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Regulation of Staphylococcus aureus-mediated activation of interleukin-18 in peripheral blood mononuclear cells

Abstract

Bacteria and bacterial antigens strongly induce cytokine secretion by peripheral blood leukocytes and thereby initiate an inflammatory cascade with potentially deleterious consequences for the host. The present study focussed on receptors and signal transduction pathways involved in activation of interleukin (IL)-18 by heat-inactivated Gram-positive Staphylococcus aureus Cowan strain I (SAC). Similarly to IL-12/IL-12p40, IL-10 and IFN-gamma, SAC dose-dependently activated IL-18. Secretion of IL-18 was independent of functional activity of IL-10,

IL-12 or IFN-gamma. Lipoteichoic acid (LTA), a structural component of SAC, was not sufficient for activation of IL-18, while it dose-dependently induced IL-10. In contrast to IL-12, blockade of CD14 only partially diminished secretion of IL-18 and did not affect secretion of IL-10, suggesting involvement of other receptors (e.g., Toll-like receptors) in SAC responses. Further down-stream however, secretion of IL-10, IL-12 and IL-18 was uniformly inhibited by blockade of G-protein-mediated kinase activation by mastoparan. Secretion of IL-18 required phosphatidylinositol-3'-kinase, and secretion of IL-12 phosphotyrosine kinase activity. The data demonstrate that SAC potently activates secretion of IL-18 by peripheral blood mononuclear cells with differential involvement of cell-surface receptors and signal transduction pathways as compared to other natural killer- and T cell-promoting cytokines.

Full text

INTRODUCTION

Polymorphonuclear granulocytes and monocytes drive the host's initial cellular response to an invader. Cytokines released by monocytes secondarily activate non-specific natural killer cells as well as T and B cells. While natural killer cells mount a fast and strong immune response during acute infection, T and B cells are crucial in the chronic phase. Among the monocyte-derived cytokines are IL-12 and the recently cloned IL-18 that promote natural killer cell and T helper cell class 1-type responses by induction of IFN-gamma as well as IL-10, promoting a T helper cell class 2-type response [1-3].

IL-12 and IL-18 reciprocally up-regulate their receptors responsible for synergistic effects on Th1 development [4]; IL-18 enhances expression of the IL-12Rbeta2 sub-unit on naïve T cells [5]. Requirement of IL-18 for development of a T helper cell class 1-type response however, depends on the nature of the infectious agent, as recently demonstrated in IL-18-deficient mice [6, 7].

Studies on cytokine induction in monocytes exposed to bacteria and bacterial components have focussed on lipopolysaccharide (LPS), a glycolipid of the cell wall of Gram-negative bacteria. LPS induces IL-10 and IL-12 in monocytes primed by IFN-gamma [8], while preincubation with LPS abrogates the priming effect for IL-12 [9]. Peripheral blood mononuclear cells constitutively express IL-18 mRNA [10, 11], and IL-18 protein secretion is induced by LPS [12]. Signaling by LPS

involves binding to CD14, a 55-kDa glycoprotein that is anchored by glycosylphosphatidylinositol (GPI), association of CD14 with heterotrimeric G-proteins and phosphorylation/activation of the p38 MAP kinase [13].

Soluble factors, exotoxins and cell wall components mediate the induction of an inflammatory response by Gram-positive bacteria such as *Staphylococcus aureus*. Lactobacilli and streptococci are inducers of IL-10, IL-12 and IL-18 [14, 15], and SAC has been shown to induce IFN-gamma [16]. There is evidence that the initiation of the inflammatory response by *Staphylococcus aureus* or its components bears a similarity to the signaling cascade initiated by LPS [17]. CD14 comprises conformational binding sites for LPS and for peptidoglycan, a cell wall component of Gram-positive bacteria [18]. Peptidoglycan-stimulated IL-1 and IL-6 secretion is blocked by anti-CD14 [19], and LTA, another wall fragment of Gram-positive bacteria, induces iNOS mRNA in murine macrophages *via* CD14 [20]. However, conflicting data from experimental animal studies suggests that CD14 does not play a significant role in septic shock induced by *Staphylococcus aureus* [21], and the CD14-blocking antibody MY4 does not affect TNF-alpha secretion induced by SAC [22]. Furthermore, recent data propose that beta2-integrins are involved in cytokine induction by streptococci, *Listeria monocytogenes*, and *Staphylococcus aureus* [23].

The present study focussed on the potential of SAC to activate IL-18 in peripheral blood mononuclear cells. We investigated involvement of cell surface receptors and early signal transduction pathways. For the purpose of comparison, secretion of IL-10, IL-12 and IFN-gamma was assessed.

MATERIAL AND METHODS

Cell culture

Peripheral blood mononuclear cells were isolated from randomly selected healthy donors as described previously [24]. The study was approved by the local ethics committee.

