ROLE OF P38 AND EGR-1 IN THE MACROPHAGE RESPONSE TO GROUP B STREPTOCOCCUS

Running title: MyD88-dependent signaling in response to GBS

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ABSTRACT

Group B streptococcus (GBS), the most frequent single isolate in neonatal sepsis and meningitis, potently activates inflammatory macrophage genes via Myeloid-differentiation antigen 88 (MyD88). However, events parallel and downstream of MyD88 that instruct the macrophage response are incompletely understood. In this study we found that only MyD88, but not the TLR adapter proteins TIRAP/MAL, TRIF and TRAM, essentially mediates the cytokine (TNF, IL-6) and chemokine (RANTES) response to whole GBS organisms, although MAL, TRIF and TRAM have been shown to mediate the response to substructures from other Gram-positive and Gram-negative bacteria. The GBS-induced, MyD88-dependent phosphorylation of the MAP-kinase p38 activated the transcription factors AP-1 and the early growth response factor (Egr)-1, but not NFκB. Furthermore, phosphorylation of the Ets-like molecule (Elk)-1 was mediated by p38. However, in contrast to Egr-1 and AP-1, Elk-1 was dispensable for the transcriptional activation of TNF by GBS organisms. Studies in macrophages from Elk-1-deficient mice revealed, that Elk-1 was furthermore non essential for the TNF response to purified TLR2 and TLR4 agonists, which was in notable contrast to studies employing in vitro expression systems.

In conclusion, MyD88, p38 and Egr-1, but not Elk-1, essentially mediate the inflammatory cytokine response to GBS organisms.
INTRODUCTION

The advent of Toll-like receptors (TLRs) has revealed that the conventional synonymous use of “innate immune response” and “non-specific immune response” is flawed. Specific receptor based interactions between bacteria and immune cells result in the formation of distinct ecologic niches on the surface of the human body. Furthermore bacteria are likely to express TLR ligands in an inducible fashion therefore adapting to their specific immunological environment (12, 15). In other words, molecular variations among conserved structures might transform a ligand important for the recognition of one organism into a less important one in another species (10, 13). Accordingly, the interface between microbial particle and phagocyte is critical for the specificity of the host response. A further level of specific regulation is added by events downstream of TLRs that drive the phagocyte response towards transcriptional activation of inflammatory and/or directly antibacterial genes (8). The pivotal relay in uttermost proximity to the transmembrane TLRs is formed by a heteromer containing at least four well defined intracellular proteins that share the so-called Toll-IL-1-resistance (TIR) domain with TLRs: 1) myeloid differentiation primary response gene (MyD) 88, 2) Toll-IL-1-resistance (TIR) domain-containing adaptor-inducing IFN-beta (TRIF), 3) TRIF-related adaptor molecule (TRAM), 4) MyD88-adapter-like (MAL)/ TIR domain-containing adapter protein (TIRAP). The role of a fifth adapter SARM is less well established. SARM appears to negatively regulate the TRIF-dependent cytokine response (3). Specific combinations of these proteins are engaged by substructures from distinct bacterial species, i.e. MyD88 and MAL by bacterial lipoproteins (35), TRIF by double stranded RNA and all four by E. coli LPS
(7). With respect to GBS and other streptococci it is conceivable that the optimal macrophage activation requires MAL and TRAM, since both have been reported as signaling intermediates for the response to diacylated lipoproteins and lipoteichoic acid (LTA), which are putative TLR2/6 agonists from Gram-positive organisms (27), (34). Subsequently, inflammatory cytokines such as TNF and IL-6, chemokines and type I interferons are induced in a fashion depending on usage of individual adapter components (7). Downstream of the TLR adapter, the family of MAP kinases comprises particularly attractive intermediates of TLR activation. MAP kinases affect several, in part contrasting macrophage properties, such as formation of inflammatory and anti-inflammatory cytokines, apoptosis, cell migration and particle uptake (2, 11, 29, 37). Accordingly, the MAP kinases JNK and p38 have been found to be important for the GBS-induced cytokine formation (19, 24, 31). However, the contribution of individual TLR adapter proteins other than MyD88 to MAP kinase and cytokine activation has not been assessed for GBS organisms or any other streptococcal species. This lack in knowledge sharply contrasts that on E. coli, which has been shown to engage a variety of adapters (MyD88, TRIF, TIRAP/MAL, TRAM) via its lipoproteins, lipopolysaccharide, flagellin and DNA (6, 17, 18). Furthermore, events downstream of GBS-induced p38 activation eventually resulting in TNF formation remain largely elusive. It seems obvious that discrete knowledge on signaling events initiated by GBS is necessary for the development of adjunctive anti-sepsis strategies.

