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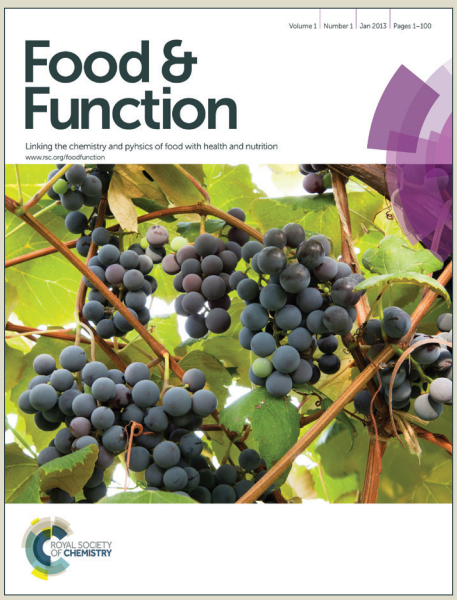


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ARTICLE

# Impact of Soyasaponin I on TLR2 and TLR4 induced inflammation in the MUTZ-3-cell model

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Previous studies have demonstrated that soyasaponin (SoSa) possesses anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated immune cells by influencing the immune sensing of toll-like receptor (TLR) 4. The aim of this study was to investigate the immune modulatory effect of SoSa I on TLR2- and TLR4-induced inflammation within the monocytic MUTZ-3-cell model. MUTZ-3 cells were stimulated with gram-negative (*Escherichia coli*) or gram-positive (*Staphylococcus aureus*) bacteria or bacterial pathogen-associated molecular patterns (PAMPs) such as LPS or peptidoglycans (PGN) alone or in combination with SoSa I. Cell morphology was characterized by raster scanning and light microscopy. Cytokine production (IL-1β, IL-6, TNF-α, IP-10, RANTES and IL-8) was measured by cytometric bead array and the expression of surface markers was assessed by flow cytometry. MUTZ-3 cells revealed a cell maturation-like alteration in morphology and increased expression of CD80, CD86, TLR2 and TLR4 after stimulation with either gram-negative and gram-positive bacteria or bacterial PAMPs. The addition of SoSa I suppressed pro-inflammatory cytokine and chemokine secretions in a dose-dependent manner regardless of TLR2 or TLR4 stimulation. Interestingly, E. coli- and S. aureus-induced inflammation was always inhibited better by SoSa I than that induced by LPS and PGN. Additionally, SoSa I reduced the expression of CD86 in PGN- or LPS-stimulated cells.
This study demonstrated that the anti-inflammatory capacity of SoSa I is based on influencing both monocytic TLR2 and TLR4 and that SoSa I inhibits more effectively whole bacteria compared to solely LPS or PGN what points to a broader role of SoSa I in the down-regulation of inflammation.

## Introduction

In general, inflammation protects the body against harmful stimuli such as pathogens by primarily sensing invading microorganisms and inducing an inflammatory response.[1 2] The first-line of protection against pathogens is the nonspecific innate arm of the immune system.[2] Macrophages and dendritic cells (DCs) in the initiation phase of the immune defense detect bacteria by Toll-like receptors (TLRs) and other germline-encoded pattern recognition receptors (PRRs).[1] These receptors sense specific evolutionarily conserved molecular structures called pathogen-associated molecular patterns (PAMPs) [1 2] like lipopolysaccharide (LPS) and peptidoglycan (PGN). In humans, ten TLRs have been characterized: TLR1, 2, 4, 5, 6 and 10 are localized extracellularly on the cell surface, whereas TLR3, 7, 8 and 9 are expressed predominantly intracellularly at the endoplasmic reticulum.[2] LPS, which is the prominent component of the outer membrane of gram-negative bacteria, e.g. *Escherichia coli,* is mainly sensed by TLR4,[2 3] whereas the predominant cell wall constituent of gram-positive bacteria, e.g. *Staphylococcus aureus*, PGN is recognized via TLR2.[2 3] The detection of PAMPs leads to activation of inflammatory pathways, for example the nuclear factor kappa-light-chain-enhancer of activated B-cells-(NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which result in the release of cytokines.[4 5] Regulation of TLR activity and pro-inflammatory cytokine release might be an important leverage point for ameliorating inflammatory and infectious diseases, especially chronic inflammatory reactions which are based on bacterial infections like *Borrelia burgdorferi,* inducing inflammatory reactions via TLRs.[6 7]

Phytochemicals, like SoSa, such as curcumin or Epigallocatechin gallate (EGCG), are thought to be promising candidates for attenuating and preventing inflammatory diseases.[6 7 8 9] If SoSa I is able to suppress the innate TLR-response against pathogens, this could be a great advantage in the therapy of chronic inflammation phenomena. SoSas are soybean (*Glycine max*)-derived oleanane triterpenoid glycosides with one or two carbohydrate moieties.[9 10] Based on their structure, SoSas are mainly categorized into three groups: A, B and E. Group A saponins with the aglycone soyasapogenol A are bisdesmoside,[10 11] whereas groups B and E are monodesmoside and their sugar chain is attached via an ether linkage to soyasapogenol B and E, respectively. Group B saponins are further divided into the subcategories SoSas I-V.[12 13] SoSa also exhibits various other physiological and pharmacological functions and anti-inflammatory properties.[9 10 11 14 15 16] It has been discussed that the hydrophilic glycosidic moieties in SoSas are mainly responsible for the anti-inflammatory properties.[14] For example, SoSa exhibits anti-inflammatory properties in

mouse (peritoneal) macrophages treated with LPS[9 10 11 14] and they are proposed to influence endotoxin recognition via TLR4.[11] Therefore, in this study, the immune modulatory properties of the monodesmoside group B saponin SoSa I, the major saponin fraction from soy,[17] on bacterial- or PAMP-stimulated MUTZ-3 cells were determined.