Peripheral blood mononuclear cells were cultured in endotoxin-free RPMI 1640 medium at 37° C and 5% CO_2. Culture was performed in 6.5 ml polystyrene tubes (Falcon, Heidelberg, Germany), for 12-24 hours immediately after cell isolation.

The following bacterial antigens, antibodies and peptides were applied: formalin-fixed, heat-inactivated *Staphylococcus aureus* Cowan strain (SAC (10^{-5}-10^{-2} weightvol/volume [w/v]; Calbiochem, Bad Homburg, Germany), lipoteichoic acid extracted from *Staphylococcus aureus* strain DSM# 20233./MAM/092194 (LTA 0.01-10 mug/ml, Sigma, Taufkirchen, Germany), anti-CD14 blocking antibody (10 mug/ml, BIG10, IgG1, Biometec, Greifswald, Germany), anti-CD14 non-blocking antibody (10 mug/ml, BIG7, IgG1, Biometec, Greifswald, Germany), anti-IFN-gamma antibody (10 mug/ml; Calbiochem), anti-IL-10 antibody (10 mug/ml, R&D Systems, Heidelberg, Germany), anti-IL-12 antibody (10 mug/ml, PharMingen, Hamburg, Germany), Ac-YVAD-chlormethylketone (20 muM; BACHEM, Heidelberg), Mastoparan (13.3 mM; Quality Controlled Biochemicals, Hopkinton, MA, USA), Mastoparan 17 (13.3 mM; Quality Controlled Biochemicals), and Genistein (20 muM), Wortmannin (10 nM), calcium ionophore A23187 (10 muM) (all Sigma). For inhibition of transcription, actinomycin D (Sigma) was added to the culture supernatant at 5 mug/ml.

Immunoassay for IL-18, IL-12, IL-12p40, IL-10 and IFN-gamma

Levels of IL-18 were determined in paired supernatant samples by a specific sandwich enzyme immunoassay (ELISA). Briefly, 96-well microtitre plates (Nunc Maxisorp, Denmark) were coated with 100 mul of the primary monoclonal antibody against IL-18 (2.5 mug/ml; MoBiTec, Göttingen, Germany), and incubated for 3 hours at room temperature. The coated plates were washed in 1 **x** PBS/Tween 20 and blocked with 200 mul 1% bovine serum albumin in PBS (pH 7.4) at 4° C overnight. Samples diluted 1:5, and serial dilutions of the recombinant IL-18 standard (MoBiTec) were added to the plates and incubated for 2 hours at RT. After washing with PBS/Tween 20 buffer, 100 mul of peroxidase-conjugated monoclonal detection antibody (working concentration 0.1 mug/ml; MoBiTec) were added for 3 hours at room temperature. The enzyme reaction was developed using 100 mul of 10 mmol/l tetramethylbenzidine (TMB), 80 mmol/l H_2O_2 and 30 mmol/l potassium citrate (pH 4.1). After incubation for 15-20 min at RT, the reaction was terminated with 50 mul of 2N H_2S0_4, and the optical density (OD) was read at 450 nm (Emax Reader, MWG, Ebersberg, Germany; reference at 650 nm). Each sample was tested in duplicate and the values determined from the standard curve (range 8-500 pg/ml), constructed using the serial dilutions of recombinant IL-18 standard. The detection limit was 7.8 pg/ml with the intra- and inter-assay variances being 5.8 ± 2.1% and 8.2 ± 2.4%, respectively. The IL-18 -specific ELISA showed no cross-reactivity with

other recombinant cytokines or cytokine receptors (IL-12, IL-2, IFN-gamma, IL-4, TNF-alpha and recombinant TNF-receptors) over a wide range of concentrations (0.1pg/ml-1 mug/ml).

Levels of IL-12, IL-12p40 and IFN-gamma were determined by ELISA as previously described [25]. Levels of
IL-10 were determined by ELISA according to the manufacturer's specifications (PharMingen).

Statistical analysis

Data were expressed as mean ± SE. Statistics were performed on a Macintosh computer using the Statview 4.5 software (Abacus Inc., Berkely, CA, USA). The Mann-Whitney-U test was used to compare data between two groups. A p value of less than 0.05 was considered statistically significant.

RESULTS

Freshly isolated PBMC were cultivated in the presence of SAC. As assessed by ELISA, SAC dose-dependently activated IL-18 (Figure 1). Similarly, IL-12, IL-12p40, and IFN-gamma were secreted into the cell culture supernatant (Figure 1). In order to demonstrate that IL-18 secretion was independent of functional activity of IL-10, IL-12 and IFN-gamma, cells were stimulated by SAC and co-incubated with specific neutralizing antibodies. IL-18 levels remained unchanged in this setting (Figure 2), indicating that SAC primarily activated IL-18. Differently to blockade of IL-10, IFN-gamma levels were reduced when IL-12 was neutralized (data not shown).

