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Role of Mitogen-Activated Protein Kinases and NF-κB in the Regulation of Proinflammatory and Anti-Inflammatory Cytokines by *Porphyromonas gingivalis* Hemagglutinin B

Ping Zhang,[1] Michael Martin,[1] Suzanne M. Michalek,[2] and Jannet Katz[1]*

Departments of Pediatric Dentistry[1] and Microbiology,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294

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Hemagglutinin B (HagB) is a nonfimbrial adhesin expressed on the surface of *Porphyromonas gingivalis* and has been implicated as a potential virulence factor involved in mediating the attachment of the bacteria to host cells. However, the molecular mechanisms underlying host responses to HagB and their roles in pathogenesis have yet to be elucidated. Mitogen-activated protein kinases (MAPKs) are activated following engagement of a variety of cell surface receptors via dual tyrosine and threonine phosphorylation and are thought to be involved in various cellular responses. The purpose of this study was to determine the role of intracellular signaling pathways including the MAPKs and NF-κB in regulating the production of proinflammatory and anti-inflammatory cytokines following stimulation of murine macrophages with recombinant HagB (rHagB). Stimulation of peritoneal macrophages with rHagB resulted in the production of the proinflammatory cytokines interleukin-12p40 (IL-12p40), gamma interferon (IFN-γ), and tumor necrosis factor alpha, as well as the anti-inflammatory cytokine IL-10. We also demonstrated the activation of extracellular signal-related kinase (ERK), c-Jun NH_2-terminal protein kinase (JNK), and p38 MAPKs by rHagB-stimulated macrophages. Furthermore, blocking of the ERK and p38 signaling pathways by using specific inhibitors revealed differential regulatory roles in the rHagB-mediated production of proinflammatory and anti-inflammatory cytokines. ERK and p38 were important in down-regulation of IL-12p40 and IFN-γ production and up-regulation of IL-10 production. The enhanced levels of IL-12p40 in rHagB-stimulated macrophages by inhibition of ERK or p38 activity were partially attributable to the inhibition of IL-10 production. Moreover, NF-κB was found to be critical for up-regulation of IL-12p40 and down-regulation of IL-10 production in rHagB-stimulated macrophages. Taken together, our results demonstrate a role for the p38 and ERK pathways and the transcription factor NF-κB in modulating key immunoregulatory cytokines involved in the development of immune responses to *P. gingivalis* HagB.

Porphyromonas gingivalis is considered to be one of the major etiological agents of human adult periodontitis, a chronic inflammatory disease characterized by the destruction of the supportive tissues surrounding teeth (35). The nonfimbrial adhesions, such as hemagglutinin B (HagB), are thought to be potential virulence factors involved in mediating the attachment of the bacteria to host cells (11, 20–22, 29, 35). We have previously demonstrated the effectiveness of recombinant HagB (rHagB) in inducing a protective immune response against *P. gingivalis* infection in an experimental rat model (19). This finding supports the potential use of rHagB as an antigen for the development of a vaccine against adult periodontitis. Furthermore, we have shown a critical role of B7 costimulatory molecules for the preferential differentiation of T-helper cells for responses to rHagB (40). However, the signaling pathways and regulatory molecules involved in host immune responses to HagB have not been delineated.

In recent years, intracellular signal transduction mechanisms responsible for inducing inflammatory gene expression have been identified. These mechanisms seem fundamental in the initiation of inflammatory responses. Products of induced inflammatory genes include cytokines, chemokines, and adhesion molecules that serve to promote the recruitment of immunocompetent cells from the circulation to the affected site (16). One of the key signaling routes is the mitogen-activated protein kinase (MAPK) signal transduction pathway. MAPKs, which belong to a large family of serine/threonine kinases, constitute major inflammatory signaling pathways from the cell surface to the nucleus (10, 16). There are three well-characterized subfamilies of MAPKs: the extracellular signal-regulated kinases (ERK), the c-Jun NH_2-terminal kinases (JNK), and the p38 family of kinases (p38 MAPKs) (16, 18). ERK activation is considered essential for entry into cell cycle and, thus, mitogenesis. Activation of the JNK pathway is associated with programmed cell death or apoptosis. The p38 MAPKs regulate the expression of many cytokines and have an important role in activation of immune response (18).

The importance of the MAPK signal transduction pathway in controlling many aspects of immune-mediated inflammatory responses has made them a priority for research related to many human diseases. The activation of intracellular signaling pathways and subsequent inflammatory cytokines has been induced by different stimuli in different cell types; however, the response induced by one stimulus cannot be extrapolated to another or by one cell type to another (30). Antigen-presenting

* Corresponding author. Mailing address: Departments of Microbiology and Pediatric Dentistry, University of Alabama at Birmingham, 845 19th Street South, BBRB 258/5, Birmingham, AL 35294-2170. Phone: (205) 934-2878. Fax: (205) 934-1426. E-mail: meow@uab.edu.

cells, such as monocytes/macrophages and dendritic cells, play an important role in directing the nature of the host immune response to microbial challenge. Previous studies have shown that a variety of stimuli, such as lipopolysaccharide (LPS) and lipoproteins, activate MAPKs in macrophages. One intriguing feature of macrophage biology is the ability of activated macrophages to produce both proinflammatory cytokines, such as interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), and IL-1, and anti-inflammatory cytokines, including IL-10 and transforming growth factor β. The balance of proinflammatory and anti-inflammatory cytokine expression is of central importance for understanding how the immune system regulates responses to pathogenic infection (7).

To gain insight into the mechanisms underlying the host response to HagB, we investigated rHagB-induced production of inflammatory cytokines by macrophages and the intracellular signaling pathways involved in the responses to rHagB. In the present study, we show that rHagB induces the production of the anti-inflammatory cytokine IL-10, as well as the proinflammatory cytokines TNF-α, IL-12p40, and gamma interferon (IFN-γ) by murine peritoneal macrophages. The ability of rHagB to activate ERK and p38 appears to mediate a differential regulation of cytokine production by macrophages. In addition, we demonstrate that the transcriptional nuclear factor-κB (NF-κB) is part of the intracellular signal transduction pathways leading to rHagB-induced production of inflammatory cytokines.

