MAPK p38 antagonism as a novel method of inhibiting lymphoid immune suppression in polymicrobial sepsis

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Song, Grace Y., Chun-Shiang Chung, Irshad H. Chaudry, and Alfred Ayala. MAPK p38 antagonism as a novel method of inhibiting lymphoid immune suppression in polymicrobial sepsis. Am J Physiol Cell Physiol 281: C662–C669, 2001.—Although studies indicate that a shift from a Th1 to a Th2 response contributes to a marked suppression of cell-mediated immunity during sepsis, the mechanism by which this occurs remains unknown. Given that the mitogen-activated protein kinase (MAPK) p38 plays a critical role in the activation and function of immune cells, the aim of this study was to determine the contribution of MAPK p38 activation to the immune dysfunction seen in polymicrobial sepsis. To study this, polymicrobial sepsis was induced in C3H/HeN male mice by cecal ligation and puncture (CLP). Splenic lymphocytes and purified T cells were harvested 24 h post-CLP, pretreated with the specific MAPK p38 inhibitor SB-203580, and then stimulated with a monoclonal antibody against the T cell marker CD3. The results indicate that interleukin (IL)-2 release is markedly depressed while the release of the immunosuppressive mediator, IL-10, as well as mRNA levels of IL-10 and IL-4, are augmented after CLP. Inhibition of MAPK p38 suppressed in vitro IL-10 levels as well as IL-10 and IL-4 gene expression while restoring the release of IL-2. To determine whether these in vitro findings could be translated to an in vivo setting, mice were given 100 mg of SB-203580/kg body wt or saline vehicle (intraperitoneal) at 12 h post-CLP. Examination of ex vivo lymphocyte responsiveness indicated that, as with the in vitro finding, septic mouse Th1 responsiveness was restored. In light of our recent finding that delayed in vivo SB-203580 treatment also improved survival after CLP, we believe that these results not only illustrate the role of MAPK p38 in the induction of immunosuppressive agents in sepsis but demonstrate that SB-203580 administration after the initial proinflammatory state of sepsis significantly prevents the morbidity from sepsis.

T lymphocytes; Th1; Th2; splenocytes; mitogen-activated protein kinase p38; cecal ligation and puncture; mouse

DESPITE AGGRESSIVE operative interventions, antibiotic therapies, and antibodies against tumor necrosis factor (TNF) or endotoxin, sepsis and subsequent multiple organ failure remains the leading cause of morbidity and mortality in intensive care units. In light of this, it is even more critical to understand the precise mechanisms that underlie the development of sepsis. Studies have shown that after trauma and/or the onset of sepsis, there is a marked suppression of cell-mediated immunity that contributes to increased morbidity and mortality seen under those conditions (8, 24). Recent studies by our laboratory have indicated that there is a shift from a Th1 to a Th2 response after the initiation of polymicrobial sepsis, which contributes to this immune suppression; however, the mechanism underlying the development of this immune dysfunction in sepsis remains unknown (1, 12, 28, 33). In this regard, studies have shown that the mitogen-activated protein kinase (MAPK) p38 is critically involved in the activation and function of immune cells (17, 23).

MAPK p38 was originally described in monocytes as a signaling pathway involved in the production of proinflammatory cytokines such as TNF-α and interleukin (IL)-1β (22). Studies using the specific inhibitor SB-203580 have indicated that blockade of MAPK p38 in monocytes suppresses TNF and IL-1 release and improves survival after lipopolysaccharide (LPS)-induced endotoxic shock (6, 15). A recent study by our laboratory has further demonstrated that inhibition of MAPK p38 also improves survival in a model of polymicrobial sepsis (35). However, while studies have suggested a role for the MAPK p38 pathway in the regulation of T cell responsiveness (20, 29, 30, 37), the precise role of this pathway in the regulation of Th1 and Th2 lymphocyte responses in sepsis remains unknown. The aim of this study, therefore, was to determine whether MAPK p38 activation plays a role in the suppression of cell-mediated immunity after the onset of polymicrobial sepsis.

