Cyclooxygenase 2-mediated suppression of macrophage interleukin-12 production after thermal injury

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Schwacha, Martin G., Chun-Shiang Chung, Alfred Ayala, Kirby I. Bland, and Irshad H. Chaudry. Cyclooxygenase 2-mediated suppression of macrophage interleukin-12 production after thermal injury. Am J Physiol Cell Physiol 282: C263–C270, 2002; 10.1152/ajpcell.000357.2001.—Macrophage (Mφ) prostaglandin (PGE2) production has been implicated in immunosuppression and increased susceptibility to sepsis after thermal injury. Deficient interleukin (IL)-12 production has also been implicated in these postburn complications. The present study examined the relationship between Mφ cyclooxygenase (COX)-2 activity and IL-12 production after thermal injury. C57BL/6 female mice were subjected to a 25% total body surface area full-thickness burn. Mφ were isolated 7 days later, or the mice were subjected to sepsis by cecal ligation and puncture (CLP). IL-12 production by Mφ from injured mice was suppressed by >50%, whereas COX-2 expression and PGE2 production were increased twofold. The COX-2 inhibitor NS-398 suppressed PGE2 production and normalized IL-12 production in the injury group, whereas it had no effect on IL-10 production. Injured mice subjected to CLP had lower IL-12 plasma levels compared with sham-treated mice subjected to CLP. NS-398 treatment prevented the suppression in plasma IL-12 levels in the injury group. Thus elevated Mφ COX-2 activity, independent of IL-10, suppresses Mφ IL-12 production after thermal injury and may play an important role in the observed immunosuppression under such conditions.

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infection (14, 29, 30). Although these studies have clearly demonstrated that IL-12 is critical for maintaining postburn immune function, it remains unclear by what mechanism(s) its production is deficient under such conditions. A number of studies showed that IL-12 production is inhibited by PGE_2 (25, 33, 51). On the basis of previous observations that PGE_2 is elevated after thermal injury (21, 31, 35, 54), the aim of our study was to determine the role of PGE_2 and COX-2 expression in the regulation of macrophage IL-12 production after thermal injury.

**MATERIALS AND METHODS**

**Animals.** C57BL/6 female mice (18-20 g, 8- to 10-wk old; Charles River) were used for all experiments. The mice were allowed to acclimatize to the animal facility for at least 1 wk before experimentation. Animals were randomly assigned into either a thermal injury group or a sham treatment group. Five to seven mice per group were used for each experimental condition. The experiments in this article were performed in accordance with the National Institutes of Health guidelines for the care and handling of laboratory animals.

**Thermal injury procedure.** Mice received a third-degree scald burn as previously described (37, 39). Briefly, the mice were anesthetized by methoxyflurane inhalant, and the dorsal surface was shaved. The animal was placed in a custom insulated mold that exposed 12.5% of the total body surface area (TBSA) along the dorsum and immersed in 70°C water for 7 s. The procedure was repeated on the opposite side of the dorsum, yielding a 25% TBSA burn. The mice were then resuscitated with 2 mL of Ringer lactate administered via intraperitoneal injection and returned to their cages. The cages were placed on a heating pad (set on medium) for 2 h until the mice were fully awake, at which time they were returned to the animal facility. Sham treatment consisted of resuscitation with Ringer lactate only. Lethality in this thermal injury model was not significant.

**Cecal ligation and puncture procedure.** Polymicrobial sepsis was induced at 7 days after thermal injury or sham procedure with the model of cecal ligation and puncture (CLP) described by Baker et al. (4). Briefly, mice were lightly anesthetized with methoxyflurane and a 2.5-cm midline laparotomy was performed. The cecum was identified and ligated just below the ileocecal valve. The cecum was then punctured twice with a 22-gauge needle, a small amount of bowel contents was extruded through the puncture holes, and the cecum was returned to the peritoneal cavity. After application of xylocaine on the incision sites, the abdominal incision was closed in two layers using 6-0 Ethilon sutures and the cecum was returned to the peritoneal cavity. Sham treatment was performed without cecal ligation and puncture.

