptides in the trypsin treated samples (Figure 6D). It has been reported that macrophages also can be activated by bacterial peptidoglycan in a CD14-dependent fashion (Gupta et al., 1996), and that the interaction of CD14 with TLR2 can lead to a synergistically enhanced activation, triggered by Gram-positive bacteria (Yoshimura et al., 1999). Additionally, Henneke et al. suggested that TLR2 and CD14 act as co-receptors for Group B streptococcal secreted products (Henneke et al., 2001). With this background, the impact of CD14 on SIC-mediated monocyte activation was investigated and blocking of the receptor with specific antibodies completely abolished SIC-mediated NF-κB activation (Figure 6E).

To investigate whether SIC directly interacts with these host surface receptors, we performed surface plasmon resonance analysis. In this set of experiments, we immobilized either TLR2 or CD14 (ligands) on a CM5 chip and used different SIC concentrations as analyte. Binding studies of SIC generated K_D values of 1.29 x 10^{-10} M for TLR2 (Figure 6F) and 2.518 x 10^{-8} M for CD14 (Figure 6G), showing affinity of SIC for both TLR2 and CD14.

**SIC activates primary CD14 + monocytes in a TLR2-dependent fashion**

Finding that SIC from S. pyogenes activated THP1 monocytes, acute monocytic leukemia cells, we sought to investigate the effect of SIC on primary blood derived monocytes. We therefore purified CD14+ monocytes from leukocyte concentrate via density gradient centrifugation and magnetic bead labeling. Similar to the results observed for the THP1 cells, a pre-incubation for 30 min with trypsin strongly reduced the phosphorylation of p38 MAPK triggered by SIC in the presence of plasma (Figures 7A and 7B). Incubation of CD14+ cells with SIC significantly triggered the secretion of TNFα (Figure 7C), which was significantly diminished by the pre-treatment of the cells with trypsin (Figure 7C). To test whether trypsin treatment has any detrimental effect on the cells, LDH release was measured. The 30 min pre-incubation of CD14+ cells with 100 μg/mL trypsin had no effect on the secretion of LDH (Supp. Figure 1B). To analyze a possible involvement of TLR2, the primary cells were incubated with the neutralizing antibody Pab-hTLR. Here, the block of TLR2 significantly reduced the phosphorylation of p38 MAPK mediated by SIC in the presence of plasma (Figures 7D and 7E). Additionally, the TNFα release triggered by SIC was significantly diminished by the neutralizing antibody (Figure 7F).

**Only M1-SIC triggers immune responses in THP1 monocytes**

Distantly related SIC (DRS) homologs are produced by S. pyogenes strains of M serotypes 12 and 55 (Hartas and Sriprakash, 1999) (Hartas and Sriprakash, 1999)[36] and similar to SIC from M1 isolates these homologs...
Figure 6. SIC interacts with TLR2 and CD14 to activate THP1 cells

(A) THP1 cells were pre-incubated for 30 min with IgG1 and Fc fragment and then incubated with 5 μg/mL SIC +/− 2.5% plasma (P). NF-κB activation was analyzed at 655 nm.

(B) Cells were pre-incubated with a neutralizing antibody against TLR2 and then incubated with 5 μg/mL SIC +/− 2.5% plasma (P). Activation of NF-κB was measured at 655 nm.

(C) Quantification of phosphorylation of Western blots of THP1 cells treated with a neutralizing antibody against TLR2. The band intensities of p-p38 samples were normalized to loading control (p38 band) and values analyzed.

(D) Quantitative DIA-MS analysis of THP1 cells before and after trypsin treatment.

(E) THP1 cells were pre-incubated with antibodies against CD14 and then incubated with 5 μg/mL SIC +/− 2.5% plasma (P). NF-κB activation was analyzed.

(F) Surface plasmon resonance data show interaction of different SIC concentrations to immobilized TLR2. Representative original data are displayed in colors, 1:1 Langmuir fitted curves are presented in black.
bind complement components and also compete with SIC for this binding, but in contrast to SIC they do not activate complement (Binks and Sriprakash, 2004). To investigate if a DRS had similar effects on monocytes as M1-SIC, DRS was purified from the growth medium of an S. pyogenes MSS strain. The purified DRS showed no cytotoxicity on THP1 cells as judged by LDH release (Figure S1C). When the effect of DRS on phosphorylation of p38 MAPK, release of TNFα and NF-κB activation was compared to SIC, DRS had no impact, neither alone nor in the presence of plasma, on the phosphorylation of p38 MAPK (Figures 8A and 8B) or the release of TNFα and NF-κB activation (Figures 8C and 8D).

