

# Protective Effects of Early Interleukin 10 Antagonism on Injury-Induced Immune Dysfunction

Ann Lyons, MB, BCh; Andreas Goebel, MD, PhD; John A. Mannick, MD; James A. Lederer, PhD

**Hypothesis:** Interleukin 10 (IL-10) plays a central role in the development of postinjury immune suppression, and early in vivo IL-10 antagonism can be protective.

**Design:** Male A/J mice underwent sham or burn injury and were treated with monoclonal anti-IL-10 antibody or control antibody at 1 day or 3 days after injury. Their ability to survive polymicrobial sepsis induced by the cecum ligation and puncture (CLP) technique was then tested. The response of sham- and burn-injured mice and burn-injured mice treated with anti-IL-10 to immunization with a T-cell-dependent antigen, trinitrophenyl (TNP)-haptened ovalbumin (TNP-OVA) was also assessed.

**Main Outcome Measures:** Mortality was monitored for a total of 7 days after CLP to assess the effect of anti-IL-10 therapy on the survival of burn-injured, immune-compromised mice. Serum antibody isotype formation was measured in sham- and burn-injured mice and burn-injured mice treated with anti-IL-10 to determine how IL-10 antagonism influenced helper T-cell responses in vivo. In vitro cytokine production by antigen-

stimulated spleen cells was assessed to study the effect of blocking IL-10 activity at 1 day vs 3 days after burn injury.

**Results:** Treating mice with anti-IL-10 at 1 day after injury significantly improved CLP survival, whereas delaying treatment 3 days had no beneficial effect. The analysis of T-cell function in vivo as determined by serum antibody isotype formation indicated that anti-IL-10 treatment at 1 day or 3 days after injury increased T helper cell 1-type antibody formation to sham injury levels by day 10. Moreover, these treatments restored the injury-induced reduction of antigen-stimulated IL-2, interferon  $\gamma$ , and IL-10 production.

**Conclusions:** Interleukin 10 plays an early role in the development of burn injury-induced immune suppression. Its in vivo inhibition at 1 day after injury may be a useful approach toward preventing the development of injury-induced immune dysfunction and may do so by restoring T-cell function and cytokine production.

*Arch Surg.* 1999;134:1317-1324

**S**EVERE INJURY caused by burns, multiple trauma, major surgery, and hemorrhage can markedly alter the immune status of the injured hosts, reducing their ability to combat opportunistic infections.<sup>1-6</sup> Decades of research involving injured patients and animal models have demonstrated that the development of postinjury complications involves dramatic changes in the balanced control of the cells and mediators of both the innate and adaptive immune system.<sup>7</sup>

Using a mouse model for burn injury and polymicrobial sepsis, it has been shown that the development of postinjury immune suppression coincides with an altered pattern of T-cell cytokine production.<sup>8,9</sup> In particular, mitogen-stimulated spleen cells harvested from burn-injured mice produce relatively

higher levels of the helper T cell type 2 ( $T_H2$ )-type cytokines, interleukin 4 (IL-4), and IL-10 than those from sham-injured mice.<sup>10</sup> This increase in  $T_H2$  cytokine production correlates directly with the increased susceptibility of burn-injured mice to polymicrobial sepsis induced by the cecum ligation and puncture (CLP) technique.<sup>8</sup> Given the anti-inflammatory activity of these cytokines and their established immune-suppressive activity, this observation suggested to us that  $T_H2$ -type cytokines may play a central role in inducing or maintaining injury-induced immune suppression.<sup>11,12</sup>

Recent work addressing the influence of burn injury on T-cell activation and function demonstrated that burn injury suppresses  $T_H1$  function in vivo.<sup>13</sup> This was shown using an immunization approach that allowed us to follow the develop-

From the Department of Surgery, Section of Immunology, Harvard Medical School, Brigham and Women's Hospital, Boston, Mass.

## MATERIALS AND METHODS

### MICE

Male A/J mice (The Jackson Laboratory, Bar Harbor, Me) were acclimated for at least 1 week before being used for experiments at 7 to 9 weeks of age. The mice were maintained in an accredited viral antibody-free animal facility in accordance with the guidelines of the National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research.<sup>9</sup>

### REAGENTS

Chicken ovalbumin fraction IV (OVA), rat IgG, and picrylsulfonic acid (2,4,6-trinitrobenzene sulfonic acid) were purchased from Sigma-Aldrich Corporation (St Louis, Mo). The trinitrophenyl (TNP)-haptenated OVA (TNP-OVA) was prepared for immunization by mixing an equal weight of picrylsulfonic acid with a 10-mg/mL solution of OVA made in 0.1-mol/L borate buffer (pH 9.0) for 4 hours at room temperature in the dark. This mixture was dialyzed extensively against phosphate-buffered saline solution (PBS; pH 7.2). Freund complete adjuvant (DIFCO Laboratories, Detroit, Mich) was mixed 1:1 with TNP-OVA to prepare an emulsion for immunization. Culture medium referred to as complete-5 was prepared for in vitro experiments using RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS), 1-mmol/L glutamine, 1-mmol/L sodium pyruvate, 100- $\mu$ mol/L nonessential amino acids, 10-mmol/L HEPES, a combination of penicillin and streptomycin, and  $2.5 \times 10^{-5}$ -mol/L  $\beta$ -mercaptoethanol (Life Technologies, Grand Island, NY). Anti-IL-10 antibody was purified from hybridoma (JES-2A5) supernatant by affinity chromatography using HiTrap protein G columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Its IL-10 binding activity was tested and standardized using enzyme-linked immunosorbent assays (ELISA).

### MOUSE BURN-INJURY MODEL

The mouse burn protocol was performed as described previously and as approved by the National Institutes of Health and the Harvard Medical School Standing Committee on Animal Research.<sup>9</sup> Before receiving sham or burn injury, mice were randomized into 4 groups and anesthetized using intraperitoneal (IP) injection with 60 mg/kg of pentobarbital sodium (Abbott Laboratories, Chicago, Ill). Once mice were fully anesthetized, the dorsa were shaved, and mice were placed in a plastic mold that exposes 25% of their total body surface area. The mice were then subjected to scald burn injury by exposure to 90°C water for 9 seconds. Sham-injured mice were treated in a fashion identical to burned mice except they were exposed to 25°C rather than 90°C water.