MATERIALS AND METHODS

Antibodies and reagents. Antibodies (Abs) used for the detection of total p38 and the phosphorylated forms of ERK (Thr202/Tyr204), p38 (Thr180/Tyr182), stress-activated protein kinase (SAPK)/JNK (Thr183/Tyr185), and MAPKAPK-2 (Thr334) were obtained from Cell Signaling Technology (Beverly, Mass.). Abs against NF-κB p65, phospho-NF-κB p65 (Ser536), phospho-NF-κB p105 (Ser933), and the horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin G (IgG) Ab were also obtained from Cell Signaling Technology. The selective inhibitor of p38 MAPK SB203580, the MEK-1 inhibitor PD98059, and the NF-κB inhibitor SN50 and its inactive analog SN50 M were obtained from Calbiochem Biosciences Inc. (La Jolla, Calif.). The MEK-1/2 inhibitor U0126 was obtained from Biomol Research laboratories Inc. (Plymouth Meeting, Pa.). Rat anti-mouse IL-10 (clone JES5-2A5) and its isotype control Ab were purchased from BD Biosciences Parmingen (San Diego, Calif.).

rHagB. rHagB was purified as previously described (38, 40). Briefly, rHagB was purified from the soluble fraction of lysates of *Escherichia coli* JM 109 expressing the *hagB* gene by using a His-bind resin column, according to the manufacturer's instruction (Novagen, Madison, Wis.). The purity of rHagB was confirmed by silver staining and Western blot analysis using a rabbit anti-rHagB antibody. The concentration of rHagB was estimated by a bicinchoninic acid protein determination assay (Pierce, Rockford, Ill.), using bovine serum albumin as the standard.

Isolation of mouse peritoneal macrophages. C57BL/6 female mice, 6 to 8 weeks of age, were used as the source of macrophages. Mice were injected by the intraperitoneal route with 3 ml of 3% thioglycolate broth (Becton Dickinson Microbiology Systems, Sparks, Md.). Mice were sacrificed after 4 days, and cells were harvested by flushing the peritoneal cavity with 10 ml of phosphate-buffered saline (PBS). The peritoneal exudate cells were washed, resuspended in RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1.5 mg/ml of sodium bicarbonate, 50 μg/ml of gentamicin sulfate, 25 mM HEPES, 50 U/ml of penicillin, and 50 μg/ml of streptomycin (complete medium), and counted in a hemacytometer. Cell viability was estimated by trypan blue exclusion. The cells were placed into tissue culture plates, and the macrophages were allowed to adhere overnight at 37°C in a humidified 5% CO_2 incubator. The nonadherent cells were then removed by washing the plates three times with complete medium.

Cytokine-specific ELISA. Peritoneal macrophages (5×10^5 cells/well) were incubated with or without selective inhibitors for 2 h at 37°C in 96-well tissue culture plates. In some experiments, a neutralizing monoclonal Ab (MAb) to IL-10 or its isotype control was added to the cultures together with the inhibitor. The cultures were then incubated with or without rHagB for 20 h. The levels of IL-10, IL-12p40, IL-12p70, IFN-γ, and TNF-α in the culture supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (IL-10, IL-12p40, and IL-12p70 ELISA kits from BD Biosciences and IFN-γ and TNF-α ELISA kits from eBioscience, San Diego, Calif.).

RNA purification and real-time PCR. Peritoneal macrophages were stimulated with or without rHagB in the presence or absence of selective inhibitors and/or anti-IL-10 MAb. Total RNA from 3×10^6 cells was isolated at the indicated time points (see Results) by using an RNeasy Mini kit (QIAGEN, Valencia, Calif.) and following the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA by reverse transcription using an Omniscript RT kit (QIAGEN) and an oligo(dT)$_{16}$ primer (Applied Biosystems, Foster City, Calif.). The cDNA was subsequently amplified for IL-10, IL-12p35, IL-12p40, IFN-γ, TNF-α, and β-actin. The PCR primers used in this study are as follows: for IL-10, GCTCTTACTGACTGGCATGAG (forward) and CGCAGCTCTA GGAGCATGTG (reverse); for IL-12 p35, CTGTGCCTTGGTAGCATCTATG (forward) and GCAGAGTCTCGCCATTA TGATTC (reverse); for IL-12 p40, TGGTTTGCCATCGTTTTGCTG (forward) and ACAGGTG AGGTTCACT GTTTCT (reverse); for IFN-γ, ATGAACGCTACACACTGCATC (forward) and CCATCCTTTTGCCAGTTCCTC (reverse); for TNF-α, CCCTCACACTC AGATCATCTTCT (forward) and GCTACGACGTGGGCTACAG (reverse); and for β-actin, CCGCATCCTCTT CCTCCCTGGAGAA (forward) and GGA GGGGCCGGACTCATCGTACTC (reverse). Real-time PCR was performed by using a Lightcycler (Roche Molecular Biochemicals, Indianapolis, Ind.) with a FastStart DNA Master SYBR Green I reagent (Roche Applied Science, Germany), according to the manufacturer's instructions. Samples were subjected to 36 cycles of amplification at 95°C for 10 s, followed by 60°C for 20 s and 72°C for 40 s. Relative quantities of the cytokine mRNAs were normalized to β-actin mRNA. The normalized data were expressed using the comparative cycle threshold method (1).

Cell extract preparation. Peritoneal macrophages (3×10^6 cells/well) were pretreated with or without selective inhibitors for 2 h in 24-well tissue culture plates. Cells were then incubated with complete medium only or with rHagB for the indicated time. To prepare whole-cell lysates, cells were washed with PBS and then lysed on ice for 10 min in radioimmunoprecipitation assay lysis buffer (Upstate, Lake Placid, N.Y.) freshly supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM NaF, and 1 μg/ml of protease inhibitor cocktail (Roche). The whole-cell lysates were transferred to tubes and incubated on ice for an additional 20 min, and then following centrifugation, the supernatants were collected. To prepare cytoplasmic and nuclear extracts, a nuclear extract kit (Active Motif, Carlsbad, Calif.) was used according to the manufacture's protocol. Specifically, cultured macrophages were washed with PBS-phosphatase inhibitors and harvested by centrifugation. The cell pellet was then resuspended in hypotonic buffer and incubated on ice for 15 min. Detergent was then added, and cells were mixed vigorously before centrifugation. The cytoplasmic extract was collected, and the nuclear pellet was resuspended in complete lysis buffer and rotated at 150 rpm on ice for 30 min. The nuclear fraction was collected after centrifugation at 14,000 rpm for 10 min at 4°C.

Western blotting. Equivalent amounts of protein samples from cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% Tris-HCl gel (Bio-Rad Laboratories, Hercules, Calif.). Protein was electrotransferred to immobilon-P transfer membranes (Millipore, Bedford, Mass.) and probed with specific Abs against the phosphorylated forms of ERK, p38, SAPK/JNK, MAPKAPK-2, NF-κB p65, and NF-κB p105. Detection of bands was carried out using HRP-linked rabbit IgG Ab, followed by ECL Western blotting detection reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). In some experiments, the same blot was used for total p38 or NF-κB p65 detection after stripping of the previous Ab with Restore Western blot stripping buffer (Pierce). Densitometer scans of the blots were performed using the AlphaImager 2000 documentation and analysis system (Alpha Innotech, San Leandro, Calif.).