**DISCUSSION**

In this investigation (the main findings are summarized in the graphical abstract) SIC was found to bind to TLR2/CD14 at the surface of THP1 monocytes and primary CD14+ monocytes resulting in the activation of the cells and the release of pro-inflammatory cytokines. SIC is known to bind clusterin and HRG in human plasma, and the presence of whole plasma or these individual plasma proteins, enhanced the activation, indicating that SIC-driven cytokine release could be particularly relevant in blood. This notion is further supported by the finding that a SIC homolog from a strain of the M55 serotype showed no pro-inflammatory activity. It is also noteworthy that the M12 and M55 serotypes are associated with post-streptococcal glomerulonephritis (Ferrieri et al., 1970; Dillon, 1979), an important sequelae of S. pyogenes infection, but not with invasive disease. The activation of NF-κB and MAPK in macrophages/monocytes by S. pyogenes (Wu et al., 2016) leads to the production of pro-inflammatory cytokines like TNFα in a MyD88-dependent fashion (Gratz et al., 2008). Similar to our finding that SIC-containing bacterial growth medium triggered activation of both the NF-κB and p38 MAPK pathways, growth medium of other Gram-positive bacteria, like S. aureus, triggered the release of antimicrobial peptides via the same pathways (Zhu et al., 2013).

S. pyogenes are internalized by macrophages where they replicate (O’Neill et al., 2016), and trigger inflammation (Valderrama et al., 2017). M1 protein has been discussed as a possible invasion factor, facilitating the uptake of the bacteria (Dombek et al., 1999; Ochel et al., 2014). Furthermore, the internalization of S. pyogenes has been reported to involve actin polymerization, with F-actin associated with intracellular streptococci (Dombek et al., 1999; Nobbs et al., 2007). Phagocytic activity, and thus the uptake of bacteria, is inhibited by cytochalasin D (Elliott and Winn, 1986; Kapellos et al., 2016). In relation to this work, it is interesting that Hoe and colleagues found that SIC was internalized by lung epithelial cells within few minutes, and the authors suggested that SIC interacts with intracellular proteins (Hoe et al., 2002). This study showed that SIC was taken up by the monocytes, an uptake that was inhibited by cytochalasin D and incubation on ice, indicating that SIC may interact with proteins involved in the cytoskeletal rearrangement, similar to whole streptococci of the M1 serotype (Dombek et al., 1999).

In addition to a possible intracellular interaction with cytoskeletal proteins, SIC was demonstrated to interact with monocyte surface receptors TLR2 and CD14. CD14, a GPL-anchored protein, is expressed by myeloid cells, like monocytes, macrophages, and neutrophils (Van Amersfoort and Kuiper, 2007). The relatively low expression levels of this receptor is increased by stimulation with LPS or DMSO (Zamani et al., 2012). M1 protein activates human peripheral monocytes via TLR2 interaction, leading to IL-6 secretion (Pählman et al., 2006). However, M1 might not be a direct TLR agonist, but rather operating through interactions with other secreted streptococcal products (Valderrama et al., 2017), triggering the subsequent cytokine release. Similar to CD14, the expression of TLR2 can be induced by bacterial products and whole bacteria, like S. pyogenes (Wu et al., 2016). However, the primary interaction of the bacterial product can be with CD14 followed by the involvement of a TLR (Van Amersfoort and Kuiper, 2007). The data of this study indicate that this could be valid for SIC, and that TLR2 and CD14 act as co-receptors for SIC. Based on these findings, we hypothesize that the interaction of SIC with the surface receptors,
rather than the uptake into the cells might lead to the signaling and activation of the NF-kB and p38 MAPK pathways.