## Results and Discussion

### Characterization of MUTZ-3 cells by microscopy and flow cytometry

In order to specify the phenotype of the acute human myeloid leukemia MUTZ-3 cell line, which is an accepted cell model for general and LPS-induced inflammatory studies[18 19], the profile of relevant surface markers and TLRs was characterized by flow cytometry. Figure 1 (panel A) shows the phenotype of MUTZ-3 cells examined by flow cytometry. MUTZ-3 cells expressed CD14 (22.11% ±4.68), CD123 (97.71% ±28.20), CD11c (32.23% ±2.67) and HLA-DR (77.74% ±7.16). Although MUTZ-3 cells exhibit monocyte-like features concerning phenotype and morphology,[20] it has been reported that they rather resemble precursors of immature DCs than monocytes by expressing only low levels of the monocyte marker CD14. [19] Phenotypic characterization of MUTZ-3 cells confirmed low expression levels of CD14 and moderate expression of CD11c suggests that the MUTZ-3 cells used in this study were rather DC precursors than monocytes, as previously proposed by Larsson and colleagues. [19]

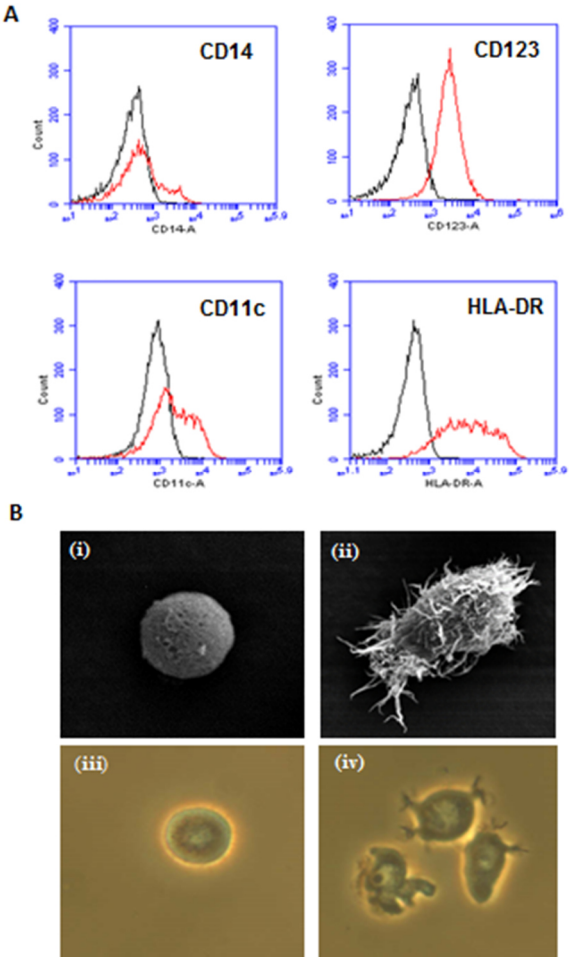


Figure 1: A: Surface expression levels of CD14, CD123, CD11c and HLA-DR on MUTZ-3 cells characterized by flow cytometry (black line = unstained control, red line = stained sample). Data show one representative out of three independent experiments. B: Morphological changes of MUTZ-3 cells in culture scanned by REM (i, ii) (magnification: x5000; pictures) and light microscopy (iii, iv). Images show MUTZ-3 cells in inactivated (i, iii) and LPS-stimulated (ii:, iv) shapes.

In culture, MUTZ-3 cells were not plastic adherent and grew floating in suspension. In order to visualize the distinct maturation steps of LPS-stimulated MUTZ-3 cells, the altered cell morphology was documented by REM (panel B (i and ii)) and light microscopy (panel B (iii and iv)), as illustrated in Figure 1. In culture without antigen stimulation, MUTZ-3 cells revealed a round regular shape (panel B (i, iii), Figure 1) and represent immature monocytes. With LPS stimulation MUTZ-3 cells altered into an irregular, wrinkled structure, with defined DC-like pseudopodia (panel B (ii and iv), Figure 1) because of the phagocytic effect and represented mature monocytes. Corresponding to the observations concerning morphological changes after stimulation with LPS or PGN, MUTZ-3 cells showed expression of the activation marker molecules CD80 and CD86 and the TLR2- and the TLR4-receptor (Figure 2, panel A). In regard to inflammatory stimulation with the TLR2- and TLR4-ligands PGN and LPS, respectively, expression levels of CD80, CD86, TLR2 and TLR4 on MUTZ-3 cells were measured before and after stimulation with LPS (1 µg/mL) and PGN (100 µg/mL) (Figure 2, panel B). Unstimulated MUTZ-3 cells were positive for $TLR2^+$ (97.54% ±1.67) and $TLR4^+$ (98.07% ±1.67). Expression of CD80 was 49.04% ±9.58 and of CD86 was 50.97% ±3.99. After stimulation with LPS we found a slight up-regulation of TLR4 by 32.37% ±7.85, increased expression of CD80 by 37.92% ±9.16 and CD86 by 21.92% ±10.37. In contrast, stimulation with PGN led to an up-regulation of TLR2 by 13.30% ±14.17, TLR4 by 57.26% ±4.67, CD80 by 73.89% ±23.95 and CD86 by 59.43% ±18.90. TLR-expression on MUTZ-3 cells has not been well described to date. Whilst Larrson et al. found that TLR4 was not expressed on MUTZ-3 DCs, but TLR2 was expressed with a fluorescence signal intensity value from 100 to 1000, respectively[19], our data showed TLR2 as well as TLR4 expression in undifferentiated MUTZ-3 cells. In accordance with Kim et al., we found a failed up-regulation of TLR2 after LPS-stimulation.[21] In previous studies, phenotypic characterization of MUTZ-3 cells displayed the expression of low CD80 and CD86 levels.[22] Maturation of MUTZ-3 DCs induced by pro-inflammatory stimulants resulted in up-regulated CD80 and CD86 expression levels.[19 23] Our data confirmed the low basal expression of CD80 and CD86, which could be enhanced after LPS or PGN stimulation. In summary, MUTZ-3 cells provided an excellent model to investigate the impact of Soyasaponin I on TLR2- and TLR4-induced inflammation.