MATERIALS AND METHODS

Animals. Male inbred C3H/HeN (Charles River Laboratories, Wilmington, MA) mice 6–7 wks old and weighing 18–21 g were used in all experiments. Mice were acclimatized for 1 wk after their arrival at the Rhode Island Hospital Central Research Laboratory’s animal facilities and provided food and water ad libitum. The studies performed were carried out in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
out in accordance with the National Institutes of Health guidelines on the use of laboratory animals and were approved by the Rhode Island Hospital committee on animal use and care.

**Cecal ligation and puncture.** Polymicrobial sepsis was produced according to the method of Baker et al. (7) as previously described by Ayala et al. (5). In brief, mice were lightly anesthetized using Metofane (methoxyflurane; Pitman-Moore, Mundelein, IL). After laparotomy, the latter third of the cecum was ligated and punctured twice with a 22-gauge needle, and a small amount of the bowel contents was extruded through the punctured holes. The midline incision was then closed in layers with 6-0 Ethilon sutures (Ethicon, Somerville, NJ), and the animals were resuscitated with saline (40 ml/kg body wt) subcutaneously. Sham controls underwent the same surgical procedures, i.e., laparotomy and resuscitation, but the cecum was neither ligated nor punctured. Previously, it was demonstrated that blood cultures taken from mice are positive for both gram-negative (e.g., *Bacteroides fragilis*, *Escherichia coli*, *Klebsiella*, and *Pseudomonas mirabilis*) and gram-positive (e.g., *Streptococcus bovis*) bacteria as little as 1 h after cecal ligation and puncture (CLP) of the polymicrobial nature of this septic model (7).

**Cell preparations.** Mice were killed by overanesthetizing them with Metofane at 24 h after CLP or sham CLP operations. Splenocytes were isolated aseptically from the spleens of these animals according to the methods previously outlined by Meldrum et al. (26). After isolation, splenocytes were washed twice and counted, and their viability was assessed before suspension in RPMI 1640 medium (GIBCO BRL, Grand Island, NY). Cells taken from all animals were typically >95% viable by trypan blue exclusion. Splenocytes were depleted of macrophages by adherence to plastic. Nonadherent splenic lymphocytes were washed and then re-suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum to a final concentration of 2 × 10^6 cells/ml (GIBCO BRL). To determine the MAPK p38 activity and activation in purified T cells, macrophage-depleted splenic lymphocytes were further depleted of their B cell population by mixing 3 × 10^6 cells with 4 × 10^7 rat anti-mouse B220-coated dynabeads (Dynal, Great Neck, NY) according to the method previously described by Ayala et al. (4).

**Lymphocyte activation.** Purified T cells were preincubated for 30 min with either 10 μM of the MAPK p38 inhibitor SB-203580 or DMSO vehicle (Calbiochem, San Diego, CA) and subsequently transferred to polystyrene tissue culture dishes coated with monoclonal antibody to the T cell marker CD3 [plates were precoated with 15 μg anti-CD3/ml (clone 145-2C11, Pharmingen) for 1 h]. Cells and supernatants were harvested after 4 or 24 h of stimulation with anti-CD3 monoclonal antibody. SB-203580 had no significant effect on cell viability compared with controls as assessed by trypan blue exclusion.

**Cytokine determination.** The release of cytokines by cells stimulated for 24 h with anti-CD3 into supernatants was determined by the sandwich ELISA technique as described by us previously with antibodies supplied by Pharmingen [IL-10, clone JES-5; interferon (IFN)-γ, clone R4-6A2; IL-2, clone JES6-1A12] (1). The optical density of 405 nm for each well was determined on a CERES UV900C microplate reader (Bio-Tek), and the concentration of IL-10, IFN-γ, or IL-2 in the supernatant samples was determined by interpolation against a standard curve produced with murine recombinant IL-10, IFN-γ, or IL-2, respectively (Pharmingen).