### CECUM LIGATION AND PUNCTURE

The CLP technique was used to induce polymicrobial sepsis in mice 10 days after sham or burn injury and was performed as described previously.<sup>9</sup> In brief, mice were anesthetized using IP injection with 60 mg/kg pentobarbital sodium. A midline incision was made on their shaved abdomen to expose the cecum. The cecum was ligated at its base using silk suture and then punctured through using a 25-gauge needle. The punctured and ligated cecum was reinserted into the peritoneal cavity, and the incision was closed using 4-0 nylon suture. All mice were then resuscitated with an IP injection of 1 mL of sterile isotonic sodium chloride solution. Mortality was monitored daily for a total of 7 days after CLP.

### IMMUNIZATION AND ANTI-IL-10 ANTIBODY TREATMENT

Mice were immunized subcutaneously in the anterior abdominal wall with 100  $\mu$ L of TNP-OVA (1 mg/mL) emulsified 1:1 in Freund complete adjuvant at the time of burn injury. Anti-IL-10 antibody or control rat IgG was

ment of T<sub>H</sub>1 and T<sub>H</sub>2 responses against a defined T-cell-dependent antigen. In addition to demonstrating that burn injury suppressed T<sub>H</sub>1 function as judged by the formation of antigen-specific T<sub>H</sub>1-type antibody isotype, IgG2a, this work also showed that treating mice with anti-IL-10 antibody at the time of injury and at 2 days after injury could correct the injury-induced loss of T<sub>H</sub>1 function in vivo.<sup>13</sup> This is an important issue, since T<sub>H</sub>1-type responses help to protect the injured host from development of postinjury infections. This occurs because T<sub>H</sub>1-type cytokines promote efficient cell-mediated immunity, and T<sub>H</sub>1-type antibodies are efficient at clearing microorganisms by activating efficiently the complement immune defense system. Given the correlation between the perturbed balance of T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production and the development of postinjury immune suppression, we wanted to test whether in vivo IL-10 antagonism could potentially prevent the development of injury-induced immune dysfunction. Hence, we examined the effect of blocking IL-10 function at early time

points after injury to determine if the increased susceptibility to infection could be prevented in burn-injured mice. We also used the antigen immunization approach to examine the influence of IL-10 antagonism on alterations in helper T-cell function in vivo and antigen-specific T-cell cytokine production in an attempt to correlate directly improved in vivo T-cell function with better survival of CLP-challenged, immune-compromised, burn-injured mice. Our findings suggest that IL-10 plays an early role in the development of postinjury immune suppression and that IL-10 antagonism may be another therapeutic approach to consider for correcting suppressed immunity against opportunistic infections after severe injury.

## RESULTS

Anti-IL-10 antibody treatment 1 day after burn injury helps protect mice from development of postinjury immune suppression. To determine if in vivo inhibition of IL-10 activity could prevent the development of burn in-

injected IP (0.250 mL of a 1-mg/mL solution) at 1 day or 3 days after injury, depending on the experiment.

#### SERUM TNP-SPECIFIC ANTIBODY ISOTYPE ELISA

The TNP-specific antibodies were assayed using an ELISA as described previously.<sup>13</sup> In brief, ELISA-grade microtiter plates (Nunc MaxiSorb; Nalge Nunc International Corporation, Tastrupgaardsvej, Denmark) were coated with 50  $\mu$ L of TNP-haptenated bovine serum albumin (BSA; 10  $\mu$ g/mL) prepared in a manner similar to TNP-OVA at 37°C for 1 hour. The plates were washed with wash buffer (PBS, pH 7.4, and 0.1% polysorbate 20 [Tween 20; ICI Surfactants, Wilmington, Del]), then blocked for at least 15 minutes with blocking buffer (PBS containing 1% BSA). Plates were then washed, and serial 1:3 dilutions of serum initially diluted 1:50 were added to TNP-BSA-coated ELISA plates. The samples were incubated at 4°C overnight, the plates were washed, and alkaline phosphatase-conjugated antibodies specific for murine IgM, IgG1, IgG2a, or IgE (Life Technologies) were added at 1  $\mu$ g/mL. After incubation at 37°C for 1 hour, the plates were washed thoroughly, and alkaline phosphatase substrate (Sigma-Aldrich Corporation) was added to detect the bound isotype-specific antibodies. The absorbance at 405 nm was determined with an ELISA plate reader (Molecular Devices Corporation, Sunnyvale, Calif) to detect the relative levels of TNP-specific antibody isotypes in the serum of TNP-OVA-immunized mice.

#### SERUM HARVEST AND IN VITRO SPLEEN CELL STIMULATIONS

Ten days after immunization, mice were killed using carbon dioxide asphyxiation, and blood samples were collected via cardiac puncture. Serum was harvested from the clotted blood samples by centrifugation at 700g for 10 minutes. All serum samples were stored at 4°C before being analyzed for TNP-specific antibody titers using ELISA. Spleens were removed using sterile technique, and single-cell suspensions were prepared by mincing them in complete-5 medium. After 2 washes by centrifugation, the spleen cells were counted, then

cultured at a density of  $5 \times 10^5$  cells per well in 96-well plates (Corning Costar Corporation, Cambridge, Mass) in the absence or presence of 30  $\mu$ g/mL of TNP-OVA. After a 48-hour incubation period at 37°C, culture supernatants were harvested for cytokine analyses using ELISA.

#### CYTOKINE ELISA

The ELISA plates were coated for 1 hour at 37°C with capture anti-cytokine antibodies (PharMingen, San Diego, Calif) diluted to 1  $\mu$ g/mL in carbonate-coating buffer. The plates were then washed in wash buffer, and nonspecific protein binding was prevented by incubating the plates at 37°C for 30 minutes in 100  $\mu$ L of blocking buffer. The plates were washed, then serial dilutions of cytokine standards (Genzyme, Cambridge, Mass) and the unknown samples were added to individual wells in triplicate. The plates were incubated at 37°C for 2 hours, after which they were washed, and biotinylated detection antibody (PharMingen) diluted in blocking buffer was added to the plate. After 1 hour, the plates were washed, a 1:2000 dilution of avidin-alkaline phosphatase conjugate (Sigma-Aldrich Corporation) was added, and the plates were incubated at room temperature for 30 minutes. After washing extensively, alkaline phosphatase activity was detected by adding substrate (Sigma-Aldrich Corporation) prepared in carbonate-coating buffer containing 1-mmol/L magnesium chloride. The ELISA plate reader set to read an absorbance wavelength at 405 nm was used to assess the alkaline phosphatase activity. The results were analyzed using the accompanying computer software program (SOFTmax PRO Version 1.1; Molecular Devices Corporation).