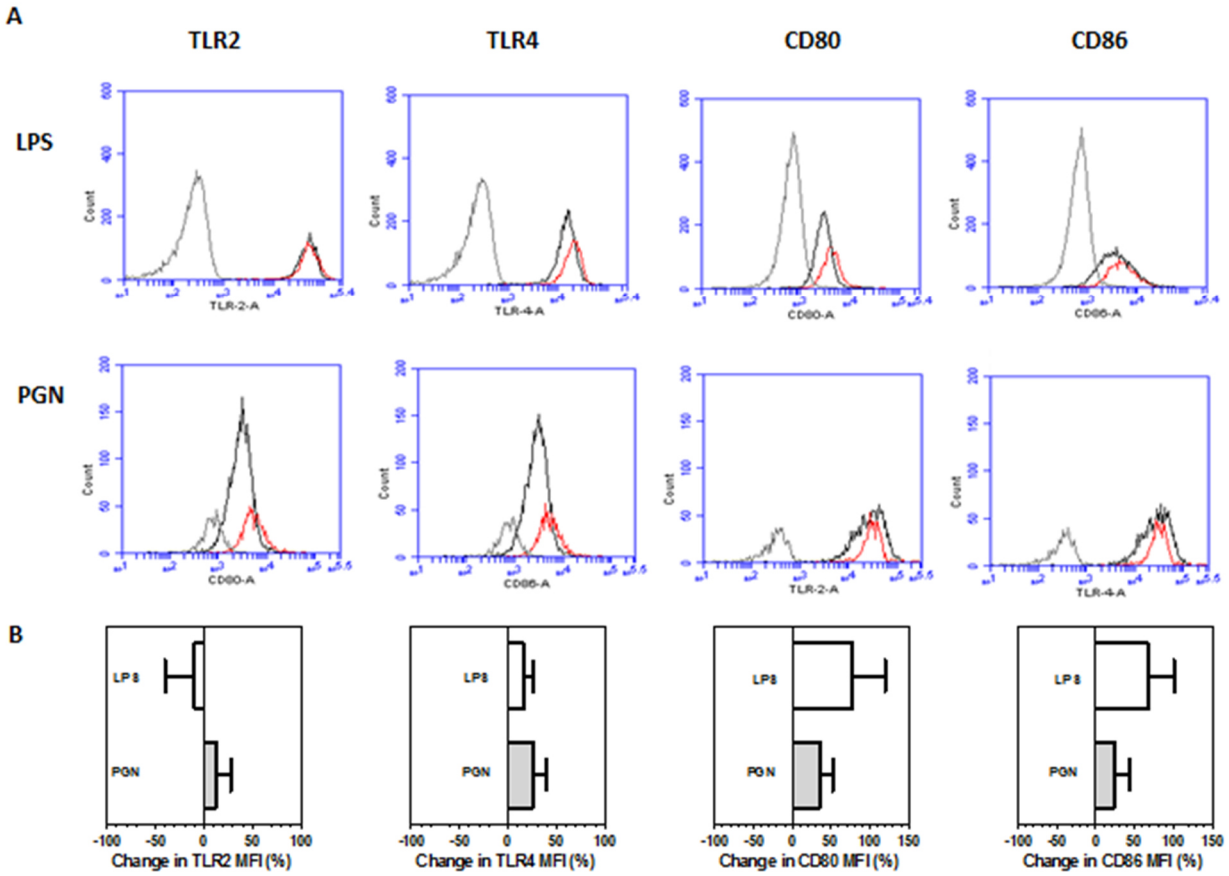


Figure 2: A: The effect of PAMP-treatment on surface marker expression levels was determined by incubating MUTZ-3 cells with LPS (1 µg/mL) or PGN (100 µg/mL) (grey line = unstained MUTZ-3 cells, black line = stained unstimulated MUTZ-3 cells, red line = MUTZ-3 cells stimulated with LPS or PGN). B: The percent influence of LPS or PGN on the Mean fluorescence of Surface markers (above named) compared to negative control. Expression levels of TLR2, TLR4, CD80 and CD86 were determined by flow cytometry. Data show one representative out of three independent experiments.

**Effect of SoSa I on LPS- or E. coli-induced cytokine and chemokine release**

Toll-like receptors (TLRs) importantly contribute to the innate immune response to bacterial and viral infections and play a pivotal role in systemic inflammation and in contributing to the promotion of systemic lupus erythematosus (SLE), asthma, Crohn's disease, multiple sclerosis, type 1 diabetes and rheumatoid arthritis (RA).[7] Previous studies have demonstrated that SoSa I possesses anti-inflammatory properties on lipopolysaccharide (LPS)-stimulated immune cells by influencing the endotoxin-binding to the toll-like receptor (TLR) 4 and therefore SoSa I could open a new avenue in the therapy of chronic inflammation phenomena.[9 10 11 14 15].

In order to investigate the immune-modulatory potential of SoSa I on the distinct primary inflammatory reaction, MUTZ-3 cells were stimulated with the gram-negative bacteria *E. coli* (1 x $10^6$) or LPS (1 µg/mL) and co-incubated with different concentrations of SoSa I (5, 50 and 100 µg/mL).

Both stimulants led to a significant pro-inflammatory response for all of the measured cytokines and chemokines compared to unstimulated controls. Only in the case of IL-1β *E. coli* did induce a significant higher cytokine level than LPS ($p < 0.05$) (Figure 3).

The addition of SoSa I inhibited *E. coli*-induced cytokine/chemokine release rather than LPS-induced inflammation (Figure 3). All cytokine and chemokine responses to *E. coli* were significantly inhibited by SoSa I in a dose-dependent manner. Except for IL-6, which was inhibited at a concentration of 100 µg/mL (*E. coli*: 221.8 pg/mL ±69.3; +SoSa I: 46.5 pg/mL ±15.5, $p < 0.05$), IL-1β, TNF-α, IP-10, RANTES and IL-8 were already inhibited at a concentration of 5 µg/mL SoSa I (IL-1β from 26.3 pg/mL ±3.2 to 15.8 pg/mL ±2.8, $p < 0.01$; TNF-α from 23.0 pg/mL ±2.6 to 10.3 pg/mL ±1.2, $p < 0.001$; IP-10 from 491.5 pg/mL ±52.3 to 301.8 pg/mL ±37.6, $p < 0.01$; RANTES from 816.5 pg/mL ± 67.7 to 494.3 pg/mL ±72.5, $p < 0.001$ and IL-8 from 53334.5 pg/mL ± 4359.5 to 35657.3 pg/mL ±5651.2; $p < 0.01$). In contrast, LPS-induced responses could be only reduced for IL-1β, TNF-α RANTES and IL-8, and, except for IL-8, higher SoSa I concentrations were necessary to reduce LPS-induced cytokine/chemokine responses compared to *E. coli* (IL-1β from 15.0 pg/mL ±2.1 to 8.0 pg/mL ± 0.8 (+SoSa

I 100 µg/mL), $p < 0.01$; TNF-α from 18.0 pg/mL ±1.6 to 12.0 pg/mL ± 0.9 (+SoSa I 50 µg/mL), $p < 0.05$; RANTES from 585.8 pg/mL ±72.3 to 277.0 pg/mL ± 10.8 (+SoSa I 100 µg/mL), $p < 0.001$ and IL-8 from 43176.8 pg/mL ±3245.7 to 33963.5 pg/mL ± 1578.4 (+SoSa I 5 µg/mL), $p < 0.05$). Using heat-inactivated whole bacteria such as *E. coli* as well as LPS, respectively, we further compared whole bacteria against their appropriate PAMPs. Stimulation via TLR4 induced the MyD88-dependent as well as the MyD88-independent pathway. After *E. coli* stimulation, both cytokines and chemokines were significantly inhibited by SoSa I in a dose-dependent manner. Interestingly, SoSa I more efficiently inhibited *E. coli* than LPS-triggered inflammation. Earlier studies by Lee et al. proposed blocking of TLR4 by SoSa I.[11] Taking into account the fact that the induction of inflammation by *E. coli* is not only due to LPS but also different structures like the outer membrane proteins (OMPs), the inhibitory effect seems to be similar but also more general. To test this hypothesis, we further stimulated the MUTZ-3 cells via the TLR2 receptor using *S. aureus* and PGN.