Here we analyzed the role of the individual components of the TLR adapter heteromer and the downstream MAP kinase axis in the cytokine response to GBS...
organisms. We found that MyD88 and p38 constitute a master regulator signaling pathway in the GBS-induced cytokine and chemokine response via control of the transcription factors c-Fos/ AP-1 and Egr-1.
EXPERIMENTAL PROCEDURES

Reagents

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. PBS, Dulbecco’s modified Eagle's medium, G418, and trypsin/versene mixture were from Cambrex (Walkersville, MD). Low endotoxin fetal bovine serum was from (HyClone, Erembodegem, Belgium). LPS derived from Escherichia coli strain 0111:B4 was purchased from Sigma-Aldrich and twice re-extracted by phenol chloroform. SB203580 was from CalBiochem (San Diego, CA).

Generation of ethanol fixed GBS

GBS type III strain COH1, initially isolated from a newborn infant with sepsis has been previously described (26). The strain was grown on blood agar plates (Remel, Lenexa, KS). Bacterial colonies were removed from the plates after overnight culture, washed three times in PBS, and then used to inoculate chemically defined medium prepared from endotoxin-free water [for GBS (25)] and grown to mid-log phase (ABS = 0.27-0.30). Subsequently, bacteria were harvested by centrifugation and ethanol (70% ETOH, 45 minutes, on ice), heat (45 min at 70°C) or antibiotic (90 min, DMEM supplemented with ciprofloxacin) fixed, washed once with water and resuspended in pyrogen-free water at a concentration of 20 mg/ml (corresponding to approximately 1 x 10^8 organisms/ml as determined by CFU/ml before inactivation). Accordingly, 200 μg/ml dry weight corresponds to 1 x 10^8 organisms/ml as depicted
in the figures). The entire procedure was performed under pyrogen-free conditions, resulting in preparations that were essentially free of endotoxin as determined by a highly LPS sensitive reporter system (CHO-CD14 with ELAM promoter driven CD25, lower limit of detection 10-100 pg/ml).

**Macrophages**

8 wks old MyD88−/−, MAL/ TIRAP−/−, TRIF−/−, TRAM−/− mice [engineered as described in (30)], Elk−/− mice (4) and C57Bl/6 mice (The Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with 2.5 ml of a 3% thioglycollate solution (Remel, Lenexa, KS). After 72 to 96h, peritoneal exsudate cells were harvested exactly as described (14). Peritoneal macrophages were washed with medium, counted in a hemocytometer, and plated. After 2 h, non-adherent cells were removed by washing with medium and adherent cells were stimulated.

Bone marrow-derived macrophages were generated from bone marrow stem cells cultured on 100 mm-diameter dish for 7 to 10 days in DMEM medium containing 10 mg/ml ciprofloxacin and supplemented with 10% fetal bovine serum and 10 ng M-CSF (R&D Systems, Minneapolis MN) per ml. Differentiated bone marrow macrophages were counted, and plated in 96-well plates and stimulated as indicated. All cultures were kept at 37°C in a 5% CO₂ in air atmosphere.

RAW 264.7 mouse macrophages were cultured in DMEM medium containing 10% FBS and 10 µg of ciprofloxacin /ml
Transfection of RAW264.7 macrophages and reporter constructs.

RAW 264.7 cells were seeded into 96-well tissue culture plates at a density of $10^5$ cells per well. The following day, cells were transiently transfected with luciferase reporter constructs comprising Egr-1 ($pEGR1.600$.Luc, a kind gift by Dipika Gupta, Northwest Center for Medical Education, Indiana University School of Medicine, Gary, IN, (1)) or NFκB (κB3.Luc, Stratagene, La Jolla, CA) minimal promoter constructs or a human wild type TNF promoter ($TNF.WT$.Luc, kindly provided by Nigel Mackman, Scripps Research Institute, La Jolla, CA) using Fugene (Roche, Basel, Switzerland) or Superfect (Qiagen, Hilden, Germany) per the manufacturers’ recommendations. The following day, the cells were stimulated as indicated. After 4-6 h of stimulation, the cells were lysed in passive lysis buffer (Promega), and reporter gene activity was measured using a plate reader luminometer (MicroLumat Plus; Berthold Detection Systems, Pforzheim, Germany). In all cases, the data shown represent one of at least three separate but similar experiments and are presented as the mean values ± S.D. of triplicate samples.