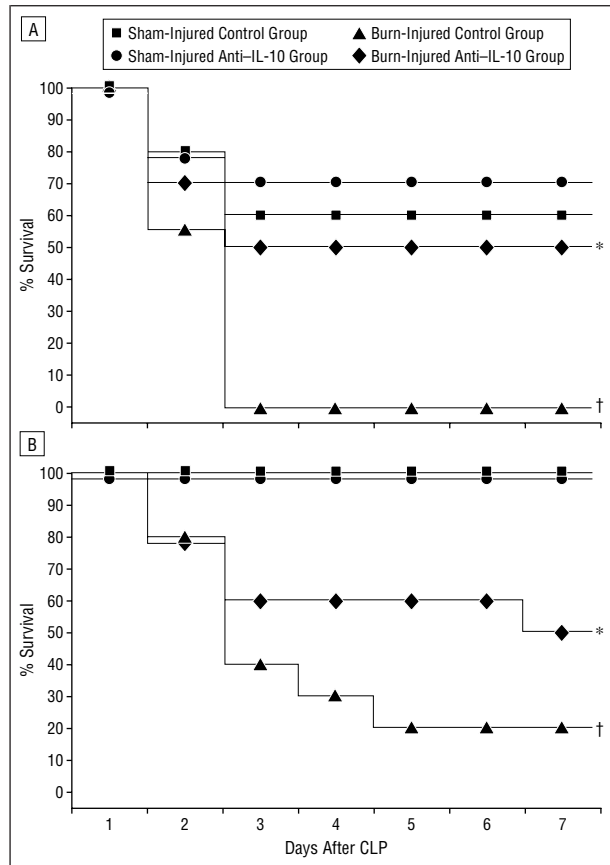
#### STATISTICAL ANALYSIS

Survival after CLP was compared using the Fisher exact test. Cytokine and antibody ELISA results were analyzed by analysis of variance and the Tukey multiple comparison test. Both analyses were performed with the InStat2 software program (Graphpad, San Diego). Values of  $P < .05$  were considered significant.

jury-induced immune suppression, we tested the effect of treating burn-injured mice with anti-IL-10 antibody on survival of CLP challenge at 10 days after injury. As shown in **Figure 1**, we observed that burn-injured mice displayed poor survival following CLP challenge at 10 days after injury. However, in 2 independent studies, we observed that treating mice with anti-IL-10 monoclonal antibody at 1 day after injury significantly improved the ability of burn-injured mice to survive CLP-induced polymicrobial sepsis. This finding indicates that early inhibition of IL-10 function *in vivo* protects burn-injured mice from development of immune suppression as judged by CLP survival. In addition, we found that IL-10 antagonism did not affect the survival of burn-injured mice, since we observed no increased burn-induced mortality in burn-injured mice treated with anti-IL-10 antibody.

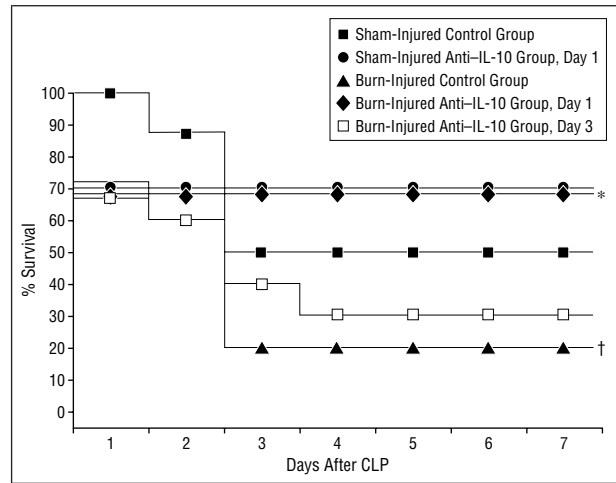
Delaying anti-IL-10 antibody treatment until 3 days after burn injury does not protect mice from development of injury-induced immune suppression. To address the kinetics of IL-10 antagonism on the develop-

ment of burn injury-induced immune suppression, we compared the effectiveness of blocking IL-10 activity at 1 day vs 3 days after injury on CLP-induced survival. We reasoned that this experimental approach would reveal whether IL-10 acts early to promote the development of injury-induced immune suppression or if anti-IL-10 antibody treatment acts simply to improve the survival of CLP-challenged mice by acting during the infectious challenge phase of our studies. As shown in **Figure 2**, delaying anti-IL-10 treatment until 3 days after injury had no significant effect on survival of CLP challenge, whereas we again observed that inhibiting IL-10 at 1 day was beneficial. Taken together, this outcome suggests that IL-10 may indeed act as an early mediator to promote the development of postinjury immune dysfunction and that the beneficial effects of anti-IL-10 antibody therapy on CLP-induced survival of burn-injured mice probably does not involve residual anti-IL-10 antibody effects mediated at the time of CLP challenge.



**Figure 1.** The effect of anti-interleukin 10 (IL-10) treatment at 1 day after injury on cecum ligation and puncture (CLP)-induced survival. Sham- or burn-injured mice (10 mice per group) were given 250  $\mu$ g of anti-IL-10 antibody or control IgG by intraperitoneal injection at 1 day after injury. One death occurred in the burn-control group in experiment 2 (B), before performing CLP. The CLP procedure was performed 10 days after injury, and survival was monitored daily for a total of 7 days. The results from 2 independent experiments (A and B) are shown. Asterisk indicates that the survival of the burn-injured anti-IL-10 group is significantly different ( $P \leq .01$ ) from that of the burn-control group; dagger, survival of the burn-control group is significantly different ( $P < .001$ ) from that of the sham-injured control group.