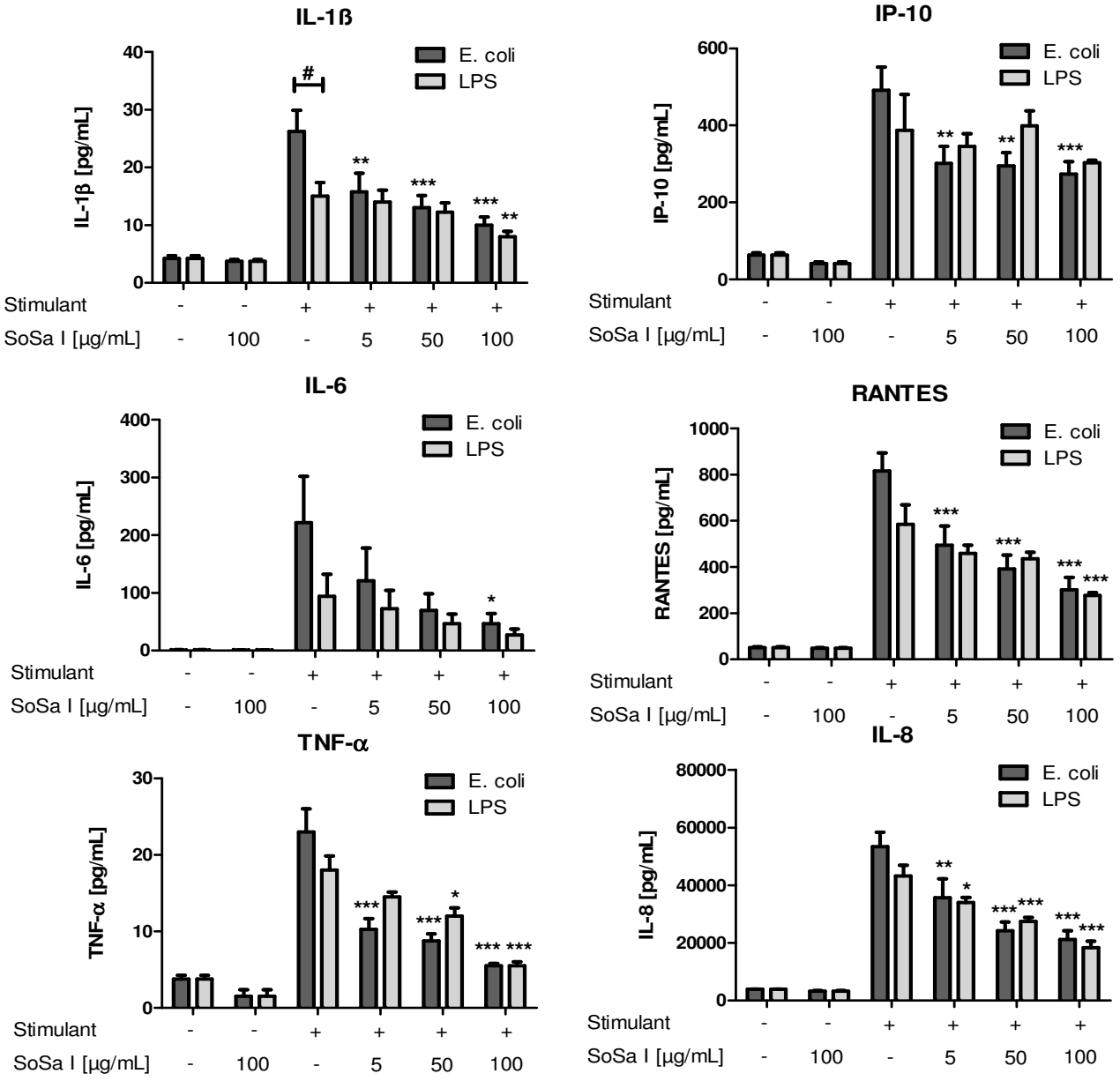


Figure 3: Modulatory effect of SoSa I on the cytokine release of LPS- and *E. coli*-stimulated MUTZ-3 cells. Stimulants are divided into LPS (1 µg/mL) and *E. coli* ($10^6$ cells/mL). Determination of IL-1β, IL-6, TNF-α, IP-10, RANTES and IL-8 levels was performed by CBA. Values are represented as mean ± SEM (n=4; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ respectively, t-test; *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ respectively, One-way ANOVA).

**Effect of SoSa I on the cytokine and chemokine release induced by PGN and the gram-positive bacteria *S. aureus***

The aim of the present study was to investigate the role of SoSa I on TLR2 and TLR4 triggered inflammation. Therefore we stimulated MUTZ-3 cells not only with LPS and *E.coli* which trigger the TLR4-response, but also with PGN and *S.aureus* for triggering the TLR2-response. PGN is the predominant immune-stimulatory PAMP of gram-positive bacteria, e.g. *S. aureus*.[5] In order to specify the primary immune response against the antigen complex of total bacteria, MUTZ-3 cells were distinctively stimulated with 100 µg/mL PGN or 1 x $10^8$ cells/mL *S. aureus* and co-incubated with different concentrations of SoSa I (5-100 µg/mL). The resulting secretion of IL-1β, IL-6, TNF-α cytokines and of IP-10, RANTES and IL-8 chemokines was measured by CBA. Unstimulated cells and MUTZ-3 cells incubated only with 100 µg/mL SoSa I were used as a negative control.

Both stimulants significantly activated the secretion of primary pro-inflammatory mediators compared to the negative control (Figure 4). Except for IL-6 and IP-10, PGN induced a significantly higher cytokine level for all measured cytokines than *S. aureus*. In contrast to the TLR4-triggered inflammation, PGN, as PAMP, induced a higher inflammatory response compared to the whole bacteria *S. aureus*. These results are in