Measurement of proinflammatory activity

For determination of cytokine formation in mouse macrophages were seeded at a density of $1x10^6$ cells /ml in 96-well dishes in RPMI 1640 with 10% FBS plus 10 µg of ciprofloxacin /ml and incubated over 16 h at 37°C in a 5% humidified CO₂ environment. Supernatants were processed directly for the determination of released...
cytokines by ELISA (R&D Systems, Minneapolis, MN) per the manufacturer's protocols.

Analysis of MAPK activation

Phosphorylation of MAPK was evaluated according to standard protocols. Briefly, following stimulation cell lysates were prepared, separated by SDS-PAGE, and analyzed by Western immunoblotting using nitrocellulose membranes (HyClone, Erembodegem, Belgium). Primary Abs directed the phosphorylated (activated) forms of MAPK p38 (Cell Signaling Technology, Beverly, MA), cJun (Cell Signaling Technology, Beverly, MA), Elk-1 (Cell Signaling Technology, Beverly, MA) and intracellular IκB (Santa Cruz Biotechnologies, Heidelberg, Germany). Identity of the bands was confirmed using molecular mass markers and positive controls.

RNA isolation

Macrophages from MyD88 deficient or wild type mice were seeded onto 24well plates at a density of 300000 cell per well. Cells were stimulated with heat fixed GBS strain COH1 (10^7 /ml) over six hours. Subsequently cells were washed with ice-cold PBS and samples were frozen till RNA isolation.
Quantitative real-time PCR

Total RNA was extracted from samples using the RNAeasy mini kit, according to instruction manual (Qiagen). For quantitative two-step RT-PCR, 2 µg of total RNA were reverse-transcribed to first-strand cDNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Aliquots of 20 ng of first-strand cDNA were subsequently used as a template for quantitative PCR with gene-specific primers. The mouse specific GAPDH gene served as a control for constitutive gene expression. GAPDH expression was consistent after bacterial inoculation when compared with the amount of 18S ribosomal RNA. Amplifications were performed in 20µl of SYBR green JumpStart Taq ReadyMix (Sigma–Aldrich, Munich, Germany) with 350 nM oligonucleotides, using an Eppendorf realplex thermal cycler (Eppendorf, Hamburg).

After an initial activation step at 95°C for 7 min, 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s) were performed, and a single fluorescent reading was obtained after the 82°C step of each cycle. A melting curve was determined at the end of cycling to ensure amplification of only a single PCR product. Ct values were determined with the Realplex V1.5 software supplied with the instrument. Comparative expression levels \(2^{\Delta\text{Ct}}\) were calculated according to (LIVAK and SCHMITTGEN 2001). Expression levels are relative to the level of GAPDH expression, which was constant in all RNA samples used and was set to 1. Values are the representative of six samples of two biological experiment assayed by quantitative PCR in triplicates. The oligonucleotides used were as follows: MoGAPDH (accession no BC145812), 5′ ACTCCACTCAGGCAATTC 3′ and 5′ TCTCCATGGTGGTGAAGACA 3′ MoCfos (accession no NM010234) 5′
CCTGCCCTTCTCAACGAC 3' and 5' GCTCCACGTTGCTGATGCT 3' MoEGR1
(accession no NM007913), 5' AGGAAGTTTGCCAGGAGTGA 3' and 5'
TGGGTAGGAGGTAGCCACTG 3'.