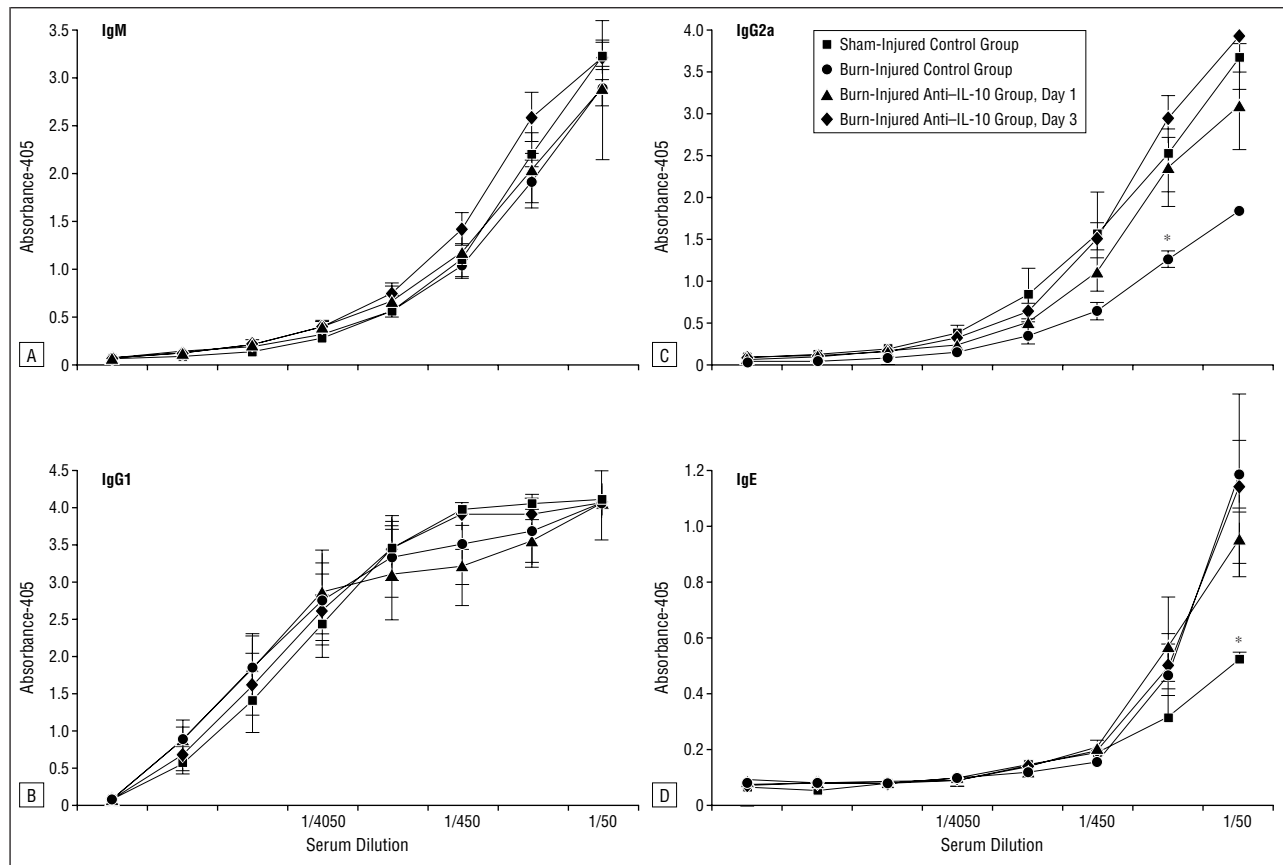
Anti-IL-10 antibody treatment at days 1 and 3 after injury restores burn injury-induced suppression of  $T_H1$  function in vivo. We next used an immunization approach to investigate a mechanistic basis for the beneficial effect of day 1 postinjury anti-IL-10 therapy. To accomplish this, sham- and burn-injured mice and burn-injured mice treated with anti-IL-10 were immunized with TNP-OVA. Ten days later, TNP-specific serum antibody responses were assessed to determine how burn injury and anti-IL-10 antibody treatment affected T-cell help for antibody isotype formation in vivo. As shown in **Figure 3**, C, burn injury in the absence of anti-IL-10 therapy caused a significant reduction in formation of the  $T_H1$ -dependent antibody isotype, IgG2a, whereas anti-IL-10 antibody treatment given at 1 day or 3 days after injury corrected this burn-induced loss of  $T_H1$  function. In contrast, burn injury had no significant effect on IgM or IgG1 antibody isotype production (Figure 3, A and B, respectively). However, as illustrated in Figure 3, D, we observed a significant increase in IgE formation in burn-injured mice immunized with TNP-



**Figure 2.** The effect of day 3 vs day 1 postinjury anti-interleukin 10 (IL-10) treatment on survival of cecum ligation and puncture (CLP). Control treatment consisted of IgG. Sham- and burn-injured mice and burn-injured mice treated with anti-IL-10 antibody at 1 day or at 3 days after injury (10 mice per group) were tested for their ability to survive CLP challenge at 10 days after burn injury. Survival was monitored at daily intervals for 7 days. Asterisk indicates that the survival of the burn-injured anti-IL-10 group is significantly different ( $P = .02$ ) from that of the burn-injured control group; dagger, survival of the burn-injured control group is significantly different ( $P = .02$ ) from that of the sham-injured control group.

OVA. These findings indicate the inhibiting in vivo IL-10 activity at 1 day or 3 days after injury can prevent the burn injury-induced reduction in  $T_H1$  function.