NF-κB DNA binding assay. NF-κB activation in the nuclear extracts was quantified by a TransAM NF-κB assay kit (Active Motif) based on an ELISA principle. Specifically, an immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) was bound to microwell plates. The active form of NF-κB present in nuclear extracts specifically binding to this oligonucleotide was detected using a primary antibody that recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. Following the addition of an HRP-conjugated secondary antibody, the plates were read on an automated plate reader, and the level of nuclear NF-κB p65 was expressed as the absorbance at 450 nm (A_{450}).

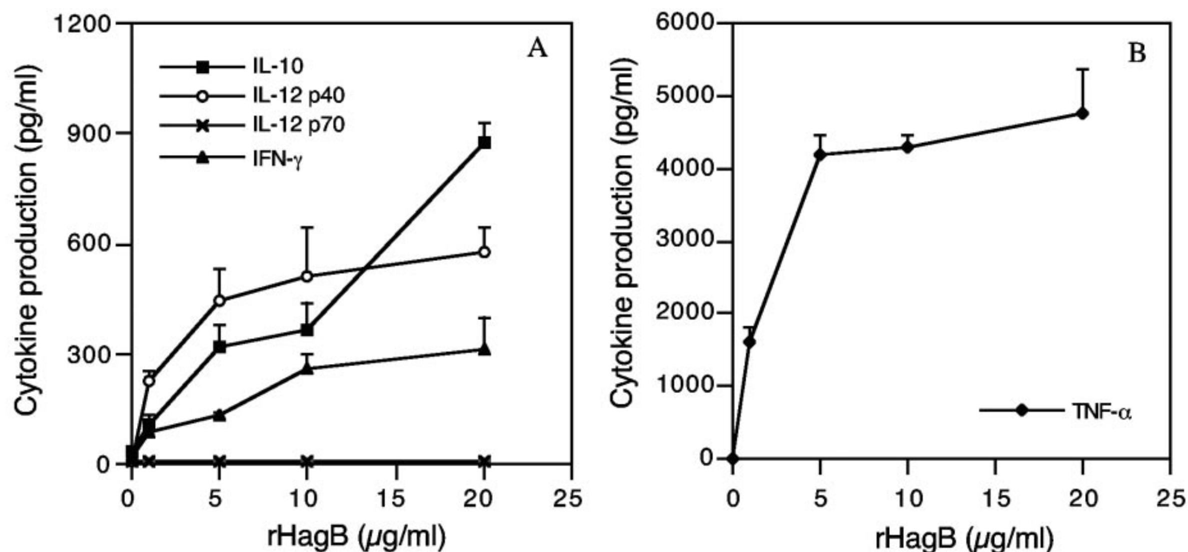


FIG. 1. rHagB induces cytokine production by murine macrophages. C57BL/6 peritoneal macrophages (5×10^5 cells) were stimulated with rHagB (0 to 20 μg/ml) for 20 h. The levels of IL-10, IL-12p40, IL-12p70, IFN-γ, and TNF-α in culture supernatants were determined by ELISA. Data represent the means ± standard deviations of triplicates. The results are representative of three separate experiments.

Statistical analysis. Statistical significance between groups was evaluated by analysis of variance and the Tukey multiple-comparisons test using the InStat program (Graphpad Software, San Diego, Calif.). Differences between groups were considered significant at the level of $P < 0.05$.

RESULTS

Activation of cytokine production by rHagB. To determine the ability of rHagB to induce cytokine production by mouse peritoneal macrophages, cells were incubated with various concentrations of rHagB for 20 h, and then the culture supernatants were assessed for the levels of IL-10, IL-12p40, IL-12p70, IFN-γ, and TNF-α. Incubation of macrophages with rHagB resulted in an increase in the levels of IL-10, IL-12p40, IFN-γ, and especially TNF-α in a dose-dependent manner (Fig. 1). As little as 1 μg/ml of rHagB resulted in a significant increase in the production of these cytokines. TNF-α was the cytokine with the highest level of production, followed by IL-12p40, IL-10, and IFN-γ. At 20 μg/ml of rHagB, however, IL-10 levels increased about threefold from those seen at 5 and 10 μg/ml of rHagB and were higher than the levels for IL-12p40 and IFN-γ. For the rest of the experiments in this study, 20 μg/ml of rHagB was chosen as the dose. No IL-12p70 production was detected at any time.

The induction of cytokine expression following stimulation with rHagB was confirmed by monitoring cytokine mRNA levels using real time reverse transcription-PCR at various times over a 24-h activation period (Fig. 2). The levels of IL-10, TNF-α, IFN-γ, and IL-12p40 mRNAs peaked within 2 h after rHagB stimulation, whereas rHagB-induced IL-12p35 mRNA expression peaked at 12 h. Furthermore, the amounts of IL-10, TNF-α, IFN-γ, and IL-12p40 proteins induced by rHagB (Fig. 1) were proportional to the levels of cytokine mRNA (Fig. 2).

Activation of MAPK pathways by rHagB. To determine the role of the MAPK pathways in response to rHagB, macrophages were stimulated with rHagB (20 μg/ml) for 0 to 120 min and then analyzed for activation of ERK, JNK, and p38 MAPK by Western blotting (Fig. 3). The phosphorylation of the three MAPKs following stimulation with rHagB was detected within 10 min. The maximal level for p38 phosphorylation was seen 30 min after stimulation and persisted through 120 min, as assessed by densitometer scans of the blots of total p38 and of the phosphorylated form of p38. The levels of phosphorylated p44/42 MAPK and of p54/p46 SAPK/JNK were dramatically decreased by 60 min after stimulation. These results demonstrate that rHagB induces early phosphorylation of ERK, p38, and JNK in mouse peritoneal macrophages.

Specific effect of selective inhibitors on rHagB-induced MAPK activation. To determine the functional significance of the MAPK signaling pathways in rHagB-induced cytokine production, we next assessed the effect of specific pharmacological inhibitors of ERK (PD98059 or U0126) and p38 MAPK (SB203580) on downstream events. Initially, we determined the specificities and effect of the dose of each inhibitor on the inhibition of MAPK activation. Macrophages were pretreated with different concentrations (1 to 50 μM) of the inhibitors for 2 h, followed by stimulation with 20 μg/ml of rHagB for 30 min. The concentrations of inhibitors used in this experiment did not affect cell viability as determined by trypan blue exclusion (data not shown). U0126, as well as PD98059, inhibited the phosphorylation of p44/42 MAPK (ERK) in a dose-dependent manner (Fig. 4A and B), whereas no inhibition of p38 or JNK phosphorylation was seen (data not shown). Similarly, SB203580 inhibited the phosphorylation of MAPKAPK-2, a direct target of p38 MAPK, in a dose-dependent manner (Fig. 4C), without affecting the activation of either ERK or JNK (data not shown).