**Analysis of gene expression.** mRNA was prepared as previously described by Chung et al. (10) from splenic lymphocytes derived 24 h after CLP or sham protocol, which were then treated in vitro with or without SB-203580 and stimulated for 24 h more with anti-CD3. Multiprobe synthesis and ribonuclease protection assay for IL-10 and IL-4 gene expression in the extracted lymphocyte mRNA was then performed as previously outlined by Chung et al. (10). In brief, 32P-labeled cRNA multiprobe was synthesized by using a cDNA template for the mouse mCK-1 set from the RiboQuant RPA kit (Pharmingen). A standard curve plotted with the undigested probe markers was used to identify the bands corresponding to IL-10 and IL-4 genes in the experimental samples. Quantitation of mRNA was performed by densitometric scanning of the autoradiogram (MOCHA image analysis/SigmaGel work station; Jandel Scientific, Corte Madera, CA). To quantify the individual cytokine mRNA levels, two housekeeping gene (L32 and glyceraldehyde-3-phosphate dehydrogenase) transcripts were also included, and the sample values were corrected (indexed; % of housekeeping gene) to the extent of the housekeeping gene expression.

**Immunoblottting.** Cell lysates of lymphocytes from 24-h cultures (prepared as described for 4 or 24 h with or without anti-CD3 in vitro, were prepared according to methods previously described in our laboratory (36). The protein concentration of each cell lysate was determined using a Nano-Orange detection assay (Molecular Probes, Eugene, OR). Western immunoblotting was performed with antibody specific to the dually phosphorylated form of MAPK p38 (New England Biolabs, Beverly, MA) (36). To determine basal expression of MAPK p38 in the samples, membranes were stripped and reprobed with antibody against total MAPK p38 (New England Biolabs). In a separate set of animals, the time course of MAPK p38 activation was determined by isolating splenic T cells 24 h after sham or CLP insult and stimulating the cells in vitro for 2, 4, 8, or 24 h with anti-CD3 as previously described. To determine the effect of SB-203580 on cyclooxygenase-2 (COX-2) expression, splenic T cells were again harvested 24 h after sham or CLP insult, and the cells were stimulated for 4 h with anti-CD3. Immunoblotting was then performed with antibody specific to COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Immunoprecipitation and kinase assay.** To determine the activity of MAPK p38 and the inhibition of MAPK p38 by SB-203580 in lymphocyte samples, an in vitro kinase assay (New England Biolabs) was used according to the manufacturer’s instructions. Cell lysates containing 200 μg of total protein were incubated with MAPK p38 antibody conjugated to protein A-Sepharose beads (Pharmacia, Piscataway, NJ). The beads were then pelleted, and the precipitated kinase was incubated with activating transcription factor (ATF)-2 fusion protein and ATP for 30 min at 30°C. After the reaction was terminated by being boiled in SDS sample buffer, samples were centrifuged and the pellets discarded. Sample (20 μl) was separated by electrophoresis and transferred onto nitrocellulose. Membranes were probed with antibody specific to phosphorylated ATF-2, and the resultant signal was detected by chemiluminescence.

**Densitometric analysis.** Molecular weight (kilodaltons) of the protein of interest was determined by comparison with low-molecular-weight standards included in each gel. To quantify the differences present in various samples, the band intensities were assessed densitometrically by using a Mocha image analysis/SigmaGel work station.

**Assessment of the effect of SB-203580 on immune function: in vivo administration of SB-203580.** To determine the effect of in vivo MAPK p38 inhibition on the septic host’s immune responsiveness, male C3H/HeN mice were randomly assigned into one of three groups (n = 5–6 animals/group). The
first group was subjected to sham CLP. The second group received 100 mg of SB-203580/kg body wt intraperitoneally at 12 h post-CLP. To deliver the MAPK p38 inhibitor in vivo, SB-203580 was first solubilized in DMSO at a stock concentration of 100 mg/ml and was then further diluted in warm saline plus 0.03 N HCl at a final concentration of 1 mg/0.2 ml. One hundred milligrams of SB-203580/kg body wt were then administered at 12 h post-CLP (this was calculated to be ~2 mg of SB-203580 for each 20-g animal). The third group received vehicle alone at 12 h post-CLP. The in vivo dosage of SB-203580 was chosen on the basis of an in vivo experiment by Badger et al. (6) in which this dose was shown to markedly increase survival in endotoxemic mice. We chose to administer SB-203580 at 12 h post-CLP because we have recently shown that treatment of animals with this agent at 12 h but not 0 h post-CLP had a marked salutary effect on septic mouse survival (35). No significant toxicity was observed with in vivo SB-203580 treatment. Splenic lymphocytes were then harvested 24 h post-CLP and stimulated for 24 h with anti-CD3 to assess cytokine release.