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RESULTS

MyD88, but not MAL/TIRAP, TRIF, TRAM, or the interleukin-1 receptor, is essential for the formation of TNF and IL-6 in response to GBS. Peritoneal macrophages from wild type mice were potently activated by fixed GBS organisms to form both TNF and IL-6 (Fig.1) starting at concentrations of dry bacterial mass as low as 200 ng/ml. Deletion of MAL/TIRAP remained without effect on IL-6 and TNF formation despite its putative role in the recognition of Gram-positive cell wall constituents LTA and lipoproteins. Similarly, TRIF and TRAM were dispensable for a robust cytokine response to GBS organisms (Fig 1 A-D). As previously reported, the TNF and IL-6 responses were abrogated in macrophages from mice with a targeted deletion of the MyD88 gene (Fig. 1 B, D) (23). The essential role of MyD88 was independent of the mode of GBS fixation (ethanol, heat, antibiotics) and was similarly observed in response to live bacteria (Fig 1G). In contrast to GBS, all four tested adapter proteins were essential for the cytokine response to LPS from E. coli (Fig.1E). In addition, inhibition of the interleukin-1-converting enzyme (ICE) by N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD x fmk) remained without effect on GBS-induced TNF formation (Fig. 1F). Furthermore, GBS induced cytokine formation in IL-1 receptor 1 deficient macrophages was indistinguishable from cytokine formation in wild type macrophages (data not shown). Accordingly, induction of IL-1/18 by GBS organisms cannot explain the role of MyD88 in this context, although MyD88 is an adapter for the IL-1 and IL-18 receptors.
P38 is essential for the transcriptional activation of TNF by GBS organisms (Fig. 2). GBS organisms robustly phosphorylated the mitogen activated protein (MAP) kinase p38 in wild-type but not in MyD88 deficient macrophages (Fig. 2A). Activation of p38 was absolutely required for GBS-induced cytokine formation since inhibition of p38 by SB203580 (30 µM) abrogated the formation of TNF (Fig. 2B) and IL-6 (Fig. 2C). The effect of p38 on GBS-induced formation of TNF occurred at the transcriptional level, since SB203580 negatively affected activation of a TNF-promoter driven luciferase reporter in a dose-dependent fashion (Fig. 2D).

P38 is essential for the MyD88-dependent induction of the transcription factor c-fos/ AP-1 by GBS, but is dispensable for NFκB activation (Fig. 3). NFκB has been shown to be essential for GBS-induced formation of TNF and to require the presence of MyD88 (14, 19, 31). Moreover, p38 has previously been shown to promote the transactivation of the NFκB subunit p65 (16). Accordingly, we tested whether SB203580 inhibited GBS-induced transcriptional activation of NFκB. However, in contrast to our expectations, SB203580 concentrations as high as 100 µM did not inhibit NFκB-activation in response to GBS organisms, clearly indicating the p38-independent nature of this pathway (Fig 3A). In contrast, inhibition of the MAP kinase JNK by the anthrapyrazolone SP600125 led to inhibition of NFκB-dependent transcriptional activation at concentrations as low as 20 µM (Fig. 3B).

Another signature substrate of p38 is c-fos which constitutes an important element of the dimeric transcription factor activator protein 1 (AP-1). GBS-induced AP-1
activation was dependent on p38, since SB203580 inhibited an AP-1-dependent minimal promoter in a dose-dependent fashion (Fig. 3C). In conclusion, the GBS-induced MyD88-p38 pathway mediates TNF-formation via c-Fos/AP-1, but not NFκB activation.

GBS induces the de novo synthesis of the transcription factor Egr-1 in a MyD88- and p38-dependent fashion (Fig. 4). Next to NFκB and AP-1, Egr-1 is an essential transcription factor that triggers TNF production and has been previously associated with p38 activation. However, data on the role of MyD88 in Egr-1 induction by streptococci were not available. Accordingly, we wondered whether Egr-1 was a second MyD88- and p38-dependent branch of GBS-induced transcriptional activation of cytokine genes. First, we assessed whether egr-1 was induced by GBS organisms in macrophages and transcribed in dependence of MyD88. We found a strong dose-dependent egr-1 induction in wild type macrophages (Fig 4 A). In contrast, egr-1 was not induced by GBS in MyD88-deficient mouse macrophages as assessed by quantitative PCR (Fig. 4B). Furthermore, p38 was essential for egr-1 transcriptional activation since SB203580 inhibited GBS-induced egr-1 reporter activation (Fig. 4C). Next we wondered whether Egr-1 was an essential transcription factor for GBS-induced TNF production. We found that a point mutation in the TNF-promoter, which abolishes Egr-1 interaction with its canonical binding site at the TNF-promoter, abrogates transcriptional activation of TNF by GBS organisms (Fig. 4D). In contrast, a mutation that abrogates the binding of AP-2 to the TNF promoter region did not alter the transcriptional activity induced by GBS on the TNF promoter.
Accordingly, GBS organisms induce Egr-1 via p38 in a MyD88-dependent fashion. Egr-1 is essential for TNF-induction in response to GBS organisms.

In contrast to TLR4 signaling, RANTES induction by GBS occurs in a MyD88- and p38-dependent fashion (Fig. 5).