**Preparation of splenic macrophage cultures.** The mice were killed by methoxyflurane overdose at 7 days after injury, and the spleens were removed aseptically. Splenocyte suspensions were prepared in complete medium (RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 5 μg/mL gentamicin, and 100 μg/mL streptomycin and penicillin; Gibco BRL, Grand Island, NY) as described elsewhere at a concentration of 1 × 10^7 cells/mL (37, 38). Splenic macrophages were purified by adherence. Briefly, 1 × 10^7 splenocytes were added per well and allowed to adhere for 2 h. Nonadherent cells were removed by vigorous washing with warm PBS. More than 90% of the adherent cells displayed typical macrophage morphology. The adherent cells were cultured in a final volume of 500 μL/well containing 1 μg/mL lipopolysaccharide (LPS). Cell-free supernatants were collected at various times in culture and frozen at −80°C until analysis. In certain experiments the COX-2-specific inhibitor NS-398 (Cayman Chemical) was added to the cultures at a final concentration of 5 μM.

**Determination of IL-10, IL-12, and PGE_2 concentrations.** Immunoreactive IL-10 and IL-12 p70 in cell-free supernatants and plasma were determined by commercial sandwich ELISA according to the manufacturer’s recommendations (OptiEIA; BD Pharmingen, La Jolla, CA). PGE_2 levels in cell-free supernatants and plasma were determined using an enzyme-linked immunoassay (ELISA) kit according to the manufacturer’s recommendations (Cayman Chemical). PGE_2 in plasma was purified using a two-column extraction procedure under vacuum as previously described (12). In brief, 300 μL of plasma, acidified to pH 3.5 and containing 10 000 dpm of 3H-labeled PGE_2 (specific activity 200 Ci/mmol), was applied to a 100-μg Bond-Elut C18 column (Varian, Harbor City, CA) that was prepared by washing with methanol and acidified water (pH 3.5). A 500-μg Bond-Elut SI column (Varian) was prepared by washing with benzene-ethyl acetate (80:20). The C18 column, with the sample loaded, was washed consecutively with 2 mL of acidified water, 2 mL of 15% methanol, and 2 mL of petroleum ether. The C18-bound PGE_2 was eluted with 1 mL of ethyl acetate, and the eluate was collected in the SI column. The SI column was then washed with 1 mL each of benzene-ethyl acetate (80:20), benzene-ethyl acetate (40:60), benzene-ethyl acetate-methanol (40:60:2), and benzene-ethyl acetate-methanol (40:40:10). The PGE_2 was eluted with 3 mL of benzene-ethyl acetate-methanol (40:40:30) and dried under nitrogen. The samples were stored at −20°C until being assayed. Samples were reconstituted in EIA buffer, and PGE_2 concentrations were determined by EIA according to the manufacturer’s instructions. The PGE_2 concentrations obtained were corrected for percent recovery (~50% for all samples) from the SI column based on the disintegrations per minute of 3H[PGE_2 present in the eluted samples.

**Preparation of macrophage cellular lysates and electrophoresis and Western blot.** Macrophages were incubated with LPS (1 μg/mL) for 24 h. The adherent cells were removed with the plunger from a 1-mL syringe, pelleted by centrifugation, and washed three times with ice-cold PBS. Macrophage cell lysates were prepared by solubilizing the pellet in RIPA lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid, 2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 2.5 mM sodium orthovanadate, 1 μM protease inhibitor cocktail (Boehringer, Lewes, UK), PMSF, and 1 μg/mL sodium orthovanadate, 1 μM protease inhibitor cocktail (Boehringer, Lewes, UK), and 1 μg/mL sodium orthovanadate, 1 μM protease inhibitor cocktail (Boehringer, Lewes, UK).
The blots were exposed to manufacturer’s recommendations (Pierce Chemical, Rockford IL). ‘signal chemiluminescent substrate according to the manufacturer’s instructions. The membranes were probed for COX-2 by reacting with monoclonal anti-COX-2 (Transduction Laboratories, Lexington, KY) followed by reaction with anti-mouse IgG-horseradish peroxidase conjugate (Transduction Laboratories). Protein bands were detected by using Pierce Super IgG-horseradish peroxidase conjugate (Transduction Laboratories), reacting with monoclonal anti-COX-2 (Transduction Laboratories). COX-2 protein expression is shown in Fig. 1B. In the absence of stimulation COX-2 protein was not observed in cell lysates from either group, whereas LPS stimulation induced significant levels of COX-2 protein expression in both groups. Additionally, densitometry analysis of the resultant protein bands in all experiments (n = 3) determined that COX-2 expression was significantly higher (P < 0.05) in the injury group compared with sham-treated animals (Fig. 1C).