Presentation of data and statistical analysis. The data are presented as means ± SE of the mean for each group. Differences in the experimental means were considered significant if P < 0.05 as determined by Mann-Whitney's U-test.

RESULTS

The capacity to activate MAPK p38 in vitro was increased after CLP. In our preliminary studies, we observed that the activation of MAPK p38 in T cells from septic mice appeared to be maximal at 4 h of anti-CD3 stimulation with sustained expression through 24 h (Fig. 1A). In contrast, sham T cells exhibited only very low levels of activated MAPK p38 from 2 to 24 h, compared with CLP (Fig. 1A). Thus in our subsequent studies, we restricted our assessment of MAPK p38 activation to the 4-h poststimulation time point. Repeated densitometric assessment of 4 h of anti-CD3 stimulation in vitro indicated that the levels of activated (phosphorylated) MAPK p38 were significantly higher in septic mouse T cells compared with shams (Fig. 1B). Compartively, 4 h of stimulation produced no difference in the expression of MAPK p38 protein in splenic T cells harvested from septic and sham C3H/HeN animals (Fig. 1C). To further confirm the contribution of T cells to the activation of MAPK p38, we stimulated purified T cells with 2.5 µg/ml of the T cell mitogen concanavalin A. Our results indicated that MAPK p38 activation occurred in a similar fashion in response to these stimulants compared with anti-CD3 stimulation (data not shown). No activated MAPK p38 was detectable in the absence of either anti-CD3 or concanavalin A stimulation.

MAPK p38 phosphorylation of ATF-2 was increased in lymphocytes from septic animals. To the extent that increase in phosphorylated MAPK p38 detected in these cells represented a functional increase in MAPK p38 activity, lymphocyte samples were further assessed by examining the capacity of the cell lysates to phosphorylate the MAPK p38 substrate, ATF-2, using an in vitro kinase assay. Our results indicated that there was a significant increase in MAPK p38 activity in splenocytes after the induction of sepsis (Fig. 2). Furthermore, pretreatment of lymphocytes with the MAPK p38 inhibitor SB-203580 markedly suppressed the phosphorylation of ATF-2.

In vitro MAPK p38 inhibition restored the ability of lymphocytes from septic animals to release the Th1 cytokine IL-2. Anti-CD3 in vitro-stimulated splenileymphocytes, derived from 24-h post-CLP mice, showed a marked suppression in the ability to release the Th1 cytokines, IL-2 and IFN-γ, while releasing markedly higher levels of the Th2 cytokine, IL-10, compared with shams (Fig. 3). Pretreatment with a 10-µM concentration of the MAPK p38 inhibitor SB-203580 restored the ability of lymphocytes to release IL-2 to sham levels. There were no changes in the absence of stimulation (data not shown).

In vitro MAPK p38 inhibition suppressed release of the Th2 cytokine IL-10 by lymphocytes from septic an-
imals and also suppressed the gene expression of both IL-10 and IL-4. Cells harvested 24 h after CLP exhibited a marked augmentation in their in vitro capacity to express mRNA for both IL-10 and IL-4 in response to stimulation with anti-CD3 (Fig. 4). Pretreatment of cells with SB-203580 prevented the increase in IL-10 and IL-4 gene expression in lymphocytes from septic animals.

**SB-203580 had no affect on the expression of COX-2 in splenic T cells.** In splenic lymphocytes obtained 24 h post-CLP, the expression of COX-2 was not significantly altered in in vitro-stimulated T cells that had been treated with SB-230580 (Fig. 5).