Transcription was required for SAC-induced cytokine secretion, as determined by co-incubation with actinomycin D, an inhibitor of transcription (Figure 3), leading to significant reduction of IL-18, IL-12, IL-12p40 and IFN-gamma contents in cell-free supernatants. To confirm that in our system IL-18 was actively processed rather than passively released, cells were stimulated in the presence of Ac-YVAD-chlormethylketone (Ac-YVAD-CMK). Ac-YVAD-CMK specifically and irreversibly blocks caspase-1 activity required for cleavage of the IL-18 pro-form [26]. IL-18 secretion was specifically reduced down to 25%, while the content of IL-12, IL-12p40, IFN-gamma and IL-10 in the supernatant remained unaffected (Figure 4). The data indicated that IL-18 was not the IFN-gamma-inducing factor

in SAC-stimulated peripheral blood mononuclear cells. Distinct cell wall components characterize Gram-positive bacteria. We assessed whether LTA carried the IL-18 activating structure of SAC. Strikingly, IL-18 was not secreted in response to LTA, while IL-10 was dose-dependently induced (data not shown).

The receptors and signal transduction pathways involved in SAC-mediated activation of IL-18 are incompletely understood. To analyze involvement of CD14, we applied specific antibodies to CD14 with (BIG10) and without (BIG7) LPS neutralizing activity. Both antibodies abrogated induction of IL-12 by SAC and reduced secretion of its sub-unit p40 (Figure 5). Also, both antibodies reduced IFN-gamma levels by more than 50% at 10^{-3} w/v SAC and down to the baseline at 10^{-4} w/v SAC (Figure 5). In contrast, the amount of IL-18 in the supernatant was diminished by less than 50% (Figure 5), and secretion of IL-10 remained unaffected (Figure 5). The data suggested that CD14 was essential for SAC-mediated induction of IL-12 and IFN-gamma, but only partially or not involved in SAC-mediated secretion of IL-18 and IL-10, respectively. Confirming the data presented in Figure 4, induction of IFN-gamma was dependent on the presence of IL-12.

We then explored whether activation of early signal transduction pathways was required for SAC-mediated activation of IL-18. Cell activation upon binding of bacterial components to cell surface receptors involves activation of kinases leading to nuclear translocation of transcription factors and cytokine gene expression. G-protein dependent activation of the p38 MAP kinase is essential for CD14-mediated cytokine induction by LPS, and it is inhibited by the peptide Mastoparan [13]. In our study, Mastoparan significantly inhibited SAC-induced secretion of IL-12, IL-12p40, IL-18, and IL-10, and it reduced mean levels of IFN-gamma levels by more than 70% (Figure 6). The control substance Mastoparan 17 had no effect. Liberation of calcium by the ionophore A23178 reduced secretion of the p40 subunit of IL-12, while there was only a trend towards lower levels of IL-12 (Figure 7). Interestingly, only Wortmannin, a phosphatidylinositol-3'-kinase inhibitor, selectively reduced IL-18 levels (Figure 7). Secretion of IL-12, IL-12p40 and IFN-gamma was dependent on phosphotyrosine kinase activity as assessed in the presence of its inhibitor Genistein (Figure 7).

DISCUSSION

The host's innate response to bacterial infections comprises activation of phagocytic immune cells that, beyond their immediate bactericidal capability, express cytokines, which secondarily attract and activate natural killer, T- and B-lymphocytes. Initiation and perpetuation of this cascade by both Gram-negative and Gram-positive bacteria can itself constitute a serious condition for the host leading to septic shock [27]. Recently, our understanding of the cell surface receptors and signaling pathways involved in immune activation by LPS from Gram-negative bacteria has been substantially elucidated. Cell activation comprises binding of LPS to cell surface receptors such as CD14, enhanced by LBP, stimulation of Gi and Go heterotrimeric G proteins, phosphorylation and activation of the p38 MAPK and eventually cytokine gene expression [13]. In terms of Gram-positive bacteria, the structure of lipoteichoic acid determines the ability of a bacterium to cause shock and multiple organ failure, while peptidoglycan amplifies the response induced by lipoteichoic acid [28]. In our study, we investigated the potential of Gram-positive *Staphylococcus aureus* Cowan strain, an important cause of sepsis in immuno-compromised patients [29, 30], to induce IL-18 in peripheral blood mononuclear cells.

