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

MARTIN G. SCHWACHA, CHUN-SHIANG CHUNG, ALFRED AYALA, KIRBY I. BLAND, AND IRSHAD H. CHAUDRY

Center for Surgical Research, Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama 35294; and Division of Surgical Research, Department of Surgery, Brown University School of Medicine, Providence, Rhode Island 02903

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Address for reprint requests and other correspondence: M. G. Schwacha, Univ. of Alabama at Birmingham, Center for Surgical Research, G094 Volker Hall, 1670 Univ. Blvd., Birmingham, AL 35294-0019 (E-mail: Martin.Schwacha@ccc.uab.edu).

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.00357.2001.—Macrophage (Mφ) prostaglandin (PG)E2 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 injured 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 injured 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.

prostaglandin E2; interleukin-10; immunosuppression; sepsis; burns

IT IS WELL ESTABLISHED that thermal injury induces a profound suppression in cell-mediated immune responses in burn patients (5, 15) and experimental animal studies (11, 26, 39). Furthermore, thermal injury-induced immunosuppression correlates with the severity of the injury and susceptibility to subsequent sepsis and multiple organ failure (26, 34). An important aspect of the host response to thermal injury is the hyperproduction of eicosanoids that have profound immunomodulatory properties (15, 24, 36, 38, 54). A causal relationship between prostaglandins (PGs) and thermal injury-induced immune dysfunction is supported by the observations that 1) systemic prostaglandin levels are elevated after burn injury (21); 2) macrophages from injured animals produce elevated amounts of PGE2 on stimulation (31, 35, 54); and 3) inhibitors of cyclooxygenase (COX) can restore various aspects of immune function after thermal injury and can improve survival (15, 42).

PGs are generated by COX, an enzyme that has been identified in two isoforms: COX-1, the constitutive form, and COX-2, the inducible form (52). COX-1 is constitutively expressed in most tissues and is responsible for the regulation of a number of “housekeeping” functions including vascular homeostasis and gastroprotection (19, 28). Conversely, COX-2 expression is primarily expressed in monocytes, macrophages, neutrophils, and endothelial cells after induction by a number of mediators including cytokines and endotoxin at sites of inflammation and/or injury (13, 16, 19, 27). COX-2 generates high levels of PGs that mediate the inflammatory process, pain, and immunosuppression. Recent findings with specific COX-2 inhibitors demonstrated that this isoform of the enzyme is responsible for some of the deleterious consequences associated with thermal injury, as well as other forms of traumatic injury (42, 46).

Interleukin (IL)-12 is a heterodimeric cytokine produced primarily by antigen-presenting cells such as macrophages, monocytes, and dendritic cells. IL-12 has potent immunomodulatory properties that include stimulation of cytolytic activity in various immune cell populations and induction of interferon (IFN)-γ production by T and NK cells, which contributes to a Th-1 response that supports cell-mediated immunity (7). Studies also suggest that IL-12 acts as both a proinflammatory cytokine and an immunomodulator that bridges the innate and adaptive immune responses (47).

Recent studies showed that IL-12 production is deficient after thermal injury and that treatment of injured animals with recombinant murine IL-12 improves survival after subsequent sepsis and/or...
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 PGE2 (25, 33, 51). On the basis of previous observations that PGE2 is elevated after thermal injury (21, 31, 35, 54), the aim of our study was to determine the role of PGE2 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 to 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 approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham and 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 (Ethicon, Somerville, NJ). Normal saline solution (20 ml/kg body wt) was administered subcutaneously at that time. Animals were treated with either subcutaneous vehicle (17% ethanol) or subcutaneous NS-398 (10 mg/kg body wt; Cayman Chemical, Ann Arbor, MI) immediately after CLP and 12 h thereafter (42). Previous studies demonstrated that blood cultures taken from mice after CLP are positive for gram-positive (e.g., *Streptococcus bovis*) as well as gram-negative (e.g., *Bacteroides fragilis, Escherichia coli, Klebsiella, Proteus mirabilis*) organisms (4).

Twenty-four hours after the induction of CLP the mice were killed by methoxyflurane overdose. Whole blood was obtained by cardiac puncture and placed in Microtainer tubes (Becton Dickinson). The tubes were then centrifuged at 16,000 g for 15 min at 4°C. Plasma was transferred to microcentrifuge tubes and stored at −80°C until assayed for IL-12 and PGE2 levels as described in Determination of IL-10, IL-12, and PGE2 concentrations.

**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 and stored 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 PGE2 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). PGE2 levels in cell-free supernatants and plasma were determined using an enzyme-linked immunoassay (ELISA) kit according to the manufacturer’s recommendations (Cayman Chemical). PGE2 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 PGE2 (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 PGE2 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 methanol (60:40:2), and benzene-ethyl acetate-methanol (60:40:10). The PGE2 was eluted with 3 ml of benzene-ethyl acetate-methanol (60:40:30) and dried under nitrogen. The samples were stored at −20°C until being assayed. Samples were reconstituted in EIA buffer, and PGE2 concentrations were determined by EIA according to the manufacturer’s instructions. The PGE2 concentrations obtained were corrected for percent recovery (−50% for all samples) from the SI column based on the disintegrations per minute of 3H[PGE2 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× protease inhibitor cocktail (Boehringer, Lewes, UK),
and 100 mM tetrasodium pyrophosphate]. The lysate was vortexed, incubated on ice for 30 min, and centrifuged at 14,000 g for 10 min. The clear supernatant was collected and stored at −80°C. The protein content of the cell lysate was determined by Bio-Rad Dc protein assay (Bio-Rad, Hercules, CA).

Electrophoresis was performed on precast 4–10% Bis-Tris polyacrylamide gels in MOPS running buffer containing 10% SDS (Novex, San Diego, CA) for 60 min at 180 V as previously described (38). Each lane was loaded with 10 μg of protein. Western transfer to nitrocellulose membranes (Novex) was carried out for 60 min at 25 V, 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 SuperS IgG-horseradish peroxidase conjugate (Transduction Laboratories, Lexington, KY) followed by reaction with anti-mouse IgG-horseradish peroxidase conjugate (Transduction Laboratories). Protein bands were detected by using Pierce SuperSignal chemiluminescent substrate according to the manufacturer’s instructions (Pierce Chemical, Rockford IL). The blots were exposed to film for 10 min, and images were captured with a MOCHAII Image Analysis workstation (Jandel Scientific, San Rafael, CA). Densitometry analysis of the images was performed with ChemiImager 5500 (Alpha Inotech, San Leandro, CA). The intensity of the positive control band on each blot was arbitrarily set at 100 densitometric units.

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 the resultant protein bands from all experiments are 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.

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 an ~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...
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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