Interleukin 10 antagonism restores cytokine production by antigen-specific T cells. The ability of anti-IL-10 antibody treatment to restore  $T_H1$  function in burn-injured mice suggested to us that blocking IL-10 activity in vivo may help restore injury-induced alterations in T-cell cytokine production. Thus, to test the effects of IL-10 antagonism on cytokine production by antigen-specific T cells, spleen cells were prepared and harvested from sham- and burn-injured mice and burn-injured mice treated with anti-IL-10 undergoing immunization with TPA-OVA, then stimulated in culture with the immunogen, TNP-OVA. As illustrated in **Figure 4**, burn injury caused a significant suppression in antigen-induced IL-2, interferon  $\gamma$  (IFN- $\gamma$ ), and IL-10 production compared with induced cytokine levels in sham-injured mice. We did, however, observe a burn injury-related increase in TNP-OVA-induced IL-4 production. Comparing the TNP-OVA-induced cytokine production levels in spleen cell cultures established from burn-injured mice given anti-IL-10 at 1 day or 3 days after injury revealed that blocking IL-10 activity in vivo helped restore IL-2, IFN- $\gamma$ , IL-10, and even IL-4 production to near levels in sham-injured animals. Taken together with the antibody isotype formation results shown in Figure 3, these findings indicate that IL-10 plays a role in the suppressed  $T_H1$  function and altered T-cell cytokine production observed after burn injury. However, the observation that day 3 anti-IL-10 antibody administration improved T-cell responses in burn-injured mice, but failed to improve the survival of CLP-challenged, burn-injured mice, suggests that other factors besides restored T-cell function are responsible for the improved survival of CLP-challenged, burn-injured mice given anti-IL-10 at 1 day after injury.



**Figure 3.** Anti-interleukin 10 (IL-10) antibody treatment effects on T-cell-dependent antibody isotype formation in vivo. Control treatment consisted of IgG. Sham- and burn-injured mice (4 mice per group) were immunized at the time of injury with the T-cell-dependent antigen, trinitrophenyl (TNP) haptenated ovalbumin (TNP-OVA), then given anti-IL-10 antibody at 1 day or at 3 days after injury. Ten days later, serum samples were prepared from cardiac blood and tested for TNP-specific antibody titers by enzyme-linked immunosorbent assay using the indicated mouse antibody isotype detection reagents. Each point represents mean  $\pm$  SEM of serum tested in duplicate for antibody levels from 4 individual mice. Asterisk indicates significant differences between other experimental groups ( $P = .009$  for IgE and  $P < .001$  for IgG2a). Similar results were obtained in 3 independent experiments.

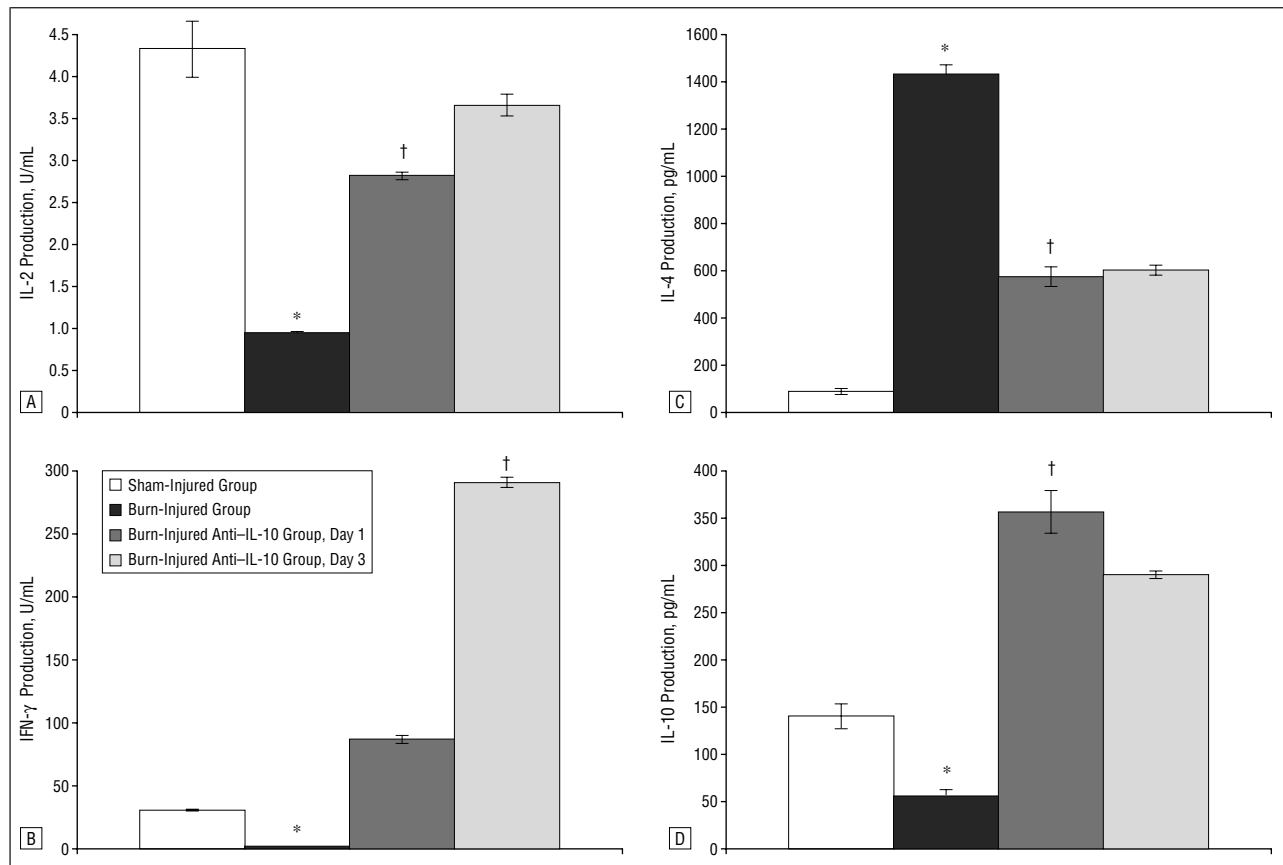
## COMMENT

The suppressed immunity seen in patients after severe injury is an ongoing clinical problem that is complex in nature.<sup>1,4,7</sup> The development of the mouse burn-injury experimental model has allowed us and others to study the influence of injury on the immune system in a more controlled fashion than can be attained in clinical studies. Nevertheless, evidence suggests that the mouse burn-injury model mimics many of the changes in immunity seen in severely injured patients.<sup>3,10,14,15</sup> These alterations include suppressed mitogen-induced T-cell proliferation, reductions in IL-2 and IFN- $\gamma$  production, increases in IL-4 and IL-10 production, increased susceptibility to infection, and changes in the balance of proinflammatory cytokine production.<sup>7</sup>