Role of MAPKs on rHagB-induced cytokine production. To investigate the possible regulatory roles of rHagB-induced activation of MAPKs on the production of proinflammatory and anti-inflammatory cytokines by macrophages, cells were treated with different concentrations of inhibitors (1 to 50 μM) for 2 h, prior to stimulation with 20 μg/ml of rHagB. Culture supernatants were harvested at 20 h for analysis of cytokine production

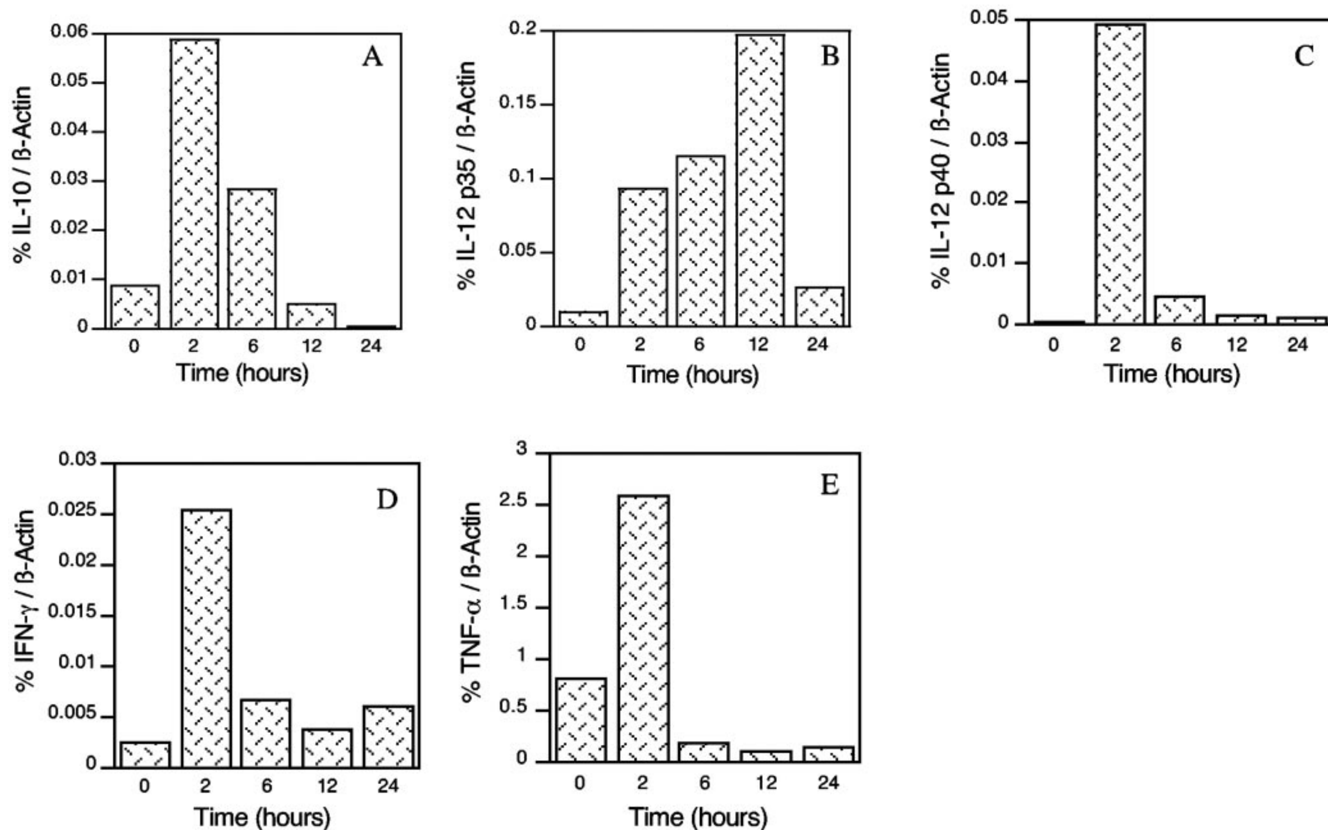


FIG. 2. rHagB induces cytokine mRNA expression by murine macrophages. C57BL/6 peritoneal macrophages (3×10^6 cells) were stimulated with rHagB (20 μg/ml) for 0 to 24 h. Total RNA was isolated and the presence of mRNA for IL-10, IL-12p35, IL-12p40, IFN-γ, TNF-α, and β-actin in each sample was assessed by real-time PCR using SYBR green. β-actin was used as the endogenous control. The results are representative of three independent experiments.

(Fig. 5). rHagB-induced IFN-γ and IL-12p40 production were significantly increased ($P < 0.01$ to $P < 0.001$) in a dose-dependent manner by inhibition of ERK activation; however, the inhibitors PD98059 and U0126 rendered somewhat different effects. On the other hand, IL-10 production was suppressed by U0126 in a dose-dependent manner and was almost completely abolished with a 50 μM concentration of inhibitor ($P < 0.001$). The addition of PD98059 to the cell cultures resulted in suppression of IL-10 production at all concentrations tested ($P < 0.001$). U0126, but not PD98059, had an inhibitory effect on TNF-α production, but the effect was not significant.

The incubation of macrophages with rHagB in the presence of the p38 inhibitor SB203580 resulted in a significant reduction ($P < 0.001$) in IL-10 levels, whereas IL-12p40 levels were increased by 100% to 200%. SB203580 caused only a slight enhancement in IFN-γ production but had essentially no effect on TNF-α production.

We also tested the effect of the specific inhibitors SB203580 (10 μM), PD98059 (25 μM), or U0126 (25 μM) on cytokine production by macrophages when stimulated with different concentrations of rHagB (1 to 20 μg/ml). In general, a similar pattern was observed to that seen at 20 μg/ml of rHagB stimulation (data not shown).