We confirm that inactivated SAC substantially and dose-dependently induces both cytokines driving a NK (IFN-gamma), T helper cell class 1 (IL-12 and IL-18) and T helper cell class 2 (IL-10) response. Both IL-12 and IL-18 are important inducers of IFN-gamma, therefore IFN-gamma levels were determined in parallel. Interestingly, in the presence of IL-12, induction of IFN-gamma did not require extracellular IL-18. Since IL-18 is synthesized as a pro-form stored in the intracellular compartment and cleaved for secretion by caspases [31], we confirmed that SAC-stimulated IL-18 secretion required both gene transcription and caspase activity, suggesting that SAC is a potent inducer of caspase-1 - although not proven by measurement of caspase-1 activity - as has been shown for LPS [26]. Previous studies have shown that SAC-induced IFN-gamma is produced by NK cells and not by T-lymphocytes [16]. In viral infection, the presence of IL-12 is mandatory for IFN-gamma production [32, 33]. Our data support requirement of IL-12 and not IL-18 for SAC-induced IFN-gamma secretion.

Involvement of CD14 in SAC-mediated cytokine induction has been suggested [17]. Furthermore, blockade of CD14 inhibits the production of IL-10 in monocytes stimulated by *Haemophilus influenzae, Streptococcus pneumoniae* or *Borrelia burgdorferi* [34, 35]. Moreover, it was recently shown that *Staphylococcus*

aureus antigens bind to Toll-like receptor-2 followed by activation of the transcripton factor NF-kappaB [36, 37]. We utilized specific monoclonal antibodies that bind to different epitopes of CD14 with and without LPS-antagonizing activity. The heterogenous effects on SAC-stimulated cytokine expression in our study - abrogation of IL-12, less than 50% reduction of IL-18 levels - suggest that in fact, different surface receptors are involved in SAC-signaling, potentially stimulated by different components of SAC, comprising protein A and lipoteichoic acid. Since interaction of CD14 and Toll-like receptor-2 has been shown to be essential for signal transduction activation, we hypothesize that the binding sites of both anti-CD14 antibodies (BIG10 and BIG7) applied in this study take part in the CD14/Toll-like receptor-2 complex required for SAC-mediated induction of IL-12/IL-12 p40. Involvement of Toll-like receptors in SAC responses will be investigated in future experiments, since specific neutralizing antibodies were not available in our study.

Differences at the receptor levels were followed by differential activation of signal transduction pathways. While G-protein-mediated kinase activation was needed for induction of all cytokines investigated, IL-12 and IL-18 secretion required phosphotyrosine and phosphoinositol-3'-kinase activity, respectively. Inhibition of G-protein-mediated p38 MAPK as part of the CD14-signaling cascade, supports our finding of CD14-dependent induction of IL-12/IL-12 p40 by SAC. However, inhibition of SAC-mediated IL-10 secretion by mastoparan but not by blockade of CD14 demonstrates that SAC-driven p38 MAPK activity is not dependent on functional CD14.

CONCLUSION

In summary, we have presented experimental data demonstrating activation of IL-18 and induction of other T-helper cell targeting cytokines in peripheral blood mononuclear cells by SAC. Differential involvement of cell surface receptors and signal transduction pathways was revealed. A better understanding of the regulatory mechanisms responsible for stimulation of the immune system by bacterial components, as broadened by our study, will help to develop specific intervention strategies in these conditions.

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U.B and T.M. contributed equally to the study.

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Figures

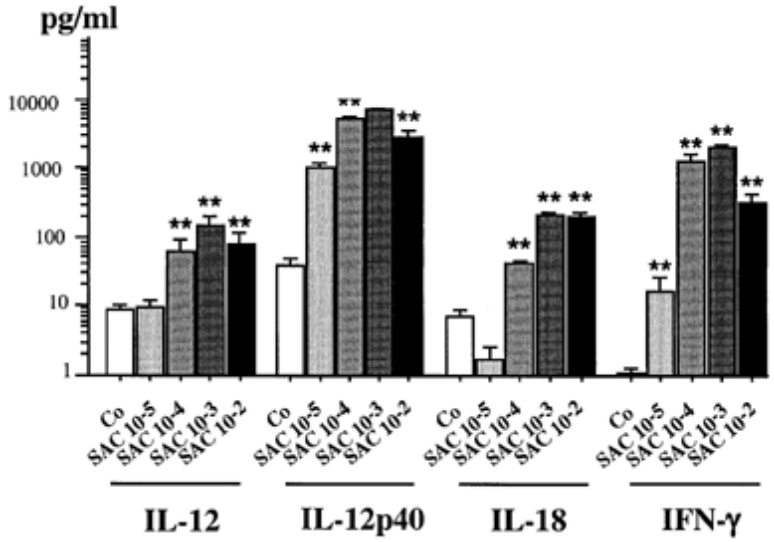


Figure 1
Dose-dependent cytokine induction in peripheral blood mononuclear cells by SAC. Peripheral blood mononuclear cells (3 **x** 10^6/ml) were stimulated for 12 hours by 10^{-5} to 10^{-2} w/v SAC prior to ELISA of cell-free supernatant for indicated cytokines. Note logarithmic scale. Co: Unstimulated control. N = 4; ** $p < 0.01$.