**In vivo MAPK p38 inhibition improved lymphocyte immune function.** In vivo treatment of the animals with SB-203580 restored the ex vivo ability of lymphocytes to produce the Th1 cytokine IL-2 (Fig. 6A), partially restored the ability to secrete IFN-γ (Fig. 6B), and also prevented the increased IL-10 release by splenic lymphocytes (Fig. 6C).

**DISCUSSION**

Previous studies by our laboratory have indicated that there is a marked suppression in cell-mediated immune responses after the induction of polymicrobial sepsis by CLP (1). This immune suppression is defined as an inability to respond to mitogenic or antigenic challenge either in vivo or in vitro (3, 18, 38). In this respect, we as well as others have demonstrated that there is a shift from a Th1 to a Th2 response that appears to contribute to this developing dysfunction (1, 12, 14, 27, 28). However, the underlying mechanism responsible for this remains unknown.

We hypothesized that this shift toward a Th2 response may be due in part to alterations in the signal transduction pathways involved in translating the nature of the stimulus perceived at the cell surface receptor to the initiation of gene transcription at the level of the nucleus. In this regard, our results indicate that the phosphorylation and activity of MAPK p38 increased in septic animal lymphocytes and T cells compared with shams. These changes correlated with a significant increase in IL-10 protein and IL-10 and IL-4 mRNA, as well as a suppression in the release of Th1 cytokines IL-2 and IFN-γ in these same cell populations. In vitro pretreatment with the MAPK p38 inhibitor (SB-203580) markedly suppressed IL-10 release as well as IL-10 and IL-4 gene expression while restoring the ability of these cells to release IL-2. IFN-γ release remained unchanged. With regard to IL-4, we unfortunately experienced difficulties in measuring IL-4 pro-
tein levels in mice of the C3H/HeN background used in these studies.

Although these data demonstrate the efficacy of MAPK p38 inhibition for restoring Th1 responsiveness in an in vitro setting, the effect of MAPK p38 inhibition in vivo remains unknown. In this respect, we have recently reported (35) that mice treated with SB-203580 either immediately post-CLP (0 h) or 12 h post-CLP (12 h) show divergent survival patterns. In that study, we chose those two time points to more closely determine the physiological significance of inhibiting MAPK p38 activation during the two stages of sepsis: 0-h time point represented the beginning of the proinflammatory stage of sepsis in this model (2, 5, 13, 16), and 12 h represented a time point after which much of the proinflammatory stage had occurred (1, 3). We found that although there was a trend toward improvement in survival with immediate treatment (from 30% survival in untreated animals to 56% survival at 10 days after CLP with immediate SB-203580 treatment), only delayed treatment (12 h) with the MAPK p38 inhibitor markedly increased survival rates (from 30 to 81% at 10 days after CLP) (35). These data suggested that the critical events mediated by MAPK p38 phosphorylation occur late, after the initial proinflammatory state. Therefore, to address the role of MAPK p38 inhibition in vivo on immune function, we chose to give SB-203580 only at the 12-h time point.

To the extent that MAPK p38 inhibition in vivo had an effect comparable to that seen in our in vitro studies, our results illustrate that in vivo treatment of

Fig. 4. Lymphocytes from septic mice express significantly higher levels of mRNA for the Th2 cytokines IL-10 (A) and IL-4 (B) compared with shams. In vitro pretreatment of cells with 10 μM of the MAPK p38 inhibitor SB-203580 markedly suppressed the increase in IL-10 and IL-4 gene expression seen in sepsis. Cells were isolated from mice 24 h post-CLP or sham CLP and stimulated for 24 h with anti-CD3 MAb. To quantify the individual cytokine mRNA levels, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript was also included, and the sample values were corrected (indexed; % of GAPDH) to the extent of the housekeeping gene expression. Insets: typical blots from 2 separate animal splenic T cells. Histograms: cumulative data from no less than 3–4 mice in each group. Results are given as means ± SE. *Significance by P < 0.05 with respect to sham control as determined by the Mann-Whitney’s U-test.