Egr-1 has previously been linked to the chemokine RANTES ("regulated upon activation, normal T cell expressed and secreted") mRNA. RANTES is a signature cytokine of the MyD88-independent pathway with respect to some TLRs (TLR3, TLR4) (7). Data on RANTES induction by whole Gram-positive bacteria were not available prior to our study. Accordingly it seemed important to assess, whether RANTES was induced by GBS organisms in a MyD88-dependent fashion. This was indeed the case, since GBS organisms induced large amounts of RANTES in wild-type, but not in MyD88-deficient macrophages (Fig. 5A). In contrast, LPS from E. coli induced RANTES irrespective of MyD88 expression. Similar to TNF and IL-6, GBS-induced formation of RANTES was largely dependent on p38, since SB203580 potently reduced the response (Fig. 5B).

GBS-induced p38 mediates activation of Elk-1. However, Elk-1 is dispensable for the TNF-activation by GBS (Fig. 6). The observation that p38 and Egr-1 were essential for GBS-induced transcriptional activation of TNF prompted us to analyze the putative role of Elk-1 in transcriptional activation of TNF. Elk-1 is a transcription factor for both the Egr-1 and TNF promoter; hence it might mediate GBS-induced
cytokine formation through transcriptional activation of cytokine genes on several levels. First, we found that GBS organisms induced phosphorylation of the transcription factor Elk-1 in mouse macrophages in a dose-dependent fashion (data not shown). Elk-1 activation by GBS organisms was dependent on p38 activation, since inhibition of p38 prevented phosphorylation of Elk-1 by GBS (Fig. 6A). Interestingly, the kinetics of p38 and Elk-1 phosphorylation clearly differed. Whereas p38 was activated at 30 minutes only, Elk-1 was phosphorylated at 30 and 90 minutes, both of which were prevented by inhibition of p38 (Fig. 6A and data not shown).

Since Elk-1 was activated by GBS organisms and Elk-1 had been previously implicated in the inflammatory response to TLRs, we addressed its specific role in GBS-induced cytokine formation. To this end, we analyzed GBS-induced cytokine formation and GBS phagocytosis in wild type and Elk-1-deficient macrophages. In contrast to our expectations, we did not observe a role of Elk-1 in GBS-induced cytokine formation (Fig. 6B) or phagocytosis of FITC-labeled GBS (data not shown). Next, we assessed whether Elk-1 deficiency resulted in the abrogation of the inflammatory response to LPS, since all available data generated by in vitro analysis of reporter expression indicated a role of Elk-1 downstream of TLR4. However, and in apparent contrast to previous studies in other cellular models (36), Elk-1 was not involved in LPS-induced cytokine formation (Fig. 6C). Accordingly, although Elk-1 is phosphorylated by GBS (via p38) and by LPS, Elk-1 is dispensable for the subsequent cytokine formation.
DISCUSSION

This study provides novel insights into the macrophage response to GBS organisms. First, MyD88 is the key TLR adapter protein for the formation of inflammatory cytokines and chemokines by macrophages, whereas the TLR adapter proteins MAL/TIRAP, TRIF and TRAM are dispensable for this response. Second, downstream of MyD88, the MAP kinase p38 controls at least two modes of transcriptional activation, c-fos/ AP-1 and EGR-1, which essentially mediate cytokine formation. In contrast, Elk-1, which had previously been suggested as an essential signaling intermediate in TLR signaling, is dispensable for GBS-induced cytokine formation.

The discrete delineation of TLR-adapter usage by whole streptococci seems important, since available studies on the TLR adapters (other than MyD88) are largely limited to purified bacterial toxins. The absolute dependence of GBS inflammatory signaling on MyD88, but not any other well defined TLR adapter protein, is consistent with the notion that GBS organisms activate TNF and IL-6 in macrophages via a pathway clearly distinct from that engaged by TLR2 and TLR4 (14). With respect to the essential role of MyD88 alone, GBS mimics CpG-DNA. Indeed, GBS has been reported to interact with the CpG receptor TLR9. However, the overall cytokine response to GBS is largely TLR9-independent, whereas it is entirely dependent on MyD88 [(14) and unpublished observations]. It seems that this GBS-induced MyD88-dependent pathway, which does not involve any of the single TLRs commonly associated with Gram-positive bacteria, is a common signaling route for many Gram-positive bacteria, since recent data found a similar mode of signal
activation in response to *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Bacillus anthracis* (9, 22).