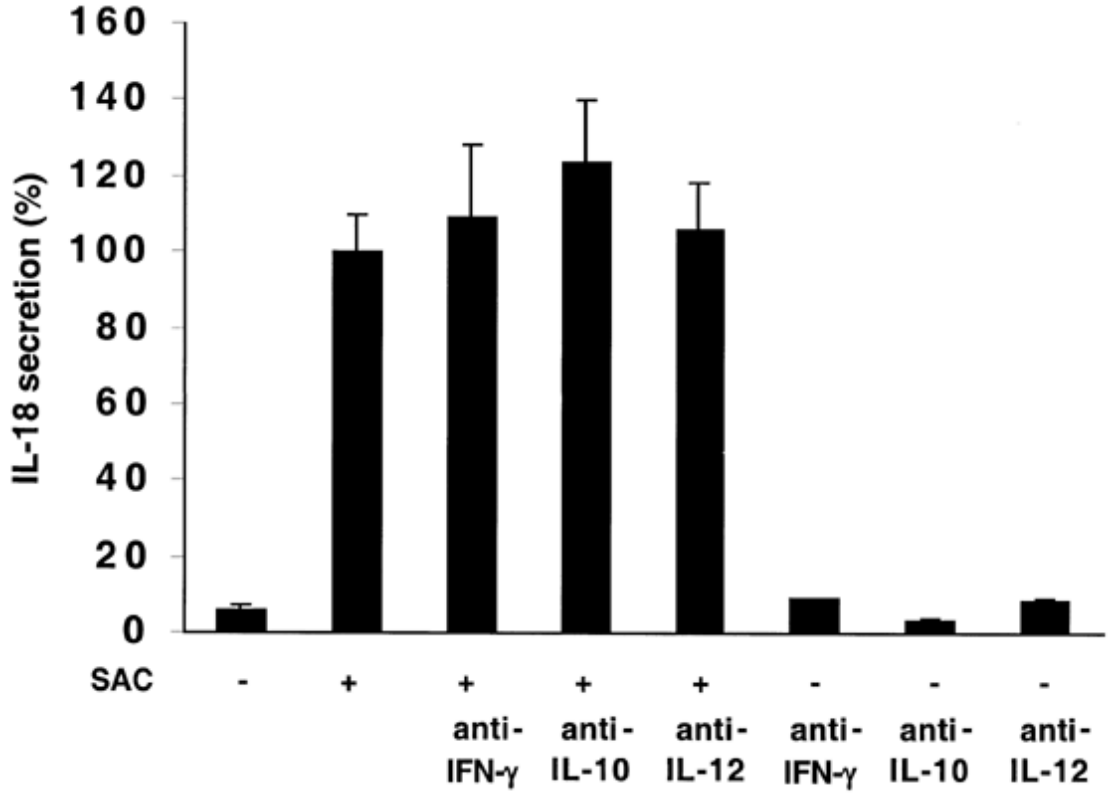


Figure 2

Induction of IL-18 by SAC is independent of functional presence of IFN-gamma, IL-10 or IL-12. Peripheral blood mononuclear cells were stimulated for 12 hours by 10^{-3} w/v SAC in the absence or presence of neutralizing antibodies. IL-18 secretion by SAC-stimulated cells in the absence of specific antibodies was considered 100%. N = 4.

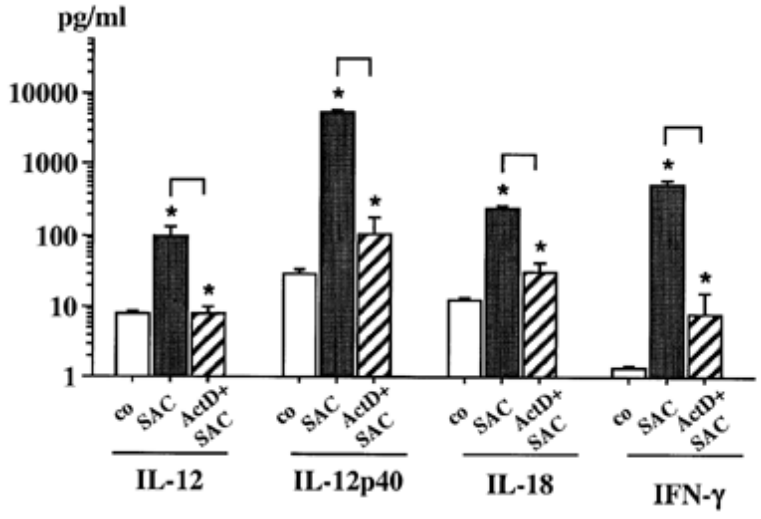


Figure 3

Secretion of SAC-induced cytokines is dependent on transcriptional activity. Peripheral blood mononuclear cells were pretreated for 1 hour with 5 mug/ml Actinomycin D and stimulated by 10^{-3} w/v SAC in the presence of Actinomycin D for additional 8 hours Co: Unstimulated control. N = 4; * p < 0.05.