Fig. 5. Expression of cyclooxygenase (COX)-2 was not significantly altered in in vitro-stimulated T cells that were treated with SB-203580. Cells were isolated from mice 24 h post-CLP or sham CLP and stimulated for 4 h with anti-CD3 MAb.
direct effect on IFN-γ observed that although MAPK p38 inhibition had little may augment IL-2 release. In the present study, we indicated that in human T cells, SB-203580 treatment IL-2 release (25). In contrast, Koprak et al. (20) have indicated T cells, MAPK p38 inhibition appears to suppress IL-10 and IL-4 release. With regard to Th1 responses, activation contributes to increased (30) that demonstrates that, in murine splenic Th2 activation in the release of immune suppressive mediators confirm a recent study by Schafer and colleagues significance by *P < 0.05 with respect to sham control; significance by #P < 0.05 with respect to CLP control as determined by the Mann-Whitney's U-test; n = 5–6 animals/group.

septic mice with SB-203580 markedly suppressed the release of IL-10 by lymphocytes while restoring the ability to release IL-2 and partially restoring the ability to release IFN-γ.

These results illustrating the role of MAPK p38 activation in the immune suppression of macrophage-depleted lymphocyte cultures with SB-203580 had no effect on IFN-γ release suggest that the suppression of IFN-γ release observed after sepsis is not a direct result of MAPK p38 activation in lymphocytes. However, it was interesting to note that in vivo inhibition of MAPK p38 resulted in a partial restoration of IFN-γ release in these same cell populations. A possible explanation for this is that improved macrophage responsiveness may impact lymphocyte function. In this regard, results by our laboratory suggest that MAPK p38 inhibition increases LPS-inducible release of IL-12 by septic macrophages (35). Because IL-12 has been shown to stimulate the release of IFN-γ, primarily by activating STAT4, it is possible that the enhanced in vivo IL-12 release by local macrophages may have resulted in improved IFN-γ release in the ex vivo setting where the cells were stimulated.

MAPK p38 was originally identified as an enzyme regulating the production of proinflammatory cytokines (15). However, recent studies have also indicated a role for MAPK p38 in the induction of immune suppression (11, 31, 32). Kumar et al. (21) as well as others (19) have illustrated that MAPK p38 activity is necessary for human immunodeficiency virus (HIV)-1 gene expression and that inhibition of MAPK p38 may help prevent the immune suppression seen in HIV infection. It is also interesting to note that in these studies, the immune-suppressive effects required a sustained activation of MAPK p38, as opposed to short-duration changes in the activation state typically associated with proinflammatory cytokine stimulation. Our results likewise suggest an important role for the delayed and sustained activation of MAPK p38. In vitro studies examining the phosphorylation kinetics of MAPK p38 have indicated that the augmentation in T cells and macrophages (35) from septic animals was evident after 2 h of stimulation and was sustained up to 24 h of stimulation. Furthermore, only delayed treatment in vivo with the MAPK p38 inhibitor SB-203580 outside of the proinflammatory stage significantly improved immune responsiveness and increased survival (35). It should also be noted that this alteration in signaling through MAPK p38 was not apparent in vivo or ex vivo and was only seen in response to activating stimuli (unpublished data).

Recent findings indicate that the pyridinyl imidazole compounds, including SB-203580, may directly inhibit COX-2 expression (9). In view of this, it may be warranted to compare the present results with those obtained by treatment with ibuprofen or indomethacin following CLP. In this respect, our results indicate that in vitro SB-203580 treatment had no significant effect on COX-2 expression. Although these data imply that the use of this inhibitor did not alter COX-2 expression at the transcriptional/translational level, we cannot rule out the possibility that SB-203580 treatment might have directly or indirectly affected COX-2 enzymatic activity, and thus, PGE2 levels. However, along
In summary, these data document for the first time that MAPK p38 inhibition may not be as tightly limited by time point of treatment as are treatments that target specific mediators alone.

In summary, these data document for the first time that the MAPK p38 signaling pathway plays a significant role in the development of immune suppression seen in the septic host and that an inhibitor of MAPK p38 markedly improves survival rates when administered after the initial proinflammatory stage of sepsis. Therefore, the use of specific inhibitors to MAPK p38 may represent a novel therapeutic approach for the treatment of sepsis.

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