Beyond the formation of inflammatory cytokines, MyD88 was essential for GBS-induced RANTES formation. This was intriguing, since, to our best knowledge, the role of MyD88 in the context of RANTES formation by Gram-positive organisms had not been examined previous to this study. In contrast, RANTES formation in response to Gram-negative bacteria occurs independently of MyD88 through engagement of TRIF and TRAM (7).

Why MyD88 functionally interacts with MAL/TIRAP, TRIF and TRAM for some ligands but not others is currently not understood. Docking studies suggest that TLRs, MyD88 and MAL/TIRAP form heterotetrameric complexes. Specifically, MAL and MyD88 bind to different regions in TLRs, and interact at a third non-overlapping site (8). It is unlikely that MyD88 can mediate TNF-formation independently of other adapter proteins in the case of GBS, flagellin and CpG, but requires interaction with one adapter protein (plus MAL/TIRAP) for the cytokine response to bacterial lipoproteins and four adapter proteins (plus MAL/TIRAP, TRIF and TRAM) for the response to LPS. Thus it is tempting to speculate on the existence of at least one further currently unknown TLR adapter protein, which serves as a signaling partner of MyD88 in the inflammatory response to GBS organisms. The current paradigm holds that, subsequent to TLR-MyD88 interaction, the IL-1 receptor–associated kinases (IRAK)–1, 2, 4 and M are recruited. Phosphorylation of IRAK-1 by IRAK-4 results in the dissociation of IRAK-1 from the receptor complex and interaction with TNF receptor–associated factor 6 (TRAF6) through its COOH terminus. TRAF6 activates
the evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) that in turn activates downstream MAP kinase cascades (20, 21). However, several technical issues like the early lethality of the knockout mice currently preclude absolute certainty on the role of ECSIT between TRAF-6 and MAP kinase kinases (33). MAP kinases are a family of serine/threonine protein kinases. Among these p38 can be regarded as a signaling relay that funnels various upstream signals and allows downstream diversification in dependence of the immediate cellular environment. P38 itself is activated through phosphorylation on both threonine and tyrosine residues at a Thr-Gly-Tyr dual phosphorylation motif in the kinase catalytic domain. Of the four known p38 isoforms α, β, γ and δ only the first two are significantly inhibited by SB203580, a pyridinyl-imidazole that exerts its relatively high kinase specificity by binding to the ATP binding pocket of p38 (28). Since SB203580 inhibits GBS-induced cytokine formation, p38 α and β are critical for GBS-induced inflammatory signaling and the and isoforms cannot compensate for the inhibition of α and β.

Notably, the signaling pathways of p38 and other MAP kinases are interrelated on several levels. As an example, Elk-1 can be phosphorylated both by p38 and JNK. However, our data indicate that, in the absence of p38 activity, JNK is not sufficient for GBS-induced Elk-1 phosphorylation. JNK and p38 signaling furthermore converge on the level of AP-1 transcription factors, which are homo- or heterodimers of the Jun family, Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) and members of activating transcription factor family (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) subfamilies. While cJun proteins are mainly activated by the cJun-N-terminal Kinase (JNK), Fos
and ATF proteins are phosphorylated downstream of p38 and other MAPKs. In our hands, AP-1 activation is similarly reduced by the p38 inhibitor SB203580 or the JNK inhibitor SP600125 (Fig. 3A and unpublished observations).

Two transcription factor heteromers (c-Jun/c-Fos, c-Jun/ATF2) have previously been shown in more detail to have a particularly high affinity to preferentially bind to the AP-1 binding site within the TNF promoter. Hence cJun and therefore JNK activity is essential for the activation of AP-1 as a transcription factor for \textit{tnf}. Cross inhibition of JNK substrates by p38 inhibition is unlikely to be relevant in this context, since JNK (19), but not p38 (current study) mediates transactivation of NFκB in response to GBS. It seems notable that GBS differs from IL-1β in this context, since the latter engages p38 for the transactivation of the NFκB subunit p65 (through aminoterminal phosphorylation) (16). Accordingly, GBS engages a specific MyD88-dependent pathway that comprises the MAP kinases p38 and JNK as essential inflammatory intermediates with overlapping, but clearly distinct regulatory functions.