Invasive *S. pyogenes* infections, particularly streptococcal toxic shock syndrome and necrotizing fasciitis, are feared clinical conditions, and the prevalence of invasive diseases has been reported to be at least 663,000 cases annually worldwide with fatality rates around 25 percent. However, the large majority of *S. pyogenes* diseases are relatively uncomplicated superficial infections of the throat (pharyngitis) and skin (impetigo and erysipelas) with 700 million estimated cases per year (Carapetis et al., 2005). In addition, children and youngsters are often healthy carriers of the bacterium in the pharynx without showing any
symptoms of pharyngitis which further underlines that the likelihood to develop invasive disease among individuals colonized with *S. pyogenes* is very low. This and the high mortality rate of invasive diseases indicate that the adaptation of *S. pyogenes* to its human host (*S. pyogenes* is a strict human pathogen) has evolved on mucosal surfaces and that the very rare invasive state is accidental with weak or no evolutionary impact.

Starting in the 1980s, a marked increase of invasive *S. pyogenes* infections was recorded in Europe and the USA (Kaplan, 1991), and since then M1 strains have been the most frequently serotype recovered from patients with invasive disease in this part of the world and in Australia (O’Grady et al., 2007; Nelson et al., 2016; Gherardi et al., 2018). However, it is important to stress that in these countries M1 is the most common serotype also in superficial pharyngitis, indicating that M1 bacteria have developed selection advantage in the ecological niche most important for *S. pyogenes*. SIC was discovered in the M1 strain (AP1) used in this study, and during the 25 years we have used AP1 bacteria and the SIC protein in different experiments *in vitro*, *ex vivo* and *in vivo*, we have not observed any variations in size or sequence of the SIC protein or its gene (*sic*). This is not surprising since our experiments have not put a selective pressure on *sic*. However, analysis of strains from two consecutive M1 epidemics revealed that each epidemic wave was composed of several new SIC variants and that natural selection had contributed to the variation (Stockbauer et al., 1998). In a follow-up study, the same group analyzed 240 random M1 isolates from patients with pharyngitis and found 58 SIC variants. This ratio of variants was almost identical to what was found for invasive M1 isolates, showing that penetration into sterile sites does not drive selection (Hoe et al., 1999).

Together with the observation that repeated intraperitoneal passage in mice did not generate new SIC variants (Stockbauer et al., 1998), and the low number of invasive compared to non-invasive infections discussed above, this demonstrates that sic selection takes place in the pharynx mucosa.

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**Figure 8. An M55-SIC homolog shows no affinity for clusterin and HRG and does not activate THP1 cells**

(A) Lysates of THP1 cells incubated with 5 μg SIC/ml or DRS +/− 2.5% plasma (P) were subjected to SDS-PAGE analysis and Western blotting. Membranes were probed with antibodies against p38 MAPK (p38) and phosphorylated p38 MAPK (p-p38).

(B) Band intensities of the Western blot in B were analyzed.

(C) Release of TNFα from THP1 cells incubated with SIC or DRS in presence and absence of plasma (P). Absorbance detected at 450 nm.

(D) Activation of NF-κB in cells incubated with 5 μg SIC/ml or DRS in presence and absence of 2.5% plasma (P). Absorbance was detected at 655 nm. All data represent mean ± SEM of 3-4 independent experiments, one-way ANOVA, Dunnett’s multiple comparison test, with single pooled variance. *p < 0.05, ***p < 0.001, ****p < 0.0001.
It is not clear what evolutionary pressure drives the rapid and extensive selection of new SIC variants, but a possible explanation could be found in a unique property of SIC; its capability to inhibit a large number of antimicrobial proteins and peptides which are important components of innate immunity and with bactericidal activity against *S. pyogenes* (Fernie-King et al., 2002; Frick et al., 2003; Egesten et al., 2007). The complement system, and the contact system (Frick et al., 2006), are two additional branches of innate immunity which when activated also generate antibacterial peptides (C3a of complement and fragments of H-kininogen of the contact system) that kill *S. pyogenes* but are inhibited by SIC (Frick et al., 2011). C3a is a pro-inflammatory peptide and activation of the contact system results in the release of bradykinin, which is also a potent pro-inflammatory peptide. SIC reduces contact activation and thereby bradykinin release (Åkesson et al., 2010). This means that SIC will protect the bacteria against various antibacterial proteins and peptides, and because of the interference with the complement and contact systems, the protein will simultaneously have an anti-inflammatory effect. However, in pharyngitis streptococcal colonization has induced an inflammatory host response causing vascular leakage and the recruitment of leukocytes, including monocytes, antibacterial proteins, and peptides, and complement and contact proteins, creating an environment with considerable evolutionary pressure and a hotbed for new SIC variants. In addition, several studies have found that spontaneous mutations in genes encoding the CovR/S two-component global regulatory system may transform M1 bacteria into a more invasive genotype [for more references see (Cole et al., 2011)]. For the present investigation it is striking that among the genes upregulated by CovR/S mutations are the genes encoding SIC and M1 protein. Significant amounts of SIC (Åkesson et al., 1996) and M1 (Åkesson et al., 1994) are produced and released by M1 bacteria into the growth medium, suggesting that the invasive genotype could produce even higher amounts. It is therefore noteworthy that released M1 protein form complexes with fibrinogen in blood and plasma which activate neutrophils to release heparin-binding protein (Herwald et al., 2004), a potent inducer of vascular leakage (Gautam et al., 2001).