Thermal injury suppresses macrophage IL-12 production. LPS stimulation induced similar levels of IL-12 production after an incubation period of 1.5–6 h in the two groups (Fig. 2). After 12–24 h in culture, however, IL-12 levels continued to rise in the sham-treated group whereas no further increase was observed in the injury group and levels were significantly lower (P < 0.05) compared with the sham-treated group.

Statistical analysis. Data are expressed as means ± SE, and comparisons were analyzed with one-way ANOVA followed by Student’s t-test or Tukey’s test for multiple comparisons. A P value of <0.05 was considered to be statistically significant for all analyses.

RESULTS

Macrophage COX-2 activity and expression are increased after thermal injury. LPS stimulation induced significant PGE2 production by macrophages from both sham-treated and injured mice (Fig. 1A). Maximal levels of PGE2 in the supernatants were reached after 12 h in culture in the injury group. In contrast, PGE2 levels continued to rise in the sham-treated group until 48 h; however, they remained significantly lower (P < 0.05) than those of the injury group. A representative Western blot analysis (of 3 separate experiments) for COX-2 protein expression is shown in Fig. 1B. In the absence of stimulation COX-2 protein was not observed in cell lysates from either group, whereas LPS stimulation induced significant levels of COX-2 protein expression in both groups. Additionally, densitometry analysis of the resultant protein bands in all experiments (n = 3) determined that COX-2 expression was significantly higher (P < 0.05) in the injury group compared with sham-treated animals (Fig. 1C).

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Inhibition of COX-2 activity restores IL-12 productive capacity by macrophages from injured mice. The COX-2-specific inhibitor NS-398 was added to the macrophage cultures immediately before LPS stimulation, and PGE2 and IL-12 production was determined (Fig. 3). NS-398 completely inhibited PGE2 production in both the sham treatment and injury groups (Fig. 3A). Levels of PGE2 in supernatants from unstimulated and LPS-stimulated cells with NS-398 were not significantly different. Addition of NS-398 to cultures in the absence of LPS had no effect on PGE2 levels (data not shown). Analysis of the same macrophage cultures for
IL-12 concentrations showed that NS-398 prevented the suppression in IL-12 production in the injury group (Fig. 3B). NS-398 did not significantly alter IL-12 production in the sham-treated group. NS-398 also had no effect on IL-12 release in the absence of LPS in either group (data not shown).

COX-2-mediated suppression of macrophage IL-12 production is independent of IL-10. Previous studies showed that PGE2 is capable of upregulating IL-10 production (3, 10, 45). IL-10 has also been shown to suppress IL-12 production (2, 8). In light of these observations, the effect of COX-2 inhibition on macrophage IL-10 production was assessed. The results in Fig. 4 show that LPS-stimulated macrophages from injured mice produced significantly higher ($P < 0.05$) amounts of IL-10 than cells from sham-treated animals. Addition of NS-398 to the cultures did not significantly alter IL-10 production in either group. To verify whether PGE2 was capable of upregulating IL-10 release in our system, LPS-stimulated macrophages from sham-treated and injured mice were cultured with increasing concentrations of exogenous PGE2. Exogenous PGE2 caused a $50\%$ increase in IL-10 production in cells from the injury group (Table 1).

COX-2 inhibition in vivo alters systemic IL-12 levels after polymicrobial sepsis. The effect of COX-2 inhibition in vivo after the induction of polymicrobial sepsis by CLP was determined. Mice were subjected to CLP 7 days after sham treatment or burn injury, and plasma samples were collected 24 h thereafter. Plasma levels of PGE2 were significantly higher ($P < 0.05$) in the mice subjected to thermal injury and CLP (Fig. 5A). Treatment of the mice with NS-398 profoundly suppressed the systemic increase in PGE2 levels in both groups after CLP. Although CLP induced significant levels of IL-12 in the plasma of both sham-treated and injured mice (Fig. 5B), the plasma IL-12 levels in the sham-treated group were significantly greater ($P < 0.05$). Not only was the deficient IL-12 response in the injury group after CLP prevented by NS-398 treatment, but IL-12 plasma levels were significantly greater ($P < 0.05$) than those of sham-treated animals subjected to CLP and treated with NS-398. NS-398 treatment did not significantly alter IL-12 levels in sham-treated mice after CLP.