More recently, the mouse burn-injury model and an immunization approach were used to demonstrate that burn injury causes a marked diminution of  $T_H1$  function in vivo, whereas  $T_H2$  function persists.<sup>13</sup> In this same study, it was demonstrated that the anti-inflammatory and immune-suppressive cytokine, IL-10, played a role in the reduced in vivo  $T_H1$  function seen in burn-injured mice. This suggested that IL-10 may play a central role in the immune-suppressive state and increased susceptibility to opportunistic infections that

often occur after severe injury. Therefore, the present study was undertaken to test the potential usefulness of IL-10 antagonism in restoring the lowered resistance to infection after burn injury. We used the CLP technique to induce polymicrobial sepsis at a time point after injury when burn-injured mice are known to be maximally immune suppressed to determine the effect of inhibiting in vivo IL-10 activity on survival. The usefulness of this experimental approach has been confirmed in several other studies demonstrating the beneficial effects of using other immune-enhancing therapeutic approaches, including IL-1, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-12 treatment.<sup>9,16-18</sup> We demonstrate herein that the in vivo inhibition of IL-10 activity at 1 day but not 3 days after burn injury significantly improved the survival of burn-injured mice challenged by the CLP method at 10 days after injury. This finding provides direct evidence to suggest that IL-10 plays a role in the development of injury-induced immune suppression. However, its role is as an early mediator, since delaying treatment to 3 days after injury did not improve the survival of CLP-challenged burn-injured mice.

We also found that giving anti-IL-10 antibody at 1 day or 3 days after burn injury had no adverse effects on the survival of burn-injured mice. This is an important



**Figure 4.** The influence of anti-interleukin 10 (IL-10) antibody treatment on antigen-specific cytokine production by splenic T cells from trinitrophenyl (TNP)-haptenated ovalbumin (TNP-OVA)-immunized sham- and burn-injured mice. Sham- and burn-injured mice (4 mice per group) were immunized at the time of injury with the T-cell-dependent antigen, TNP-OVA, then given anti-IL-10 antibody at 1 day or at 3 days after injury. Ten days later, spleen cells were restimulated with TNP-OVA *in vitro* to assess antigen-induced cytokine production. After 48 hours, culture supernatants were harvested, then tested for the indicated cytokines by enzyme-linked immunosorbent assay. No cytokines were detected in unstimulated spleen cell culture supernatants. The results are representative of 3 experiments and are illustrated as mean  $\pm$  SEM of triplicates. Asterisk indicates significant differences between sham- and burn-injured groups ( $P = .009$ ); dagger, significant differences between the burn-injured control group and burn-injured group treated with anti-IL-10 ( $P < .001$ ) and interferon  $\gamma$  (IFN- $\gamma$ ) day 1 vs day 3 anti-IL-10 treatment ( $P < .001$ ).

finding, since detrimental effects of IL-10 antagonism have been described in other inflammatory conditions such as endotoxin-mediated shock and CLP-induced polymicrobial sepsis.<sup>19,20</sup> This result also suggests that IL-10 antagonism may be a safer therapeutic approach for preventing injury-induced immune suppression than had been anticipated.

To provide a mechanistic basis for IL-10 antagonism and its beneficial effects at preventing the development of postinjury immune suppression, we wanted to determine how anti-IL-10 treatment given at 1 day vs 3 days after injury affected the development of T-cell-dependent immunity. To accomplish this, we used an established immunization approach that allowed us to follow the development of an immune response against a defined T-cell-dependent antigen in sham- vs burn-injured mice and in burn-injured mice given anti-IL-10 at 1 day or 3 days after injury. The outcome of this work demonstrated that burn injury caused the expected suppression in  $T_H1$ -type antibody isotype formation and an increase in the production of IgE, which is the  $T_H2$ -dependent antibody isotype. Treating mice at 1 day or at 3 days after injury with anti-IL-10 antibody restored the *in vivo*  $T_H1$  response, which indicates that IL-10 is

clearly involved in suppressing  $T_H1$ -type immune responsiveness after burn injury. The restoration of  $T_H1$  function *in vivo* and the improved survival of CLP-challenged mice given anti-IL-10 antibody at 1 day after injury suggests that the loss of  $T_H1$  function after injury may also be involved in the reduced resistance to infection after injury. However, we did observe that delaying anti-IL-10 treatment to 3 days after injury did not improve CLP survival, but did restore  $T_H1$  function *in vivo*. This leads us to the conclusion that blocking IL-10 activity at 1 day after injury has other effects beyond restoring  $T_H1$  function that contribute to improving postinjury CLP survival. These findings highlight the importance of considering the kinetics of enhancing or suppressing inflammatory or T-cell-mediated responses when designing therapies to control systemic inflammatory responses or immunity after severe injury.

Since IL-10 is known to counteract proinflammatory events by acting to suppress the production of inflammatory cytokines such as IL-1, IL-12, tumor necrosis factor (TNF), and IFN- $\gamma$ , we were initially concerned that treatment would lead to a lethal inflammatory state in burn-injured mice.<sup>12</sup> Results to support this hypothesis come from other mouse models of injury and in-

### Statement of Clinical Relevance

After burn or traumatic injury, there is an upset in the regulation of the immune system such that severely injured patients can become highly susceptible to development of infectious complications. Recent advances in our understanding of this process indicate that changes in cytokine production by cells of the immune system can mediate many of these injury-induced changes in the immune system. This study uses a mouse model of injury to study how altering the early cytokine response to injury affects the development of postinjury immune suppression. We demonstrate herein that blocking the activity of a cytokine with immune suppressive activity, IL-10, can prevent the development of postinjury immune dysfunction. Our studies show that treating mice with anti-IL-10 antibody at 1 day after injury can significantly improve their ability to survive polymicrobial infection. Moreover, this protective effect of anti-IL-10 antibody treatment correlated directly with improved in vivo T-cell-mediated (adaptive) immunity. Taking all results together, this report suggests that therapies designed to block the early activity of the immune-suppressive cytokine, IL-10, may help to prevent the development of postinjury immune dysfunction.