Massive inflammation and vascular leakage are characteristic symptoms in severe invasive *S. pyogenes* disease. In these patients isolates of the M1 serotype are the most frequent, and they release M1 protein and SIC. The demonstration here that SIC, like M1, has powerful pro-inflammatory properties, indicate that both proteins contribute to the particular virulence of the M1 serotype and the pathophysiology of a very serious clinical condition. The results also identify SIC and its interactions with monocytes as new potential therapeutic targets.

**Limitations of the study**

As a potential caveat of our work presented here, one could consider the used concentration of SIC. Since we found 5 μg/mL in THY growth medium, all our experiments were performed with this concentration. We added additional information about lower concentrations (Figure 3I), decreasing the protein concentration down to 1.25 μg/mL. This still resulted in a significant response in the monocytes. However, future experiments are needed to determine the SIC concentration released by *S. pyogenes* during ongoing infections.

The use of CD14 + magnetic beads for the isolation of a specific monocyte subpopulation from human blood could possibly affect the CD14 + receptor. Thus, a potential false lower response in primary monocytes cannot be excluded.

**Resource availability**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ariane Neumann (ariane.neumann@med.lu.se).

**Materials availability**

No unique reagents have been generated and there are no restrictions on availability.

**Data and code availability**

No custom code, software, or algorithm was used here.

**METHODS**

All methods can be found in the accompanying Transparent methods supplemental file.
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102339.

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AUTHORS CONTRIBUTIONS
Conceptualization: A.N., I.-M.F. and L.B. Methodology: A.N. and W.B. Validation: A.N., I.-M.F., and L.B. Formal analysis: A.N., L.H., and C.K. Investigation: A.N., L.H., C.K., and I.-M.F. Data curation: L.H. and C.K. Writing – Original draft: A.N., I.-M.F. and L.B. Writing – Review & Editing: A.N., L.H., C.K., W.B., I.-M.F. and L.B. Visualization: A.N. Supervision: A.N. and L.B. Project administration: A.N. and L.B. Funding acquisition: A.N., I.-M.F. and L.B.

DECLARATION OF INTERESTS
The authors declare no competing interest.

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Supplemental information

Streptococcal protein SIC activates monocytes and induces inflammation

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Supplemental items

Supplementary figure + legend

Supplementary figure 1. M1-SIC and M55-SIC display no cytotoxic effect on host cells. A) related to Fig. 3, B) related to Fig. 7, C) related to Fig. 8

A: THP1 and Detroit 562 cells were incubated with 5 µg/ml SIC +/- 2.5% plasma (P) for 18 h and LDH release was detected by reading absorbance at 490 nm. Triton X-100 served as lysis control.

B: CD14+ cells were incubated with 100 µg/ml Trypsin for 30 min. LDH release was measured at 450 nm. No increase of LDH release was detected after Trypsin treatment.

C: THP1 cells were incubated with 5 µg/ml of distantly related SIC (DRS) +/- 2.5% plasma (P) and release of LDH was measured. Triton X-100 served as lysis control.

Transparent Methods

Bacterial strains, purification of proteins and antibodies

S. pyogenes strain AP1 (40/58) serotype M1 was from WHO Collaborating Centre for references and research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic. The ΔSIC strain was generated from AP1 as described in (Frick et al., 2003)
allelic replacement mutagenesis strategy. *S. pyogenes* strain W38 (GT 71-154) serotype M55 was a kind gift of the late Dr. Wannamaker. Bacteria were grown in TH medium + 2% yeast at 37 °C overnight and 150 µg/ml Kanamycin was added for the ΔSIC strain. Supernatants from the cultures were collected at an OD<sub>620nm</sub> of 0.5. M1-SIC and M55-SIC from *S. pyogenes* strains AP1 and W38, respectively, were purified by precipitation of the culture medium with 30% ammonium sulphate, followed by an ion-exchange chromatography on a MonoQ column (Amersham). Purity of M1-SIC was analysed via quantitative MS analysis. For infection experiments, overnight cultures of the bacteria were spun down and resuspended in RPMI medium (Gibco) + 10 % FCS (Thermo). Bacteria were grown until OD<sub>620nm</sub> of 0.5, the supernatant was collected, sterile filtered, concentrated and stored at -20 °C until further usage. For analysis of SIC expression in the growth medium, proteins were precipitated with TCA and the protein concentration was analysed by direct ELISA.

Mouse monoclonal IgG antibodies against p38 MAP kinase and phosphorylated p38 MAP kinase were from Cell signalling, and polyclonal rabbit IgG antibodies against TLR2 and CD14 were from Invivogen.

**Cell culture**

THP1 cells (ATCC® TIB-202™) were cultivated in RPMI + 10% FBS and 1% Anti/Anti (all from Thermo). THP1 XBlue CD14<sup>+</sup> cells (Invivogen) were cultivated in RPMI-FBS-Anti/Anti with 250 µg/ml G418 (Invivogen) and 200 µg/ml Zeocin (Invivogen). Detroit 562 cells (ATCC® CCL-138™) were grown in DMEM medium (Thermo) with 10% FBS and 1% Anti/Anti. All cell lines were used in low passage numbers. Primary CD14<sup>+</sup> monocytes (ethical permit 2020:24, Lund University) were isolated by density gradient centrifugation and magnetic bead separation. Briefly, 10 ml of leukocyte concentrate were diluted 1:1 with 0.9% NaCl. 20 ml of that mixture was layered onto 20 ml of Lymphoprep™ (AxisShield) and centrifuged for 20 min at 700 x g without brake. Erythrocytes were lysed with H<sub>2</sub>O for 15 s. Purified PBMCs were resuspended in MACS buffer and CD14 microbeads (both Miltenyi Biotec) were added. Cells were then sorted with a LS column using a MACS separator (both Miltenyi Biotec). After the separation, cells were resuspended in RPMI without supplements (Thermo) and cell numbers were adjusted to respective experiments. For all experiments, RPMI supplemented only with 10% FBS was used. Pre-incubation with 100 µg/ml trypsin was performed in un-supplemented RPMI, cells were subsequently spun and resuspended in RPMI + 10% FBS. For inhibition studies, cells were pre-incubated with blocking IgG antibodies or IgGFc fragments: human IgG1 and Fc fragments (in house; 10 µg/ml), Pab-hTLR2 (20 µg/ml)
and anti-CD14 (10 µg/ml) both from Invivogen. Pre-incubation was performed for 15-60 min at 37 °C 5% CO₂, prior to stimulation with purified SIC.

Detection of inflammatory cytokines and chemokines

Cells were seeded in 96-well plates with 5*10⁵ cells/ml and incubated with 5 µg/ml purified SIC, 2.5% plasma or both for 2 h at 37 °C and 5% CO₂. After incubation, the supernatant was collected and stored at -80 °C until further analysis. For an overall cytokine profiling, samples were analysed using the Bio-Plex Pro™ Human Cytokine 27-plex Assay (Biorad). Additionally, the secretion of TNFα, was analysed according to the manufacturer’s recommendation with a human TNFα uncoated ELISA (Invitrogen).