DISCUSSION

Recent evidence indicates that IL-12 production is deficient after thermal injury and suggests that this deficiency is a causative factor in the increased susceptibility to sepsis observed under such conditions (14, 29, 30, 49). Our findings are consistent with these observations, in that we observed suppressed macrophage IL-12 production after thermal injury as well as suppressed systemic levels after subsequent sepsis. The role of IL-12 in the induction of a cellular immune response is well documented (47). IL-12 is required for the development of a Th-1 type immune response and

Fig. 2. Interleukin (IL)-12 production by splenic MΦ from sham-treated and injured mice. Splenic MΦ were isolated from mice 7 days after injury and stimulated with LPS (1 μg/ml). IL-12 p70 concentrations in cell-free supernatants were determined by ELISA. Data are means ± SE for 3–5 mice/group. *$P < 0.05$ vs. sham.

Fig. 3. Effect of the COX-2-specific inhibitor NS-398 on PGE2 (A) and IL-12 (B) production by splenic MΦ from sham-treated and injured mice. Splenic MΦ were isolated from mice 7 days after injury and cultured for 48 h in the absence of stimulation with LPS (1 μg/ml) or with LPS and NS-398 (5 μM). PGE2 concentrations in cell-free supernatants were determined by EIA, and IL-12 concentrations were determined by ELISA. Data are means ± SE for 6–7 mice/group. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. LPS.
is a potent inducer of IFN-γ production (18, 41). Furthermore, IL-12 plays a crucial role in the development of immunity against a range of pathogens, and its neutralization increases susceptibility to such pathogens (7, 40, 48). With regard to thermal injury, the efficacy of IL-12 treatment after injury in reducing susceptibility to sepsis appears to be in part related to the induction of IFN-γ (29). Additionally, the promotion of a Th-1 immune response by IL-12 after sepsis is also associated with improved bacterial clearance and prolonged survival (44, 53, 55).

Our results suggest that thermal injury alters the macrophage phenotype to support an immunosuppressive Th-2 immune response (i.e., increased IL-10 and decreased IL-12 production). Other studies also support the concept that thermal injury induces a Th-2 response that negatively impacts resistance to subsequent infectious challenges (17, 22, 23, 30). The COX-2 product PGE2 has been suggested to be an important regulatory factor in inducing a Th-2 type response (1, 10, 20, 25, 33, 45, 51). The effect of PGE2 on T cell responses is opposite of that of IL-12, in that the production of Th-1 cytokines (IL-2, IFN-γ) is markedly more sensitive to inhibition by PGE2 than production of Th-2 cytokines (IL-4, IL-10) (6, 43, 50). Th-1 and Th-2 cytokines negatively cross-regulate each other's production, suggesting that inhibition of Th-1 responses by PGE2 can result in an immunosuppressive Th-2 response (32). Van der Pouw Kraan et al. (51) were the first to demonstrate that PGE2 inhibited IL-12 production. Our findings extend these observations by demonstrating that under a pathological condition (i.e., thermal injury) enhanced COX-2 activity is a causative factor for the observed deficiency in IL-12 production and, therefore, potentially the induction of an immunosuppressive Th-2 response.

Table 1. Effect of PGE2 on LPS-stimulated macrophage IL-10 production

<table>
<thead>
<tr>
<th>PGE2 Added to Culture</th>
<th>0 nM</th>
<th>0.1 nM (35 pg/ml)</th>
<th>1 nM (350 pg/ml)</th>
<th>10 nM (3,500 pg/ml)</th>
<th>100 nM (35,000 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>569 ± 17</td>
<td>554 ± 60</td>
<td>468 ± 24</td>
<td>639 ± 187</td>
<td>536 ± 63</td>
</tr>
<tr>
<td>Injury</td>
<td>978 ± 145*</td>
<td>1,005 ± 46*</td>
<td>1,368 ± 76*</td>
<td>1,528 ± 41†</td>
<td>1,345 ± 219*</td>
</tr>
</tbody>
</table>