flammation demonstrating a high mortality of CLP-challenged mice treated with anti-IL-10 antibody.<sup>20</sup> Several other reports<sup>19,21,22</sup> suggest that the anti-inflammatory activity of IL-10 helped to protect against endotoxin-induced shock, experimentally induced peritonitis in mice, or, in a mouse model, multiple organ dysfunction syndrome. However, we report herein that the temporal inhibition of IL-10 activity in vivo does not affect the survival of burn-injured mice. Therefore, we conclude that the anti-inflammatory activity of IL-10 is not essential for survival after burn injury. Instead, our findings support the hypothesis that the immune-suppressive activity of IL-10 may be its dominant function in the injured host. Its inhibition early after injury may help prevent development of immune dysfunction in burn-injured mice by augmenting the production and release of proinflammatory cytokines such as IL-1, IL-12, IFN- $\gamma$ , and TNF or by blocking the inhibitory effects of IL-10 on antigen-presenting cell functions.<sup>12,23</sup> All of these potential effects of early IL-10 antagonism may then act in concert to promote a more inflammatory, T<sub>H</sub>1-like immunity in vivo. Although promoting this type of proinflammatory response after injury may seem counterintuitive, evidence supports the idea that these inflammatory cytokines that are counterregulated by IL-10 can protect mice from infection by boosting functional attributes of the innate and adaptive immune systems.<sup>24-26</sup> The usefulness of IL-10 antagonist therapy for preventing injury-induced immune dysfunction is supported by our work; however, more extensive analysis of the mechanisms involved and the universality of its effects in other injury models are needed before considering this type of a therapeutic approach.

This work was supported in part by grant RO1 GM775033 from the National Institutes of Health, Bethesda, Md, and

by the Julian and Eunice Cohen and David Brook Funds for Surgical Research, Boston, Mass.

Presented at the 19th Annual Meeting of the Surgical Infection Society, Seattle, Wash, April 29, 1999.

Reprints: James A. Lederer, PhD, Department of Surgery, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St, Boston, MA 02115 (e-mail: jlederer@rics.bwh.harvard.edu).

### REFERENCES

1. Baker CC, Miller CL, Trunkey DD. Predicting fatal sepsis in burn patients. *J Trauma*. 1979;19:641-648.
2. Esrig BC, Frazee L, Stephenson SF, Polk HCJ, Fulton RL, Jones CE. The predisposition to infection following hemorrhagic shock. *Surg Gynecol Obstet*. 1977;144:915-917.
3. Faist E, Kupper TS, Baker CC, Chaudry IH, Dwyer J, Baue AE. Depression of cellular immunity after major injury: its association with posttraumatic complications and its reversal with immunomodulation. *Arch Surg*. 1986;121:1000-1005.
4. Miller SE, Miller CL, Trunkey DD. The immune consequences of trauma. *Surg Clin North Am*. 1982;62:167-181.
5. Ninnemann JL. Trauma, sepsis, and the immune response. *J Burn Care Rehabil*. 1987;8:462-468.
6. Xu YX, Ayala A, Chaudry IH. Prolonged immunodepression after trauma and hemorrhagic shock. *J Trauma*. 1998;44:335-341.
7. Lederer JA, Rodrick ML, Mannick JA. The effects of injury on the adaptive immune response. *Shock*. 1999;11:153-159.
8. Moss NM, Gough DB, Jordan AL, et al. Temporal correlation of impaired immune response after thermal injury with susceptibility to infection in a murine model. *Surgery*. 1988;104:882-887.
9. O'Suilleabhain C, O'Sullivan ST, Kelly JL, Lederer J, Mannick JA, Rodrick ML. Interleukin-12 treatment restores normal resistance to bacterial challenge after burn injury. *Surgery*. 1996;120:290-296.
10. O'Sullivan ST, Lederer JA, Horgan AF, Chin DH, Mannick JA, Rodrick ML. Major injury leads to predominance of the T helper-2 lymphocyte phenotype and diminished interleukin-12 production associated with decreased resistance to infection. *Ann Surg*. 1995;222:482-492.
11. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature*. 1996;383:787-793.
12. Howard M, O'Garra A. Biological properties of interleukin 10. *Immunol Today*. 1992;13:198-200.
13. Kelly JL, Lyons A, Soberg CC, Mannick JA, Lederer JA. Anti-interleukin-10 antibody restores burn-induced defects in T-cell function. *Surgery*. 1997;122:146-152.
14. Mack VE, McCarter MD, Naama HA, Calvano SE, Daly JM. Dominance of T-helper 2-type cytokines after severe injury. *Arch Surg*. 1996;131:1303-1308.
15. Lyons A, Kelly JL, Rodrick ML, Mannick JA, Lederer JA. Major injury induces increased production of interleukin-10 by cells of the immune system with a negative impact on resistance to infection. *Ann Surg*. 1997;226:450-460.
16. O'Riordain MG, Collins KH, Pilz M, Saporoschetz IB, Mannick JA, Rodrick ML. Modulation of macrophage hyperactivity improves survival in a burn-sepsis model. *Arch Surg*. 1992;127:152-158.
17. Molloy RG, Holzheimer R, Nestor M, Collins K, Mannick JA, Rodrick ML. Granulocyte-macrophage colony-stimulating factor modulates immune function and improves survival after experimental thermal injury. *Br J Surg*. 1995;82:770-776.
18. Gough DB, Moss NM, Jordan A, Grbic JT, Rodrick ML, Mannick JA. Recombinant interleukin-2 (rIL-2) improves immune response and host resistance to septic challenge in thermally injured mice. *Surgery*. 1988;104:292-300.
19. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin-10 protects mice from lethal endotoxemia. *J Exp Med*. 1993;177:1205-1208.
20. van der Poll T, Marchant A, Buurman WA, et al. Endogenous IL-10 protects mice from death during septic peritonitis. *J Immunol*. 1995;155:5397-5401.
21. Lane JS, Todd KE, Lewis MPN, et al. Interleukin-10 reduces the systemic inflammatory response in a murine model of intestinal ischemia/reperfusion. *Surgery*. 1997;122:288-294.
22. Ferrer TJ, Webb JW, Wallace BH, et al. Interleukin-10 reduces morbidity and mortality in murine multiple organ dysfunction syndrome (MODS). *J Surg Res*. 1998;77:157-164.
23. Bogdan C, Vodovotz Y, Nathan C. Macrophage deactivation by interleukin 10. *J Exp Med*. 1991;174:1549-1555.
24. Czuprynski CJ, Brown JF, Young KM, Cooley AJ, Kurtz RS. Effects of murine recombinant interleukin-1 alpha on the host response to bacterial infection. *J Immunol*. 1988;140:962-968.
25. Roll JT, Young KM, Kurtz RS, Czuprynski CJ. Human rTNF alpha augments antibacterial resistance in mice: potentiation of its effects by recombinant human IL-1 alpha. *Immunology*. 1990;69:316-322.
26. Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol*. 1995;13:251-276.