Several publications suggest that the microbial engagement of Toll-like receptors leads to the activation of Elk-1 (5, 32) and that this event in turn mediates transcriptional activation of TNF (36). However, conclusive data on the cytokine response to TLR agonists, such as data generated in cells from in Elk\(^{-/-}\) mice were not available. We report here that GBS and LPS induced normal amounts of TNF in Elk\(^{-/-}\) macrophages. Accordingly, Elk-1 is dispensable for the response to both stimuli. Furthermore, and in contrast to the upstream kinase p38, Elk-1 was not essential for GBS phagocytosis (19). We conclude that signaling events upstream or parallel of Elk-1 mediate the p38 effect on transcriptional activation of \textit{tnf} and phagocytosis.
Several lines of evidence suggest that, next to Elk-1, neither AP-1 nor NFκB account for the antimicrobial effects of p38 activation. First, JNK mediates AP-1 activation but its inhibition does not interfere with phagocytosis or bacterial killing. Furthermore, GBS-induced NFκB activation is not dependent on p38 activation. Therefore, antimicrobial activity and TNF formation are independently regulated events downstream of p38. The Rab proteins are attractive intermediates in the p38-dependent antimicrobial pathway, since they modulate the endocytic traffic downstream of p38 (2).

In summary MyD88 and p38 are parts of a distinct and functionally pivotal pathway activated by streptococci. AP-1 and Egr-1 inhabit key downstream positions in this highly inflammatory process (Fig. 7). Since p38 exerts desirable antimicrobial functions, interventional strategies that aim to target cytokine formation need to modulate molecules further downstream in the signaling cascade.
FIGURE LEGENDS

Figure 1

MyD88, but not MAL/TIRAP, TRIF or TRAM is essential for GBS-induced TNF and IL-6 formation. Bone marrow derived macrophages from mice with a targeted deletion of TRIF and TRAM (A and C), MAL/TIRAP and MyD88 (B and D) (open symbols) and from wild-type C57B/J6 (closed symbols) mice were stimulated with escalating doses of ethanol fixed GBS (strain COH1) or (E) LPS over 16 h. Then TNF and IL6 concentrations in the supernatants were determined by ELISA. Depicted are means + SD of triplicate wells from one representative experiment out of three. (F) RAW 264.7 macrophages were preincubated with N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD x fmk, 20 µM), or a vehicle control and subsequently stimulated with GBS strain NEM 316 as indicated. (G) Macrophages from wild type, MyD88 or TLR2 deficient mice were stimulated with ethanol, heat or antibiotic killed GBS or live bacteria. Then TNF and IL6 concentrations in the supernatants were determined by ELISA. Depicted are means + SD of triplicate wells.

Figure 2

GBS requires activation of the MAP kinase p38 for proper formation of TNF. A. Macrophages from mice with a targeted deletion of MyD88 or wild-type mice were stimulated with GBS over 30 min. The cellular lysates were probed with antibodies specific for the phosphorylated form (Tyrx and Thrx) of p38 (pp38). B. and C. RAW
264.7 cells were stimulated with ethanol fixed GBS in the presence of SB203580 (30 µM, open symbols) or vehicle control (closed symbols) over 16 h. Then TNF (B.) and IL-6 (C.) concentrations were determined by ELISA. Depicted are means ± SD of triplicate wells from one representative experiment out of three. D. RAW 264.7 cells were transfected with the wild type TNF luciferase reporter (TNF.WT.Luc) and stimulated with 10⁸ GBS/ml in the presence of escalating dosages of the p38 inhibitor SB203580 over 5 h and cellular lysates were analyzed by luminometry for reporter activity. Depicted are light units (means ± SD from triplicate wells) as fold activation over background (medium control).

**Figure 3**

*P38 mediates GBS-induced transcription of c-fos/ AP-1, but does not interfere with NFκB activation.* A. and B. RAW 264.7 cells were transfected with an NFκB luciferase reporter construct. Then, cells were stimulated with or without fixed GBS (10⁸ /ml) plus an inhibitor for (A.) p38 (SB203580) or (B.) JNK (SP600125) and reporter activity was determined by luminometry. C. RAW 264.7 cells were transfected with an AP-1 luciferase reporter construct (AP1.Luc). Then, cells were stimulated with a vehicle control or GBS (10⁸ /ml) plus an inhibitor for p38 (SB203580) and reporter activity was determined by luminometry. Depicted is one representative out of three experiments.
Figure 4