Data are means ± SE of 3 animals/group. Macrophage cultures were stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 48 h in the presence of various concentrations of exogenous prostaglandin (PG)E2. Interleukin (IL)-10 levels in cell-free supernatants were determined as described in MATERIALS AND METHODS. *P < 0.05 compared with respective sham-operated group; †P < 0.05 compared with 0 nM PGE2.
Previous studies showed that PGE$_2$ can upregulate IL-10 production (3, 10, 45). The enhanced macrophage IL-10 observed after thermal injury, however, was independent of COX-2 activity, because inhibition of COX-2 with NS-398 had no effect on IL-10 productive capacity. In contrast, addition of PGE$_2$ to the cultures was capable of enhancing IL-10 production in the injury group. The apparent discrepancy between these two results is likely related to the kinetics of macrophage PGE$_2$ production after LPS stimulation. It can be speculated that high levels of PGE$_2$ at the time of LPS stimulation are necessary for an enhancing effect on IL-10 production to be observed. This concept is supported by the observation that LPS stimulation of macrophages did not induce stimulatory levels of PGE$_2$ in the supernatant (10 nM; 3,500 pg/ml) up to 24 h (Fig. 5). In contrast, when exogenous PGE$_2$ was added at the same time as LPS (Table 1), an enhancing effect on IL-10 production was observed. These in vitro observations, however, do not preclude the possibility that elevated systemic PGE$_2$ levels after thermal injury (21) might contribute to a subsequent enhancement of macrophage IL-10 productive capacity. Clearly, additional studies are warranted to precisely elucidate the relationship between PGE$_2$ and IL-10 after thermal injury.

The notion that Th-2 cytokines are responsible for the suppression of postburn IL-12 responses is supported by recent findings by Utsunomiya et al. (49). This study suggests that the suppressed IL-12 response observed after thermal injury is mediated by IL-4. In contrast, our findings here suggest that PGE$_2$, independent of Th-2 cytokines, is responsible for the suppression of IL-12 production. The difference between our findings and those of Utsunomiya and coworkers may be related to the fact that they induced an in vivo IL-12 response with LPS, whereas our study used CLP, which represents a polymicrobial insult. Moreover, the study by Utsunomiya et al. examined the IL-12 response 1–6 h after LPS administration, whereas we collected plasma samples at 24 h after CLP. The different intervals at which IL-12 responses were examined might also contribute to a different conclusion.

Studies by Mannick and coworkers (14, 29, 30) indicate that thermally injured mice display a suppressed IL-12 response to CLP that is associated with an increase in mortality. Administration of IL-12 under such conditions has been shown to restore resistance to sepsis. Our findings are consistent with those of Mannick and coworkers in that we observed a suppressed IL-12 response to CLP. We have extended their observations by demonstrating that the deficiency in IL-12 production is due to elevated COX-2 activity. Interestingly, we observed that COX-2 inhibition actually increased plasma IL-12 levels in the thermal injury group subjected to CLP above that of sham-treated animals subjected to CLP. In contrast, no such differences in the IL-12 response to LPS in vitro were observed. The likely reason(s) for the differences between our in vivo and in vitro findings are related to differences in the response of macrophages to in vitro LPS stimulation versus polymicrobial stimulation in vivo. In addition, it should be noted that the in vitro response represents a purified macrophage population from the spleen, whereas the in vivo response involves multiple cells from various tissues (i.e., spleen, liver, blood, peritoneal cavity, etc). Previous studies demonstrated that the in vivo immune responses to LPS and CLP differ markedly (9). These previous studies support our finding that the in vitro IL-12 response to LPS differed from the in vivo response to a polymicrobial challenge of CLP. Although recent findings by Strong et al. (46) demonstrated that in vivo inhibition of COX-2 activity after hemorrhage and traumatic injury markedly improved survival of a subsequent septic insult, it remains to be determined whether COX-2 inhibition after thermal injury would improve survival after a subsequent septic challenge to a similar extent.

In conclusion, our findings suggest that enhanced macrophage expression of COX-2 activity after thermal injury contributes to the suppression of IL-12 production. The suppressed IL-12 responsiveness leads to the expression of an immunosuppressive Th-2 phenotype that contributes to an increased susceptibility to subsequent septic complications. Thus elevated postburn COX-2 activity would appear to be an important causative factor in the observed increased morbidity and mortality under such conditions.

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REFERENCES