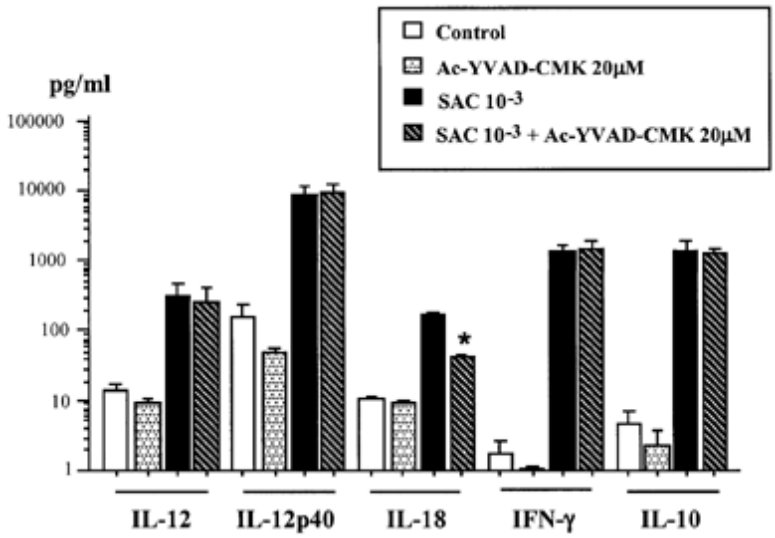


Figure 4

Inhibiton of SAC-stimulated IL-18 secretion by caspase-1 inhibitor. Peripheral blood mononuclear cells were stimulated by 10^{-3} w/v SAC for 20 hours in the absence or presence of the caspase-1 inhibitor Ac-YVAD-CMK at 20 mM. Cytokine levels were determined by ELISA. Note logarithmic scale. N = 3; * p < 0.05.