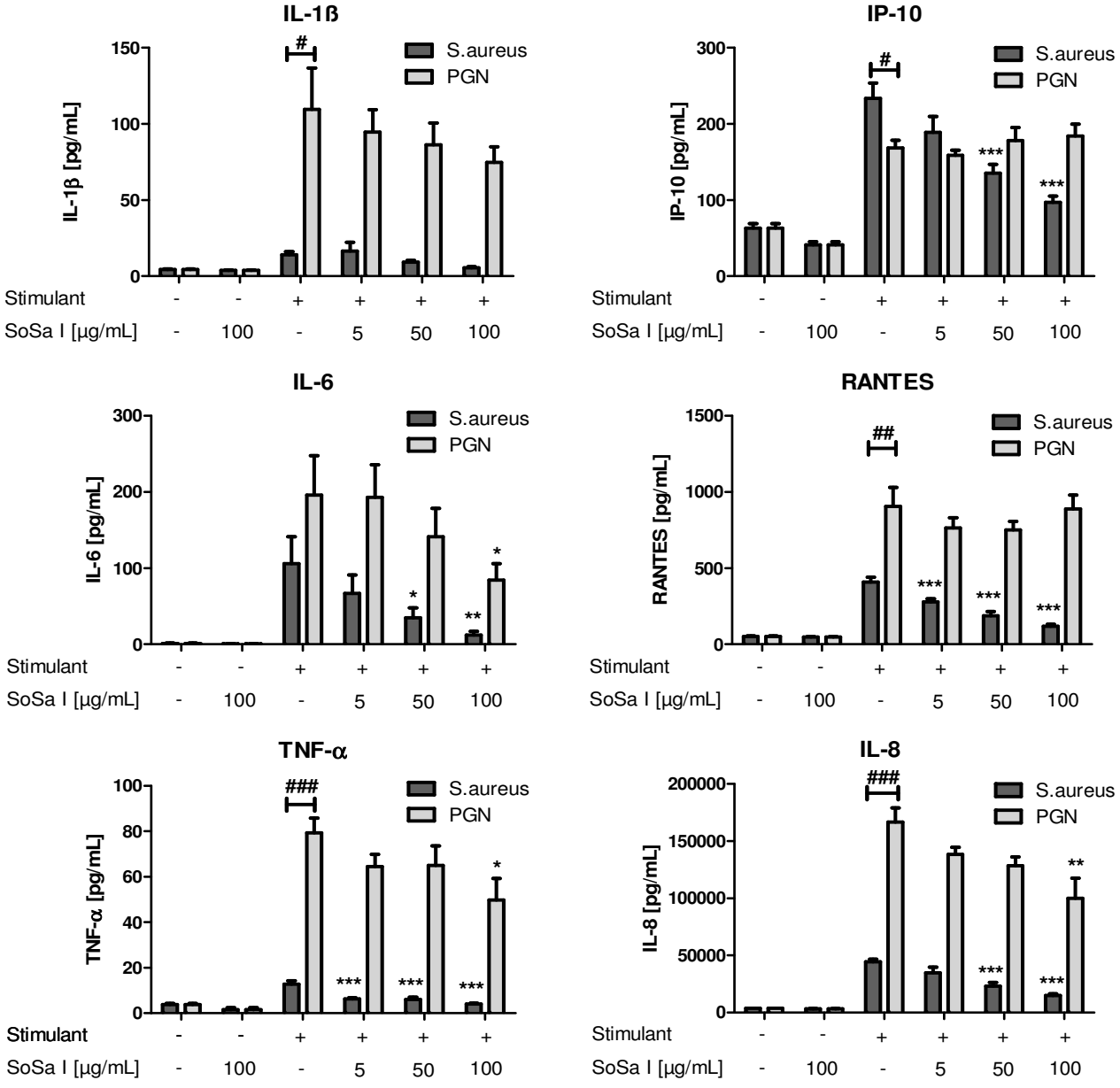


Figure 4: Modulatory effect of SoSa I on the cytokine release of PGN- and *S. aureus*-stimulated MUTZ-3 cells. Stimulants are divided into PGN (100 µg/mL) and *S. aureus* ($10^8$ cells/mL). Determination of IL-1β, IL-6, TNF-α, IP-10, RANTES and IL-8 levels was performed by CBA. Values are represented as mean ± SEM (n=4; #p < 0.05, ##p < 0.01 and ###p < 0.001 respectively, t-test; *p < 0.05, **p < 0.01 and ***p < 0.001 respectively, One-way ANOVA).

line with Kadowaki et al., who showed higher IL-6 and TNF-□ production of PGN than LTA in human precursors of dendritic cells.[24]

Whilst no differences in IL-6 secretion were seen between *S. aureus* and PGN stimulation, IP-10 release was significantly higher in *S. aureus*-stimulated samples. Whilst PGN triggers the MyD88-dependent pathway (Il-1ß, IL-6, IL-8, TNF-a), *S. aureus* also led to a strong IP-10 induction of the MyD88-independent pathway, indicating the participation of TLR4 or TLR3 in an *S. aureus*-provoked response.[2] Although IP-10 and RANTES secretions are mediated independently of MyD88 via the TRIF-dependent signaling cascade,[25] Fitzgerald and colleagues proposed that the MyD88-independent pathway also triggers NF-κB activation because RANTES and IP-10 promoters need NF-κB to be completely active.[25] Likewise, here, the release of RANTES was strongly inhibited in PGN-treated samples, but not in *S. aureus*-treated samples.

SoSa I showed a stronger reduction of cytokine/chemokine levels to *S. aureus*-stimulated cells compared to PGN activation. In detail, SoSa I inhibited the release of IL-6, TNF-α, IP-10, RANTES and IL-8 after *S. aureus* stimulation (IL-6 from 105.8 pg/mL ±30.7 to 34.7 pg/mL ±11.3 (+SoSa I 50 µg/mL), $p < 0.05$; TNF-α from 12.8 pg/mL ±1.3 to 6.0 pg/mL ± 0.9 (+SoSa I 5 µg/mL), $p < 0.001$; IP-10 from 233.5 pg/mL ±17.5 to 135.3 pg/mL ±10.1 (+SoSa I 50 µg/mL), $p < 0.001$ RANTES from 407.5 pg/mL ±28.5 to 277.8 pg/mL ±17.4 (+SoSa I 5 µg/mL), $p < 0.001$ and IL-8 from 44430.0 pg/mL ± 2015.2 to 23258.0 pg/mL ±2684.6 (+SoSa I 50 µg/mL), $p < 0.001$). In contrast, SoSa I inhibited solely IL-6, TNF-α and IL-8 production after PGN stimulation at a concentration of 100 µg/mL (IL-6 from 196.0 pg/mL ±44.8 to 84.3 pg/mL ±18.7 $p < 0.05$; TNF-α from 79.3 pg/mL ±5.7 to 49.8 pg/mL ±8.2, $p < 0.05$; and IL-8 from 166711.0 g/mL ±10696.2 to 100060.5 pg/mL ±15114.7 $p < 0.001$). The release of IL-1β could not be reduced by SoSa I, neither in PGN-stimulated cells nor in *S. aureus*-stimulated cells and TNF-α was also hardly suppressed by SoSa I after PGN stimulation: In contrast we could describe a down regulation for LPS and *E.coli* -stimulated MUTZ-3 cells, agreeing with the results of Lee et al., who also described a down regulation of IL-1β, IL-6 and TNF-α in LPS-stimulated macrophages.[11] For the release of TNF-α, we could describe that SoSa I not only strongly inhibit the TNF-α release for LPS- and *E.coli*-stimulated cells but also for *S. aureus*-treated MUTZ-3 cells. In contrast, we have found that SoSa I could not reduce the release of IL-6 in LPS-stimulated cells. Also SoSa I could not reduce the cytokine release of IP-10 in LPS-stimulated MUTZ-3 cells. Among the measured cytokines and chemokines, IP-10 levels were not influenced or affected by SoSa I in PAMP-treated MUTZ-3 cells. This slight reduction can also be observed with RANTES or IL-1β in samples stimulated by the isolated cell wall components. A very high induction of IL-8 production could be observed in all samples, but especially in PGN-treated samples. However, PGN-induced IL-8 release was reduced by SoSa I almost at the lowest concentration. One explanation could be the favored secretion of IL-8 upon cell activation via TLR2 which activates the MyD88-dependent pathway and by gram-positive bacteria, respectively.[26 27]