Taken together, these results indicate that the ERK and p38 MAPK signaling pathways play differential regulatory roles in

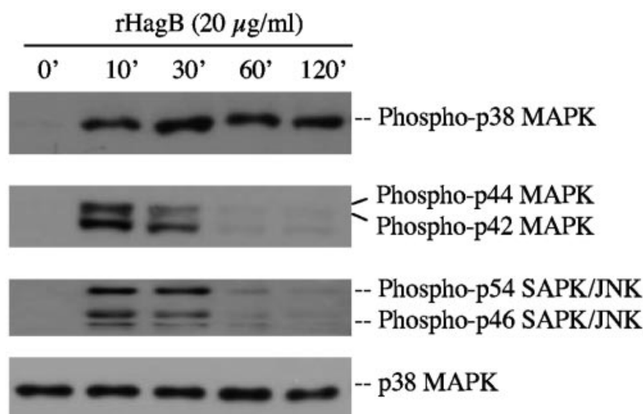


FIG. 3. Early activation of MAPKs by rHagB. C57BL/6 peritoneal macrophages (3×10^6 cells) were stimulated with rHagB (20 μg/ml) for 0 to 120 min. Equal amounts of whole-cell lysates were subjected to electrophoresis on a 10% Tris-HCl gel, and Western blots were done using a specific Ab against the phosphorylated form of MAPKAPK-2 (Thr334), ERK (p44/42; Thr202/Tyr204), or JNK (SAPK/JNK; Thr183/Tyr185). Total p38 in each sample was used to ensure equal protein loading. Blots are representative of results obtained from three separate experiments.

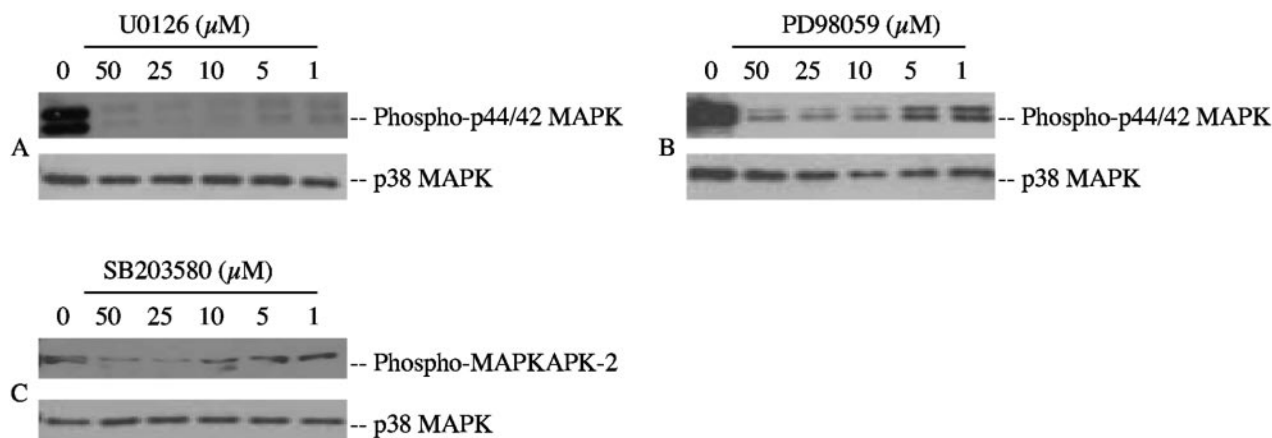


FIG. 4. Specific inhibitory effects of MAPK inhibitors. C57BL/6 peritoneal macrophages (3×10^6 cells) were pretreated with U0126, PD98059, or SB203580 (1 to 50 μM) for 2 h and then stimulated with rHagB (20 μg/ml) for 30 min. Equal amounts of whole-cell lysates were subjected to electrophoresis on a 10% Tris-HCl gel, and Western blots were done using a specific Ab against the phosphorylated form of p38 (Thr180/Tyr182) or ERK (p44/42; Thr202/Tyr204). The levels of total p38 in the samples were determined by stripping the blots and reprobing with a specific anti-p38 Ab. Blots are representative of results obtained in three separate experiments.

rHagB-mediated production of proinflammatory and anti-inflammatory cytokines by macrophages. ERK appears to play an important role in the down-regulation of IFN-γ and IL-12p40 production and up-regulation of IL-10 production, whereas p38 appears to be critical for down-regulation of IL-12p40 and up-regulation of IL-10 production by mouse peritoneal macrophages stimulated with rHagB. IL-12p70 was not detected in any of the tested culture supernatants.

To investigate whether the differential regulatory roles of ERK and p38 on rHagB-induced cytokine production was related to the levels of cytokine mRNAs, we next assessed mRNA levels in macrophage cultures after 2 h of rHagB (20 μg/ml) stimulation with or without the inhibitors. An increase in the levels of mRNA for IL-10, IL-12 p35, IL-12 p40, IFN-γ, and TNF-α was observed in macrophage cultures incubated with SB203580 (10 μM) or U0126 (25 μM) (data not shown). These results suggest that the differential regulatory effects of ERK and p38 on rHagB-induced cytokine production take place, at least in part, at the posttranscriptional level.

Role of IL-10 in the enhancement of IL-12 production. It is well known that IL-10 acts as an anti-inflammatory cytokine by inhibiting the production of IL-12 by macrophages (17). In the present study, we have shown that inhibition of p38 and ERK results in a down-regulation of IL-10 and an up-regulation of IL-12p40 production. Therefore, we next investigated whether the increase in IL-12p40 production by the inhibition of ERK or p38 was the result of suppressing IL-10 production. Macrophages were pretreated with a neutralizing MAb to IL-10 in the presence or absence of SB203580 or U0126 and then stimulated with rHagB (20 μg/ml). The pretreatment of cultures with a neutralizing MAb to IL-10 resulted in a significant increase ($P < 0.05$) in IL-12p40 production (Fig. 6). When the neutralizing MAb to IL-10 was added to macrophage cultures along with SB203580 or U0126, a slightly higher level of IL-12p40 was observed in comparison to cultures pretreated with SB203580 or U0126 only; however, the difference was not significant. These results indicated that p38 or ERK has the

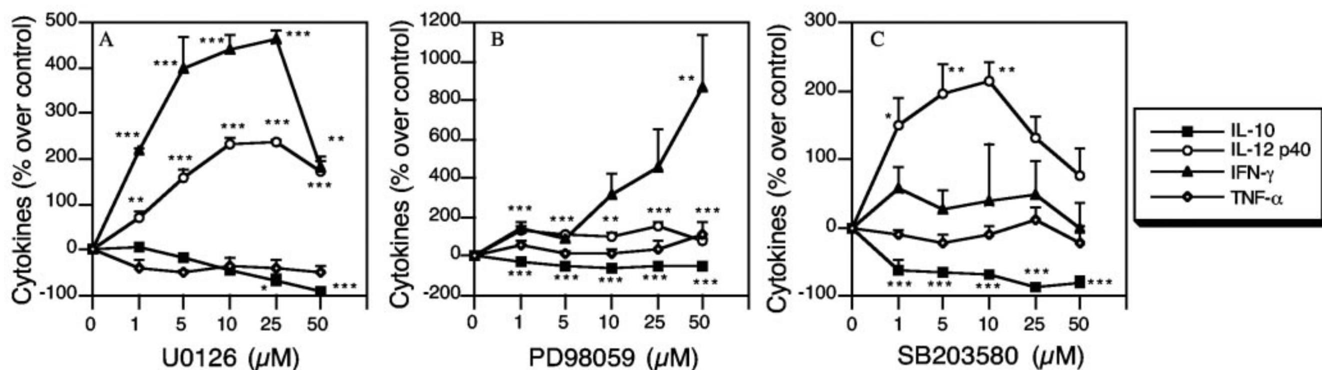


FIG. 5. Dose-dependent effect of ERK and p38 inhibitors on rHagB-induced cytokine production. C57BL/6 peritoneal macrophages (5×10^5 cells) were pretreated with U0126, PD98059, or SB203580 (1 to 50 μM) for 2 h and then stimulated with rHagB (20 μg/ml) for 20 h. The levels of IL-10, IL-12p40, IFN-γ, and TNF-α in culture supernatants were determined by ELISA. Data are expressed as the mean percent increase over that seen in rHagB-stimulated culture with no inhibitor ± standard error of the means of six separate experiments. Significant increases (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) were seen when results were compared with levels in macrophages incubated with rHagB only.

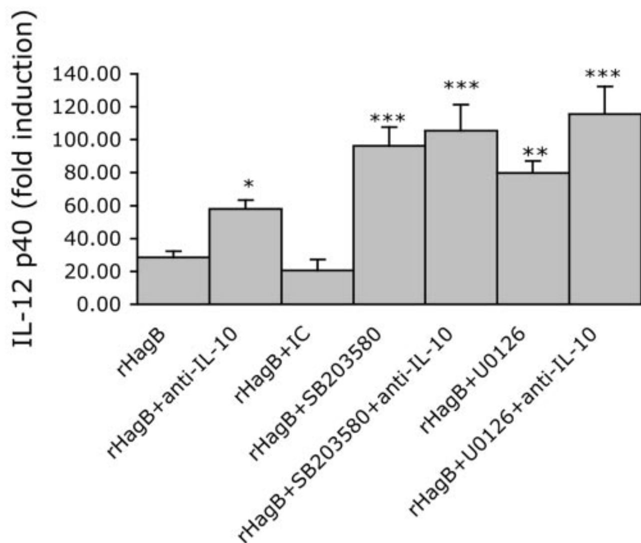


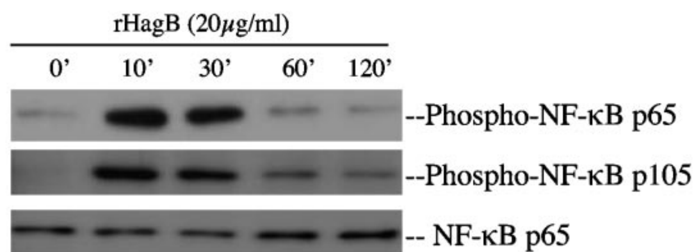
FIG. 6. Role of IL-10 in the enhancement of IL-12p40 production. C57BL/6 peritoneal macrophages (5×10^5 cells) were pretreated with a neutralizing MAb against IL-10 (10 μg/ml) or with its isotype control (IC) in the presence or absence of SB203580 (10 μM), U0126 (25 μM), or PD98059 (25 μM) for 2 h and then stimulated with rHagB (20 μg/ml) for 20 h. The levels of IL-10, IL-12p40, IFN-γ, and TNF-α in culture supernatants were determined by ELISA. Data are expressed as the mean of the increase in induction (*n*-fold) over unstimulated control ± standard error of the mean of six separate experiments. Values were significantly different when P was <0.05 (*), <0.01 (**), or <0.001 (***) in comparison to values for macrophages incubated with rHagB only.

effect of down-regulating IL-12p40, which seems to be mediated at least partly by IL-10.

Role of NF-κB in regulating rHagB-stimulated cytokine production. It has been suggested that the transcriptional factor NF-κB has a critical role in regulating cytokine-mediated inflammation (2, 5). Therefore, to further understand the cellular mechanism(s) mediating the activation of cytokine production by rHagB, we next investigated the role of NF-κB in rHagB-induced cytokine production and the effect of ERK and p38 on the activation of NF-κB. rHagB induced the phosphorylation of both NF-κB p65 and NF-κB p105 (Fig. 7A). Maximal activation was observed at 10 to 30 min of stimulation. The results of the TransAM assay further confirmed that NF-κB p65 in the nuclear extracts was indeed activated by rHagB and indicated that maximal DNA-binding activity of NF-κB p65 occurs at 30 min (Fig. 7B). In regards to the effect of the ERK or p38 signaling pathway on rHagB-mediated NF-κB activation, neither SB203580 nor U0126 showed an effect on rHagB-induced phosphorylation or DNA-binding activity of NF-κB p65 (data not shown).

The involvement of NF-κB in rHagB-induced cytokine production was then assessed by using the specific NF-κB inhibitor SN50, which inhibits the translocation of activated NF-κB to the nucleus. SN50 M, an inactive analog of SN50, was used as a negative control. SN50 greatly enhanced IL-10 production by rHagB-stimulated macrophages (Fig. 8A). The increase in rHagB-induced IL-10 production in cultures incubated with SN50 was significantly higher than that seen in cultures incubated with rHagB alone ($P < 0.01$) or with rHagB and SN50 M ($P < 0.05$). Conversely, inhibition by SN50 resulted in a very significant ($P < 0.001$) reduction in the level of IL-12p40 production (Fig. 8B). No significant effect on rHagB-induced IFN-γ or TNF-α production was seen in the presence of SN50 ($P > 0.05$) (Fig. 8C and D). These results indicated that NF-κB is critical for up-regulation of IL-12p40 and down-regulation of IL-10 production in rHagB-stimulated macrophages.