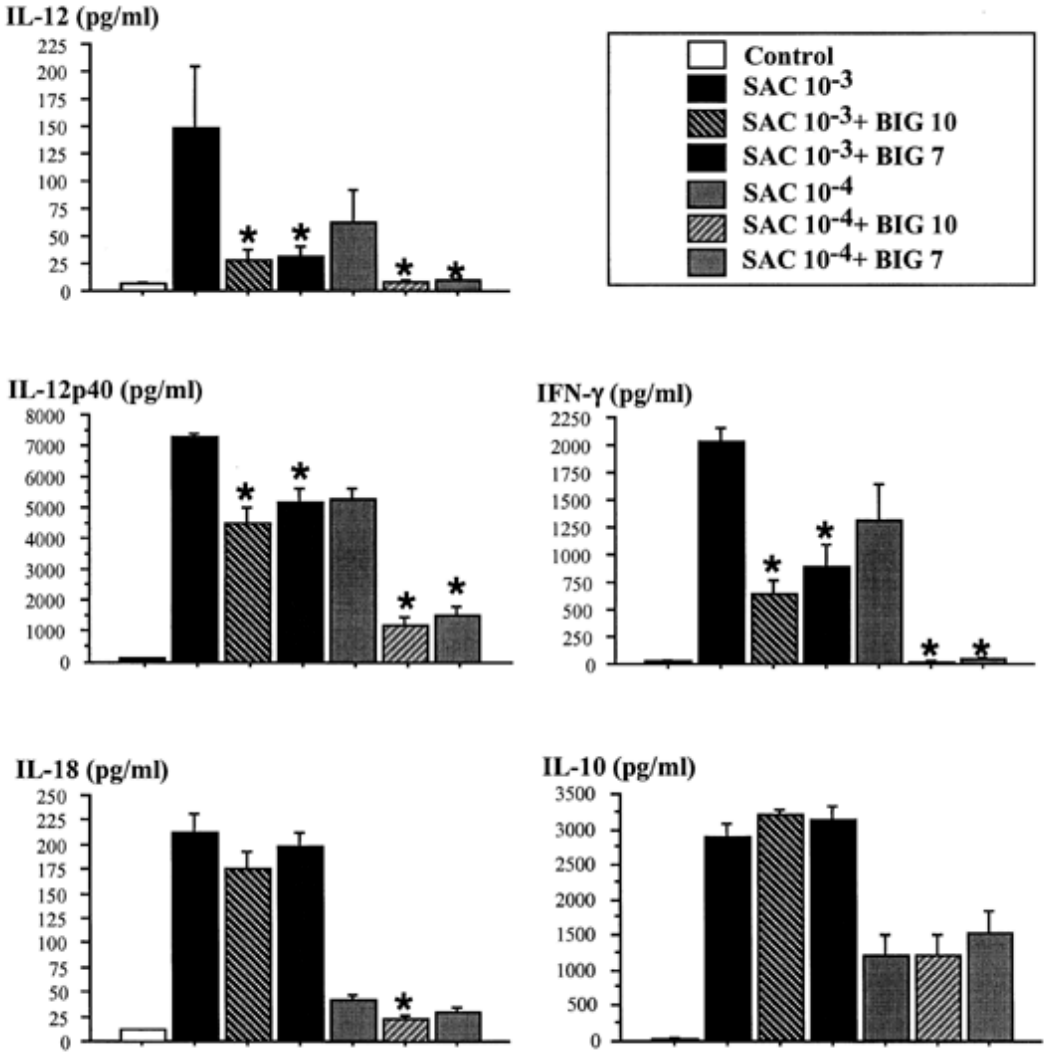


Figure 5

Functional importance of CD14 for SAC-stimulated cytokine induction. Peripheral blood mononuclear cells were stimulated by 10^{-3} w/v SAC for 12 hours in the presence or absence of anti-CD14-antibodies with (BIG 10) and without (BIG7) LPS neutralizing activity. N = 4; * $p < 0.05$.

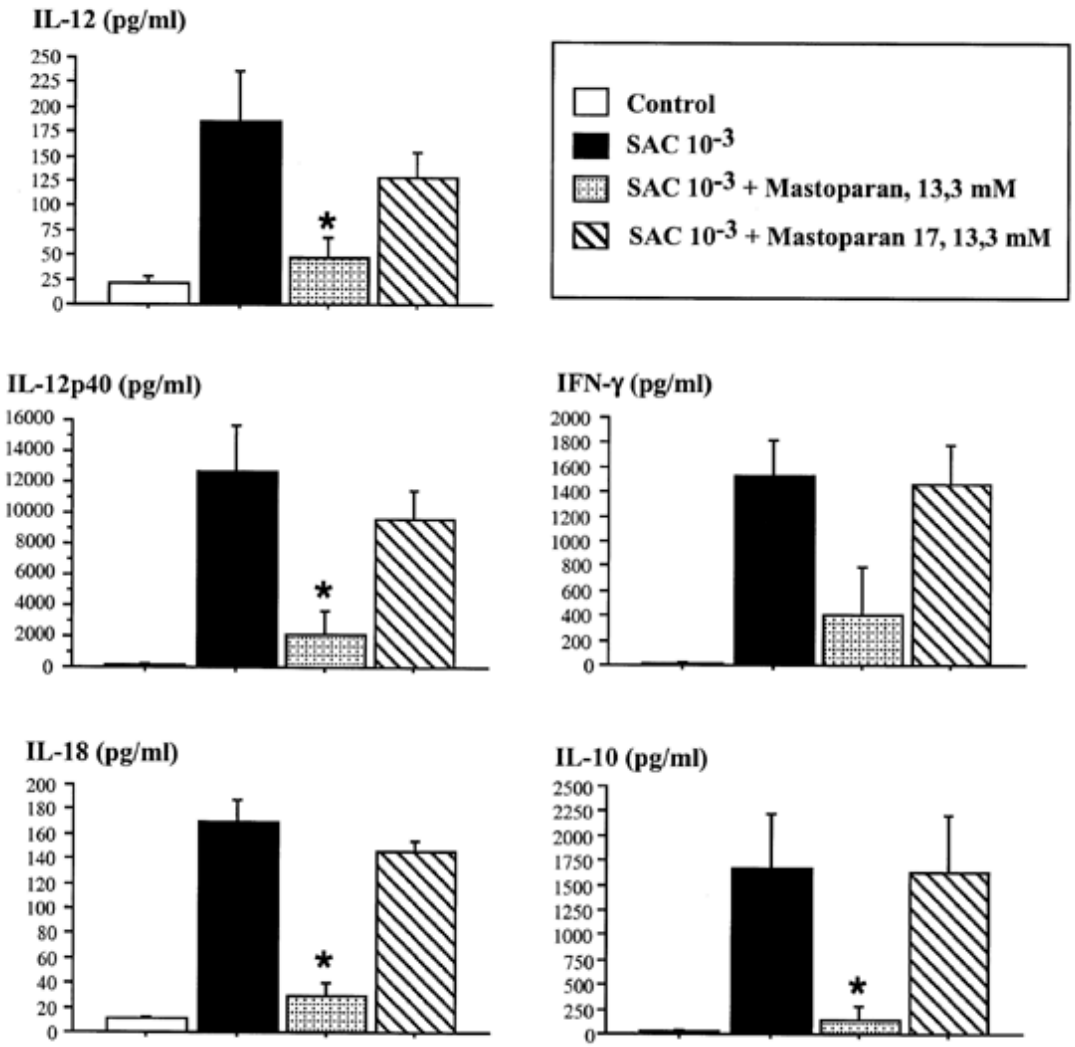


Figure 6

Requirement of G-protein-mediated kinase activation for SAC-induced cytokine induction. Peripheral blood mononuclear cells were pretreated with 13.3 mM Mastoparan or with the functionally inactive control substance Mastoparan 17 for 1 hour, and then stimulated by 10^{-3} w/v SAC for 12 hours prior to assessment of cell-free supernatant by ELISA. N = 4; * $p < 0.05$.