Regarding stimulation of the MUTZ-3 cells, our measurements indicated that LPS could not be consulted to predict the response of MUTZ-3 cells to the corresponding *E. coli* whole bacteria. The missing comparability of cell activation induced by purified pathogen patterns and whole bacteria, particularly in the case of gram-negative bacteria, was also addressed in previous studies.[26] Tietze et al. considered that whole pathogens and their isolated cell wall components might trigger different incomparable responses; thus, they only determined and matched bacterial-stimulated cytokine release.[26] Hessle and colleagues found similar cytokine levels in *E. coli*- and LPS-treated samples but this was not applicable for *S. aureus* and PGN in accordance to our data. They proposed that IL-1β and TNF-α secretion is particularly dependent on a special form of PGN and an intact bacterial cell wall. Gram-negative bacteria exhibit besides LPS a thin PGN layer[2] and cell activation is also mediated in a TLR2-dependent manner,[2 3 26] especially at higher doses of gram-negative bacteria or in cells lacking TLR4.[3] Albeit, this leads to the assumption that SoSa I hampers not only LPS attachment to TLR4 or PGN to TLR2 or the whole bacteria respectively, but also the recognition of other PAMPs by their appropriate TLRs or rather to a co-receptor like MD-2 or CD14.[28]

To the best of our knowledge, we are the first to describe a broader role of SoSa I in the down-regulation of TLR-induced inflammation taking PGN and whole bacteria into consideration. In contrast to earlier findings, our data provide evidence that SoSa I not only inhibits TLR4- but also TLR2-induced inflammation. In addition, as shown for *E. coli*, we assume that SoSa I might be involved in the reduction of OMP-induced stimulation. If SoSa I does not inhibit the immune response through binding directly to the TLRs, this could be one explanation for why PGN is more difficult to suppress than *S. aureus*, which stimulates not only by activating TLR2 alone, but also other PRRs. An explanation of this phenomena, not considering the SoSa I binding site, might be the size of the stimulating agents. Whole bacteria such as *E. coli* and *S. aureus* are much larger than their counterparts LPS and PGN, respectively, which are only one particular part of their cell membrane. Whole bacteria are more sterically inhibited than the appropriate PAMPs by the unspecific binding of SoSa I to the outer membrane of MUTZ-3 cells.

**Influence of SoSa I on the Cell Viability and Cell Proliferation of MUTZ-3 cells**

Chemically synthetized Saponin is used as a reagent for permeabilization at concentrations of 0.1-0.5% (w/v).[29] To exclude the possibility that SoSa I has a cell damaging effect, cell viability-assays were performed using the MTT-Test (3-(4,5-Dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium, bromide) with different concentrations of SoSa I (5, 50 and 100 µg/mL). In Figure 5, it is illustrated that SoSa I does not decrease cell viability at any concentration used. These results were similar to those observations previously described by Kang et al., who used concentrations of 30-100 µg/mL SoSa I for the co-incubation of



 

peritoneal macrophages with LPS and could exclude a cell damaging effect.[9]

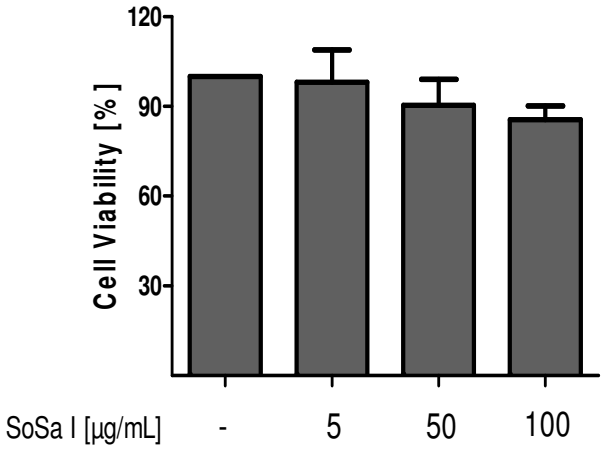


Figure 5: Influence of SoSa I (5, 50 and 100 µg/mL) on the Cell Viability [%] of MUTZ-3 cells. The Optical Density was photometrically determined by MTT-Test. Values are represented as mean ± SEM (n=3, *p < 0.05, One-way ANOVA).

**Effect of PAMP- and SoSa I-treatment on the surface marker expression profile of MUTZ-3 cells**

Generally, pro-inflammatory stimulation triggers cytokine secretion and leads to up-regulation of co-stimulatory and antigen-presenting molecules on the cell surface and to altered TLR expression.[19] [30] Figure 6 panel A shows the cell surface markers TLR2 and TLR4, and CD80 and CD86 after stimulation (black line) with LPS (1 µg/mL) or PGN (100 µg/mL) and in combination with 100 µg/mL SoSa I (red line) to investigate the influence of SoSa I on surface markers.

As shown before, incubation with LPS or PGN modulated the expression of TLR2, TLR4 CD80 and CD86 (see Figure 2). Figure 6 panel B shows that SoSa I had no effect on the expression of CD80, TLR2 and TLR4 in LPS- or PGN-stimulated MUTZ-3 cells. In contrast, expression of CD86 on MUTZ-3 cells was down-regulated by 31.99% ±21.06 after LPS-stimulation and by 27.56% ±9.22 after PGN stimulation following co-incubation with SoSa I. Obviously, only CD86 levels were reduced by SoSa I, what may be attributable to differences in the intracellular signaling pathways. CD86 up-regulation on DCs which were stimulated with a member of the TNF family is reported to be predominantly mediated via the NF-

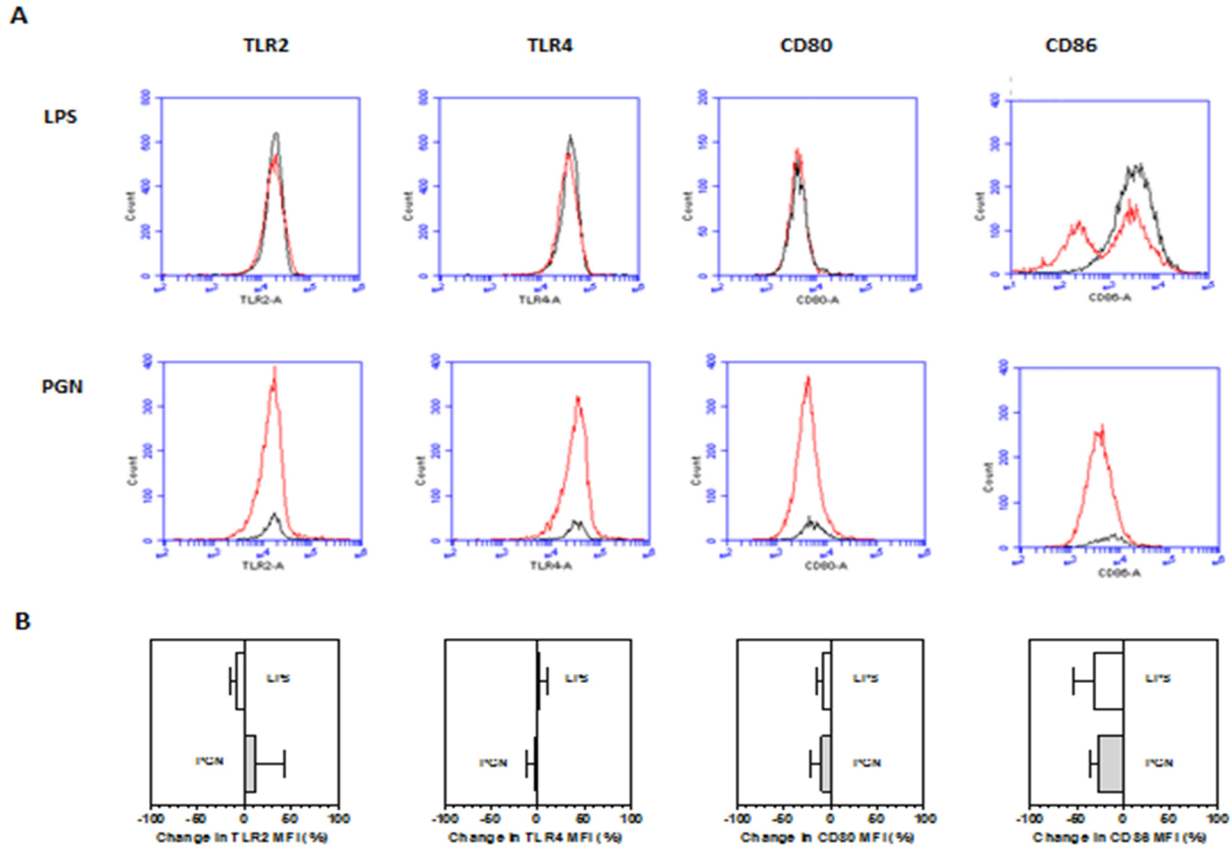


Figure 6: Panel A shows the effect of SoSa I on PAMP-treatment relating to surface marker expression levels. MUTZ-3 cells were incubated with LPS (1 µg/mL) or PGN (100 µg/mL) alone or in combination with SoSa I (100µg/mL (black line = LPS or PGN alone, red line = LPS or PGN in combination with SoSa I). Expression levels of TLR2, TLR4, CD80 and CD86 were determined by flow cytometry. Data show one representative out of three independent experiments. Panel B shows the percent influence of SoSa I on the Mean fluorescence of Surface markers (above named) compared to the PAMP-treated-positive control. Expression levels of TLR2, TLR4, CD80 and CD86 were determined by flow cytometry. Values are represented as mean ± SEM (n=3).

κB signaling pathway.[23] Previous studies determined the NF-κB pathway to be the main target of SoSa[9 10 11], and mentioned that MAPK signaling is only weakly suppressed by SoSa.[10 11] Thus, the predominant MAPK-mediated regulation of TLR2 and TLR4 expression on MUTZ-3 cells would explain the lower effect of SoSa I on the TLR levels.

## Experimental

### MUTZ-3 cell line & Cultivation

The human acute myeloid leukemia-derived cell line MUTZ-3 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The MUTZ-3 cell line was chosen because in the literature they are described as professional APC expressing pattern recognition receptors (PRR: e.g TLR) and as the most suitable cell line for LPS-studies according to information of the DSMZ. [18]

The cells were cultured in minimum essential medium alpha with ribonucleosides, deoxyribonucleosides and L-glutamine (α-MEM; Life Technologies, Darmstadt, Germany). α-MEM was supplemented with 20% (v/v) heat-inactivated (56°C for 30 min.) fetal bovine serum (FBS; Biochrom, Berlin, Germany), 10 ng/mL recombinant human (rh) granulocyte macrophage colony-stimulating factor (GM-CSF; Biochrom, Berlin, Germany) and 100 U/mL/ 100 µg/mL penicillin/streptomycin (Biochrom, Berlin, Germany). MUTZ-3 cells were incubated in 24-well plates (VWR, Darmstadt, Germany) at 1.5 - 8 x $10^5$ cells per mL and per well and cultivated in a humidified incubator (CB150, Binder, Tuttlingen, Germany) at 37°C with 5% $CO_2$. After 3-4 days of incubation, the cells were transferred into fresh medium as follows: cells were centrifuged at 300 x g for 5 min., the supernatant was removed and the cell pellet was resuspended in fresh culture medium. The cell viability was analyzed by standard 0.5% (w/v) trypan blue cell staining (Biochrom, Berlin, Germany).

### Cultivation of bacterial stimulants

*Escherichia coli* K12 and *Staphylococcus aureus* were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. Both were cultured aerobically in 9 mL standard I broth (Carl Roth, Karlsruhe, Germany) at 30°C.

### Microscopic methods

MUTZ-3 cells were visualized using REM and light microscopy. For the REM preparation, the MUTZ-3 cell culture was centrifuged for 5 min. at 300 x g. The cell pellet was resuspended in a 2.5% glutardialdehyde solution (Merck Schuchardt, Hohenbrunn, Germany) with 0.05 M HEPES (Biochrom, Berlin, Germany), incubated for 2 h at 37°C and stored over night at 2-8°C. Afterwards, the cells were washed with 2 mL 1 M HEPES and dehydrated by incubating the cells in a cascade of increasing ethanol concentrations (30%, 50%, 70% and 99.5%) with 10 min. maintenance at each step and with at least 20 min. in 99.5% ethanol, respectively. Afterwards, the cells were dried with the CPD2 PELCO Critical Point Dryer Cat. No. 2400 (Ted Pella, Inc., USA), covered with gold in an Edwards S150B sputter coater (Edwards High Vacuum Inc., Crawley, West Sussex, UK) and examined under the raster electron microscope (Leica S420, Leica Microsystems, Wetzlar, Germany).