Lena M. Napolitano, MD, Baltimore, Md: Accumulating evidence suggests that the anti-inflammatory effects of IL-10 at the cellular level can be manipulated as a therapeutic strategy to impact both the immune and the inflammatory responses associated with injury, sepsis, and other disease states. Deciphering the role of this important cytokine in sepsis, however, has been very difficult: first, due to the numerous animal models that have been utilized; second, due to the different tissues and sources of immunocytes; and third, due to the different outcome variables studied.

I would like to congratulate Dr Lederer and his colleagues and Dr Mannick's esteemed group for the extension of their previous work investigating the effects of early IL-10 antagonism on burn-induced immune dysfunction in a very clinically relevant model of polymicrobial sepsis (CLP).

I have several specific questions, since I think the results of these studies unfortunately add to the confusion and controversy regarding whether IL-10 is beneficial or harmful in sepsis.

First, anti-IL-10 antibody treatment on day 1 or day 3 post-burn resulted, as you nicely showed, in increased IL-10 secretion by immunocytes. How do you reconcile these data? Should we conclude that the antibody was not efficacious in blocking IL-10 production in this model? How do we interpret the mortality data in light of these cytokine data, ie, animals with increased IL-10 production after treatment with anti-IL-10 antibody manifested improved survival?

Second, the splenocyte cytokine profile of CLP animals has been characterized previously by a number of groups by marked suppression of IL-2 and INF- $\gamma$  release and a significant increase in IL-4 and IL-10. Did you investigate specifically the alterations in splenocyte cytokine production in the burn animals who underwent CLP 10 days after burn injury? That really has not been clearly identified in the past.

Third, other investigators have carefully studied the role of IL-10 in CLP-induced sepsis and have reported variable results, either improved survival, no difference in survival, and in 1 study, blockade of endogenous IL-10 demonstrated a decrease in survival and increased mortality. To my knowledge, this is the very first study that has documented an improved survival in CLP with anti-IL-10 therapy. Is that true? Have you investigated the efficacy of anti-IL-10 therapy in CLP alone, not with prior burn injury?

And finally, recent studies have determined that IL-10 plays a very critical role in reversing the inhibition of neutrophil apoptosis that has been identified in patients with systemic inflammatory response syndrome and sepsis. Should there not be concern that administration of anti-IL-10 antibody will result in increased suppression of activated neutrophils to undergo apoptosis, and thus induce failure to terminate the inflammatory response? Do you have any preliminary data regarding apoptosis in this model in either burn plus CLP or CLP alone?

**Dr Lederer:** Very good questions. First of all, the increased IL-10 production in response to antigen. What your group has shown is different from what we have shown in the burn injury, probably because the models are different. I know that you have a femur fracture model. So your question is, how can I explain that IL-10 increase when I give anti-IL-10? I think the easiest way to explain it is that we are correcting what has been referred to as a T-cell anergy. And I think that the role of

IL-10, or the effect of anergy on IL-10 production, has not been clearly looked at, and what happens routinely in all these studies that we have done and we have reported on is that we can bring up not only the proinflammatory cytokine productions like IL-2 and INF- $\gamma$ , but we also bring up IL-10; and in fact IL-4 sometimes is increased in response to polyclonal antigens such as phytohemagglutinin antigen or concanavalin A or anti-CD3.

The next question is, have we looked at CLP cytokines? No, we have not in this context. We have begun looking at what happens in the IL-12 therapy work that we have done, and we see that the production of TNF after CLP is markedly suppressed. But, really, I mean, we are using CLP not as purely a way to study the response to sepsis. We are trying to document the immune suppression that occurs after injury. So it is a little different approach from what other people have done, such as Lowry.

The improved survival, again, the Lowry paper that you are referring to vs our work, yes, in fact this is the first time that anyone has shown that you can improve survival giving anti-IL-10; however, as you recall, giving anti-IL-10 at day 3 didn't have an effect at improving survival. We have not particularly done that experiment, giving anti-IL-10 at the time of CLP. I feel that that experiment has been done and it was well done by that group. However, we are working with IL-10 knockout mice, and we hope to have a clearer picture on that phenomenon.

And last, your question on apoptosis, we have done CD4, CD8 stains, and whatnot at various times after injury, and we really don't see changes in the peripheral lymphoid tissues in CD4, CD8 ratios; so I can't really comment on the effects on apoptosis.

As far as anti-IL-10 being dangerous, as I mentioned, we never saw any detrimental effects of giving the antagonist; however, in later experiments, which I have not presented here, we found that when giving it at day 7, the mice got very sick. So I think the timing of when you inhibit IL-10 is extremely important in this model, and that really is something we have to consider when we are trying to figure out what is happening after injury.

**Robert G. Sawyer, MD, Charlottesville, Va:** Following up with 1 more question about the CLP model and anti-IL-10 use, do you know what the half-life of your antibody is, because there is a time lag between when you actually give the antibody and when the CLP is given? I was wondering if you think there is any anti-IL-10 either circulating or somewhere else available?

Could you comment on the possibility of macrophages rather than T cells being the source of your IL-10 when you measure it in vivo?

**Dr Lederer:** As far as we know, the half-life of our antibody in the plasma and serum is about 6 hours. I don't think that the effects that we are seeing in the CLP are due to residual effects of anti-IL-10. It is acting very early in the response, particularly when we are showing at day 3 we don't have any benefit.

As far as the cell source of IL-10, it depends on the tissue. We see early IL-10 in the spleen; however, in the lymph nodes we see it coming from T cells. It is a very complex problem that we are working on right now, and it is essential to figure that out. We are trying to figure out the basic response to injury and how it affects the immune system, and then we can go from there to try to sort out the particulars.