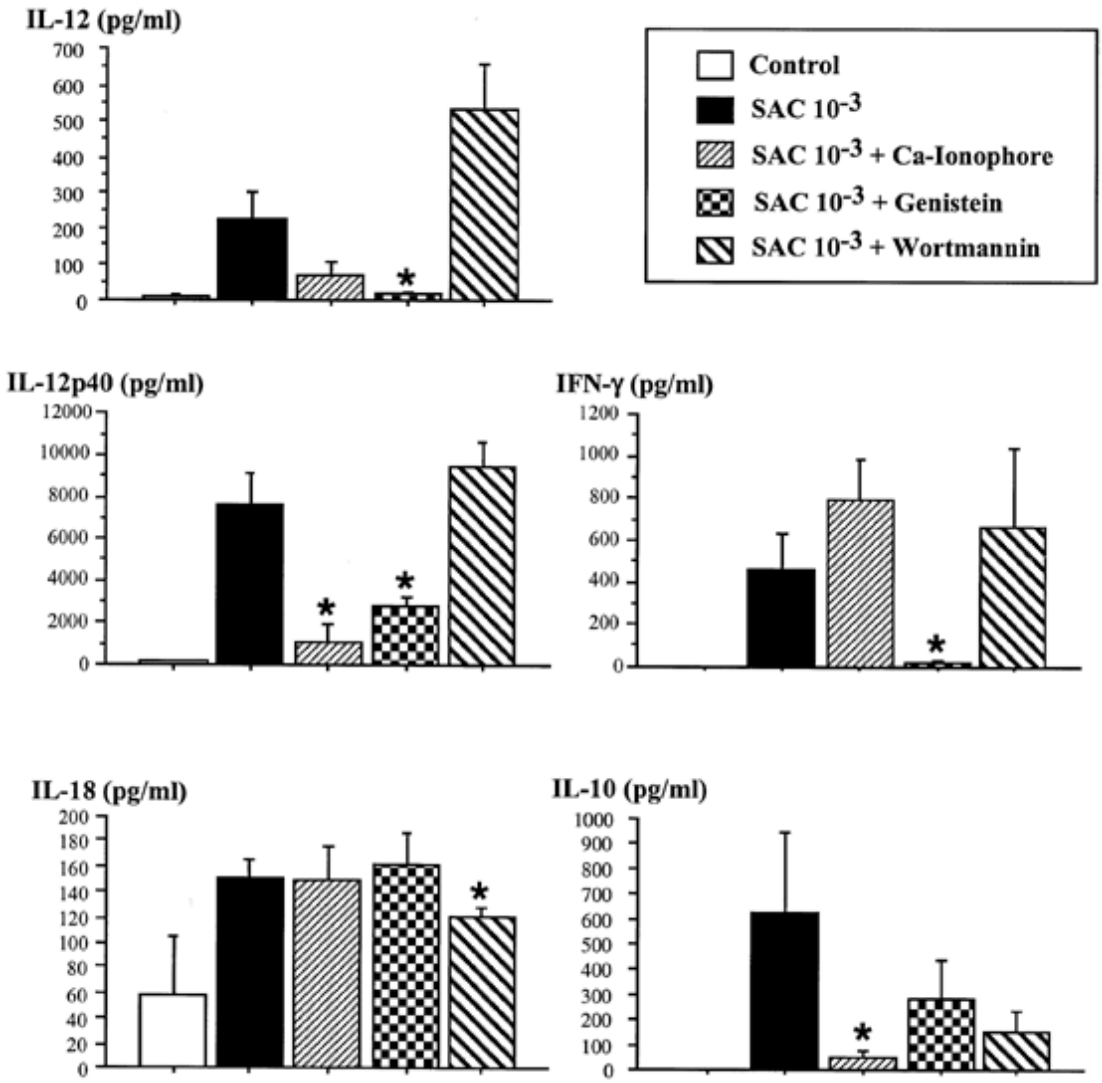


Figure 7

Inhibition of SAC-mediated activation of signal transduction pathways. Peripheral blood mononuclear cells were pretreated with Calcium-Ionophore A23187 (10 muM), Genistein (20 muM) or Wortmannin (10 nM) for 1 hour prior to stimulation by 10^{-3} w/v SAC for 12 hours and analysis of cell-free supernatant for cytokine content by ELISA. N = 3; * $p < 0.05$.

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