Morphological changes of MUTZ-3 cells were also routinely observed under the Olympus BH-2 microscope (Olympus Optical Co., LTD, Tokyo, Japan) and recorded with the ColorView 12 digital camera (Soft Imaging System SIS, Münster, Germany) in combination with analySIS FIVE software (SIS, Münster, Germany).

### Flow cytometry

The expression of specific cell surface markers on MUTZ-3 cells was determined by a BD Accuri™ C6 flow cytometer (BD Bioscience, San Jose, USA). Cell suspensions were transferred to FACS tubes and washed with phosphate buffered saline (PBS; Life Technologies, Darmstadt, Germany). Then, 5 x $10^5$ MUTZ-3 cells in 100 µL PBS were stained for 20 min. in the dark at room temperature with the following antibodies: lin1-FITC (BD Biosciences, San Jose, USA), CD86-FITC (PharMingen, San Diego, USA), CD14-APC, CD123-APC, CD11c-PerCP-Cy7 (BD Pharmingen, San Diego, USA), CD80-FITC (Immunotech, Marseille, France), HLA-DR-PE (BD Immunocytometry Systems, San Jose, USA), anti-TLR2-PE and anti-TLR4-PE (BioLegend, San Diego, USA). Afterwards, 300 µL PBS were added and samples were measured using the BD Accuri™ C6. A BD CSampler (BD Bioscience, San Jose, USA) was used to analyze the data.

### Stimulation of MUTZ-3 cells

Bacterial cultures were diluted with 1% (v/v) PBS at a concentration of 1 x $10^7$ bacteria/mL for *E. coli* and 1 x $10^9$ bacteria/mL for *S. aureus,* heat-inactivated for 10 min. at 80°C, aliquoted and stored at –80°C. LPS from *E. coli,* PGN from *S. aureus* and SoSa I from *Glycine max* (soybean) were purchased from Sigma-Aldrich (Taufkirchen, Germany). LPS and PGN were diluted with α-MEM at a concentration of 1 mg/mL and SoSa I at 2 mg/mL. Reagents were stored at –30°C.

To determine the secretion of pro-inflammatory mediators upon inflammatory stimulation, 100 µL of MUTZ-3 cells (1 x $10^6$/mL) were treated in 96-well plates (VWR, Darmstadt, Germany) with LPS (1 µg/mL), PGN (100 µg/mL), *E. coli* ($10^6$ cells/mL) or *S. aureus* ($10^8$ cells/mL). In order to investigate the immune modulatory effect of SoSa I, the cells were incubated either alone or with increasing concentrations of SoSa I (5–100 µg/mL) referring to Kang et al. and Zha et al.[9 14] Each well was filled to a total volume of 200 µL with α-MEM supplemented with 20% (v/v) heat-inactivated FBS, 10 ng/mL rhGM-CSF and 100 U/mL/ 100 µg/mL penicillin/streptomycin. After 24 h of incubation at 37°C, 5% $CO_2$ and 95% humidity, supernatants were collected, immediately frozen, and stored at -80°C. The optimal concentrations of the stimulants were determined in previous experiments (data not shown).



 

To investigate the influence of inflammatory stimulation on surface marker and TLR expression, 500 µL of MUTZ-3 cells (1 x $10^6$/mL) were treated in 24-well plates (1 mL/well) with LPS (1 µg/mL) or PGN (100 µg/mL). The highest SoSa I concentration (100 µg/mL) was added to determine the effect of SoSa I on the expression levels. After 18 h of incubation at 37°C, 5% $CO_2$ and 95% humidity, cells were assessed by flow cytometry.

### Cytokine measurement with CBA

To study the primary inflammatory response of MUTZ-3 cells to different treatments, the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and the chemokines IP-10, RANTES and IL-8 were analyzed using the BD™ CBA Flex Sets (BD Bioscience-PharMingen, USA). Frozen supernatants were thawed at room temperature and centrifuged at 10000 rpm for 10 min. at 2°C. To measure IL-8, supernatants were adequately diluted in a range of 1 to 5 for determining negative controls and *S. aureus*-induced levels, 1 to 40 for measuring LPS- and PGN-triggered secretion and 1 to 100 for investigating *E. coli*-induced release. For this, 50 µL of Flex Set Standards (10-2500 pg/mL) and samples (50 µL) were incubated with 50 µL capture bead mixture and 50 µL phycoerythrin detection reagent in 96-well plates at room temperature in the dark for 3 h. After washing, samples were measured with the flow cytometry BD FACS Array™ (BD Bioscience-PharMingen, USA). FCAP Array Software (BD Bioscience-PharMingen, USA) was used to analyze the data.

### Cell proliferation Assay (MTT)

Cell proliferation-assays were performed by the MTT-Test (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium, bromide) as described in the product information of Dojindo Molecular Technologies (Gaithersburg, USA). Therefore, MUTZ-3 cells ($10^6$ cells/mL) were treated with SoSa I (100 µg/mL) and Saponin (100 µg/mL) purchased by Sigma (Taufkirchen, Germany) in 96-well plates for 24 h. Concentrations of SoSa I and Saponin equate to about 10% (w/v) in the system. The OD was determined by Tecan Reader (Mainz, Germany).

### Statistical analysis

Data display the mean ± standard error of mean (SEM) of three or more measurements. The data were evaluated by GraphPad Prism 5 (GraphPad Software, La Jolla, USA). Comparison between two groups was performed using a two-tailed Mann-Whitney or two-tailed unpaired tests with values of $p < 0.05$ considered statistically significant. Comparison between $\geq 3$ groups were made using an ANOVA with Bonferroni's post hoc analysis for relevant comparisons. Differences were defined as significant when *P*-values < 0.05

## Conclusions

In summary, this study showed that the anti-inflammatory activity of SoSa I was higher in whole bacteria- than in LPS- or PGN-stimulated samples in the MUTZ-3-cell model. The more effective inhibition of whole bacteria compared solely to LPS or PGN points to a broader role of SoSa I in the down-regulation of inflammation. In addition, SoSa I also influenced expression of the activation marker CD86. Further studies have to be performed to investigate the anti-inflammatory activity of SoSa I in more detail.

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## Notes and references

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