P38 mediates GBS-induced transcription of egr-1, which is essential for the transcriptional activation of TNF. A. Wild-type mouse macrophages were transfected with an egr-1 luciferase reporter construct (Egr-1.Luc). Then, cells were stimulated with BS in the indicated concentrations and reporter activity was determined by luminometry. B. Wild-type or MyD88-deficient mouse macrophages were stimulated with the indicated concentrations of fixed GBS and transcriptional activation of egr-1 was assessed by quantitative PCR. C. RAW 264.7 cells were transfected with an egr-1 luciferase reporter construct (Egr-1.Luc). Then, cells were stimulated with GBS (10^8 /ml) in the presence of escalating doses of an inhibitor for p38 (SB203580) and reporter activity was determined by luminometry. Depicted is one representative out of three experiments. D. RAW264.7 were transfected with either the wild-type TNF luciferase reporter construct or a TNF promoter construct mutant in the Egr-1 or AP-2 binding site. Subsequently cells were stimulated as indicated and analyzed by luminometry.
Figure 5

GBS requires MyD88 and p38-activation for the formation of the chemokines RANTES. A. Macrophages from mice with a targeted deletion of MyD88 or wild-type mice were stimulated with fixed GBS for 10 hours. B. RAW 264.7 cells were stimulated with escalating concentrations of GBS in the presence or absence of the p38 inhibitor SB203580 (30 µM). RANTES concentrations in the macrophage supernatants were determined by ELISA. Depicted are means ± SD of triplicate wells from one representative experiment out of three.

Figure 6

MyD88 and p38 mediate GBS-induced activation of Elk-1, but Elk-1 is not essential for GBS induced formation of TNF or IL-6. A. RAW 264.7 cells were stimulated with 10^8 GBS/ml with or without SB203580 over the indicated time periods. Then cellular lysates were subjected to western blot analysis with antibodies against Phospho-Elk-1. B. Bone marrow derived macrophages from mice with a targeted deletion of elk-1 (open symbols) and from wild-type C57B/J6 mice (closed symbols) were stimulated with escalating doses of ethanol fixed GBS (strain COH1) over 16 h. Then TNF and IL-6 in the supernatants were determined by ELISA. Depicted are means ± SD of triplicate wells from one representative experiment out of three.
Figure 7

The signaling pathway MyD88 ⇒ P38 ⇒ EGR-1, AP-1 is essential for GBS-induced transcriptional activation of TNF. In response to GBS, MyD88 serves as a master signaling molecule, which essentially mediates p38 phosphorylation. In turn, p38 activates Egr-1 and AP-1, critical transcription factors for TNF. Similarly, Elk-1 is activated by p38, however it is not required for TNF formation.

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REFERENCES


Manner by Enhancing the Ability of the p65 Subunit to Transactivate Gene Expression. 10.1074/jbc.275.5.3114. J. Biol. Chem. 275:3114-3120.


Figure 1:

A: TNF-α (ng/ml)

B: TNF-α (ng/ml)

C: IL-6 (ng/ml)

D: IL-6 (ng/ml)

E: IL-6 (ng/ml)

F: TNF-α (ng/ml)
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G: TNF (ng/ml)

- WT
- TLR2 KO
- MyD88 KO

Vehicle
Ethanol-inactivated
Heat-inactivated
Antibiotic inactivated
Live

Group B streptococcus (/ml)
Figure 2:

A: Wild type vs. MyD88 -/-

pp38

p38

0 10^7 10^8 GBS/ml

0 10^7 10^8 GBS/ml

B: TNF-α (ng/ml)

Vehicle

SB 203580

GBS (10^6/ml)

C: IL-6 (ng/ml)

Vehicle

SB 203580

GBS (10^6/ml)

D: Fold Activation (TNF.WT.Luc)

vehicle

GBS 10^6/ml

SB203580 (µM/ml)
Figure 3:

A: Fold Activation (NFkB)

B: Fold Activation (NFkB)

C: Fold activation (AP-1.Luc)

Vehicle

SB 203580 30µM
Figure 4:

A: Fold Activation (Egr-1.Luc)

B: Fold Activation (egr-1 gene expression)

C: Fold Activation (Egr-1.Luc)

D: Fold Activation
Figure 5:

A: RANTES (ng/ml)

B: RANTES (ng/ml)
Figure 6:

A: c-Jun

B: TNF [ng/ml]  IL-6 [ng/ml]

C: TNF [ng/ml]  IL-6 [ng/ml]

WT  Elk-/-

0 10^6 10^7 10^8  GBS/ml

0 1 10 100  ng LPS/ml

0 1 10 100  ng LPS/ml
Figure 7: