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Requirement of Mitogen-Activated Protein Kinases and IκB Phosphorylation for Induction of Proinflammatory Cytokines Synthesis by Macrophages Indicates Functional Similarity of Receptors Triggered by Glycosylphosphatidylinositol Anchors from Parasitic Protozoa and Bacterial Lipopolysaccharide[1]

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In the present study, we evaluated the ability of GPI-anchored mucin-like glycoproteins purified from *Trypanosoma cruzi* trypomastigotes (tGPI-mucin) to trigger phosphorylation of different mitogen-activated protein kinases (MAPKs) and related transcription factors in inflammatory macrophages. Kinetic experiments show that the peak of extracellular signal-related kinase (ERK)-1/ERK-2, stress-activated protein kinase (SAPK) kinase-1/mitogen-activated protein kinase (MAPK) kinase-4, and p38/SAPK-2, phosphorylation occurs between 15 and 30 min after macrophage stimulation with tGPI-mucin or GPI anchors highly purified from tGPI-mucins (tGPI). The use of the specific inhibitors of ERK-1/ERK-2 (PD 98059) and p38/SAPK-2 (SB 203580) phosphorylation also indicates the role of MAPKs, with possible involvement of cAMP response element binding protein, in triggering TNF-α and IL-12 synthesis by IFN-γ-primed-macrophages exposed to tGPI or tGPI-mucin. In addition, tGPI-mucin and tGPI were able to induce phosphorylation of IκB, and the use of SN50 peptide, an inhibitor of NF-κB translocation, resulted in 70% of TNF-α synthesis by macrophages exposed to tGPI-mucin. Finally, the similarity of patterns of MAPK and IκB phosphorylation, the concentration of drugs required to inhibit cytokine synthesis, as well as cross-tolerization exhibited by macrophages exposed to tGPI, tGPI-mucin, or bacterial LPS, suggest that receptors with the same functional properties are triggered by these different microbial glycoconjugates. ***The Journal of Immunology*****, 2001, 166: 3423–3431.**

The cellular compartment from the innate immune system has low levels of specificity and can be activated immediately after infection through the involvement of cell receptors specific for dominant structures (e.g., LPS, lipopeptides, lipoteichoic acid, repetitive mannose structures, and DNA CpG motifs) that are unique and characteristic molecules from certain groups of specific pathogens (1–7). Cells of the macrophage lineage exposed to such microbial components will synthesize high levels of proinflammatory cytokines that induce multiple activities in other cells of the immune system. Notably, cells from macrophage lineage exposed to protozoan parasites produce IL-12 and TNF-α that are responsible for initiating IFN-γ synthesis by NK cells (8–10). In agreement, different studies indicate that during the early stages of infection, before the establishment of acquired protective immunity, the cellular compartment of the innate immune system plays a crucial role in host resistance against different intracellular protozoa (8–10).

To better understand the early stimulation of the innate immune system by parasitic protozoa, studies performed in our laboratories and elsewhere have focused on the identification and chemical characterization of the protozoan products that trigger the proinflammatory and effector functions of macrophages. These studies indicate that tGPI,[3] a GPI anchor purified from the mucin-like glycoprotein (tGPI-mucin) of *Trypanosoma cruzi* trypomastigotes, has an essential role in triggering various macrophage functions (11–16), similar to the importance of LPS in infection with Gram-negative bacteria. Comparable results were obtained with the GPI anchors purified from *Plasmodium falciparum* and *Trypanosoma brucei* (Refs. 17 and 18; see review in Ref. 19).

Recent studies have suggested that protozoan GPI anchors may have two signaling portions (i.e., the glycan core and inositolphospholipid) that trigger different signaling components responsible for cytokine and NO synthesis by mammalian host cells (20–23).

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[3] Abbreviations used in this paper: tGPI, GPI anchor purified from tGPI-mucin; tGPI-mucin, GPI-anchored mucin-like glycoproteins derived from *Trypanosoma cruzi* trypomastigotes; CREB, cAMP response element binding protein; ATF, activating transcription factor; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; JNK, c-jun N-terminal kinase; iNOS, inducible NO synthase; SAPK, stress-activated protein kinase; SKK, SAPK kinase; MKK, MAPK kinase; TLR, Toll-like receptor.

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However, not enough information is available regarding the macrophage receptor(s) and signaling pathways that are triggered by protozoan-derived GPI anchors. Different studies indicate a similarity in gene expression and functions displayed by macrophages exposed to either tGPI anchors, tGPI-mucin, or LPS (11–14). LPS has been reported to stimulate signal transduction through the mitogen-activated protein kinases (MAPKs) (24–27). The MAPKs comprise an important group of serine/threonine signaling kinases that transduce a variety of extracellular stimuli through a cascade of protein phosphorylations, which lead to the activation of transcription factors (28–31). There are at least three distinct MAPK pathways in mammals, including the extracellular singal-related kinases (ERK-1/ERK-2), the c-jun N-terminal kinases (JNKs), and the stress-activated protein kinase (SAPK)-2, also named p38 (30–35). Here, we compared in a systematic way the kinetics of phosphorylation of these different members of the MAPK family, as well as of the inhibitor of the NF-κB transcription factor, IκB, in macrophages exposed to tGPI-mucin, tGPI, or LPS derived from *Escherichia coli*. In addition, we compared the ability of drugs that are specific inhibitors of the activation of ERK-1/ERK-2 and/or SAPK-2/p38, as well as NF-κB translocation, to inhibit the induction of IL-12(p40), TNF-α, and NO synthesis by macrophages exposed to the above-mentioned microbial glycolipids. Our results show a striking similarity in the macrophage response to LPS and tGPI-mucin (or tGPI), indicating that the receptors used by these distinct microbial glycolipids may transduce common signaling pathways.

Materials and Methods

Animals

Five- to 6-wk-old C57BL/6 or C3H/HeJ were obtained from the animal house of Fundaçao Oswaldo Cruz (Rio de Janeiro, Brazil) and maintained under standard conditions in the animal house of the Centro de Pesquisas René Rachou-Fundaçao Oswaldo Cruz (Belo Horizonte, Brazil).

Reagents and Abs

Reagents used were obtained from Sigma (St. Louis, MO) unless indicated otherwise. SN50 peptide inhibitor of NF-κB translocation and SN50M control peptide; PD 98059, 2′-amino-3′methoxyflavone, specific inhibitor of ERK-1/ERK-2 phosphorylation; SB 203580, (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole, specific inhibitor of SAPK-2/p38 phosphorylation, were all purchased from Calbiochem (San Diego, CA). Abs were obtained from the following sources: anti-IL-12(p40) Abs (clones C17.15 and C15.6, as capture and detection, respectively) were a generous gift from Dr. Giorgio Trinchieri (Wistar Institute, Philadelphia, PA); anti-TNF-α and IL-12(p70) kits (Duoset ELISA Development System) were purchased from Genzyme (Cambridge, MA); and Abs against MAPK family members (i.e., ERK-1/ERK-2, SAPK kinase (SKK)-1/MAPK kinase (MKK)-4, and SAPK-2/p38), I-κB, cAMP response element binding protein (CREB)/activating transcription factor (ATF)-1, and ATF-2 were obtained from New England Biolabs (Hertfordshire, U.K.).

Purification of T. cruzi*-derived tGPI*

The tGPI-mucin (GPI-anchored glycoprotein) was isolated from tissue culture trypomastigotes as described previously (16, 36) by using sequential organic extraction followed by hydrophobic-interaction chromatography in octyl-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) and elution with a propan-1-ol gradient (5–60%). The tGPIs were obtained after treatment of tGPI-mucin with proteinase K followed by hydrophobic-interaction chromatography in octyl-Sepharose column and elution with a propan-1-ol gradient (5–60%). Purified tGPI-mucin and tGPI were quantified by myo-inositol analysis (37). The presence of *Mycoplasma* contaminants in the tGPI-mucin preparations were checked by Edman sequencing and mass spectrometry (matrix-assisted laser desorption ionization-time of flight-mass spectrometry), which did not reveal any contamination with bacterial lipopeptides (16).

Murine macrophage preparation

Thioglycollate-elicited peritoneal macrophages were obtained from either C3H/HeJ or C57BL/6 by peritoneal washing (11). Adherent peritoneal macrophages were cultured in 96-well plates (2×105 cells/well) at 37°C/5% CO_2 in DMEM (Life Technologies, Paisly, U.K.) supplemented with 10% heat-inactivated FCS (Life Technologies), 2 mM L-glutamine, and 40 μg/ml of gentamicin. Cells were incubated with inhibitors of different MAPK cascade, i.e., PD 98059, an inhibitor of the ERK-1/ERK-2 activation; SB 203580, an inhibitor of the SAPK-2/p38 activation; or SN50, an inhibitor of the NF-κB translocation. The inhibitors were used on cells at the indicated concentrations for 30 min before stimulation with LPS (50 ng/ml), tGPI-mucin (10 nM), or tGPI (10 nM) with or without IFN-γ (50 IU/ml). Culture supernatants were collected 18 and 48 h after the addition of the microbial product for the evaluation of TNF-α and IL-12(p40)/IL-12(p70) or NO production, respectively.

Cytokine measurement

IL-12(p40) was determined by ELISA with 5 μg/ml of anti-IL-12(p40) mAbs: clone C17.15 as the capture Ab and biotinylated anti-IL-12 (clone C15.6) diluted 750-fold as the detecting Ab. The development was made with streptavidin-peroxidase conjugate. The plates were read at 405 nm, and IL-12(p40) concentration was calculated by reference to a standard curve for murine rIL-12 (11). TNF-α and IL-12 were quantified in 18 and 48 h supernatants, respectively, by ELISA with the Genzyme Duoset kit.

Nitrite measurement

Nitrite concentrations in culture supernatants were assayed at 48 h after macrophage activation by the Griess reaction (38). Plates were read at 550 nm, and NO_2 concentration was determined with reference to a standard curve with sodium nitrite in culture medium.

Cell viability assay

To assess toxic effects of the used inhibitors and cell viability we used MTT as described previously (39). Briefly, cells were incubated with 100 μl/well of supplemented medium containing 0.5 mg/ml MTT overnight at 37°C and 5% CO_2. Cells then were washed and treated with 100 μl/ml 10% SDS in dimethylformamide:H_2O (1:1). Absorbance was read at 570 nm. Cell viability was calculated as relative index of control cells (100% viable cells). No evidence of toxic effects were observed when PD 98059, SB 203580, or SN50 peptide were evaluated in the concentrations used in our experiments.

Lysate preparation

Peritoneal macrophages were cultured and stimulated with either LPS (50 ng/ml), tGPI (10 nM), or tGPI-mucin (10 nM) at the times shown. Where indicated, PD 98059 and/or SB 203580 were added before macrophage stimulation. Cells were washed and lysed on ice in lysis buffer (20 mM Tris-acetate, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10 mM sodium glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 4 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM benzamidine, 0.1% v/v 2-ME, and 2 μM microcystin-LR). Lysates were scraped, collected into Eppendorf tubes, and centrifuged at $13,000 \times g$ for 20 min at 4°C (40).

Electrophoresis and immunoblotting

Cell lysate samples were separated by 10% acrylamide SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked overnight at 4°C with PBS containing 5% (w/v) defatted milk and 0.1% Tween 20. Membranes were washed three times with PBS containing 0.1% Tween 20, then incubated with rabbit polyclonal Abs anti-phosphorylated MAPKs or transcription factors in PBS containing 5% (w/v) BSA and 0.1% Tween 20. After washing, the membranes were incubated with HRP-conjugated anti-rabbit Ab and assayed by the ECL chemiluminescent system (Amersham-Pharmacia Biotech, Little Chalfont, U.K.) according to the manufacturer's instructions.

Results

Activation of ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38 pathways by tGPI-mucin and tGPI

Bacterial LPS is a potent inducer of proinflammatory cytokines by macrophages, and it recently has been demonstrated that it activates three different groups of MAPK (ERK-1/ERK-2, JNKs, and SAPK-2/p38) in cells of the macrophage lineage (24–27). In our

previous studies, we have shown that the tGPI-mucin or the highly purified tGPI also activate inflammatory macrophages leading to the production of proinflammatory cytokines as well as NO (11–16). Similar results are obtained with the macrophage cell line, named RAW 264, resident macrophages, and bone marrow-derived macrophages from mouse origin (unpublished data). Here, we examined whether the tGPI-mucin or tGPI were capable of activating these MAPKs by examining their phosphorylation in immunoblots by using phospho-specific Abs. To be consistent with our previous publications, we used the inflammatory macrophages. As shown in Fig. 1*A*, ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38 phosphorylation were all stimulated by LPS, tGPI-mucin, or tGPI to a similar extent. The maximum levels of phosphorylation induced by LPS, as indicated by Western immunoblotting was observed at 30 min for SKK-1/MKK-4 and SAPK-2/p38, whereas the ERK-1/ERK-2 activity peaked at 15 min poststimulation. The tGPI-mucin strongly activated ERK-1/ERK-2, which was maximal at 15 min before declining toward the basal level within 30 min. SKK-1/MKK4 and SAPK-2/p38 were both stimulated by tGPI-mucin within 15 min and sustained for up to 60 min. Likewise, tGPI stimulated the three MAPKs. These results clearly show that either tGPI-mucin or tGPI stimulate all three classes of MAPKs in murine macrophages.

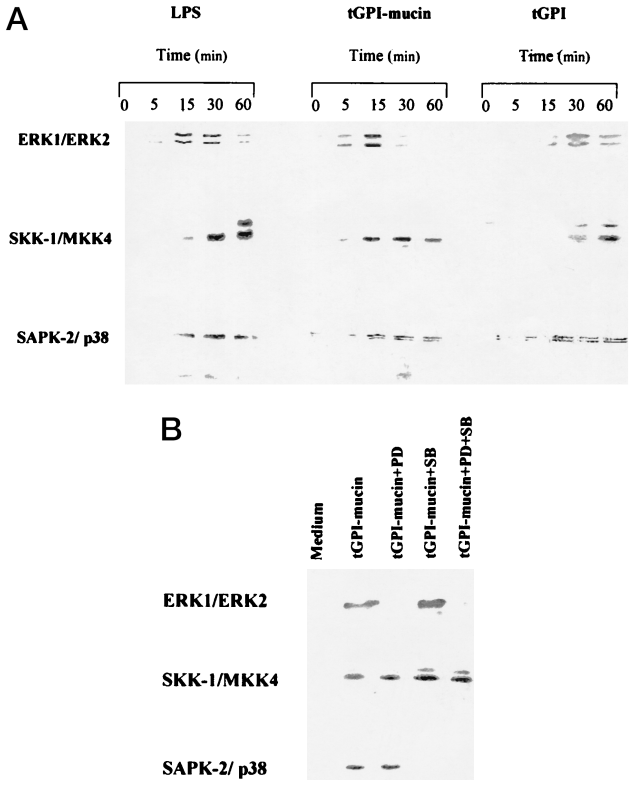

FIGURE 1. *A*, Time-course of LPS-, tGPI-mucin-, or tGPI-induced phosphorylation of MAPKs. Peritoneal macrophages from C57BL/6 mice were stimulated with either LPS (50 ng/ml), tGPI-mucin (10 nM), or tGPI (10 nM) and harvested at the indicated times (0–60 min). Cell lysates were resolved by 10% acrylamide SDS-PAGE followed by immunoblotting with a set of Abs that recognize either phosphorylated ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38. Data are representative of three experiments. *B*, Effects of PD 98059 and/or SB 203580 on tGPI-mucin-induced MAPK phosphorylation in murine inflammatory macrophages. Peritoneal macrophages from C57BL/6 were pretreated with PD 98059 (40 μM) and/or SB 203580 (10 μM), or medium alone for 30 min before stimulation for 15 min with tGPI-mucin (10 nM). The cells were harvested and the cell lysates resolved in 10% acrylamide SDS-PAGE followed by immunoblotting with a set of Abs that recognize either of the phosphorylated MAPKs. Data are representative of three experiments.

Specific effect of MAPK inhibitors on ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38 phosphorylation

We considered that it was important to verify whether the specific inhibitors PD 98059 and SB 203580 appropriately blocked phosphorylation of different MAPKs in macrophages activated with tGPI-mucin. Macrophages were pretreated with a fixed concentration of PD 98059 (40 μM) and/or SB 203580 (10 μM) for 30 min and stimulated with tGPI-mucin for 15 min. As shown in Fig. 1*B*, PD 98059 and SB 203580 inhibited ERK-1/ERK-2 and SAPK-2/p38 phosphorylation, respectively. PD 98059 (40 μM) significantly inhibited the tGPI-mucin-stimulated ERK-1/ERK-2 phosphorylation but had no effect on the phosphorylation of SAPK-2/p38 and SKK-1/MKK-4. In turn, SB 203580 completely abrogated the SAPK-2/p38 activity at 10 μM, and at the same time appeared to increase the levels of ERK-1/ERK-2 and SKK-1/MKK-4 phosphorylation.

Effect of drugs inhibiting phosphorylation of ERK-1/ERK-2 and SAPK-2/p38 on the production of IL-12, TNF-α, and NO

To discriminate between the roles of ERK-1/ERK-2 and SAPK-2/p38 on the production of cytokines and NO after stimulation with tGPI-mucin and tGPI, we investigated the effects of PD 98059 (34), a specific inhibitor of the MAPK cascade that leads to ERK-1/ERK-2 phosphorylation, and SB 203580 (35), an inhibitor of SAPK-2/p38 phosphorylation and activity. Monolayers of macrophages were pretreated with PD 98059 or SB 203580 30 min before the addition of LPS, tGPI-mucin, and tGPI. As shown in Fig. 2*A*, neither PD 98059 nor SB 203580 inhibited NO production in response to stimulation by LPS, tGPI-mucin, or tGPI. This is in agreement with a previous study showing that the NO production induced by TNF-α or LPS in murine macrophages was not affected by PD 98059 or SB 203580 pretreatment (41, 42). In contrast, the LPS-induced TNF-α production was inhibited in 30% by PD 98059 at 40 μM. The TNF-α inhibition observed in tGPI-mucin- or tGPI-treated macrophages was lower, reaching ~25% at the highest dose of PD 98059. Preincubation with SB 203580 of LPS-, tGPI-, or tGPI-mucin-treated macrophages resulted in a significant inhibition of TNF-α production in a dose-dependent manner. The maximum inhibitory effect was ~60% at 10 μM.

To assess the relative role of ERK-1/ERK-2 and SAPK-2/p38 on LPS-, tGPI-mucin-, or tGPI-mediated IL-12 induction, we also investigated whether PD 98059 or SB 203580 affected IL-12(p40) production in murine macrophages. As reported previously (43), the results presented in Fig. 2*A* show enhanced IL-12(p40) production, up to 40% in the PD 98059-treated cells. In contrast, SB 203580 had a small inhibitory effect on IL-12(p40) production by the stimulated macrophages in a dose-dependent manner with IC_{50} >10 μM. Similar results were obtained for the synthesis of IL-12(p70) (data not shown).

In other experiments, IFN-γ-primed or unprimed macrophages were treated with a combination of PD 98059 (40 μM) and SB 203580 (10 μM) followed by LPS, tGPI, or tGPI-mucin stimulation and monitoring nitrite and cytokine production (Fig. 2*B*). Although there was no inhibition of NO synthesis with both inhibitors, the combination of PD 98059 and SB 203580 resulted in 85% inhibition of TNF-α production. As mentioned above, PD 98059 appeared to increase IL-12 production by primed macrophages

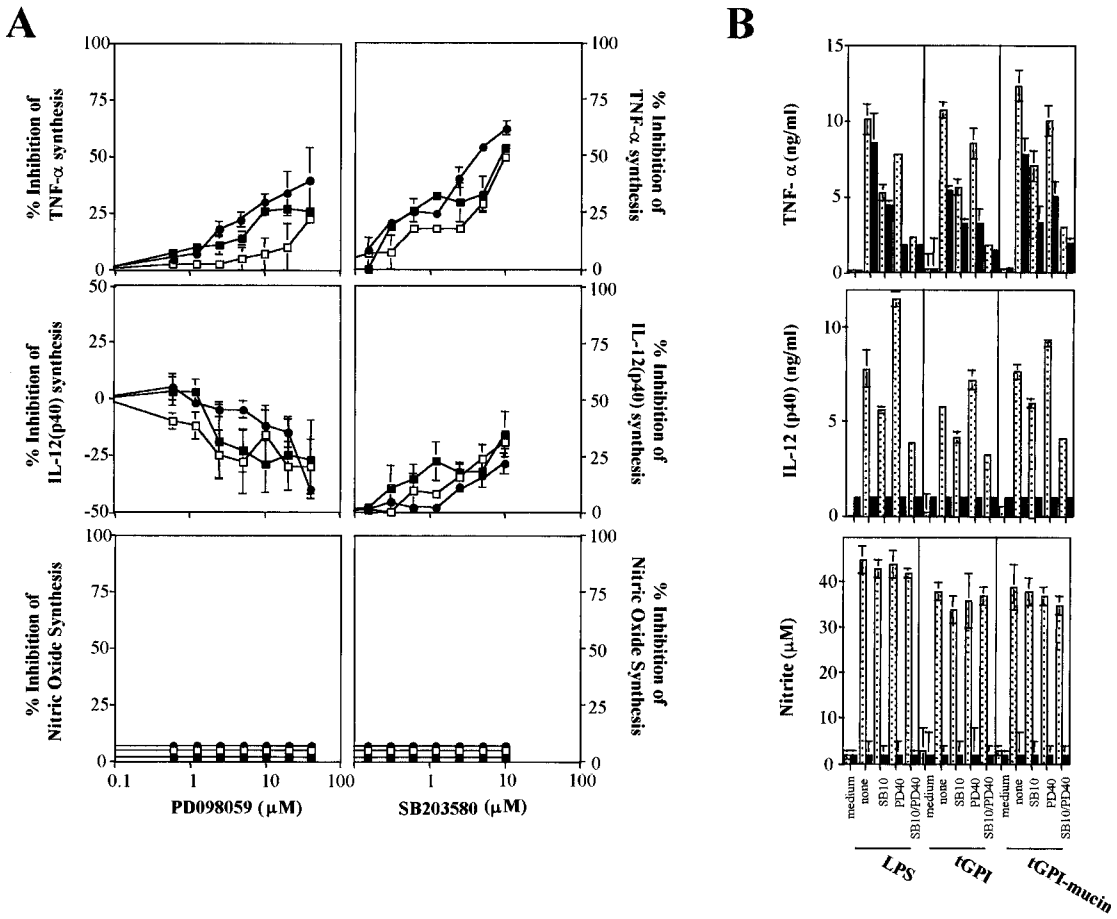

FIGURE 2. Effects of PD 98059 and SB 203580 on LPS-, tGPI-mucin-, or tGPI-induced TNF-α, IL-12, or NO synthesis. *A*, Inflammatory macrophages from C57BL/6 were primed with 50 IU/ml of IFN-γ and pretreated with various concentrations of PD 98059 or SB 203580 followed by stimulation with tGPI-mucin (10 nM; ■), tGPI (10 nM; □), or LPS (50 ng/ml; ●). Levels of TNF-α, IL-12(p40), and NO were measured in the cell culture supernatants 18 and 48 h later, respectively. The results are expressed as percent inhibition of cytokine or NO synthesis. *B*, Inflammatory macrophages from C57BL/6 were primed with 50 IU/ml of IFN-γ (▨) or not (■) and pretreated with PD 98059 (40 μM) or SB 203580 (10 μM) or with the association of PD 98059 and SB 203580 at 40 and 10 μM, respectively, and the different stimuli were added (50 ng/ml of LPS, 10 nM tGPI-mucin, and 10 nM tGPI). The level of TNF-α, IL-12(p40), and NO were measured in the cell culture supernatants 18 and 48 h later, respectively. Data are means obtained from duplicate samples and are representative of three experiments.

after LPS, tGPI, or tGPI-mucin stimulation but enhanced SB 203580-mediated inhibition of IL-12 production.

Activation of CREB and ATF-2 in macrophages stimulated with tGPI-mucin

The activation of the MAPK pathways results in changes in gene expression mediated by activating various transcription factors. So we investigated the activation of CREB, ATF-1, and ATF-2, which can be activated by SAPK/JNK and SAPK-2/p38 in response to inflammatory cytokines and stress stimuli. As shown in Fig. 3*A*, tGPI-mucin induced the phosphorylation of CREB and ATF-2. The phosphorylation of CREB and ATF-2 peaked at 30 min, and the signal was sustained up to 60 min after macrophage stimulation. The Ab we use to detect activation of CREB also recognizes phosphorylated ATF-1. However, in the conditions in which our experiment were performed, we were unable to detect ATF-1 activation in macrophages exposed to tGPI-mucin.

We also evaluated the specific effects of SB 203580 and PD 98059 on CREB as well as ATF-2 activation. Both SB 203580 and PD 98059 presented an inhibitory effect on tGPI-mucin-induced CREB phosphorylation (Fig. 3*B*). Consistent with the results shown on cytokine synthesis (Fig. 2), SB 203580 presented a greater inhibitory effect than PD 98059 on CREB phosphorylation. More important, SB 203580 and PD 98059 had an additive inhibitory effect on tGPI-mucin induced CREB phosphorylation. By contrast, the phosphorylation of ATF2 was unaffected by the use of PD 98059 or SB 203580.

Involvement of NF-κB in cytokine production by macrophages in response to tGPI-mucin

We have also investigated whether the tGPI-mucin is capable of activating the NF-κB transcription factor. For this purpose, peritoneal macrophages were stimulated by LPS, tGPI, or tGPI-mucin for different intervals of time, and NF-κB release was indirectly evaluated through IκB phosphorylation. As shown in Fig. 4*A*, IκB phosphorylation occurred rapidly after LPS, tGPI (not shown), or tGPI-mucin stimulation. To examine the involvement of NF-κB in tGPI-mucin- or LPS-induced cytokine and NO secretion by macrophages, we used SN50, a cell-permeable peptide, which inhibits the NF-κB translocation to the cell nucleus. The NF-κB nuclear translocation is maximally inhibited at 18 μM (44). As depicted in Fig. 4*B*, SN50 inhibited ~70% of the TNF-α production at 18 μM. To verify the specificity of the effect observed, we used SN50M, the peptide control, which did not affect the TNF-α production. By

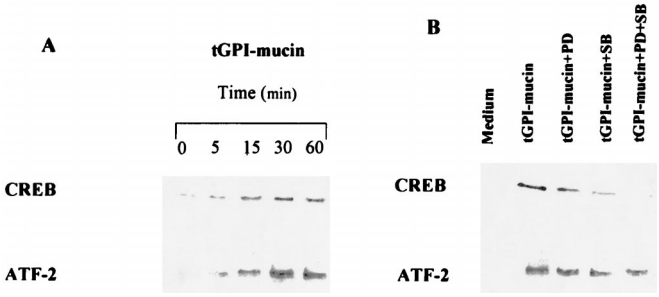

FIGURE 3. *A,* Time-course of tGPI-mucin-induced phosphorylation of CREB and ATF-2. Peritoneal macrophages from C57BL/6 mice were stimulated with tGPI-mucin (10 nM) and harvested at the indicated times (0–60 min). Cell lysates were resolved by 10% acrylamide SDS-PAGE followed by immunoblotting with a set of Abs that recognize either phosphorylated ATF-2 or CREB. Data are representative of two experiments. *B,* Effects of PD 98059 and/or SB 203580 on tGPI-mucin-induced CREB and ATF-2 phosphorylation in murine inflammatory macrophages. Peritoneal macrophages from C57BL/6 were pretreated with PD 98059 (40 μM) and/or SB 203580 (10 μM), or medium alone for 30 min, before stimulation for 30 min with tGPI-mucin (10 nM). The cells were harvested and the cell lysates were resolved in 10% acrylamide SDS-PAGE followed by immunoblotting with a set of Abs that recognize either of the phosphorylated transcription factors. Data are representative of two experiments.

contrast, SN50 appeared to have a marginal effect on the IL-12(p40) synthesis with a maximal inhibition at 18 μM. Preincubation of the cells with SN50 had no effect on the release of NO after stimulation with LPS, tGPI (not shown), or tGPI-mucin. These data suggest that NF-κB is a main transcription factor involved in induction of TNF-α, but not IL-12(p40) or inducible NO synthase (iNOS) transcription.

tGPI-mucin and LPS exhibit a cross-tolerization effect on cytokine synthesis by inflammatory macrophages

Previous studies have revealed that pretreatment of macrophage in vitro with LPS also induce a refractory state subsequent to stimulation with LPS, which includes the inhibition of MAPKs (i.e., ERK-1/ERK-2, JNKs, and SAPK-2/p38) and IκB phosphorylation (45). This study also showed cross-tolerance between IL-1β and LPS that use functionally similar receptors. Therefore, we decided to perform desensibilization experiments to investigate whether tGPI-mucin and LPS exhibit cross- tolerization. Macrophages from C57BL/6 mice were pretreated with LPS or GPI-mucin for 20 h, restimulated with either LPS or tGPI-mucin, and TNF-α, IL-12(p70), and NO synthesis evaluated thereafter. The results presented in Fig. 5 demonstrate that pretreatment with LPS or GPI-mucin resulted in partial and complete inhibition of TNF-α and IL-12 in response to the second stimulation, independent of the nature of the microbial stimuli. Collectively, these results further suggest that LPS and tGPI-mucin use functionally similar receptor to induce both TNF-α and IL-12 synthesis by inflammatory macrophages. In contrast, no desensibilization was observed in terms of NO production.

Phosphorylation of MAPKs and IκB in C3H/HeJ-derived macrophages exposed to tGPI

LPS was shown to mediate cellular activation by a member of the human Toll-like receptor (TLR) family (46). TLR4 and TLR2 have been implicated in the response of cells to LPS and other bacterial glycolipids/lipopeptides, respectively (47, 48). Because the pattern of macrophage activation by the protozoan-derived GPI is analogous to that by LPS, we speculated that signal transduction by TLR might also be triggered by GPI anchor binding. The TLR4 has

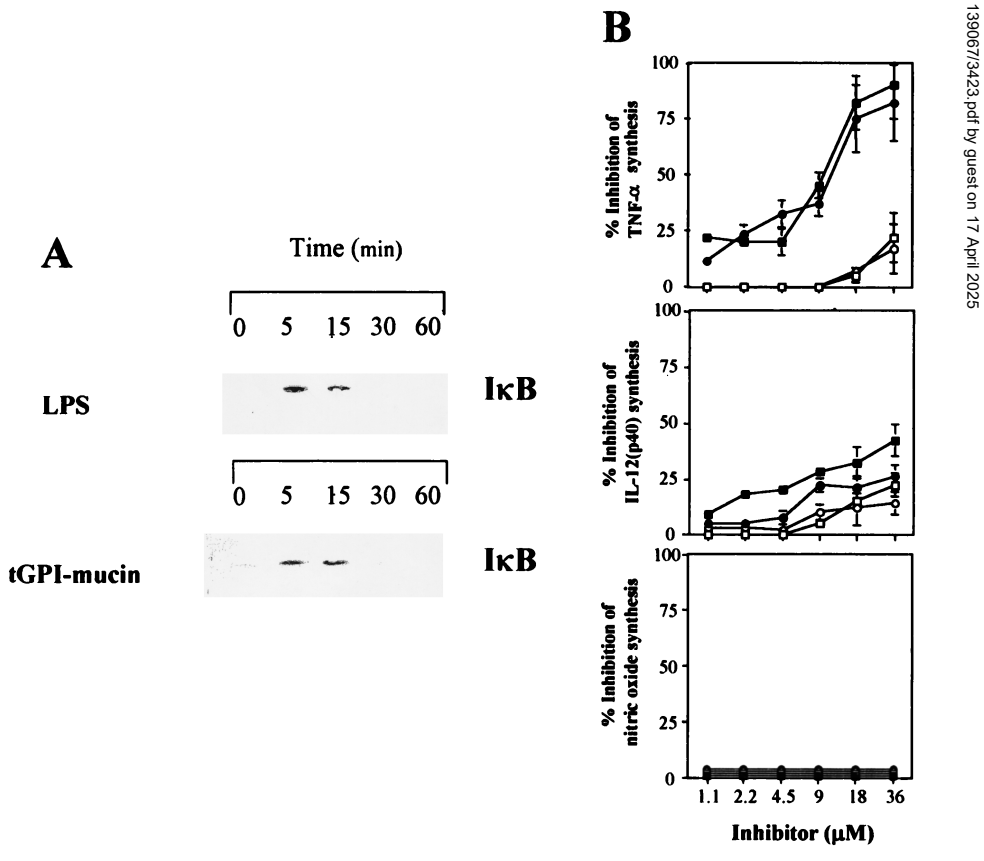

FIGURE 4. Effects of SN50 on the LPS- or tGPI-mucin-induced TNF-α, IL-12, or NO synthesis. *A,* Time-course of IκB phosphorylation after stimulation of inflammatory macrophages with LPS (50 ng/ml) or tGPI-mucin (10 nM). Peritoneal macrophages from C57BL/6 mice were stimulated with the different microbial products and harvested at the indicated times (0–60 min). Cell lysates were resolved in 10% acrylamide SDS-PAGE followed by immunoblotting using an Ab that recognizes phosphorylated IκB. Data are representative of two experiments. *B,* Inflammatory macrophages from C57BL/6 mice were primed with 50 IU/ml of IFN-γ and pretreated with various concentrations of SN50 (closed symbols) or SN50 M (control peptide; open symbols) for 30 min before stimulation with tGPI-mucin (10 nM; square) or LPS (50 ng/ml; circle). Levels of TNF-α, nitrite, and IL-12(p40) were measured in the cell culture supernatants 18, 48, and 48 h later respectively. The results are expressed as percent inhibition of cytokine or NO synthesis. Data are means obtained from duplicate samples and are representative of three experiments.

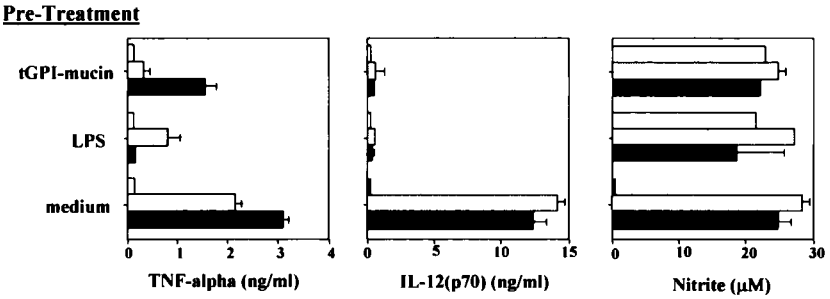

FIGURE 5. tGPI-mucin and LPS cross-tolerization effect on cytokine and NO synthesis by C57BL/6-derived inflammatory macrophages. IFN-γ-primed inflammatory macrophages were pretreated with tGPI-mucin (10 nM), LPS (50 ng/ml), or medium alone. Twenty hours after pretreatment, the cells were washed, the medium replaced, and macrophages cultured for additional 48 h, in the absence (□) or presence of tGPI-mucin (10 nM; ▨) or LPS (50 ng/ml; ■). The levels of TNF-α, IL-12(p70), and nitrite were measured at 18, 48, and 48 h after the second stimulation, respectively.

been shown to be mutated in C3H/HeJ mice, which are low responders to LPS (49). Therefore, we assessed the tGPI-mucin ability to induce MAPK activation in macrophages from C3H/HeJ mice. As shown in our previous studies (11, 12, 16) and in Fig. 6*A*, high levels of TNF-α, IL-12, and NO are produced by macrophages from C3H/HeJ mice exposed to tGPI-mucin or tGPI, but not to LPS. C3H/HeJ macrophages then were treated with LPS or tGPI-mucin for 15 or 30 min, and cell lysates were tested for ERK-1/ERK-2, SKK-1/MKK-4, SAPK-2/p38, and IκB activation by measuring their respective phosphorylation. As expected, LPS did not induce any MAPK or IκB phosphorylation in these cells. However, the absence of functional TLR4 receptor did not affect the tGPI-mucin-induced MAPK and IκB phosphorylation (Fig. 6*B*).

Discussion

To better understand the host-parasite relationship and the disease outcome during infection with *T. cruzi*, our studies have focused on the identification and characterization of the chemical nature of the protozoan products involved in triggering proinflammatory cytokines by macrophages. As reported previously for other parasitic protozoa such as *T. brucei* and *P. falciparum* (17–23), our studies indicate that GPI anchors are the main component from trypomastigote forms of *T. cruzi* parasites capable of inducing the synthesis of cytokines by murine inflammatory macrophages (11–16). To induce cytokine synthesis, the optimal concentration of tGPI-mucin is 1.0 pmol/2 × 10^5 macrophages, being equivalent to 1–10 parasites per macrophage and thus considered highly physiologic

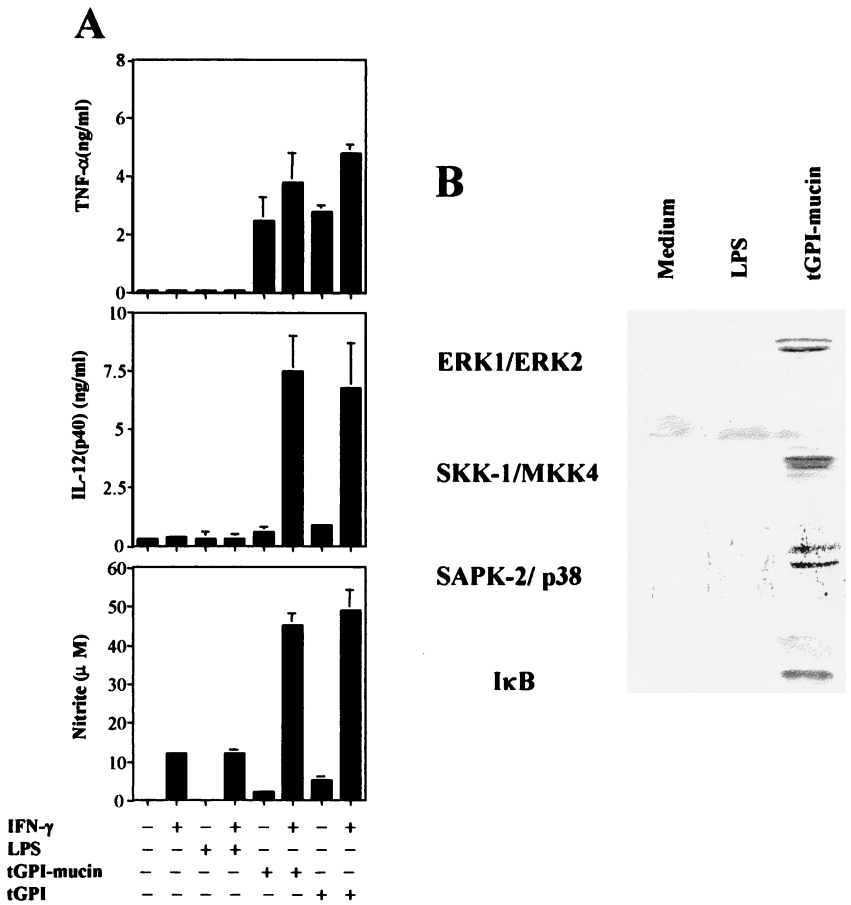

FIGURE 6. MAPKs and I-κB phosphorylation induced by tGPI-mucin but not by LPS in inflammatory macrophages from C3H/HeJ mice, which are hyporesponsive to LPS. *A*, TNF-α (*top*), IL-12(p40) (*middle*), and NO (*bottom*) production by unprimed or IFN-γ-primed C3H/HeJ-derived inflammatory macrophages stimulated with either LPS, tGPI, or tGPI-mucin. *B*, Macrophages from C3H/HeJ mice were stimulated with either tGPI-mucin or LPS and harvested at 15 or 30 min later. Cell lysates were resolved by 10% acrylamide SDS-PAGE followed by immunoblotting using a set of Abs that recognize specifically each of the different phosphorylated MAPKs.

(11, 12, 16). Furthermore, we have consistently shown that live *T. cruzi* trypomastigotes and tGPI-mucins have similar activity on murine inflammatory macrophages (11, 12, 14, 15). On basis of tGPI fragmentation as well as comparative study of over 12 different GPI anchors with defined structure, we favor the hypothesis that a longer glycan core and the presence of unsaturated fatty acids in the sn-2 position may be essential for the extreme potency of the trypomastigote GPIs in triggering NO and cytokine synthesis by macrophages (11, 12, 16).

To use a more defined system to investigate the signaling pathways involved in cytokine synthesis by macrophage exposed to *T. cruzi*, we tested the ability of tGPI-mucin or highly purified tGPI to trigger phosphorylation of different MAPKs, IκB, and the involvement of these pathways on cytokine synthesis. In the present study, we demonstrated that tGPI-mucin or tGPI are capable of triggering phosphorylation of ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38, as well as IκB in mouse peritoneal macrophages. As tGPI-mucin (or tGPI) induced the same pattern of cytokine release as LPS in murine macrophages, we compared the effect of LPS or tGPI-mucin/tGPI on the kinetics of MAPK and IκB phosphorylation in these cells. The phosphorylation of different MAPKs was similar when murine macrophages were exposed to distinct microbial glycolipids. By using specific inhibitors, we investigated the contribution of ERK-1/ERK-2 and SAPK-2/p38 in the cytokine and NO synthesis induced in macrophages stimulated by bacterial or protozoan glycolipids. Taken together, our results suggest that SAPK-2/p38 and to a lesser extent the ERK-1/ERK-2 pathways are involved in the synthesis of TNF-α by stimulated macrophages. These conclusions are in agreement with published data in another system (50–53). Simultaneous inhibition of ERK-1/ERK-2 and SAPK-2/p38 resulted in 75% inhibition of TNF-α release by macrophages exposed to tGPI-mucin or tGPI. Our experiments with SB 203580 and PD 98059 also support the findings that CREB is a main physiological substrate of ERK-1/ERK-2 and SAPK/p38 (54) and the hypothesis that this transcription factor may be an important element controlling TNF-α synthesis by macrophages exposed to microbial glycolipids (55).

In our experiments, IL-12 production was only slightly sensitive to the SAPK-2/p38 inhibitor, suggesting a minor positive regulatory role of this MAPK on IL-12 synthesis stimulated by microbial glycolipids. In contrast, Lu et al. (56) have shown a defective production of the IL-12 in mitogen-activated MKK3 (specific upstream MAPK for SAPK-2/p38)-deficient mice. Thus, it is possible that MKK3 may also activate an unknown SB-insensitive pathway, which is also responsible for induction of IL-12 synthesis. In contrast, we found a stimulatory effect of PD 98059 on IL-12 production by macrophages exposed to tGPI-mucin, tGPI, or LPS, suggesting that the IL-12 synthesis is negatively regulated by the ERK-1/ERK-2 pathway. In fact, Feng et al. (43) have suggested that *Leishmania* may suppress resistance to infection by switching on the ERK-1/ERK-2-mediated negative regulation of IL-12 production, hence preventing generation of a protective Th1 immune response.

The specific role of different MAPKs on iNOS induction and NO production have produced contrasting results. Da Silva et al. have shown that SAPK-2/p38 is necessary but not sufficient for iNOS induction by TNF-α and IL-1-α stimulation (57). ERK-1/ERK-2 were shown to be necessary in the iNOS regulation by IL-1β and IFN-γ (58), but had no effect on LPS/IFN-γ induction of the enzyme (59). In glial cells, the induction of iNOS expression and NO synthesis by IFN-γ and LPS was partially blocked by inhibiting ERK-1/ERK-2 and SAPK-2/p38 with PD98059 or SB203580, respectively, and almost completely blocked in the presence of both inhibitors (60). However, in the present paper, no effect on NO production was observed by using specific antagonistic drugs of ERK-1/ERK-2 or SAPK-2/p38 phosphorylation with macrophages costimulated either with tGPI, tGPI-mucin, or LPS and IFN-γ. Our findings are in agreement with previous studies showing that the iNOS induction by LPS in macrophages is unaffected by PD98059 and/or SB203580 (41, 42).Thus, the protein kinase that is rate-limiting for iNOS transcription appear to vary from cell to cell and/or according to the stimuli used in the different studies.

The crucial role of NF-κB in cytokine induction was established by using the peptide SN50, which inhibits the translocation of NF-κB. An inhibition of ~70% of the TNF-α production was observed when tGPI-mucin or LPS-stimulated macrophages were pretreated with 18 μM of SN50. These findings are in agreement with early studies showing that NF-κB is an important transcription factor required for maximal induction of TNF-α synthesis (61). In contrast, we found that SN50 has only minor or no effect on induction of NO or IL-12(p40) synthesis by IFN-γ-primed macrophages exposed to the different microbial stimuli. A half site for NF-κB has been identified and described in the IL-12(p40) promoter (62); however, the role of NF-κB on induction of IL-12 is poorly understood. Consistent with our findings, Feng et al. (43) concluded that NF-κB binding may not be necessary or sufficient for induction of iNOs but rather reinforces the idea that IFN regulatory factor complex may be the major regulatory factor. Altogether, the results presented here suggest that in our system, NF-κB plays a major role in induction of TNF-α, but not IL-12 or iNOS expression.

The recognition system for the stimulatory GPI-mucin appears to share much in common with the recognition system for LPS. LPS and tGPI or tGPI-mucin trigger the same pattern of phosphorylation of different members of the MAPK family. In addition, similar IC_{50} values of inhibitors specific for different MAPKs and NF-κB were necessary to inhibit different functions (i.e., cytokine) in macrophages exposed to either LPS, tGPI-mucin, or tGPI. Furthermore, our study demonstrates that pretreatment of mouse macrophages with either LPS or tGPI-mucin effectively induced a state of cross-tolerance as evidenced by significantly lower TNF-α and IL-12 release in response to each one of these stimuli. The finding of cross tolerance may also indicate the similarity of the receptors triggered by LPS and tGPI-mucin in inflammatory macrophages (45).

Studies have demonstrated the importance of members of the TLR family in the macrophage response to bacterial glycolipids (46–49). The role for TLR4 in LPS-induced activation in macrophages is supported by the demonstration that a mutation in the gene for TLR4 is associated with LPS hyporesponsiveness in the C3H/HeJ (49). Indeed, the results presented here with macrophages from C3H/HeJ mice demonstrate that LPS-induced phosphorylation of ERK-1/ERK-2, SKK-1/MKK-4, and SAPK-2/p38 is dependent on functional TLR-4. Interestingly, phosphorylation of MAPKs and IκB was still observed in macrophages from C3H/HeJ mice exposed to tGPI-mucin or tGPI. Thus, our results indicate that although functionally similar, the receptors triggered by LPS and tGPI are different. Considering that various members of TLR have been cloned at the moment, we speculate that *T. cruzi*-derived GPI anchors may in fact engage a distinct member of the TLR family, the nature of which is being investigated in our laboratories.

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