We also used a neutralizing anti-IL-10 MAb to investigate whether the diminished IL-12p40 production by SN50 was the consequence of enhanced IL-10 levels. Our results indicate that when anti-IL-10 MAb was added together with SN50, an



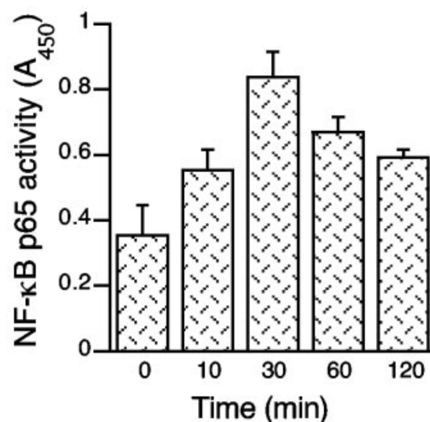


FIG. 7. Activation of NF-κB by rHagB. (A) Phosphorylation of NF-κB. C57BL/6 peritoneal macrophages (3×10^6 cells) were stimulated with rHagB (20 μg/ml) for 0 to 120 min. Equal amounts of whole-cell lysates were subjected to electrophoresis on a 10% Tris-HCl gel, and Western blots were performed using a specific Ab against the phosphorylated form of NF-κB p65 (Ser536) or NF-κB p105 (Ser933). Total NF-κB p65 in each sample was used as the equal loading control. Blots are representative of results obtained from three separate experiments. (B) Activation of NF-κB p65 translocation. C57BL/6 peritoneal macrophages (5×10^5 cells) were stimulated with rHagB (20 μg/ml) for 0 to 120 min. Equal amounts of nuclear extracts were analyzed for NF-κB p65 activity using the TransAM assay. Data represent the mean ± standard deviation of three separate experiments.

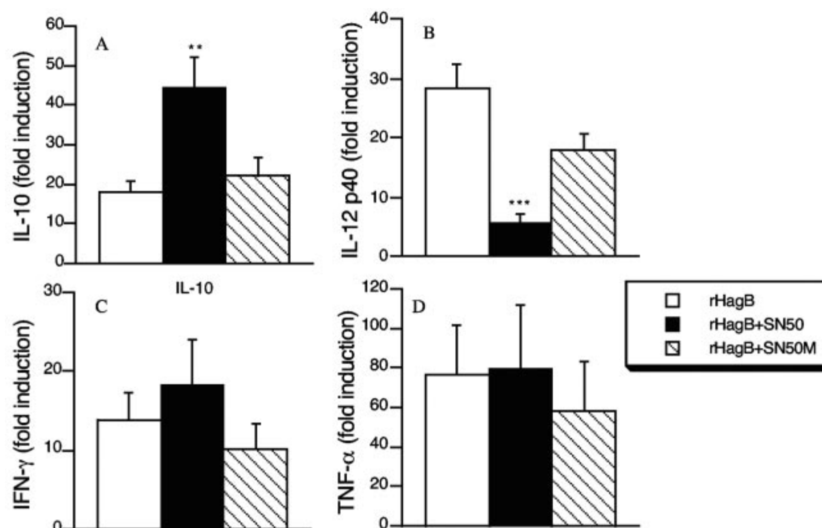


FIG. 8. Effect of NF-κB inhibition on rHagB-induced cytokine production. C57BL/6 peritoneal macrophages (5×10^5 cells) were pretreated with SN50 (10 μM) or its inactive analog SN50 M (10 μM) for 2 h and then stimulated with rHagB (20 μg/ml) for 20 h. The levels of IL-10, IL-12p40, IFN-γ, and TNF-α in culture supernatants were determined by ELISA. Data are expressed as the mean of the increase in induction (*n*-fold) over nonstimulated control ± standard error of the mean of six separate experiments. Significant differences (**, $P < 0.01$; ***, $P < 0.001$) were seen compared to macrophages stimulated with rHagB alone.

increase in the level of IL-12p40 was observed compared to cultures stimulated with rHagB in the presence of SN50 only (data not shown). These results suggested that the diminished IL-12p40 level in rHagB-treated macrophages by SN50 was mainly due to increased levels of IL-10.

DISCUSSION

In the present study, we investigated the effect of rHagB on the induction of inflammatory cytokines by antigen-presenting cells, i.e., macrophages, and the role of MAPKs and NF-κB intracellular signaling pathways in this process. Our study provides for the first time evidence for the involvement of ERK and p38 kinases and of NF-κB transcription factor on the regulation of immune activation induced by the virulence factor HagB derived from the periodontal pathogen *P. gingivalis*. An understanding of these processes will contribute to the potential development of therapeutic strategies against adult periodontal disease.

Studies have shown that rHagB induces the production of a mixed Th1- and Th2-like cytokine pattern, as determined by the production of IFN-γ, IL-2, IL-4, and IL-10 by rat splenic lymphoid cells (19) or by the production of IL-4, IL-5, and IFN-γ by mouse $CD4^+$ T cells (40). In the present study, rHagB was found to induce mRNA expression and protein production by mouse peritoneal macrophages of other proinflammatory cytokines, i.e., TNF-α and IL-12 p40, as well as IFN-γ and the anti-inflammatory cytokine IL-10. We have also demonstrated a simultaneous activation of ERK, p38, and JNK by rHagB. Furthermore, inhibition of the ERK or p38 signaling pathway by the specific ERK inhibitor U0126 or PD98059 or the p38 inhibitor SB203580 had distinct effects on rHagB-induced cytokine production by mouse peritoneal macrophages. Specifically, we showed that the p38 pathway was required for the production of the anti-inflammatory cytokine IL-10 and the down-regulation of the proinflammatory cytokines IL-12p40. Our observation that the p38 MAPK is important in IL-10 production is in agreement with Ma et al. (24), who reported that treatment of THP-1/CD14 cells with the p38 inhibitor SB203580 completely inhibited LPS-induced IL-10 production in a dose-dependent manner. However, our findings appear to be in conflict with the findings of others that showed the positive involvement of p38 on LPS (12) and CpG DNA (39) induced IL-12p40 expression. Häcker et al. (15) indicated that murine macrophages do not produce IL-12p40 in the presence of a p38 inhibitor. Conversely, Marriott et al. (25) demonstrated that p38 has a suppressive effect on LPS-induced IL-12p40 production by human monocytes/macrophages. Findings by Salmon et al. (31) also demonstrated that the p38 inhibitor SB203580 significantly enhances the production of IL-12p40 or p70 induced by LPS and IFN-γ. The authors further indicated, however, that the p38 MAPK activity is required for the production of IL-10, as well as the proinflammatory cytokines IL-12 and IFN-γ, and that the net effects of a p38 inhibitor on the production of IL-12 and IFN-γ can be positive or negative, depending on the stimuli, cell population, and levels of cytokines such as IFN-γ and IL-10 (31).