Western blot analysis

For detection of p38 MAP kinase phosphorylation, 2*10⁶/ml cells were seeded in 24-well plates with 500 µl per well and stimulated with SIC (5 µg/ml), SIC fragments (I,II, III; 5 µg/ml), DRS (5 µg/ml) and/or plasma (2.5%) or bacterial supernatant for 18 h at 37 °C and 5% CO₂. RPMI medium was used as a negative control. Cells were washed with 1 X PBS and lysed using RIPA buffer (Thermo) with a protease inhibitor mix (Thermo) for 5 min on ice. Cell lysates were boiled for 5 min at 95 °C with 4X Lämmli buffer and 10 µl samples were loaded on 4-20% TGX gel (Biorad) and run for 50 min at 150 V. Samples were transferred onto Trans-Blot® Turbo™ Mini PVDF membranes (Biorad) for 5 min at 25 V and 2.5 mA. Membranes were incubated overnight with primary antibodies (diluted 1:1000 in PBS-Tween with 5% BSA) against phosphorylated p38 MAP kinase (Phospho-p38 MAPK Thr180/Tyr182) or p38 MAP kinase (both from Cell signalling) at 4 °C, antibodies were diluted 1:1000 in PBS-Tween with 5% BSA. Secondary antibodies (goat anti-rabbit HRP-conjugated, Biorad) were diluted 1:5000 in PBS-Tween and membranes were incubated for 1-2 h at room temperature (RT). Membranes were visualised using Clarity Max™ Western ECL Blotting Substrate (Biorad). Band intensities were analysed using ImageJ software, normalising the phospho-p38 band to the according p38 band.

NF-κB activation in THP1 XBlue CD14⁺ cells

THP1 XBlue CD14⁺ cells were resuspended in RPMI + 10% FBS and seeded with 5*10⁶/ml cell in 96-well plates with 100 µl cells per well. Cells were stimulated according to manufacturer’s recommendation with 10 µl stimuli (5 µg/ml purified SIC or DRS, 2.5% plasma, 5 µg/ml M1- SIC fragments I, II and III, 10 ng/ml LPS, 10 µl bacterial growth medium)
for 18 h at 37 °C and 5% CO₂. For removal of surface proteins, cells were pre-incubated for 30 min with 100 µg/ml trypsin in un-supplemented RPMI, spun down and resuspended in RPMI + 10% FBS to continue with respective stimuli. To analyse possible binding of SIC to monocyte surface proteins THP1 cells were pre-incubated with IgG₁, Fc fragment or antibodies against TLR2 or CD14 for 20 min, followed by the addition of the various stimuli. 20-50 µl cell supernatant was transferred into a new 96-well plate containing 150-180 µl QuantiBlue solution (Invivogen) per well and incubated for 1-4 h at 37 °C. Changes in colour were measured at 655 nm.

Interaction studies with fluorescently labelled protein SIC

Purified protein SIC was labelled with an Alexa Fluor® succinimidyl ester 633 overnight. Excessive dye was removed, and the protein concentration after labelling was determined. THP1 cells were seeded in a µ-slide (ibidi) at a concentration of 2*10⁶/ml. Labelled SIC (5 µg/ml) was added and samples were incubated for 1 h at 37 °C. Live imaging was performed using a Nikon Eclipse TE300 fluorescence microscope with a PlanFluor 40X/0.60 NA objective (Bergman Labora, Lyckeby, Sweden). For quantification, samples were analysed using a BD Accuri™ C6 flow cytometer. Pre-incubation of cells with 20 µM Cytochalasin D for 30 min was used to block possible uptake/binding of the protein. Additionally, uptake/binding was blocked by incubation of the cells and labelled SIC for 1 h on ice. Cells were washed with 1 X PBS to remove excessive and unbound protein and dye before imaging and FACS analysis.

Surface plasmon resonance binding studies

SPR experiments were performed using a Biacore T100 (GE Healthcare) at 25 °C. TLR2 or CD14 were immobilised on a CM5 chip; as running buffer 100 mM HEPES, 150 mM NaCl, 0.05% Surfactant P20, pH 7.4 (GE Healthcare) was used at 10 µl/min. Immobilisation of TLR2 resulted in 250.1RU and CD14 in 237.7 RU. SIC concentrations prepared in running buffer were the following: 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml. Samples were run in flow cell 1,2 with one regeneration. 10 mM Glycine HCl, pH 2.5 was used for the regeneration with 30 sec contact time and 0 sec stabilisation period. Contact time with the analyte for 120 sec, dissociation time 180 sec. Binding affinities of SIC to either TLR2 or CD14 were calculated from rate constants obtained from fitting the data to a 1:1 Langmuir binding model. Average Kₐ values were displayed in the graphs.
Sample preparation for mass spectrometry

