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

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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)–haptenated 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 γ, 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

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. 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.

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. In particular, mitogen-stimulated spleen cells harvested from burn-injured mice produce relatively higher levels of the helper T cell type 2 (T\textsubscript{h}2)-type cytokines, interleukin 4 (IL-4), and IL-10 than those from sham-injured mice. This increase in T\textsubscript{h}2 cytokine production correlates directly with the increased susceptibility of burn-injured mice to polymicrobial sepsis induced by the cecum ligation and puncture (CLP) technique. Given the anti-inflammatory activity of these cytokines and their established immune-suppressive activity, this observation suggested to us that T\textsubscript{h}2-type cytokines may play a central role in inducing or maintaining injury-induced immune suppression.

Recent work addressing the influence of burn injury on T-cell activation and function demonstrated that burn injury suppresses T\textsubscript{h}1 function in vivo. This was shown using an immunization approach that allowed us to follow the develop-
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. 

REAGENTS

Chicken ovalbumin fraction IV (OVA), rat IgG, and picrylsulfonic acid (2,4,6-trinitobenzene 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-µg/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-µmol/L nonessential amino acids, 10-µmol/L HEPES, a combination of penicillin and streptomycin, and 2.5 × 10⁻⁵ mol/L β-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. 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. 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 µL 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

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

**SEVER TNP-SPECIFIC ANTIBODY ISOxYPE ELISA**

The TNP-spmcic antibodies were assayed using an ELISA as described previously. In brief, ELISA-grade microtiter plates (Nunc MaxiSorb; Nalgae Nunc International Corporation, Taastrupgaard, Denmark) were coated with 50 µL of TNP-haptenated bovine serum albumin (BSA; 10 µg/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 1:30 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 µg/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.

**SEUM HARVEST AND IN VITRO SPLEN 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 700 g 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 × 10⁵ cells per well in 96-well plates (Corning Costar Corporation, Cambridge, Mass) in the absence or presence of 30 µg/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 µg/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 µL 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 was used to read 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.
Anti–IL-10 antibody treatment at days 1 and 3 after injury restores burn injury–induced suppression of T\(_{\text{H}}\)1 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\(_{\text{H}}\)1-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\(_{\text{H}}\)1 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-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\(_{\text{H}}\)1 function.

Interleukin 10 antagonism restores cytokine production by antigen-specific T cells. The ability of anti–IL-10 antibody treatment to restore T\(_{\text{H}}\)1 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\(_{\text{H}}\)1 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.
The suppressed immunity seen in patients after severe injury is an ongoing clinical problem that is complex in nature. 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. These alterations include suppressed mitogen-induced T-cell proliferation, reductions in IL-2 and IFN-γ production, increases in IL-4 and IL-10 production, increased susceptibility to infection, and changes in the balance of proinflammatory cytokine production.

More recently, the mouse burn-injury model and an immunization approach were used to demonstrate that burn injury causes a marked diminution of Th1 function in vivo, whereas Th2 function persists. 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 Th1 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 Th1 function seen in burn-injured mice.

We also found that giving anti–IL-10 antibody at 1 day or at 3 days after injury had no adverse effects on the survival of burn-injured mice. This is an important finding, since delaying treatment to 3 days after injury did not improve the survival of CLP-challenged burn-injured mice.
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.\textsuperscript{19,20} 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\textsubscript{H}1-type antibody isotype formation and an increase in the production of IgE, which is the T\textsubscript{H}2-dependent antibody isotype. Treating mice at 1 day or 3 days after injury with anti–IL-10 antibody restored the in vivo T\textsubscript{H}1 response, which indicates that IL-10 is clearly involved in suppressing T\textsubscript{H}1-type immune responsiveness after burn injury. The restoration of T\textsubscript{H}1 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\textsubscript{H}1 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\textsubscript{H}1 function in vivo. This leads us to the conclusion that blocking IL-10 activity at 3 days after injury did not improve CLP survival, but did restore T\textsubscript{H}1 function in vivo.

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.\textsuperscript{12} Results to support this hypothesis come from other mouse models of injury and in-
flammmation demonstrating a high mortality of CLP-challenged mice treated with anti–IL-10 antibody. 20 Several other reports 24-26 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-γ, and TNF or by blocking the inhibitory effects of IL-10 on antigen-presenting cell functions.12,23 All of these potential effects of early IL-10 antagonism may then act in concert to promote a more inflammatory, Th1-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.24-26 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 GM773033 from the National Institutes of Health, Bethesda, Md, and by the Julian and Eunice Cohen and David Brook Funds for Surgical Research, Boston, Mass.


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REFERENCES

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 postburn 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, i.e., 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-γ 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 a 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, either improved survival, and controversy regarding whether IL-10 is beneficial or harmful in sepsis.

We see early IL-10 in the spleen; however, in the lymph nodes, 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, Charlottesvile, 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?

Dr Lederer: Yes, we have begun looking at what 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.