In regard to ERK activation, previous investigations from our group (26) and others (37) have demonstrated that ERK activation is responsible for positive and negative control of IL-10 and IL-12 production, respectively, by a variety of stimuli. In the present study, we observed that ERK activation is involved in the down-regulation of IL-12p40 and the up-regulation of IL-10 upon rHagB stimulation of macrophages. Moreover, significantly augmented IFN-γ production was seen by inhibition of ERK. Finally, we found that inhibition of ERK activation by U0126 resulted in suppression of TNF-α production. It is noteworthy that the inhibition of the ERK signaling pathway by U0126 was more effective than PD98059 in the up-regulation of IL-12p40 production and down-regulation of IL-10

and TNF-α by rHagB-stimulated macrophages. We also found the inhibiting effect of U0126 on ERK phosphorylation to be stronger than that of PD98059 at the concentrations tested. Previous studies by Häcker et al. (15) and Yi et al. (39) with CpG DNA have reported similar findings. It is likely that the difference between the two ERK inhibitors was due to the selective inhibition of both MEK-1 and MEK-2 phosphorylation by U0126 and of only MEK-1 phosphorylation by PD98059.

In this study, we also tested SP600125, a widely used JNK inhibitor in cell-based assays (4, 14, 32), to inhibit JNK activation and the subsequent regulation of cytokine production. We found that SP600125 had a potent inhibitory effect on JNK phosphorylation; however, it also inhibited rHagB-induced ERK phosphorylation, at least at concentrations greater than 1 μM, and had a suppressive effect on the production of all the tested cytokines at concentrations higher than 1 μM (data not shown). Bain et al. (3) recently reported that SP600125 was a nonspecific JNK inhibitor and showed that 13 of the 28 protein kinases tested were inhibited to a similar or greater extent as JNK. The authors suggested the need to develop more selective JNK inhibitors to evaluate whether the previously reported effects of SP600125 in vitro and in vivo result from the inhibition of JNK or other enzymes.

IL-10 is best known for mediating the down-regulation of Th1 responses by inhibiting the production of IL-12 (7). With the aid of a neutralizing MAb to IL-10, we demonstrated that rHagB-induced IL-10 production had an inhibitory effect on IL-12p40 production, which likely played a role in the ability of the ERK inhibitor U0126 or the p38 inhibitor SB203580 to up-regulate IL-12p40 levels. However, inhibition of ERK or p38 alone induced significantly enhanced levels of IL-12p40, which was similar to that seen when U0126 or SB203580 was used in combination with a neutralizing MAb to IL-10. Thus, ERK or p38 inhibition likely enhanced IL-12p40 production via both IL-10-dependent and -independent mechanisms. Taken together, our results suggest that rHagB-induced IL-12p40 production is, at least in part, regulated by an IL-10-dependent pathway that is triggered by rHagB-mediated ERK or p38 activation.

Enhancement or suppression of rHagB-induced cytokine production by inhibition of ERK or p38 could be the result of transcriptional or posttranscriptional regulation. Our data showed that in the absence of ERK or p38 activation, the changes in rHagB-induced cytokine production did not completely correlate with levels of mRNA. Thus, regulation of the rHagB-mediated cytokine production by ERK and p38 pathways might take place at both the transcriptional level and the posttranscriptional level. Our findings did show that inhibition of NF-κB with SN50 resulted in only a minor effect on rHagB-induced IFN-γ and TNF-α production. However, IL-12p40 was almost completely blocked. In contrast, rHagB-induced IL-10 production was significantly enhanced by NF-κB inhibition. These findings are in agreement with a previous study (27), which showed that blockade of the NF-κB pathway resulted in enhanced levels of LPS-induced plasma IL-10 but decreased levels of plasma IL-12. Furthermore, other studies indicate that NF-κB, a key regulator of most proinflammatory cytokine genes, is not involved in IL-10 regulation (6, 23, 27). It has been reported that the transcription factors AP-1 and CREB play a critical role in IL-10 expression (13, 28, 39). However, other investigations suggest that only the transcription factor Sp1 seems to play a prominent role in IL-10 regulation (7, 17, 24). It has been hypothesized that the molecular regulation of the anti-inflammatory cytokine IL-10 gene is fundamentally different from the regulation of proinflammatory cytokine genes (7). The selective blockade of NF-κB for proinflammatory IL-12p40 production but not anti-inflammatory IL-10 production in macrophages indicates that NF-κB is an appealing target for therapeutic strategies designed to attenuate cytokine-mediated inflammation.

The role of MAPKs in regulating NF-κB transactivation remains controversial. In the present study, we showed that the ERK inhibitor U0126 or the p38 inhibitor SB203580 did not have a significant effect on the rHagB-induced activation of NF-κB by macrophages. Similar results have been shown in a previous study indicating that inhibition of ERK phosphorylation had no effect on the phosphorylation of I-κB and NF-κB (37). Another group has also reported that neither ERK nor p38 inhibition had significant effects on CpG DNA-induced NF-κB activation at the nuclear DNA binding level (39). However, in that study, the inhibition of ERK or p38 resulted in slight inhibitory effects on the CpG DNA-induced transcriptional activity of NF-κB. It has been demonstrated that ERK and p38 regulate the transcriptional activity of NF-κB without affecting DNA binding activity in response to TNF-α or LPS (8, 36).

It is worth pointing out that we could not detect any IL-12p70 production by rHagB-stimulated macrophages in any of the experimental conditions tested. Yi et al. (39) have reported a slight induction of IL-12p70 by CpG-stimulated RAW264.7 cells and a dramatic increase in IL-12p70 levels in the absence of activated ERK. We also observed elevated levels of both IL-12p40 and IL-12p70 upon ERK inhibition in *P. gingivalis* LPS-treated human monocytes; however, *P. gingivalis* LPS alone did not induce any detectable IL-12p70 (26). While some studies have suggested that IL-12 production is controlled by the induction of IL-12p40 (9), others have reported that the IL-12p35 mRNA is a limiting factor for production of IL-12p70 (33, 34). Interestingly, our real-time PCR data showed that rHagB induced both IL-12p40 and IL-12p35 mRNA expression. However, IL-12p40 mRNA peaked at 2 h, whereas IL-12p35 mRNA peaked at 12 h. This may explain why no IL-12p70 was detected in our study. That is, since IL-12p40 translates much earlier than IL-12p35, IL-12p40 would be formed before IL-12p35. Therefore, the initial lack of IL-12 p35 and the excess of IL-12p40 could lead to the formation of IL-12p40 homodimers and not IL-12p40/IL-12p35 heterodimers.

In summary, the present study demonstrates that ERK and p38 MAPKs and the transcription factor NF-κB play differential regulatory roles in the rHagB-induced production of proinflammatory and anti-inflammatory cytokines in mouse-derived peritoneal macrophages. Because of the involvement of multiple signaling pathways and the cross talk between multiple signaling modulators, careful studies on the biologic roles of other signaling pathways/modulators activated by rHagB might provide further understanding into the immune reactions induced by rHagB. Studies are under way to delineate the role of Toll-like receptors and other signaling pathways involved in rHagB-induced immune activation.

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