THP1 were resuspended in RPMI medium without FBS and adjusted to \(5 \times 10^6\)/ml. Cells were then incubated with 100 µg/ml trypsin for 30 min, spun for 5 min and resuspended with RPMI + 10% FBS. Cells were then lysed with 300 µl RIPA buffer (Thermo) with a protease inhibitor mix (Thermo) for 5 min on ice. For analysis, 100 µg of cell lysates in triplicates were precipitated with acetone followed by an ethanol wash. The precipitated proteins were mixed with 8 M urea and 100 mM ammonium bicarbonate, and the cysteine bonds were reduced with 5 mM TCEP (37 °C for 90 min) and alkylated with 10 mM iodoacetamide (22 °C for 30 min). The samples were diluted with 100 mM ammonium bicarbonate to a final urea concentration of below 1.5 M, and sequencing grade trypsin (Promega) was added for protein digestion (37 °C for 16 h). The samples were acidified (to a final pH 3.0) with 10% formic acid, and the peptides purified with Solaµ HRP columns according to the manufacturer’s instructions (Thermo). Peptides were dried in a speedvac and reconstituted in 2% acetonitrile, 0.2% formic acid prior to mass spectrometric analyses.

Liquid chromatography tandem mass spectrometry (LC-MS/MS)

All peptide analyses were performed on Q Exactive HF-X mass spectrometer (Thermo Scientific) connected to an EASY-nLC 1200 ultra-high-performance liquid chromatography system (Thermo Scientific). Peptides were loaded onto an Acclaim PepMap 100 C18, 3 µm, 100Å pre-column (ID 75µm x 2 cm) and separated on an EASY-Spray column (Thermo Scientific; ID 75µm x 50 cm, column temperature 45 °C) operated at a constant pressure of 800 bar. A linear gradient from 4 to 45% of 80% acetonitrile in aqueous 0.1% formic acid was run for 125 min at a flow rate of 350 nl min⁻¹.

For DDA MS, one full MS scan (resolution 60000 @ 200 m/z; mass range 390–1 210m/z) was followed by MS/MS scans (resolution 15000 @ 200 m/z) of the 15 most abundant ion signals. The precursor ions were isolated with 2 m/z isolation width and fragmented using HCD at a normalized collision energy of 30. Charge state screening was enabled, and precursors with an unknown charge state and a charge state of 1 and above 6 were rejected. The dynamic exclusion window was set to 10 s. The automatic gain control was set to 3e6 and 1e5 for MS and MS/MS with ion accumulation times of 110 ms and 60 ms, respectively. The intensity threshold for precursor ion selection was set to 1.7e4.

For data independent acquisition (DIA) MS, one full MS scan (resolution 60000 @ 200 m/z; mass range from 390 to 1210m/z) was followed by 32 MS/MS full fragmentation scans (resolution 30000 @v200 m/z) using an isolation window of 26 m/z (including 0.5 m/z overlap.
between the previous and next window). The precursor ions within each isolation window were fragmented using HCD at a normalized collision energy of 30. The automatic gain control was set to 3e6 and 1e6 for MS and MS/MS with ion accumulation times of 100 ms and 120 ms, respectively.

Mass spectrometry data analysis

All raw data were converted to gzipped and NumPressed (Teleman et al., 2014) mzML using the tool MSconvert from the ProteoWizard, v3.0.5930 suite (Chambers et al., 2012). All data analyses were stored and managed using openBIS (Barillari et al., 2016). DDA acquired spectra were analysed using the search engine X! Tandem (2013.06.15.1-LabKey, Insilicos, ISB) (Craig and Beavis, 2003) and OMSSA (Geer et al., 2004) against an in-house compiled database (Quandt et al., 2014) containing the Homo sapiens Uniprot reference proteome (ID UP000005640) with 74823 entries and an equal number of reverse decoys, follow by a Trans-Proteomic Pipeline (TPP v4.7 POLAR VORTEX rev 0, Build 201403121010) using PeptideProphet (Keller et al., 2002) and false discovery rate (FDR) estimation with Mayu (v1.7) (Reiter et al., 2009). The spectral library was built with TPP Spectrast using the filtered peptide spectrum matches (PSMs) with all retention times converted to iRTs (Lam et al., 2008). All DIA MS runs were aligned using TRIC (Röst et al., 2016) to produce a final data matrix with 1% FDR at peptide level and 10% FDR at protein level.

Supplemental References

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